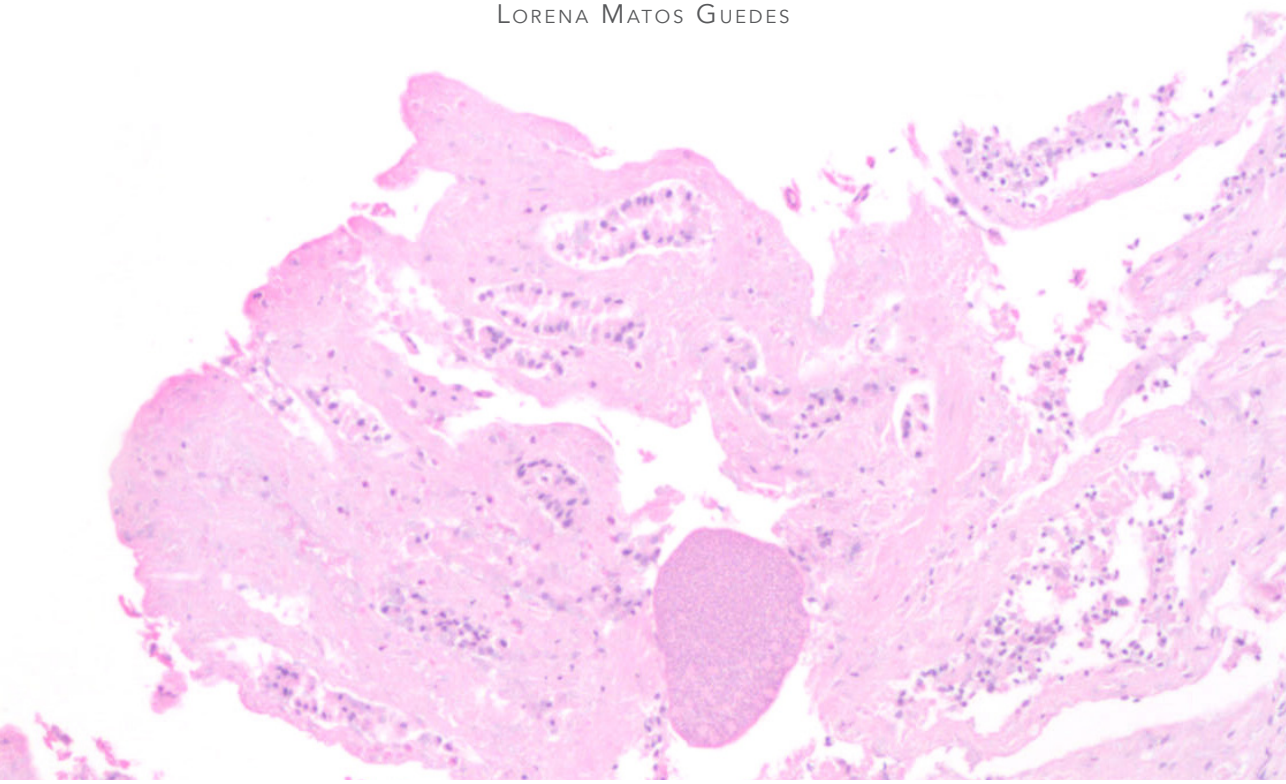
TESIS DOCTORAL 2015

ESTUDIO BIOPATOLÓGICO Y RESPUESTA INMUNE EN LA COCIDIOSIS CAPRINA PRODUCIDA POR *EIMERIA NINAKOHLYAKIMOVAE*: IMPLICACIONES EN EL CONTROL DE LA ENFERMEDAD

LORENA MATOS GUEDES



Instituto Universitario de Sanidad Animal y Seguridad Alimentaria.
Universidad de Las Palmas de Gran Canaria

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

FACULTAD DE VETERINARIA

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INFORMAN:

Que la tesis doctoral que lleva por título "**Estudio biopatológico y respuesta inmune en la coccidiosis caprina por *Eimeria ninakohlyakimovae*: implicaciones en el control de la enfermedad**" ha sido realizada por la licenciada en Veterinaria **Dña. Lorena Matos Guedes** en el Departamento de Patología Animal, Producción Animal, Bromatología y Tecnología de los Alimentos de la (Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria) bajo nuestra dirección y asesoramiento, y consideramos que cumple la normativa vigente para optar al Grado de Doctor en Veterinaria por la Universidad de Las Palmas de Gran Canaria.

En Arucas (Las Palmas) a 17 noviembre de 2015

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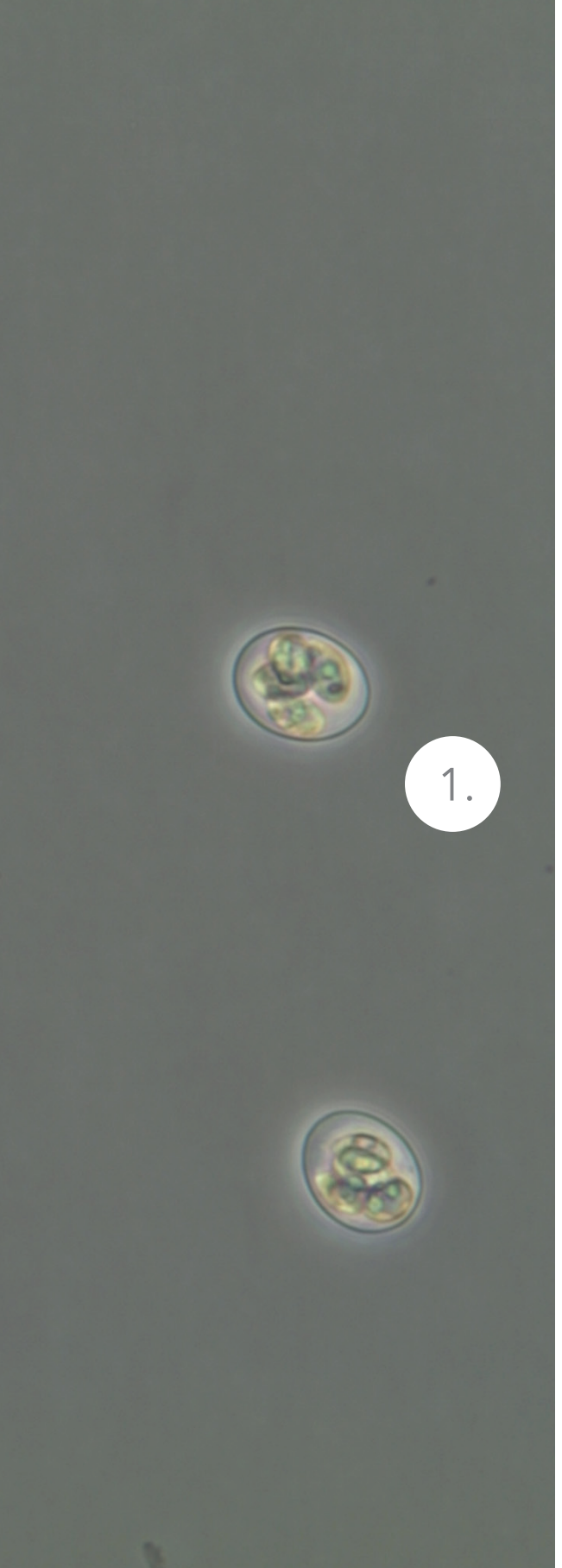

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PROTEÓMICA E INMUNOLOGÍA APLICADA A LA PROFILAXIS Y CONTROL DE LA COCCIDIOSIS CAPRINA PRODUCIDA POR *EIMERIA NINAKHOLYAKIMOVAE*

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RESPUESTA INMUNE Y MECANISMOS DE PATOGENICIDAD EN LA COCCIDIOSIS CAPRINA: IMPLICACIONES EN LA PROFILAXIS Y CONTROL DE LA ENFERMEDAD

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2.

INTRODUCCIÓN Y OBJETIVOS

2.1. INTRODUCCIÓN

Las infecciones producidas por las diferentes especies del protozoo *Eimeria*, conocidas en medicina veterinaria como coccidiosis, constituyen unas de las parasitosis más frecuentes y más ampliamente distribuidas en los sistemas de producción caprinos. La parasitación por estos enteropatógenos, que independientemente de la forma de manejo puede afectar a casi un 100% de los animales de un rebaño, representa uno de los principales motivos de pérdidas económicas para la producción caprina a nivel mundial. Los animales adultos suelen hacerse resistentes después de sobrevivir al periodo crítico durante las primeras semanas de vida, transformándose entonces en reservorios y portadores inaparentes del parásito. Por el contrario, los animales jóvenes son los más susceptibles de padecer la enfermedad, especialmente entre las 2 semanas y los 4 meses de vida.

Los coccidios están presentes en todas las ganaderías de rumiantes, aunque esto no quiere decir que en todas ellas se desarrolle la enfermedad. No se conocen con exactitud los mecanismos que desarrollan una coccidiosis patente; lo que sí están claros son los factores de riesgo: destete, transporte, entrada en cebaderos, partos múltiples, madres mal alimentadas, ubres sucias, problemas de mastitis, etc., situaciones todas ellas típicas del sistema de crianza y engorde de rumiantes.

Se han descrito al menos 18 especies diferentes de *Eimeria* que pueden infectar a cabras en todo el mundo, de las cuales *Eimeria ninakohlyakimovae* y *E. arloingi* son consideradas como las más patógenas y frecuentes en el archipiélago Canario.

La principal acción patógena de *E. ninakohlyakimovae* es la destrucción de las células epiteliales del intestino como resultado de las fases de reproducción endógena, tanto asexual como sexual. El parásito invade las células del intestino delgado, ciego y colon produciendo descamación del epitelio y atrofia de las vellosidades. Como consecuencia, se produce un síndrome de malabsorción y pérdida de fluidos, que se asocian con deshidratación y diversos grados de diarrea. Por tanto, los signos clínicos en cabritos, al igual que en otros rumiantes, incluyen retraso del crecimiento, debilidad, anorexia, diarreas y, en algunos casos, muerte.

El control de la coccidiosis caprina se basa actualmente en empleo combinado de medidas de manejo y tratamientos profilácticos con coccidiostáticos. Esto último presenta, no obstante, algunos inconvenientes, como la limitación cada vez más estricta del uso de aditivos en la Unión Europea, entre ellos los coccidiostáticos. Un problema adicional lo constituye el desarrollo cada vez más frecuente de fenómenos de resistencia. El problema de la resistencia frente a los coccidiostáticos, discutido

hace ya más de una década, proporciona un estímulo para el desarrollo de nuevos procedimientos (tales como la inmunoprotección) para el control de la coccidiosis.

Algunos aspectos de la respuesta inmune en coccidios, en especial la respuesta inmune adaptativa (humoral y celular) han sido bien caracterizados en la coccidiosis bovina, aviar y en diversos modelos de la coccidiosis en roedores. En cambio, para la coccidiosis caprina apenas existen trabajos relacionados con la caracterización de la respuesta inmune frente a este importante grupo de parásitos intestinales y, menos aún, sobre su posible repercusión en la formación de una respuesta inmunoprotectora.

Las barreras físicas, las células epiteliales, las células endoteliales y los leucocitos, componen las líneas de defensa de especificidad creciente que forman parte del sistema inmunitario innato frente a coocidiosis. Ante la infección y posterior desarrollo del parásito, las células endoteliales pueden responder modulando la transcripción génica de moléculas inmunorreguladoras de la respuesta inmune, incluyendo citoquinas, quimioquinas y moléculas de adhesión. También lo hacen los leucocitos inmunocompetentes innatos, entre ellos los polimorfonucleares neutrófilos (PMN), los monocitos, los macrófagos, las células dendríticas, las células asesinas naturales (NK), los eosinófilos, los monocitos y los leucocitos globulares. Los PMN juegan un papel muy importante en la inmunidad innata ya que, aparte de ser los leucocitos más abundantes (comprenden entre el 50 y el 80 % del total de glóbulos blancos), constituyen la primera línea de defensa frente a los agentes.

Es sabido que en las reinfecciones por coccidios es menos probable que se desencadene una enfermedad clínica, lo que confirma la importancia de la exposición anterior durante las infecciones primarias en el resultado de la inmunidad protectora. En general, las respuestas celulares y humorales que forman parte de la respuesta inmunitaria adaptativa frente a la coccidiosis intestinal en rumiantes se desarrollan rápidamente tras el primer contacto con el antígeno y su intensidad depende del número de ooquistes ingeridos. Muchos estudios indican y confirman el importante papel que juega en la inmunidad los linfocitos tipo T en la infección primaria por coccidios. En concreto, la respuesta mediada por células T se ha asociado con una menor excreción de ooquistes en animales reinfectados con *E. bovis*, estando implicados en ella los linfocitos CD4+ y CD8+, fundamentalmente. En particular, las infecciones por coccidios de rumiantes como *E. ninakohlyakimovae* o *E. bovis*, que invaden células endoteliales, pueden desarrollar reacciones inmunes que implican un gran número de células inmunoeractivas. Así, las infecciones por *E. bovis* en el ganado bovino inducen una red de regulación molecular asociada al movimiento y tráfico de leucocitos al lugar de la infección y, como resultado, se produce una fuerte reacción inflamatoria. Esta reacción coincide en el tiempo con la proliferación de células T en los terneros infectados, así como con la aparición de antígenos específicos del parásito en la superficie de las células endoteliales

parasitadas. Además de la activación de las células T, esta red atrae a células de la respuesta innata, como macrófagos y polimorfonucleares.

Cada vez son más los estudios que destacan la importancia de la respuesta humoral en la inmunidad adquirida frente a infecciones por *Eimeria*. Este tipo de respuesta se desarrolla rápidamente y se caracteriza por la aparición de un título alto de anticuerpos en el suero del animal infectado, con un incremento inicial de IgM, seguido por IgG; también pueden aparecer otras inmunoglobulinas específicas como la IgA y, en general, la cantidad de anticuerpos va aumentando si los animales están continuamente expuestos a los ooquistes. Se ha demostrado que la respuesta inmune humoral desempeña un papel importante en las reinfección por coccidios en rumiantes, aunque, por sí sola, no provoca una inmuprotección absoluta.

2.2. OBJETIVOS

1.2.1. Objetivo general

Analizar la respuesta inmune frente al coccidio caprino *Eimeria ninakohlyakimovae* mediante infecciones experimentales y su relación con los mecanismos de patogenicidad del parásito y las posibles implicaciones en la profilaxis y control de la enfermedad.

1.2.2. Objetivos específicos

Aislar una cepa de *Eimeria ninakohlyakimovae* (Islas Canarias) y analizar su capacidad infectante, patogenicidad y aspectos generales de la respuesta inmune tras su inoculación a cabritos.

Valorar el grado de protección conferido por *Eimeria ninakohlyakimovae* tras reinfecciones homólogas con altas dosis infectantes (infección aguda) y su relación con la respuesta inmune desarrollada en el curso de las infecciones experimentales.

Estudiar y caracterizar la respuesta inmune humoral (sistémica y local) tras una infección experimental de cabritos con *Eimeria ninakohlyakimovae.*

Identificar péptidos reconocidos por anticuerpos del isotipo IgG en muestras séricas y su relación el desarrollo de repuestas inmunes protectoras.

Evaluar la influencia de la edad en el desarrollo de respuestas inmunes protectoras en la coccidiosis experimental caprina por *Eimeria ninakohlyakimovae*.

Analizar y caracterizar la respuesta inmune humoral y celular en la fase prepatente de la enfermedad producida por *E. ninakohlyakimovae* en cabritos y su relación con respuestas inmunoprotectoras frente a las formas inmaduras del parásito (esquizontes inmaduros) que se desarrollan durante esta fase del ciclo endógeno.

3.

REVISIÓN BIBLIOGRÁFICA

3.1. COCCIDIOSIS CAPRINA

3.1.1. INTRODUCCIÓN

La coccidiosis es un proceso patológico causado por protozoos del género *Eimeria* (Schneider, 1875), parásitos altamente específicos para el hospedador. Las infecciones que producen constituyen unas de las parasitosis más frecuentes y más ampliamente distribuidas en los sistemas de producción ganadera de grandes y pequeños rumiantes, representando, además, uno de los principales motivos de pérdidas económicas (Rashi y Tak, 2012; Hashemnia y cols., 2014). Las pérdidas están ligadas al menor rendimiento zootécnico de los animales, incluso cuando las infecciones son moderadas y no aparecen signos clínicos. Las repercusiones económicas se reflejan, principalmente, en una disminución de las producciones y de la tasa de crecimiento, y en un aumento del índice de mortalidad que, en determinadas circunstancias, puede alcanzar tasas superiores al 20% (Lima, 1981). Estas pérdidas aumentan y se agravan cuando la eimeriosis se asocia con infecciones producidas por otros protozoos y/o a infecciones por helmintos de patogenicidad diversa. Por todo ello, las coccidiosis se engloban en lo que han venido a denominarse "enfermedades económicas". El problema puede aparecer en cualquier sistema de explotación, si bien, es en la explotación intensiva cuando la enfermedad se desarrolla de forma más manifiesta y, por consiguiente, el impacto económico es más alto, probablemente debido a la elevada densidad de animales.

Los coccidios del género *Eimeria* son parásitos intracelulares de las células epiteliales del intestino delgado y grueso de los hospedadores, aunque no exclusivamente. Entre las localizaciones extra-intestinales más frecuentes se encuentran el hígado y el riñón aunque, en menor frecuencia, también se han observado en el bazo y en el pulmón (Collins y cols. 1988; Dai y cols., 1991; Morgan y cols., 2013). Su acción patógena fundamental se debe a la destrucción celular que resulta de los ciclos de reproducción asexual y sexual de su ciclo endógeno (Stockdale, 1980).

Los animales adultos suelen hacerse resistentes después de sobrevivir al periodo crítico durante las primeras semanas de vida, transformándose entonces en reservorios y portadores inaparentes del parásito (Daugscheis y Najdrowski, 2005). Por el contrario, los animales jóvenes son los más susceptibles de padecer la enfermedad, especialmente entre las 2 semanas y los 4 meses de vida (Ruiz y cols., 2006).

Los coccidios están presentes en todas las ganaderías de rumiantes, aunque esto no quiere decir que en todas ellas se desarrolle la enfermedad. No se conocen con exactitud los mecanismos que desarrollan una coccidiosis patente; lo que sí están claros son los factores de riesgo: destete, transporte, entrada en cebaderos, partos múltiples, madres mal alimentadas, ubres sucias, problemas de mastitis, etc.,

situaciones todas ellas típicas del sistema de crianza y engorde de rumiantes, por ejemplo en corderos (**Sanz, 2000**).

En general, la coccidiosis en los rumiantes es el resultado de una compleja interacción entre huésped y parásito en la que intervienen, además, numerosos factores externos que pueden condicionar la severidad de la enfermedad, por lo que, a pesar de los avances logrados en los últimos años en el estudio de los ciclos de vida, la patogenia, la epidemiología y el control, la coccidiosis en los rumiantes presenta, aún en la actualidad, muchos aspectos sin aclarar. Además, muchos de los ciclos de vida de las especies que se consideran de menor importancia aún no se han dilucidado y existe controversia sobre la patogenicidad de algunas de ellas. En este contexto, se ha observado que los brotes de coccidiosis se producen con una frecuencia cada vez mayor, lo que contrasta con la escasa disponibilidad comercial de medicamentos para su tratamiento y prevención; asimismo, son limitadas las investigaciones que se han llevado a cabo sobre los métodos inmunológicos de control **(Taylor y Catchpole, 1994**).

Han sido descritas al menos 18 especies de *Eimeria* parasitando a los caprinos con elevada prevalencia en diferentes localizaciones geográficas **(Pellerdy, 1974; Soe y Pomroy, 1992; Smith y Sherman, 2009)**. La especie *E. ninakohlyakimovae* se considera una de las más patógenas y más ampliamente distribuidas en todo el mundo **(Balicka-Ramisz, 1999; Ruiz y cols., 2006; Taylor y cols., 2007**), motivo por el cual ha sido objeto de infecciones experimentales en caprinos, fundamentalmente orientadas al estudio de los aspectos parasitológicos y biopatológicos de la enfermedad **(Viera y cols., 1997; Dai y cols., 2006)**, pero poco se conoce sobre la caracterización de la respuesta inmune frente a ésta y otras especies caprinas de *Eimeria* y, menos aún, sobre su posible repercusión en el desarrollo de respuestas inmunes protectoras.

3.1.2. ETIOLOGÍA

Los coccidios son parásitos intracelulares altamente específicos y de ciclo directo (monoxenos) **(Witcombe y Smith, 2014)**, por lo tanto, no necesitan más de un hospedador para completar su ciclo. Se engloban dentro del phylum Apicomplexa, siendo su clasificación compleja **(Lefevre y Blancou, 2010)** por la gran cantidad de especies que se han descrito en todo el mundo. El phylum Apicomplexa está constituido actualmente por más de 300 géneros que, a su vez, incluyen cerca de 4600 especies de organismos. Los apicomplejos son parásitos intracelulares obligados durante su fase proliferativa y entre ellos se incluyen, probablemente, algunas de las especies responsables de las principales zoonosis del ser humano. Dentro de este grupo se encuentran los géneros *Eimeria* e *Isospora*, comúnmente conocidos como coccidios no formadores de quistes, así como otros parásitos de máxima importancia en medicina humana como *Plasmodium* spp. y *Toxoplasma gondii* **(Champan, 2014)**.

3.1.2.1. Encuadre taxonónico

La clasificación correcta de los Apicomplexa ha sido posible gracias a la utilización del microscopio electrónico, que ha permitido la observación detallada de la compleja estructura de estos organismos. Se han publicado diversas propuestas de clasificación para miembros de este phylum conocidos comúnmente como coccidios, siendo la siguiente la más aceptada para el género *Eimeria* **(Tabla 1) (Schneider, 1975; Soulsby, 1982; Levine, 1988; Urquhart y cols., 1996)**.

Phylum Apicomplexa
Clase Sporozoea
Subclase Coccidia
Orden Eucoccidia
Suborden Eimeriina
Familia Eimeridae
Género *Eimeria*

Tabla 1. Encuadre taxonómico de género *Eimeria*

Aunque se creyó que ovinos y caprinos compartían los mismos coccidios, hoy se reconoce que cada hospedador tiene sus propias especies de *Eimeria* (**Amstutz y cols., 2000**). De hecho, en base a la gran similitud morfológica entre las diferentes especies, se estableció una clave de determinación común para ovinos y caprinos (**Yvoré y Esnault, 1984; Yvoré y cols., 1985**). Entre las nueve especies de *Eimeria* más frecuentes en la cabra, muchas de ellas son considerablemente parecidas a las propias del ganado ovino, pero los ensayos de infección cruzada han demostrado que se trata de agentes diferentes. A raíz de tales estudios, algunas especies no guardaban el mismo nombre para los dos huéspedes, mientras que otras fueron renombradas para cada una de las dos especies animales.

Las especies reconocidas como más importantes en los caprinos son: *E. arloingi* **(Sayin y cols., 1980 -Marotel, 1905-)**, *E. hirci* **(Lima, 1980)**, *E. christenseni* **(Lima, 1981-Levine, 1962-)**, *E. ninakohlyakimovae* **(Viera y cols., 1997 -yakimoff y Rastegaieff, 1930-)**, *E. caprovina* **(Lima, 1980)**, *E. caprina* **(Lima, 1980)**, *E. alijevi* **(Kasim y cols., 1991 -Musaev, 1970-)**, *E. apsheronica* **(Musaev, 1970)**, *E. jolchijevi* **(Musaev, 1970)** y *E.kocharli* **(Musaev, 1970)**. Como posiblemente compartibles entre oveja y cabra, con reservas, se han citado estas otras: *E. marsica* **(Restani, 1971)**, identificada en caprinos españoles por **Romero (1984)** y *E. gilruthi* **(Soliman, 1970 -**

Chatton, 1910), hallada en la necropsia de una cabra y localizada en el cuajar. Sin embargo, se ha demostrado que estas dos últimas especies se desarrollan mejor en la oveja que en la cabra **(Chatton, 1910; Restani, 1971; Maratea y Miller, 2007)**.

3.1.2.2. Morfología

Morfología de los Apicomplexa

Los Apicomplexa se caracterizan por la presencia de un polo apical que se observa en las fases infectantes del ciclo del parásito, en concreto, en los esporozoítos, los merozoítos, los taquizoítos y los bradizoítos **(Jolley y Barley, 2006)**. Los caracteres de estas formas infectantes se emplean para la clasificación de las distintas especies.

El polo apical o complejo apical proporciona al parásito la orientación y es el punto focal donde tiene lugar la exteriorización de los orgánulos secretores **(Katris y cols., 2014**). Además, existen elementos estructurales (como la actina y la miosina) dentro del complejo apical que son los que proporcionan la motilidad para la invasión de la célula hospedadora **(Striepen y cols., 2007)**. Por último, el complejo apical parece actuar directamente sobre la célula hospedadora a nivel de los microtúbulos, favoreciendo la replicación intracelular del parásito **(Mehlhorn y Piekarski, 1993; Morrrissette y Sibley; 2002**). Lo componen microtúbulos, micronemas, roptrias, gránulos densos, conoides y un complejo anillo-polar **(Urquart y cols., 1996)**.

El conoide, de morfología tubular, se encuentra en el centro del complejo apical y es el encargado del proceso mecánico en la invasión intracelular **(Mehlhorn y Piekarski, 1993; Morrrissette y Sibley; 2002)**. Los micronemas, gránulos densos y roptrias secretan moléculas esenciales para la supervivencia del parásito en el interior de la célula parasitada **(Morrrissette y Sibley; 2002)**, y las roptrias, cuyo número puede variar de un género a otro dentro de los Apicomplexa **(Blackman y Bannister, 2001)**, se encuentran situadas a nivel del extremo apical y contienen proteínas que van a actuar en la adhesión a la membrana de la célula hospedadora **(Alexander y cols., 2005**). Por último, los microtúbulos son estructuras longitudinales que sirven de anclaje al anillo-polar, proporcionando estabilidad **(Morrrissette y Sibley; 2002)**.

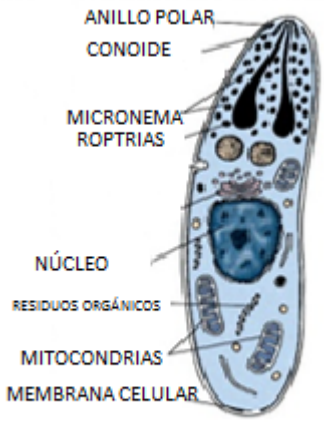


Fig. 1. Zoíto de Apicomplexa

Los ooquistes que salen en las heces de los animales infectados son los que tradicionalmente se han utilizado para diferenciar entre las distintas especies de *Eimeria* en base a sus características morfológicas. Sin embargo, hay que considerar que ésta no es más que una fase en el ciclo del parasito y que su morfología es mucho más compleja. La morfología y el tamaño de los ooquistes de las diferentes especies de *Eimeria* son muy variables, siendo las formas más comunes las esféricas, subesféricas, ovoides o elipsoides. Poseen una pared compuesta por dos o más capas (una a base de queratina y otra proteica) y, generalmente, son claras y transparentes, y presentan un contorno doble bien definido, aunque algunas especies pueden presentar una coloración que puede variar entre amarillo, marrón o, incluso, verde. El ooquiste puede o no tener una abertura en el extremo anterior que puede estar cubierto por un tapón llamado capuchón polar o cápsula micropilar. En el interior del ooquiste esporulado se desarrollan cuatro esporocistos que contienen en su interior dos esporozoítos cada uno. En algunas especies de *Eimeria,* en el extremo apical de los esporocistos puede observarse una pequeña protuberancia que recibe el nombre de cuerpo de "Stieda" **(Long, 1990)**. Los esporozoítos tienen forma de huso o de coma, y constituyen el elemento que emigra a través del hospedador e invade sus células.

Eimeria ninakohlyakimovae

En la actualidad se reconoce como especie exclusiva de los caprinos y presenta una distribución mundial. Se localiza en la porción posterior del intestino delgado, ciego y colon. Los ooquistes de *E. ninakohlyakimovae* son elipsoidales, a veces ovoides, con un tamaño medio de 23,1 µm de largo por 18,3 µm de ancho (con variaciones entre 20-28 µm) (**Christensen, 1938**). Carecen de cápsula micropilar **(Yakimoff y Rastegaieff, 1930)** y, generalmente, el micrópilo es poco evidente o incluso puede no observarse. Los ooquistes son de pared fina, lisa y transparente,

ligeramente amarillo-marronácea, y, una vez esporulados, albergan en su interior cuerpos residuales y gránulos polares; los esporocistos presentan cuerpo de Stiedae **(Ruiz y cols., 213)**. La esporulación tiene lugar en uno o dos días **(Opoku–Pare y Chineme, 1979; Deb y cols., 1981)** y los esporozoítos que resultan de ella son alargados y con un tamaño aproximado de 11,6x 6,3 µm.

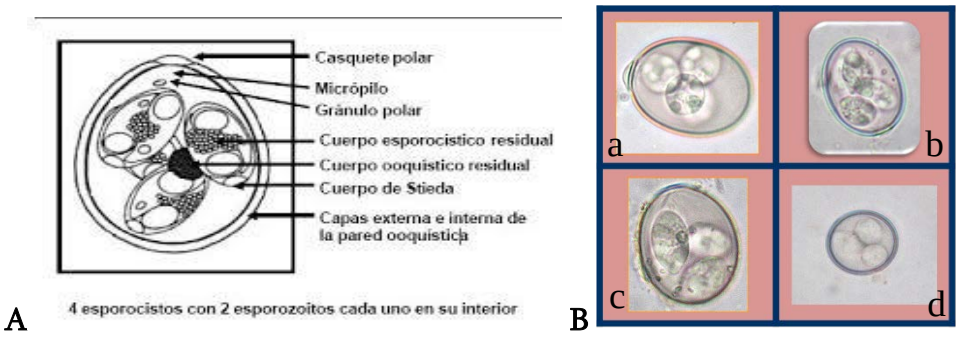


Figura 2. Representación esquemática de un ooquiste esporulado de *Eimeria* (A); ooquistes esporulados de las especies más frecuentes del género *Eimeria* en las cabras **(Ruiz y cols., 2006)**: *Eimeria arloingi* (a), *Eimeria ninakohlyakimovae* (b), *E. caprina* (c) y *E. alijevi* (d).

3.1.2.3. Biología de los coccidios

El ciclo biológico endógeno de los coccidios del género *Eimeria* es continuo y más del 70% ocurre en el intestino delgado. Una vez ingeridos los ooquistes (día 1) se reproducen rápidamente en el yeyuno e íleon. A partir de los 10-12 días invaden y se desarrollan en el intestino grueso y, transcurridas aproximadamente dos semanas desde la infección, comienzan a liberar un gran número de ooquistes con las heces, los cuales, tras esporular en el medio y ser ingeridos por otros hospedadores, darían comienzo a un nuevo ciclo **(Marshall y Williams, 1985)**.

En rumiantes, como en otras especies hospedadoras, el desarrollo tiene lugar en dos etapas **(Sánchez y cols., 2005)**: fase endógena, en la que se alternan ciclos de reproducción asexual (esquizogonia o merogonia) y sexual (gemetogonia), y fase exógena en medio, que consiste en un proceso de reproducción asexual mediante esporulación (esporogonia).

Esporogonia

El proceso de esporulación consiste en la segmentación del protoplasma en pequeños cuerpos infectantes llamados esporozoítos que se encuentran dentro de los esporocistos y éstos, a su vez, se encuentran dentro del ooquiste. Dependiendo de la especie de *Eimeria*, el tiempo de esporulación puede extenderse entre las 48 y las 104

horas. En el género *Eimeria*, en el ooquiste esporulado se desarrollan cuatro esporocistos que contienen dos esporozoítos cada uno. Para que ocurra ese proceso son necesarias determinadas condiciones de humedad, temperatura y oxígeno en el ambiente.

Merogonia

El ooquiste esporulado ingresa al organismo hospedador cuando es ingerido junto con alimentos o agua de bebida y, a nivel de la luz del tracto digestivo, se produce el desenquistamiento. Una vez en el lumen, los esporozoítos penetran en las células epiteliales del intestino (enterocitos), gracias a un complejo sistema de microfibrillas que existen en su histoarquitectura, aunque en ocasiones pueden parasitar células endoteliales en primera instancia. Ya dentro de la célula hospedadora, se transforman en trofozoítos, más redondeados y ligeramente de mayor tamaño, los cuales se replican de forma asexual por un proceso conocido como endopoligenia, término que definieron por primera vez **Pierkarski y cols. (1971)**, transformándose finalmente en esquizontes de primera generación.

Los esquizontes contienen una gran cantidad de merozoítos que son liberados a la luz intestinal tras la destrucción de la célula hospedadora, momento en el cual pueden empezar a observarse los primeros signos clínicos. Los merozoítos resultantes de la primera esquizogonia penetran otra vez en el interior de las células epiteliales colonizando de nuevo la mucosa intestinal. Estos merozoítos experimentarán de nuevo una fase asexual, en este caso por endodiogenia, creciendo en número dentro de las células epiteliales hasta formar esquizontes de segunda generación (más pequeños y con menos merozoítos que los de primera generación). Los merozoítos resultantes salen a la luz intestinal tras la ruptura de las células epiteliales y colonizarán nuevas células epiteliales para iniciar la etapa sexual **(Lucas Drugueri, 2002)**. La merogonia puede repetirse en más de dos generaciones dependiendo de la especie de *Eimeria* **(Mehlhorn, 2004; Urquhart y cols., 1996)**.

Gametogonia

Los merozoítos de la "última" generación de esquizontes penetran en nuevas células transformándose en: a) macrogamontes (la mayor parte), que se dividen y forman un microgameto cada uno, y b) microgamontes, que por división generan una gran cantidad de gametocitos biflagelados, los cuales salen de la célula para alcanzar aquellas que contienen macrogametos **(Sanchez R.O. y cols., 2008).** Los cigotos resultantes de la unión de los microgametos con los macrogametos mediante singamia sufren posteriormente una división meiótica tras la que se genera una nueva forma haploide, el esporogonio u ooquiste no esporulado **(Striepen y cols., 2007)**. Los nuevos ooquistes serán liberados al medio con las heces de los animales, reiniciándose nuevamente el ciclo **(Lucas Drugueri, 2002)**

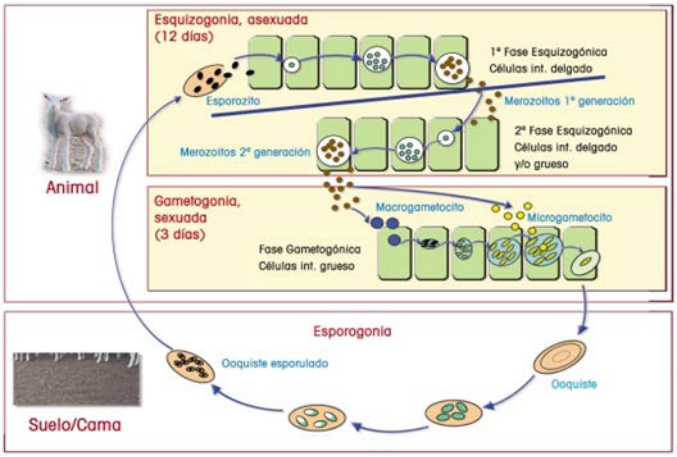


Figura 3. Esquema del ciclo evolutivo de *Eimeria* en caprino

Los datos descritos anteriormente en relación a la biología de los coccidios son de carácter general, pues cada especie de *Eimeria* presenta particularidades muy concretas en su ciclo biológico. La principal particularidad de *E. ninakohlyakimovae* es que la primera esquizogonia se desarrolla a nivel de las células endoteliales de los vasos linfáticos del intestino íleon distal, siendo, por tanto, las células endoteliales las células hospedadoras que los esporozoítos infectan en primera instancia **(Codero del Campillo y Rojo-Vázquez, 2000)**.

En el desarrollo endógeno de *E. ninakohlyakimovae* se produce dos generaciones de esquizontes; la primera generación da lugar a macroesquizontes de hasta 166µm x 124 µm **(Viera y cols., 1997)**. Estos esquizontes se desarrollan a los 10-12 días post-infección y pueden contener más de 100.000 merozoítos de primera generación. Por el contrario, los esquizontes de segunda generación son más pequeños (tamaño medio de 17 µm x 12 µm) y se desarrollan en torno al día 13 post-infección a nivel de las células epiteliales de las criptas del ciego y colon. En estas células se desarrollan también los gamontes y, entre los días 14-15 post-infección, suele comenzar la liberación de ooquistes al medio ambiente junto con las heces del hospedador **(Viera y cols., 1997)**.

3.1.3. EPIDEMIOLOGÍA

La coccidiosis en rumiantes presenta muchas características en común, pero con ciertas peculiaridades dependiendo de la especie parásita y el hospedador. La prevalencia en las diferentes especies hospedadoras varía claramente, pero, para un mismo hospedador y rango de edad suele ser constante. En todos los casos, el tipo de sistema de explotación influye decisivamente en la prevalencia y en desarrollo clínico de la enfermedad. Así, en sistemas de pastoreo extensivo en zonas áridas o semiáridas, en donde el factor humedad limita la supervivencia del parásito, hay una

dilución enorme de los ooquistes en amplias superficies y la posibilidad de contaminaciones intensas es muy baja. Sin embargo, la estabulación, normalmente asociada con una superpoblación, sea para proteger del clima frío, sea para preparar los cabritos para el mercado, es un factor de riesgo (**Soulsby, 1987**).

La presencia de diferentes especies de *Eimeria* en los caprinos ha sido descrita por varios autores con elevada prevalencia en diferentes localizaciones geográficas, en particular en zonas áridas y semiáridas rurales. Así, en Europa se han descritos prevalencias que oscilan entre el 84% para *Eimeria arloingi* y el 63% para *E.hirci* **(Koudela y Boková, 1998; Balika-Ramisz, 1999)**. Una prevalencia similar o, incluso, superior se ha encontrado en otras partes del mundo como América **(Lima, 1980; Cavalcante y cols., 2012)**, África **(Vercruysse, 1982; Chhabra y Pandey, 1991; Abo-Shehada y Abo-Farieha, 2003; Donkin y Boyazoglu, 2004)** y Asia **(Faizal y Rajapakse, 2001)**. En la Península Ibérica, en concreto en Portugal, **Silva y cols. (2014)** describen una elevada prevalencia de diferentes especies de *Eimeria* en los caprinos, siendo las especies más frecuentes *E. ninakohlyakimovae* (88%), seguida de *E. arloingi* (85%), *E. alijevi* (63%) y *E. caprovina* (63%). Otras especies observadas con menos frecuencia fueron *E. hirci, E. caprina, E. jolchijevi, E. christenseni y E. apsheronica*. En España, tanto en la Península **(de la Fuente y Alunda, 1992)** como en el Archipiélago Canario **(Ruiz y cols., 2006)** la coccidiosis también se han descrito como una parasitosis de amplia distribución y de elevada prevalencia.

La incidencia estacional de la coccidiosis está determinada por la disponibilidad de los animales jóvenes para el desarrollo del parásito (los animales adultos son por lo general inmunes), por la supervivencia de los ooquistes de una estación a la próxima, y por la producción incrementada de ooquistes durante el periodo postparto. En zonas templadas con inviernos fríos, las bajas temperaturas del invierno pueden reducir la viabilidad de los ooquistes, o bien prevenir su esporulación; consecuentemente, en estas zonas, los pastos o las naves de engorde suelen encontrarse relativamente libres de ooquistes a la llegada de la nueva estación **(Soulsby, 1987)**.

Los ooquistes son capaces de sobrevivir y mantenerse infectantes en el medio durante semanas o meses dependiendo de las condiciones ambientales. Concretamente, ambientes muy secos y fríos disminuyen su capacidad infectante. Los ooquistes mueren a temperaturas superiores a 40 °C y menores de -30°C, pero en este margen pueden permanecer viables por un año **(Foreyt, 1986**), pudiendo sobrevivir en las praderas que se utilizan de nuevo el año siguiente para el pastoreo **(Cordero del Campillo y Rojo-Vázquez, 2000)**. La humedad menor al 75%, las bajas tensiones de O_2 y la luz solar directa, condicionan la supervivencia, aún a temperaturas que permiten a los ooquistes sobrevivir varios meses **(Benavides y Romero, 2010).** Los ooquistes son más sensibles a factores físicos extremos como la congelación, exceso de calor o sequedad y a determinados agentes químicos como el amoniaco, sulfúrico, sosa caústica, etc. **(Gregory y Catchpote, 1990)**. Por último, en relación al proceso de esporulación, se ha observado que a temperaturas moderadas

en torno a 25 °C la mayoría de las especies que afectan a los bovinos esporulan en menos de una semana. Sin embargo, se ha comprobado que las temperaturas extremas (>35 °C) afectan negativamente la esporogonia en todas las especies de *Eimeria,* aunque *E. zuernii* parece ser que soporta perfectamente esos niveles en la fase de presegmentación.

Desde un punto de vista epidemiológico, la transmisión de la enfermedad se produce por la ingestión de los ooquistes que eliminan con las heces los animales infectados. No existe transmisión transplacentaria ni a través de la leche; la infección se realiza siempre por vía oral **(Merck, 2000)**. El contagio fecal-oral lo facilita la falta de higiene, como camas sucias, no renovadas, húmedas y con ooquistes esporulados que son ingeridos por los cabritos al contacto con los pezones contaminados de sus madres **(Kanyari, 1993)**. En los sistemas productivos con lactancia artificial, la infección puede provenir del periodo calostral, o por contaminación fecal del alimento o de los utensilios, pero es menos frecuente si se toman medidas higiénicas correctas y no hay excesivo hacinamiento **(Cordero del Campillo y Rojo-Vázquez, 2000)**.

En general, las infecciones suelen estar producidas por una mezcla de especies de *Eimeria*, patógenas y apatógenas, y el paso sucesivo de coccidios de un animal a otro generalmente incrementa la contaminación de los pastos y corrales, por lo que pueden aparecer coccidiosis graves dentro de las explotaciones que se creían libres de coccidiosis **(Cordero del Campillo y Rojo-Vázquez, 2000)**.

Muchos de los animales de mayor edad que ya han estado expuestos al parásito eliminan una pequeña y constante cantidad de ooquistes al medio que acaban infectando a los animales más jóvenes no inmunes. Situaciones de estrés como el destete, los cambios en la alimentación, una gran densidad de animales, unas condiciones climáticas adversas, los cambios de lote, el transporte, otras infecciones concomitantes, etc. facilitan el desarrollo de los síntomas clínicos, principalmente en los animales de menor edad **(Daugschies y Najdrowski, 2005)**.

En bovinos, la infección afecta comúnmente a los animales de hasta los 2 años de edad **(Ernst y Benz, 1986)**, siendo más susceptibles los terneros entre 3 y 6 meses que aún no han adquirido inmunidad **(Benavides y Romero, 2010)**. Así, en Colombia, **Rave y cols. (1985)** indicaron que la coccidiosis bovina es más frecuente en animales de 3 a 12 meses de edad. Otras investigaciones que relacionan la presentación de coccidiosis y la edad de los animales mostraron, igualmente, que la mayor prevalencia de la enfermedad se produce en terneros menores de doce meses (40,2%), especialmente en sistemas de producción de leche o doble propósito **(Tamasaukas, 1998)**. La coccidiosis en terneros de pocas semanas se ha asociado a sistemas de crianza artificial donde la carga de animales susceptibles por unidad de superficie es muy elevada **(Taraba, 1978; Vottero y Suárez, 1980; Guardis y Brandetti, 1986)**. Sin embargo, la creencia de que esta enfermedad sólo afecta a los terneros o animales menores de un año no es del todo correcta, lo cual ha facilitado la "colonización" del

parásito de vastas zonas, ocasionando inmensas pérdidas económicas (Lütcher, 2010). En general, ha de tenerse en cuenta que, aunque la edad de máximo riesgo oscila entre las 3 semanas y los 6 meses, la coccidiosis puede afectar a animales de cualquier edad mientras no desarrollen una inmunidad adquirida. Además, la inmunidad desarrollada por los animales que se recuperan de una infección previa está dirigida sólo frente a la especie que los afectó y no es absoluta, ya que los adultos pueden reinfectarse de forma discreta, por lo general sub-clínica, transformándose en fuente de infección para los más jóvenes, como se comentó anteriormente. Por otra parte, un estrés marcado puede quebrar la inmunidad, por lo que, en tales circunstancias, animales supuestamente inmunes pueden desarrollar nuevamente un cuadro agudo de coccidiosis clínica (Lima, 1980).

Los cabritos son particularmente susceptibles a los efectos patógenos de las infecciones por coccidios, especialmente los animales recién destetados que se mantienen hacinados en sistemas de manejo intensivos (Koudela y Bokova, 1998). Al igual que en bovinos, aunque los cabritos de entre las 3 semanas de vida y los 6 meses son los más susceptibles a padecer esta enfermedad, todos los rangos de edad y estados productivos suelen estar parasitados por diversas especies de *Eimeria* (Ruiz y cols., 2006). Sin embargo, el grado de parasitación también suele ser diferente según la edad. Así, la cantidad media de ooquistes liberados por heces oscila entre 1000-2000 OPG en animales adultos, pudiéndose alcanzar recuentos de más de 10^6 OPG en animales jóvenes. En cualquier caso, la eliminación suele ajustarse a una curva normal teniendo en cuenta todos los rangos de edad (Cordero del Campillo y Rojo-Vázquez, 2000; Ruiz y cols., 2006).

En bovinos, al igual que en ovinos y caprinos, los brotes de la enfermedad tienden a estar asociados con animales mantenidos en condiciones de hacinamiento, alta contaminación fecal del medio y ambientes húmedos y sucios (Benavides y Romero, 2010). La coccidiosis aparece habitualmente en áreas con una alta densidad de animales, tales como cebaderos y pastizales pequeños, por lo que los animales criados intensivamente están más expuestos a sufrir de coccidiosis (Sánchez, 2005). Así, se ha comprobado que los bovinos de engorde en corral y el ganado lechero son más susceptibles que los animales en pastoreo (Ernst y Benz, 1986). Del mismo modo, en los cabritos que vuelven a los cebaderos en condiciones de mala higiene suele ser frecuente también la aparición de brotes clínicos de coccidiosis (Kanyari, 1993; Ruiz y cols., 2006). Por otro lado, una inadecuada alimentación, la inmunosupresión del periparto, las infecciones víricas o bacterianas, son todas situaciones que aumentan la probabilidad de presentar coccidiosis clínica (Argüello y Cordero del Campillo, 1987; Daugschies y Najdrowski, 2005; Witcombe y Smith, 2014).

Desde el punto de vista económico, ya desde la década de los 50, 60 y 70 la coccidiosis se consideraba un problema importante en pequeños rumiantes, causa de grandes pérdidas. No obstante, la cuantificación económica de las enfermedades

causadas por coccidios se basa en datos puntuales de reducción de ganancias de peso o mortalidad correspondientes a casos clínicos particulares, proyectadas a la totalidad de la población expuesta **(Benavides y Romero, 2010)**. En general, las pérdidas económicas están relacionadas con el deterioro producido en los animales parasitados, con manifestaciones clínicas o no, lo que ocasiona un menor desarrollo corporal y pérdidas en el potencial de producción. A esto hay que sumarle la muerte de animales y los gastos de tratamiento, entre otros aspectos **(Rossanigo, 1997)**.

3.1.4. PATOLOGÍA

3.1.4.1. Patogenia

Las condiciones por las cuales los coccidios producen patología son complejas y variadas, siendo necesario tomar en consideración numerosos factores. Entre los factores relacionados con el hospedador, pueden citarse la edad, la raza y el estado de inmunidad natural o adquirida. Así, tal y como se comentó anteriormente, los animales jóvenes son más susceptibles a sufrir la enfermedad clínica que los adultos, a pesar de que no existe una verdadera resistencia por la edad. Diferentes aspectos medioambientales, fundamentalmente relacionado con las medidas de manejo, también son determinantes en el desarrollo de coccidiosis clínica, pues marcan el ritmo e intensidad de las infecciones. Por último, también han de tenerse en cuenta los factores relacionados con el parásito ya que, según las especies de *Eimeria* que estén afectando al ganado, la gravedad clínica puede variar. La patogenicidad de las especies va a depender del número de generaciones esquizogónicas, del número de merozoítos producidos por cada generación, y de la localización de los estadios endógenos del parásito en los tejidos y células del hospedador **(Rose, 1987)**. Las especies de *Eimeria* que se localizan en el intestino delgado tienen un poder patógeno menor, dado que el órgano tiene una longitud mayor y una capacidad de renovación celular intensa, lo que disminuye el impacto que supone la destrucción de las células epiteliales. Las especies más patógenas son aquellas que tienen, en alguna de sus fases, tropismo por el intestino grueso, donde, generalmente, tiene lugar la gametogonia (fase de reproducción sexual) **(Taylor y Catchpole, 1994)**. En los caprinos, se considera que la especie más patógena es *E. ninakohlyakimovae* (**Yakimoff y Rastegaieff, 1930)**, seguida por *E. arloingi*. En el ganado vacuno destacan por su patogenicidad las especies *E. bovis* y *E. zuernii*, mientras que en el ovino *E. bakuensis*, *E. crandallis* y *E. ovinoidalis* son las especies más patógenas **(Taylor y cols., 2007)**. Normalmente, aunque la mayoría de los animales del rebaño adquieren la infección, sólo una minoría desarrolla la enfermedad clínica. La capacidad de respuesta a la enfermedad por parte del hospedador, en referencia a la respuesta inmune, está determinada genéticamente e influirá en el cuadro clínico de la enfermedad **(Daugschies y Najdrowski, 2005; Witcombe y Smith, 2014)**. Por lo general, en infecciones moderadas, tras un primer contacto, se desarrolla una sólida

inmunidad específica. Por este motivo, los animales adultos son generalmente más resistentes y, a menos que la infección sea muy alta, no suelen presentar sintomatología, por lo que se considera una enfermedad autolimitante. En cambio, la introducción de animales susceptibles, generalmente jóvenes, en un grupo con portadores asintomáticos puede favorecer el desarrollo de infecciones serias y hasta fatales **(Benavides y Romero, 2010)**. En cualquier caso, habría que tener en cuenta que el desarrollo de la enfermedad está muy influenciado tanto por el ritmo de infección como por la cantidad de ooquistes ingeridos **(Gregory y Catchpole, 1990**).

Los efectos de la eimeriosis pueden exacerbarse si están presentes varias especies en diferentes partes del intestino, así como cuando hay infecciones concurrentes, bien sea por helmintos, bacterias o virus **(Taylor y Catchpole, 1994; Daugschies y Najdrowski, 2005; Witcombe y Smith, 2014)**. En general, los coccidios del género *Eimeria* ejercen su acción patógena de la siguiente forma:

- Lesionando las células epiteliales y, en ocasiones, endoteliales del intestino. Cada especie de *Eimeria* tiende a desarrollar su ciclo preferentemente sobre áreas concretas del tracto intestinal, pudiendo afectar a grandes tramos entéricos dejando al descubierto la lámina propia **(Norton, 1986; Hermosilla y cols., 2008)**.
- Modificando la microflora intestinal: se pasa de un 16% de gérmenes gram-negativos a un 76%, **(Mohamed, 2000)**, lo cual supone un agravante en la aparición de diarreas.

3.1.4.2. *Signos clínicos*

La exposición de los rumiantes a los ooquistes infectantes de *Eimeria* es constante pero, tal y como se comentó con anterioridad, dependiendo de determinados factores, la coccidiosis puede manifestarse de forma subclínica o, en el otro extremo, desencadenar una clínica severa. No obstante, en la mayoría de los casos, la infección por coccidios es bien tolerada por el animal **(Daugschies y Najdrowski, 2005; Witcombe y Smith, 2014)**. Así, en investigaciones sobre coccidiosis bovina desarrolladas por **Rave y Zaraza (1985**) en Colombia se encontró que tan sólo en el 40,6% de los casos positivos presentaban diarrea, en el 12,8% diarrea con sangre, y en el 50% no se apreció síntoma alguno que denunciara la presencia de la enfermedad. En este estudio, del total de animales analizados el 62% eran adultos **(Lütcher, 2010)**.

La única forma de poder valorar el impacto de los coccidios en las infecciones subclínicas es realizar una comparación entre lotes tratados y lotes testigos sin tratar. Se observa claramente una disminución del apetito, pérdida de peso y un aumento del índice de conversión. Las coccidiosis clínicas son más esporádicas, con manifestaciones clínicas más evidentes tales como diarreas más o menos

hemorrágicas (según la especie de *Eimeria*), retrasos evidentes del crecimiento y, eventualmente, muertes (Benavides y Romero 2010).

El primer síntoma de los animales es la presencia de diarrea de un color que puede oscilar entre amarillento y marrón oscuro, a veces con sangre, coágulos y mucus (Foreyt, 1990). Suele coincidir con el inicio de la gametogonia y está asociada a la destrucción de las células de la mucosa del intestino grueso y, por consiguiente, a la disminución de su capacidad para absorber líquidos (Norton 1986). Esta fase puede durar entre 3-4 días hasta que se restablezca la mucosa (Koudela y Boková, 1998) pero, en los casos en los que la destrucción de la pared intestinal es masiva, la recuperación podría ser de varias semanas (Reeg y cols., 2005).

La diarrea en los rumiantes puede ir acompañada de signos clínicos generales como inapetencia, letargo, pérdida de peso, debilidad, anemia, hipoproteinemia y deshidratación; también suele apreciarse mal pelaje en los animales y, en ovinos, la lana suele volverse quebradiza (Amstutz y cols., 2000). Aunque lo habitual es que se recuperen y que la mortalidad no supere el 10 % de los animales infectados, la coccidiosis puede estar asociada a una mortalidad mucho más elevada, a veces súbita y sin signos digestivos evidentes, sobretodo en animales entre los 2 y 4 meses de edad (Chartier y cols., 1994).

En el ganado vacuno, además de la coccidiosis intestinal descrita anteriormente, se han observado casos de coccidiosis nerviosa. Los signos clínicos asociados a este tipo de coccidiosis pueden variar en gravedad y frecuencia, con un rango que va desde una leve incoordinación muscular y temblores, hasta la pérdida de equilibrio con convulsiones intermitentes o continuas. Durante las convulsiones, los terneros afectados caen en decúbito lateral y exhiben una variedad de signos nerviosos (Isler y cols., 1987a). Estudios de casos de coccidiosis entérica bovina, con y sin signos nerviosos, han concluido que la concentración de Na, K, Ca, P, Mg en sangre, la carencia de vitamina A, la deficiencia de tiamina, la anemia, la intoxicación por plomo, la uremia, las meningoencefalitis bacterianas, la gravedad de la infección por coccidios propiamente dicha y las alteraciones graves en la flora bacteriana intestinal no están involucrados en la patogenia de la coccidiosis nerviosa. Ese mismo año estos investigadores (Isler y cols., 1987b) llegaron a la conclusión que los signos nerviosos podrían asociarse a reacciones de autointoxicación producidas por toxinas de los coccidios que se absorben a través del epitelio dañado.

3.1.4.3. *Lesiones*

En la necropsia, los animales muertos por coccidiosis muestran la zona perianal sucia por deyecciones diarreicas, pudiendo ser más o menos líquidas, con mucus o, incluso, contener restos entéricos (Mundt y cols., 2005; Ruiz y cols., 2014). El intestino delgado aparece dilatado, congestivo y con la mucosa inflamada, frecuentemente con hemorragias y exceso de mucus. Sobre la mucosa, es común que aparezcan placas gruesas, blancas y opacas, visibles a simple vista, que suelen

corresponderse con macroesquizontes. Microscópicamente es común observar atrofia de las microvellosidades (Koudela y Boková, 1998; Daugschies y Najdrowski, 2005; Witcombe y Smith, 2014).

En general, la localización de las lesiones varía con el tipo de ciclo endógeno de la especie o especies de *Eimeria* que estén parasitando al animal. Por ejemplo, las especies que más frecuentemente parasitan a los caprinos, *E.alijevi, E.caprina y E.ninakohlyakimovae* se localizan tanto en intestino delgado como grueso, mientras que *E. alijevi y E. christernseni* lo hacen fundamentalmente en intestino delgado (Taylor y cols., 2007).

Como ya se ha comentado anteriormente, la especie *E. ninkohlyakimovae* se considera la más patógena entre las que parasitan a los caprinos. Las lesiones macroscópicas que produce incluyen engrosamiento de la pared cecal, petequias y leve congestión del colon, zonas de enteritis y aumento considerable del tamaño de los nódulos linfáticos mesentéricos (Dai y cols., 2006). Histológicamente se observan áreas de erosión del epitelio y las criptas aparecen invadidas por esquizontes, gametocitos y ooquistes (Gregory y cols., 1989; Taylor y cols., 2003; Hashemia y cols., 2012). Norton (1986) encontró que *E. ninakohlyakimovae* destruía las células madre en las criptas del intestino ciego y/o del colon, dejando la mucosa desprovista de epitelio, comparando su patología a la de *E. ovinoidalis* en ovejas. Las células que se encuentran parasitadas presentan un tamaño alrededor de cuatro veces el habitual y de hasta 15-20 veces si en su interior se desarrolla un macroesquizonte (Ruiz y cols., 2010; Razavi y cols., 2014). Durante el estudio histológico también se han encontrado congestión y hemorragias capilares, así como una infiltración celular que corre a cargo de linfocitos, polinucleares neutrófilos y eosinófilos. Tras la muerte de los animales, después de padecer una enfermedad clínica, las poblaciones celulares que predominan son los macrófagos y los linfocitos (Long, 1990; Hashemia y cols., 2014), mientras que en los casos de muerte súbita es más marcada la infiltración de neutrófilos y eosinófilos. En infecciones primarias, esta misma infiltración eosinofílica, además de hiperplasia de las células epiteliales, fueron descritas por Dai y cols. (2006) en infecciones experimentales con *E. ninakohlyakimovae*.

Las infecciones por *E. arloingi* también pueden llegar a ser graves. Aparte de lo ya mencionado, lo más característico es la observación de placas amarillentas o blanquecinas (macroesquizontes) y formaciones papilomatoides en la mucosa (Catchpole y Gregory, 1985). Por su parte, la coccidiosis por *E. caprina* se ha descrito que provoca hemorragias en la segunda mitad del colon y en la parte anterior del recto, el cual aparece vacío de heces, pero con mucus y restos de sangre coagulada. Los frotis de la mucosa muestran gran número de gametocitos y ooquistes en todo el ciego, colon y recto, mientras que el intestino delgado aparece relativamente normal. Como también ocurre con *E. christenseni* y en otras especies, las lesiones más importantes coinciden con el desarrollo de los estados gametogónicos y la producción de ooquistes (Gregory y cols., 1989).

3.1.5. DIAGNÓSTICO

A la hora de abordar el diagnóstico ha de tenerse en cuenta la historia particular del brote de coccidiosis. Generalmente, existen evidencias de poca higiene en la explotación, tanto en los sistemas intensivos como en los extensivos, y diarreas en animales de entre 1-6 meses. No obstante, el diagnóstico ha de complementarse con la observación de las lesiones en el examen *post mortem* o con la realización de análisis coprológicos **(Daugschies y Najdrowski, 2005; Silva y cols., 2013)**. Por lo general, prácticamente todos los animales de la explotación presentan recuentos positivos, variando desde decenas de miles de ooquistes por gramo de heces (OPG) hasta varios millones en aquellos individuos que padecen coccidiosis clínica patente. Por tanto, la mera presencia de ooquistes en las heces no es un motivo suficiente para el diagnóstico de coccidiosis. Además, hay que tener en cuenta que en infecciones agudas pueden aparecer los signos clínicos antes de la eliminación fecal de ooquistes, como consecuencia del daño producido por la multiplicación asexual del parásito. En resumen, para hacer un diagnóstico correcto, es necesario apoyarse en la clínica y epidemiología, en las lesiones que aparecen en el animal muerto y en los datos que puedan aportar los análisis coprológicos.

3.1.5.1. Diagnóstico clínico y epidemiológico

Se sospecha de coccidiosis cuando se encuentran problemas digestivos, con o sin diarrea hemorrágica, en animales jóvenes criados en malas condiciones de higiene o en sistemas intensivos. Por otro lado, una mortalidad súbita hacia el destete también podría hacer sospechar de una coccidiosis sobreaguda. La disminución del crecimiento y el empeoramiento de los índices de conversión, incluso sin la presencia de signos gastroentéricos aparentes, también pueden ser indicativos de la presencia de coccidiosis en la explotación (**Sanz, 2000)**.

3.1.5.2. Diagnóstico anatomopatológico

Una buena necropsia debe permitir detectar lesiones típicas de la enfermedad, así como evidenciar las diferentes formas parasitarias del ciclo de *Eimeria* **(Gregory y Catchpole, 1990)**.

3.1.5.3. Diagnóstico laboratorial

En los análisis coprológicos se deben de incluir técnicas de concentración por flotación, por ejemplo con ClNa. También es recomendable realizar una cuantificación de los ooquistes liberados y la determinación de las especies de *Eimeria* implicadas. La cuantificación suele realizare por la técnica modificada de McMaster (**Thienpont y col., 1979; Bangoura y Daugschies, 2007**), aunque

recientemente se ha demostrado la utilidad de nuevas técnicas como el FLOTAC ® o el Mini-FLOTAC® (**O'Grady y Slocombe, 1980**). La interpretación de los resultados coprológicos no es fácil, porque existe una gran variabilidad en la eliminación de ooquistes según las especies de *Eimeria* que estén parasitando al ganado y según el animal que se esté analizando **(Chartier y cols., 1994)**. En general, la determinación del número de ooquistes por gramo de heces (OPG) permite predecir el grado de parasitación, a diferencia de lo que ocurre en algunas helmintosis, donde recuentos bajos pueden estar asociados a cargas parasitarias elevadas **(Eysker y Ploeger, 2000**). Se estima que los recuentos fecales indicativos de una coccidiosis clínica serían del orden de 50.000 a 100.000 OPG. No obstante, hay ocasiones en las que los síntomas de la enfermedad pueden aparecer antes de la excreción de ooquistes **(Wright y Coop, 2007)**.

3.1.6. TRATAMIENTO

Con el fin de evitar contagios entre los animales infectados y los no infectados, se considera importante el aislamiento y tratamiento de los animales positivos, especialmente aquellos que están padeciendo enfermedad clínica y, por tanto, eliminando gran cantidad de ooquiestes al medio **(Catchpole y col., 1993)**.

El tratamiento prematuro e individual con antibióticos de amplio espectro orales, como la penicilina, sulfamidas con trimetropin o quinolonas, brindan resultados satisfactorios, ya que reducen las posibles septicemias bacterianas debidas al debilitamiento defensivo de la barrera mucosa intestinal durante la infección **(Smith y Sherman, 2009)**. El uso combinado con antiinflamatorios no esteroideos reduciría los casos de inflamación local y dolor agudo. Del mismo modo, el empleo de analgésicos y antipiréticos podría ser considerado entre los procedimientos terapéuticos para disminuir casos de fiebre en los animales sintomáticos. En casos graves, cuando se detecten problemas de deshidratación, también se recomienda realizar un tratamiento de recomposición. El uso de suero, bien por vía oral (a través de biberones, o con sonda directamente en los casos de letargia), o incluso vía intravenosa o subcutánea (menos usado en ganadería), estaría indicado para restablecer el desequilibrio electrolítico consecuencia de la deshidratación por las diarreas, casos de anorexia o incluso infecciones crónicas secundarias a la alteración de la mucosa intestinal **(Argüello y Cordero del Campillo, 1999)**. En todos los casos, la administración de vitaminas, vía oral o intramuscular, siempre es aconsejable.

Existen numerosos fármacos específicos susceptibles de ser utilizados frente a esta enfermedad parasitaria. Entre los que caben destacar, por un lado, aquellos fármacos que se utilizan como aditivos y que se incluyen dentro de la alimentación de los animales. Estos fármacos, conocidos como coccidiostáticos, se emplean actualmente como estrategias de control de la emeriosis, actuando en las fases iniciales del ciclo del parásito. No obstante, se ha detectado cierta resistencia frente a algunos de ellos debido a la plasticidad genómica del género *Eimeria*, la cual permite

al parásito seguir replicándose en presencia de un fármaco que, en general, debería suprimir su multiplicación (Witcombe y Smith, 2014). Incluso, se han detectado efectos secundarios no deseados en ganado tratado con este tipo de coccidiostáticos debido a su baja acción farmacológica en ciertas etapas del ciclo evolutivo del parásito (Coppens, 2013).

Por otro lado, los fármacos anticocciodiósicos son aquellos compuestos químicos utilizados, no tanto para prevenir que los animales se infecten, sino para tratar aquellos que ya se encuentran infectados, por lo que paralelamente se estimula de forma positiva el desarrollo de respuesta inmunitaria (Wang, 1997). Aun así, los mejores resultados se obtienen cuando se administran en fases iniciales de la enfermedad (Foreyt y cols., 1990; Mundt y cols., 2005), ya que reducen el contacto con el parásito y, por tanto, se disminuye satisfactoriamente la clínica y las lesiones producidas a nivel del digestivo. Muchos de estos fármacos interaccionan de forma específica frente al proceso de gametogonia del parásito, por lo que se reducirá drásticamente la formación de nuevos ooquistes. Algunos de ellos son capaces de disminuir la multiplicación del parásito, pero no lo eliminan completamente, por lo que limitan el control de la enfermedad (Daugschies y cols., 2007; Smith y Sherman, 2009).

Siguiendo estas premisas, a continuación se detalla el uso de los quimioterápicos que con mayor frecuencia se emplean frente a la eimeriosis.

Sulfonamidas

Tradicionalmente, las sulfamidas han sido unos de los fármacos más utilizados en el control de la coccidiosis de rumiantes. Así, en el ganado vacuno, la sulfaguanidina usada a una dosis de 2 g/día durante seis días, se ha observado que suprime la producción de ooquistes en infecciones subclínicas y previene la adquisición de infecciones naturales subsiguientes (Foster y cols., 1941). Por otro lado, el alimento suplementado con una mezcla de aureomicina-sulfametazina (a partes iguales), en dosis de 100 mg y 500 mg/cordero/día, se ha demostrado que inhibe el desarrollo de una población de diferentes especies de *Eimeria* en corderos (Ajayi y Todd 1977). Así mismo, en el ganado caprino, el empleo de sulfonamidas (sulfametazina y sulfaquinoxalina) interfiere en la replicación asexual del parásito (Daugschies y Najdrowski, 2005) y, como consecuencia, reduce la producción de ooquistes y la gravedad de la enfermedad. No obstante, el uso reiterado de estos fármacos ha motivado la aparición de resistencias en varias zonas del mundo (Champan y cols., 1993; Peek y Landman, 2006; Smith y Sherman, 2009; Champan y cols., 2013).

Nitrofurazona

Su uso terapéutico a dosis de 10-20 mg/kg/día hasta 5 días consecutivos es utilizado en ganado vacuno, ovino y caprino **(Sanchez R. y cols., 2005**). Igualmente, se ha observado que puede ser administrada con éxito en el alimento o en el agua de bebida, previniendo la mortalidad y reduciendo la morbilidad producida por *E. ninakohlyakimovae, E. ovina, E. intrincata, E. parva, E. faurei* y *E. pallida.*

Amprolio

Estudios clásicos indican que este compuesto es eficaz en coccidiosis de ovinos y caprinos administrado a dosis de 50-62,5 mg/Kg de peso en el agua de bebida o en el alimento de ovejas, o de 100 mg/Kg en el caso de cabras, durante cuatro o más días **(Kauffma, 1996).** Más recientemente, se ha corroborado que, a altas dosis, este fármaco no produce toxicidad neurológica en cabras **(Young y cols., 2011)** y que actúa a nivel de los merontes de primera generación impidiendo su diferenciación **(Daugschies y Najdrowski, 2005)**, por lo que no tendrían lugar las subsiguientes fases sexuales del ciclo del parásito ni la esporulación de los ooquistes en el medio **(Arabkhazaeli y cols., 2013)**.

Toltrazuril y diclazuril

Se ha demostrado una alta eficacia del uso de estas drogas en la fase prepatente de la enfermedad **(Daugschies y Najdrowski, 2005; Ruiz y cols., 2012)** actuando a nivel de la gametonia y merogonia del ciclo endógeno del parásito. A una única dosis de 20 mg/kg de toltrazuril y 0,25-1,0 mg/kg de diclazuril vía oral se ha conseguido una reducción de la sintomatología de la enfermedad y un menor número de ooquistes excretados por las heces, así como una mayor tasa de crecimiento en los animales tratados **(Daugschies y Najdrowski, 2005; Taylor y cols., 2011; Ruiz y cols., 2012)**.

Decoquinato

El decoquinato es un derivado de la quinolona desarrollado inicialmente para aves de corral en 1967 **(Williams, 2006)** que presenta actividad frente a los esporozoitos y los trofozoítos de diferentes especies *Eimeria.* También parece inhibir el transporte de electrones y la fosforilación oxidativa, actuando entre los días 1 y 10 del ciclo de los coccidios **(Sanchez y cols., 2005)**. Este fármaco se ha utilizado en el control de la coccidiosis en los rumiantes domésticos por más de 20 años, tanto en el tratamiento como en la prevención de la coccidiosis en terneros **(Miner y Jensen, 1976; Conlogue y cols., 1984; Foreyt y cols., 1986b; Fitzgerald y Mansfield, 1989; Mage y cols., 1996)** y corderos **(Spelman y cols., 1989; Mage y cols., 1995a)**. El uso del decoquinato también se ha demostrado útil para prevenir la coccidiosis en cabritos **(Foreyt y cols., 1986a; Mage y cols., 1995b)**.

Monensina, lasalocid, salinomicina

Por último, el uso de estos fármacos, que actúan en las primeras fases del ciclo biológico del parásito, también ha sido muy extendido pero, en a diferencia del amprolio, a altas dosis sí pueden ser tóxicos (Smith y Sherman, 2009).

Romero y Sánchez (2010), describen las acciones de los fármacos descritos anteriormente de la siguiente forma:

- Diclazuril, decoquinato, toltrazuril: Inhibición del transporte de electrones y la fosforilación oxidativa.
- Sulfamidas: Inhibición sinérgica del metabolismo del ácido fólico.
- Amprolio: Inhibición competitiva del transporte de tiamina a través de la membrana celular.
- Salinomicina, lasalocid y monensina: destrucción de la integridad de membranas.

Pese al gran número de fármacos con actividad anticoccidiósica y su extendido uso en diversas especies animales para el tratamiento de esta importante enfermedad parasitológica, aún hoy en día, en Europa no hay ninguno registrado legalmente para su uso en caprinos y tampoco, ni con fines terapéuticos ni profilácticos. Este hecho hace imprescindible el establecimiento de unas estrategias de control rigurosas frente a coccidiosis en esta especie hospedadora.

3.1.7. CONTROL

El control de la coccidiosis caprina se basa actualmente en empleo combinado de medidas de manejo (de la Fuente y Alunda, 1992) y tratamientos profilácticos con fármacos que presenten actividad anticoccidiósica (Vihan, 2002). Esto último presenta, no obstante, algunos inconvenientes, como la limitación cada vez más estricta del uso de aditivos en la Unión Europea, entre ellos los coccidiostáticos. A este respecto, el informe presentado en pleno del Parlamento Europeo por Wiecher (2005) propone, en relación al uso de coccidiostáticos e histomostáticos como aditivos para piensos, que se permita su uso, ya que estas sustancias son imprescindibles actualmente en la avicultura. Sin embargo, establece que se prohíban a partir de 2009, si no hay legislación adicional, con el fin de ejercer presión suficiente para que se desarrollen productos alternativos adecuados. Un problema adicional lo constituye el desarrollo cada vez más frecuente de fenómenos de resistencia, más extendidos y estudiados en la eimeriosis aviar (Stephen y cols., 1997), pero igualmente extensibles a las infecciones por *Eimeria* de pequeños y grandes rumiantes. El problema de la resistencia frente a los coccidiostáticos,

discutido hace ya más de una década por **Chapman (1993)** y el resto de limitaciones descritas anteriormente proporcionan un estímulo para el desarrollo, no sólo de nuevos fármacos, sino también de nuevas alternativas de control de la coccidiosis.

En general, el control de la coccidiosis requiere de la puesta en marcha de técnicas de gestión encaminadas a reducir la contaminación del medio con ooquistes, el hacinamiento y el estrés, en combinación con un uso apropiado de coccidiostáticos y coccidiósicos eficaces disponibles en el mercado para prevenir la enfermedad clínica. Como en cualquier enfermedad parasitaria, lo primero que debe hacerse es prevenir la aparición de los signos clínicos en los animales. El segundo objetivo sería reducir la infección por coccidios durante los periodos de riesgo para asegurar un crecimiento óptimo de corderos, cabritos y terneros **(Gregory y Catchpole, 1990; Chartier y cols., 2011)**.

3.1.7.1. Profilaxis terapéutica

El control de la coccidiosis en poblaciones de rumiantes susceptibles es una propuesta bastante difícil, por lo que se han depositado grandes esperanzas en los productos administrados con fines profilácticos **(Tabla 2).** El objetivo de la profilaxis anticoccidiósica es proporcionar un nivel de protección suficiente al animal expuesto para permitir que desarrolle inmunidad propia sin padecer enfermedad. Los fármacos empleados en este sentido reducen la magnitud del contagio y, por tanto, previenen la coccidiosis clínica, pero no impiden la infección **(Daugschies y Naddrowski, 2005)**. Sin embargo, la profilaxis terapéutica no ha de ser la única medida de control a utilizar, ya que un exceso de contaminación del entorno con ooquistes y, lo que aún es más importante, un exceso de estrés sobre el hospedador, son circunstancias que no se pueden combatir ni siquiera con el mejor de los medicamentos **(Young y cols., 2011)**.

Como se mencionó anteriormente, el uso sistemático de coccidiostáticos en animales de recría no es estrictamente necesario. Un tratamiento mínimo en los momentos de riesgo puede ser lo más recomendable. Así, salvo casos raros, no se recomienda tratar a los animales antes de que cumplan un mes de edad. Es más, algunos trabajos recientes demuestran que la administración de tratamiento entre las tres y cuatro semanas retardaría la aparición de la inmunidad, lo que expondría a los animales a infecciones posteriores graves. En cabritos, por ejemplo, el primer tratamiento sistemático debería hacerse durante el destete, principal periodo de estrés y, por tanto, de riesgo. Un segundo tratamiento podría realizarse un mes más tarde con el fin de asegurar un mejor crecimiento de los animales. Determinadas situaciones, como los transportes entre granjas, la separación en lotes, la salida a los pastos, son capaces de exacerbar la infección por coccidios y, en estos casos, también puede estar justificado un tratamiento especial (**Benavides y Romero, 2010; Young y cols., 2011**).

	USO	DOSIS
Amprolio	Profiláctico Terapéutico	5-10mg/kg/día 21 días 100mg/Kg/día 5 días
Nitrofurazona	Terapéutico	10-20mg/kg/día 5 días
Decoquinato	profiláctico	0.5mg/kg 28 días (en alimento)
Lasalocid	Profiláctico	0.5-1.0mg/kg/día 6 semanas
Monensina	Profiláctico	1mg/kg durante 30 días
Salinomicina	Profiláctico	100ppm en alimento 3 semanas
Diclazuril	Terapéutico	20mg/kg tratamiento único
Sulfametazina	Profiláctico Terapéutico	5g/tonelada de alimento 50-100g/kg/día 4 días
Toltrazuril	Profiláctico Terapéutico	20mg/kg (dosis) cada 3-4 semanas 20mg/kg /tratamiento único

Tabla 2. Relación de fármacos de uso terapéutico y profiláctico más frecuentes y su estrategia de empleo **(Kauffman, 1996)**

En el contexto veterinario, la administración de fármacos puede realizarse como un procedimiento metafiláctico. La metafilaxis, al igual que la profilaxis, se aplica en forma grupal, pero no para prevenir la enfermedad sino para tratar enfermedades incipientes en determinados animales y evitar que el brote se extienda al resto. La metafilaxis aplicada al control de la coccidiosis de rumiantes debería causar significativas mejoras en las ganancias de peso, lo que resultaría en beneficios económicos para la producción **(Forey, 1990)**. Algunos compuestos han sido usados para este propósito en las últimas décadas, tanto para corderos **(Foreyt y cols., 1979; Horton y Stockdale, 1981; Gjerde y Helle, 1991)** como para terneros **(McMeniman y Elliott 1995; Hasbullah y cols., 1996; Mundt y cols., 2005)**, pero pocos ensayos se han realizado en coccidiosis caprina. Uno de los fármacos más utilizados y estudiados es el diclazuril, un derivado triazinona cuyo uso como medicamento metafiláctico se ha demostrado en ovinos, asociado a una reducción de la eliminación de ooquistes, a la

mejora de las lesiones en epitelio intestinal y a un aumento del crecimiento medio de los animales tratados (Alzieu y cols.1999; Taylor y cols., 2003; Platzer y cols., 2005). La aplicación de este compuesto en el ganado bovino resultó, igualmente, en una reducción significativa de la excreción de ooquistes y evitó los efectos negativos de los coccidios sobre el crecimiento de los terneros (Daugschies y cols., 2007).

3.1.2.2. Medidas higiénicas

El objetivo perseguido con este tipo de medidas es reducir el número de ooquistes en el ambiente para que las cantidades que los animales puedan ingerir sean mínimas y, por tanto, no llegue a desarrollarse una coccidiosis clínica (Young y cols., 2011). Para ello se recomienda seguir las siguientes estrategias:

- Situar a los animales, desde el mismo momento de su nacimiento, en lugares limpios y secos, manteniéndolos así durante toda su crianza. Otra medida sería no mezclar animales de edades diferentes y separar inmediatamente los enfermos (muy complicado en cebaderos industriales, donde las mezclas de corderos de diferentes orígenes y edades son la tónica habitual). También se considera fundamental planificar los partos para poder tener vacío sanitario de corderos o cabritos en las naves o corrales; por tanto, los sistemas de paridera continua no son los más recomendables (Catchpole y cols., 1993).

Evitar alimentos (heno, paja, pienso y agua) contaminados con material fecal.

Adecuar la densidad de los animales al espacio disponible que ofrezcan las instalaciones, ya que, cuanto mayor sea la densidad, mayores son las posibilidades de infección (Gregory y Catchpole, 1990; Smith y Sherman, 2009; Chartier y cols., 2011).

Igualmente, el retirar el estiércol con frecuencia y desinfectar correctamente los locales son medidas muy recomendables. La limpieza ha de ser rigurosa ya que el ooquiste, que es la forma de resistencia y de diseminación de la enfermedad, puede sobrevivir años en el medio exterior (Smith y Sherman, 2009) y puede resistir la acción de la mayoría de los desinfectantes a las concentraciones usuales. La limpieza y la desinfección de los locales también puede ser realizada con vapor de agua a presión, sobrecalentada a 120 °C (Karcher). El vacío sanitario es, sin duda, el medio más eficaz para cortar completamente el ciclo del parásito pero, en la mayoría de los casos, no es una medida factible por las repercusiones económicas negativas para el ganadero.

3.1.7.3. Inmunoprofilaxis

Los trabajos de inmunoprofilaxis llevados a cabo frente a las coccidiosis de los rumiantes, son escasas, en comparación con los realizados en especies de *Eimeria* aviares, donde en la actualidad hay disponibles un buen número de vacunas comerciales (Mcdonald y Shirley, 2009).

Con el fin de mejorar la inmunidad de las aves, el uso de vacunas ha sido un enfoque válido que ha contribuido a prevenir la enfermedad. Algunas de las vacunas ensayadas en esta especies hospedadoras se han fabricado con cepas virulentas de *Eimeria* (Chapman y cols., 2005) y, a nivel de campo, se han conseguido resultados satisfactorios en combinación con la profilaxis terapéutica. Sin embargo, el empleo de vacunas vivas requiere de un buen manejo, de lo contrario, se pueden originar graves reacciones por el efecto patológico de la propia cepa empleada en la vacuna. Como alternativa, también se han ensayado vacunas atenuadas, aunque su obtención es mucho menos económica que en caso anterior. En la década de 1980 se puso de manifiesto que las especies de *Eimeria* podrían ser establemente atenuadas por pases en serie en los pollos de los primeros ooquistes producidos (es decir, los primeros parásitos para completar su desarrollo endógeno) y este proceso dio como resultado el agotamiento del desarrollo asexual. A pesar de ser altamente atenuadas, las líneas precoces conservaron su capacidad inmunizante. Los trabajos posteriores llevaron a la introducción comercial de la primera vacuna viva atenuada, Paracox®, que ha estado en uso durante 20 años (McDonald y Shirley MW, 2009). La atenuación de *Eimeria* con fines vacunales también se ha conseguido por otros medios tales como la irradiación. En este sentido, diversas investigaciones han demostrado que la irradiación gamma puede ser utilizada para atenuar varias especies de coccidios aviares, como *Eimeria acervulina, E. tenella*, o *E. maxima*, y prevenir la reproducción asexual del parásito y la formación de ooquistes (Jenkins MC y cols., 1995). También el pase a través de huevos embrionados (Livacox ®) o las selección de cepas de baja patogenicidad a partir de infecciones naturales (NobilisCox ATM1 ®), ha resultado en el desarrollo de vacunas comerciales frente a la coccidiosis aviar. Finalmente, otros estudios realizados en este mismo campo se han basado en el empleo de vacunas recombinantes (Ding y cols., 2005; Ma D y cols., 2011).

Como ejemplo de ensayo de campo para la evaluación de vacunas frente a coccidiosis aviar, en Venezuela se estudió la eficacia de una vacuna trivalente de cepas atenuadas de *Eimeria acervulina, E. maxima* y *E. tenella*. La vacuna se administró el primer día de edad y, durante todo el experimento (46 días en total), se suministró alimento medicado con coccidiostáticos (monensina-nicarbazina). El ensayo a nivel de granja permitió verificar *in situ* la eficacia de la vacuna atenuada en infecciones naturales, a pesar de la dificultad que supone establecer la dosis de infección en situación de campo (Tamasaukas y cols., 2002).

Aunque sería posible inmunizar artificialmente el ganado con vacunas comerciales, en la actualidad y en el futuro inmediato parece difícil que esto sea una

alternativa real para para el control de la coccidiosis en rumiantes **(Taylor y Catchpole, 1994)**. Así, se ha demostrado que la inmunización mediante cepas de ooquistes virulentas de *E. alabamensis*, a pesar de que indujo una protección parcial, no logró suprimir el desarrollo clínico de la enfermedad tras la inmunización **(Svensson y cols., 1996)**. Por otro lado, se ha observado que el grado de inmunidad obtenido después de la aplicación de ooquistes virulentos a terneros es dependiente de la dosis. Así, utilizando una dosis de 2000 ooquistes de especies mixtas (predominantemente *E. bovis*) durante cinco días consecutivos no se observó protección en los terneros frente a una infección posterior masiva con 2×10^5 ooquistes **(Conlogue y cols., 1984)**. Al contrario, la administración de 5×10^4 ooquistes de *E. bovis,* repitiendo la infección 4 semanas después, indujo un moderando grado de protección en los terneros reinfectados de forma natural **(Daugschies y cols., 1998)**. Se ha intentado, incluso, producir una vacuna utilizando ooquistes irradiados aislados de las heces, con distintos grados de irradiación (250 Gy, Co-60), pero la vacunación dio lugar a infecciones patentes y solamente se indujo una protección parcial **(Mielke, 1993)**. En cambio, recientemente, **Ruiz y cols. (2014)** demostraron el desarrollo de respuestas inmunoprotectoras eficaces utilizando ooquistes de *E. ninakohlyakimovae* atenuados por irradiación en infecciones experimentales en cabritos.

En otras enfermedades producidas por parásitos próximos filogenéticamente a los coccidios del género *Eimeria*, como *B. besnoiti,* se han realizado ensayos de protección en bovinos utilizando vacunas vivas atenuadas mediante pases en cultivos celulares **(Pipano, 1997)**. Otro estudio que cabría resaltar en rumiantes consistió en un ensayo de inmunoprotección frente a la neosporosis bovina producida por *Neospora caninum* **(Monney y Hemphill, 2014)**. En dicho trabajo se citaba que, aunque la vacunación se consideraba una estrategia racional para evitar la neosporosis bovina, la única vacuna comercializada (Neoguard®) producía resultados ambiguos con eficacia relativamente baja, por lo que, recientemente, se ha retirado del mercado.

3.1.7.3. Otras alternativas de control

Aunque, como se comentó anteriormente, el control de la coccidiosis depende principalmente de la quimioprofilaxis terapéutica, la capacidad de gestión también es importante para obtener el máximo efecto de estos fármacos **(Tewari y Maharana, 2011)**. Además, debido al desarrollo de resistencias, se han hecho esfuerzos para desarrollar nuevas estrategias para el control de la coccidiosis, fundamentalmente en las infecciones por especies de *Eimeria* aviares, como son la búsqueda de nuevos agentes con actividad anticoccidiósica de origen natural, compuestos que se consideran más eficaces y seguros.

También se ha demostrado que la administración de suplementos dietéticos como los probióticos podría ser otro enfoque novedoso para mejorar el mecanismo

de defensa intrínseca de las aves frente a la coccidiosis y otras infecciones parasitarias intestinales **(Alfaro y cols., 2007; Hafez, 2011)**. Además, es conocido que dietas ricas en antioxidantes son importantes para el crecimiento, la supervivencia y el mantenimiento de la salud, así como para el desarrollo productivo y reproductivo de los animales **(Surai, 2002)**. Así, la vitamina A podría actuar como pro-oxidante modulando la respuesta inmune mediada por células **(Volpe, 2000)**.

En relación al uso de plantas con efecto anticoccidiósico, **Kommuru y cols. (2014)**, al investigar la eficacia de diferentes compuestos vegetales frente a la coccidiosis, encontraron menores signos clínicos en cabras alimentadas con un granulado de lespeleza serícea *(Lespedeza cuneata*), en comparación con el grupo control, además de una disminución del número de OPG del más del 90%. De forma similar, un estudio dirigido por **Molan y cols. (2009)** demostró que un extracto acuoso de pino (*Pinus radiata)* con un 35% de taninos condensados presentaba una importante actividad anticoccidiósica, con capacidad para disminuir significativamente la esporulación *in vitro* de los ooquistes de tres especies aviares de *Eimeria.* Por último, al ser las cabras muy selectivas en su alimentación, se ha sugerido que podrían auto-medicarse con plantas que contengan compuestos con efectos anticoccidiósicos mientras están pastando, tal y como se ha observado en primates parasitados con nematodos gastrointestinales **(Hoste y cols., 2008; Amit y cols., 2013).**

3.2. RESPUESTA INMUNE FRENTE A LA COCCIDIOSIS

En la actualidad, existe un considerable número de trabajos relacionados con la caracterización de la respuesta inmune frente a la coccidiosis bovina, pero aún son escasos los dirigidos a la coccidiosis propia de pequeños rumiantes, ovinos y caprinos. La fuerte inmunogenicidad que se desarrolla durante la infección hace de la coccidiosis una enfermedad típica de animales jóvenes criados en condiciones de elevada contaminación fecal de los alimentos. Sin embargo, este modelo simplificado no explica todos los casos de coccidiosis clínica, por lo que es preciso revisar también otra serie de factores, entre ellos, las particularidades de las especies de *Eimeria* implicadas, el tipo de huésped y su relación con diversas situaciones de estrés fisiológico, ambiental, nutricional o inmunológico.

3.2.1. Introducción

Para que los fenómenos de defensa se lleven a cabo, los organismos disponen de un conjunto de elementos especiales que, en su conjunto, constituyen lo que se conoce como sistema inmune. La capacidad de defensa se adquiere antes de nacer y se madura y consolida en los primeros años de la vida fuera del seno materno. La respuesta inmune inespecífica o innata es la primera barrera defensiva del organismo y no requiere sensibilización previa **(Albert y cols., 2002)**. Este tipo de respuesta es mediada por células con capacidad fagocítica y células asesinas naturales (NK). La respuesta específica o adquirida se desarrolla sólo frente a la sustancia que indujo su iniciación y en ella participan prioritariamente los linfocitos y los elementos solubles liberados por los mismos, anticuerpos y citoquinas. Generalmente, el sistema inmune responde de forma unitaria y, de hecho, la activación de esta respuesta solamente suele desencadenarse cuando la respuesta inespecífica (innata) no ha sido capaz de eliminar el patógeno **(Delves y Roitt, 2000)**, por lo que la división en respuesta inespecífica y específica es más teórica que real. Lo que sí ocurre es que, dependiendo de las circunstancias, en unos casos predomina una u otra de estas formas de respuesta. En la respuesta inmune frente a la coccidiosis se ha citado la participación tanto del sistema inmune innato **(Lillehoj y li, 2004; Rosenberg y cols., 2005; Boyseny y cols., 2006; Hermosilla y cols., 2006; Behrendt y cols., 2008; Tabauret y cols., 2009; Behrendt y cols., 2010; Aylsworth y cols., 2013)** como del sistema inmune adquirido **(Rose y Hesketh, 1982; Speer y cols., 1985; Hughes y cols., 1989; Rose y cols., 1988, 1990, 1992; Fiege y cols., 1992; Hermosilla y cols., 1999; Parmentier y cols., 2001; Wallach, 2010)**.

En condiciones de campo, la exposición natural al parásito asegura un contacto continuo que permite ir desarrollando inmunidad. Sin embargo, el grado de inmunidad depende del nivel exposición previa. La exposición a un número bajo o moderado de ooquistes puede no suponer un estímulo antigénico suficiente como para desencadenar una respuesta inmune protectora que prevenga la infección y la enfermedad posterior, particularmente en reinfecciones con un número elevado de

ooquistes **(Conlogue y cols., 1984; Bürger y cols., 1995)**. Además, aun habiéndose desarrollado inmunidad protectora, y no manifestándose la enfermedad de forma clínica, los animales reinfectados pueden seguir eliminando ooquistes por las heces (**Daugschies y cols., 1986; Svensson et al., 1996)**. A parte de estas consideraciones, al abordar la inmunología de la eimeriosis es importante subrayar la elevada especificidad que muestran las diferentes especies *Eimeria* por sus hospedadores **(Catchpole, 1993; Tabauret y cols., 2008; Sühwoldy cols., 2010)**, un fenómeno que resultaría de una combinación de factores determinados genéticamente entre el parásito y la respuesta inmune del hospedador **(Rose, 1987; Witcombe y Smith, 2014)**.

3.2.2. Respuesta inmune inespecífica

La respuesta inespecífica representa la primera barrera defensiva del organismo y tiene especial significado frente a los primeros contactos con el parásito. Las células que median esta respuesta son, fundamentalmente, los polimorfonucleares neutrófilos (PMN), los macrófagos y células NK (*Natural Killer)*. Intervienen, además, las barreras físicas **(Mowat y Viney, 1997)**, como la piel o el revestimiento epitelial del tracto respiratorio y digestivo **(Tierney y col., 2007)**.

Los **leucocitos polimorfonucleares (PMN)** juegan un papel importante en la inmunidad inespecífica y, de hecho, son las primeras células de defensa que entrar en contacto con el parásito **(Nathan y cols., 2006; Brinkmann y Zychlinsky, 2012)**. Una vez en el lugar de la infección, forman una vacuola (englobando al patógeno) que será destruida, bien por mecanismos dependientes de oxígeno o de péptidos catiónicos y enzimas tóxicas contenidas en los gránulos citoplasmáticos que albergan **(Garred y cols., 1995)**. Junto con el resto de leucocitos (macrófagos, NK y eosinófilos) interfieren los procesos de invasión de determinados patógenos, interaccionando y/o produciendo moléculas de adhesión, proinflamatorias, factores quimiotácticos y quimioquinas **(von Andrian y Mackay, 2000)**. Por ejemplo, cuando se estimulan con antígenos de *T. gondii in vitro*, generan citoquinas proinflamatorias como IL-1b, IL-10, IL-12 y TNF-α y quimioquinas IL-8, MCP-1, MIP-la y RANTES **(Denkers, 2003)**. Así mismo, se ha demostrado que los PMN responden directamente frente a los esporozoítos y antígeno del parásito *E. bovis* produciendo citoquinas proinflamatorias y quimioquinas **(Behrendt y cols., 2008a**). Por todo ello, los PMN se consideran cruciales para la respuesta innata en la coccidiosis, pudiendo actuar nada más salir de la médula ósea, incluso más allá de su vida útil **(Hermosilla y cols., 2014)**. De este modo, se ha descrito que las primo-infecciones con *E. papillata* producen una coccidiosis más severa en ratones que carecen de PMN (SCID) que en ratones normales (**Schito y Barta, 1997**). En este mismo sentido, **Pérez cols. (2015)** demostraron que los neutrófilos caprinos en presencia de esporozoítos y antígeno de ooquistes de *Eimeria ninakohlyakimovae* incrementaban la expresión génica de determinadas moléculas inmunorreguladoras, entre ellas la IL-12, el TNF-α y la IL-6.

Los **monocitos y macrófagos** también participan en las reacciones inmunes innatas que se desarrollan durante el primer contacto con el patógeno. Su papel concreto en las infecciones producidas por el género *Eimeria* es menos conocido, pero se ha demostrado que en infecciones por *E. bovis* son capaces de degenerar el macroesquizonte **(Friend y Stockle, 1980)**. En este sentido, **Taubert y cols. (2009**) describieron que las reacciones inmunológicas desarrolladas por los macrófagos pueden afectar el resultado de las infecciones primarias y son cruciales para la transición a la respuesta inmune adaptativa frente a la eimeriosis. En sus experimentos investigaron las reacciones inmunes mediadas por monocitos y macrófagos tanto *in vivo*, *in vitro* como *ex vivo* frente a *Eimeria bovis*, una de las especies de *Eimeria* más patógenas en el ganado bovino. Observaron que los macrófagos se infiltraban de manera significativa en la mucosa intestinal de los terneros infectados, sobre todo después de la infección primaria. Además, los monocitos periféricos de los animales infectados, células precursoras de los macrófagos, experimentaron *ex vivo* un incremento de su actividad fagocítica y oxidativa después de la infección y hacia el final de la merogonia. Por otro lado, en la exposición *in vitro* de los macrófagos a esporozoítos, se observó una intensa fagocitosis del patógeno, no ocurriendo así con los monocitos. La fagocitosis se produjo independientemente de la viabilidad de los esporozoítos, lo que indica que la invasión activa de los parásitos no era un factor clave en el proceso. También se observó actividad fagocitaria en ausencia de suero inmune, pero la administración de suero la incrementaba significativamente, lo que sugiere que la citotoxicidad derivada de macrófagos podría ser dependiente de anticuerpos. Estos mismos autores también demostraron que el co-cultivo de macrófagos con esporozoítos y merozoítos inducía niveles distintos de transcripción génica de determinadas citoquinas y quimioquinas. En su conjunto, todos estos resultados sugieren que las reacciones inmunes innatas mediadas por monocitos y macrófagos juegan un papel importante en la respuesta inmune temprana a las infecciones por *E. bovis* en terneros **(Taubert et., al 2009)**.

Los **eosinófilos** se consideran leucocitos multifuncionales de gran importancia en diversos procesos inflamatorios, alérgicos y parasitarios, sobre todo en las infecciones producidas por nematodos. En condiciones homeostáticas son abundantes en la lámina propia del tracto gastrointestinal, donde se ha postulado su participación en diversos procesos bilógicos de este órgano **(Jung y cols., 2014)**. Actúan tanto en la respuesta innata como adquirida. En general, en animales primo-infectados por el género de *Eimeria* se observa un aumento en los recuentos hematológicos de eosinófilos, tal y como se ha demostrado en pollos primoinfectados con *E. acervulina* **(McFarlane y cols., 1989)**, en ratones con *E. vermiformis* **(Linh y cols., 2009)** y en pavos primo-infectados con *E. adenoeides* **(Gadde y cols, 2009**). Aunque menos documentado, también en rumiantes se ha demostrado la participación de los eosinófilos en la respuesta inmune frente a la

coccidiosis, aunque no claramente en las respuestas primarias. Así, en un estudio sobre la eficacia del toltrazuril al 5% en suspensión frente a especies de *Eimeria* de rumiantes (*E. bovis* y *E. zuernii*) el examen histológico demostró que los recuentos de eosinófilos eran más comunes en el íleon y colon de los animales clasificados como resistentes y tendían a estar asociados a concentraciones más elevadas de TNF-α. Por otro lado, se ha observado que, aproximadamente, el 70% de los caprinos de raza pirenaica, en los que se encontró un porcentaje de parasitación por coccidios del género *Eimeria* del 84,32%, presentaba una marcada eosinofilia **(Fernández del Palacio y cols., 1985)**.

Las **células dendríticas** son un tipo celular especializado del sistema inmunológico, cuyas funciones principales son capturar y procesar los antígenos y presentarlos a los linfocitos B y T para que éstos inicien repuestas inmunológicas específicas. Por este motivo, se consideran mensajeras entre el sistema inmune adaptativo e innato **(Clark y cols., 2000)**. Las células dendríticas están presentes en pequeñas cantidades en los tejidos que están en contacto con el ambiente externo, por lo que se encuentran principalmente en la piel, en el revestimiento interno de la nariz, los pulmones, el estómago y, en general, en todo el tracto gastrointestinal. También pueden encontrarse en estado inmaduro en la sangre. Las células del sistema inmune, incluyendo las células dendríticas, poseen los denominados receptores de reconocimiento de patrones moleculares (*PRR-Pattern recognition receptor*), que son capaces de reconocer distintos patrones moleculares asociados a patógenos (*PAMP-Pathongen-associated molecular pattern*) presentes en virus, bacterias, hongos y protozoos, como pueden ser su material genético, lipopolisacáridos (LPS), etc. Los PRR mejor estudiados son los receptores de tipo Toll (*TLR- Toll-like Receptors*), muy importantes en la biología de las células dendríticas **(Reis y Sousa, 2004)**. Las distintas vías de activación de estas células pueden ser dependientes o independientes de los PAMPs. Además de los TLRs, responsables de señalizaciones intracelulares, existen otros PRR incluidos en la familia de las lectinas de tipo C que están siendo objetivo de numerosos estudios **(Figdor y cols., 2002; Kanazawa, 2007)**. Las células dendríticas están frecuentemente implicadas en la iniciación de la respuesta inmune temprana del hospedador frente a patógenos microbianos, pero su actividad funcional se extiende más allá de este importante papel durante el inicio de la actividad **(Denkers y cols., 2004)** ya que, una vez activadas, estas células son capaces de segregar un importante número de citoquinas proinflamatorias **(Moser y Murphy. 2000)**. Este tipo celular es fuente importante de IL-12 en el sistema nervioso central durante la infección crónica de *Toxoplasma*, lo cual podría estar relacionado con la protección conferida frente al parásito **(Fischer y cols., 2000)**. Estos resultados coinciden con otros donde se muestra que la producción continua de IL-12 durante los procesos crónicos es necesaria para evitar la reactivación del parásito **(Yap y cols., 2000)**. También se ha demostrado que las células dendríticas de ratón pueden ser activadas mediante INF-γ y desarrollar una actividad microbiana frente a *Toxoplasma* dependiente de oxígeno **(Aline y cols.,**

2002), y que antígenos proteicos de protozoos intestinales como *Eimeria* son estimuladores muy potentes *in vivo* de la liberación de citoquinas a partir de células dendríticas (Rosenberg y cols., 2005), todo lo cual subraya el papel de este tipo celular en la infecciones por Apicomplexa.

Los **mastocitos** son leucocitos derivados de precursores hematopoyéticos. Circulan en la sangre en su forma inmadura hasta que migran a tejidos vascularizados, donde se diferencian con ayuda del *stem- cell factor* y de otras citoquinas segregadas por células endoteliales y fibroblastos. Se localizan en la mayoría de los tejidos, particularmente en aquellas localizaciones que están en contacto con el exterior, por ejemplo, piel, vías aéreas y tracto gastrointestinal (Urb y Sheppard, 2013). Contienen mediadores primarios como proteasas, proteoglicanos o histamina, que pueden ser almacenados y liberados sin necesidad de sensibilización previa. Además, poseen mediadores secundarios como algunas prostaglandinas y citoquinas que se relacionan con el aumento de la permeabilidad vascular y la inducción de la respuesta inflamatoria. Los mastocitos se activan inicialmente al contactar con moléculas del parásito, o del tejido dañado, con moléculas de la vía alternativa del complemento o con bacterias (Balic y cols., 2000). Debido a su localización, su plasticidad y la variedad de mediadores que producen, los mastocitos se consideran células inmunomoduladoras y efectoras importantes que pueden ejercer un puente entre las respuestas innatas y adaptativas (Salinas y cols., 2007; Metcalfe, 2008). Confirmando su papel como células inflamatorias primarias, en infecciones por *E. tenella* y *E. acervullina* en pollo (Petrone y cols., 2002; Metcalfe, 2008) se ha observado que el número de mastocitos aumenta en la mucosa del ciego durante el proceso inflamatorio agudo. Los mastocitos intraepiteliales se consideran su última fase efectora, al haberse liberado sus gránulos con el fin de alterar el medio ambiente parasitario; en este estado, los mastocitos reciben el nombre de **leucocitos globulares** (LG) (Balic y cols., 2000).

Las **células NK** proceden de la familia de las células linfoides innatas (Spits y Di Santo, 2011) y presentan un progenitor común (Spits y Di Santo, 2011). Se consideran esenciales como parte de la respuesta inmune innata, pero también se ha descrito que colaboran en la respuesta inmune adaptativa (Vivier y cols., 2008). Además de sus acciones defensivas frente a agentes externos, las células NK tienen la propiedad de respetar las células propias de cada individuo, reconociendo así su identidad biológica. Esto se debe a la presencia en este tipo celular de moléculas HLA-I que actúan a modo de escudo protector. Por ello, cuando las propias células pierden estas moléculas, son destruidas por las células NK. Su participación en la respuesta frente a la coccidiosis se ha descrito en diversas especies de *Eimeria* aviares, entre ellas, *E. acervulina, E. tenella* o *E. maxima* (Lillhoj, 1989; Lillhoj y Bacon, 1991). Concretamente, en infecciones por *E. acervulina* se ha observado que la actividad de las células NK esplénica e intestinal se incrementa durante la fase temprana de la infección.

Entre las barreras físicas que participan en la respuesta inmune innata frente a

la coccidiosis cabe resaltar el papel del revestimiento endotelial de los vasos sanguíneos y linfáticos. El **endotelio**, además de participar en la modulación de la homeostasis metabólica y en la hemodinámica vascular e intercambio celular, debido a su ubicación, también presenta una importante función inmunorreguladora, por lo que las células endoteliales se encuentran entre las primeras células que interactúan con los patógenos externos **(Mai y cols., 2013)**. Las células endoteliales son las células hospedadoras específicas de muchos parásitos Apicomplexa *in vivo*, sobre todo, de distintas especies del género *Eimeria* que forman macroesquizontes **(Taubert y cols., 2006; Alvarez y cols., 2014)**. Durante la infección y desarrollo del parásito, las células endoteliales actúan modulando la transcripción génica de moléculas inmunorreguladoras de la respuesta inmune, como citoquinas, moléculas de adhesión y quimioquinas. Se ha demostrado, por ejemplo, que la infección *in vitro* de células endoteliales del cordón umbilical de bovinos (BUVEC) con esporozoítos de *E. bovis* y taquizoitos de *T. gondii* y de *N. caninum* **(Taubert y cols., 2006)** produce una activación de la célula hospedadora que resulta de un aumento de la transcripción de genes que codifican moléculas proinflamatorias y inmunomoduladoras (GRO-a, IL-8 e IP-10, CC2, GM-CSF, COX-2 e iNOS, entre otras), que son importantes para la respuesta inmune innata y la transcripción hacia una respuesta adquirida o específica. Como describieron **Taubert y cols. (2006)**, las diferencias que presenta *E. bovis* respecto a *T. gondii* y *N. caninum* indicarían una particular estrategia de evasión de los esporozoítos de *E. bovis* **(Taubert y cols., 2006)**, debido a la necesidad de persistir en el hospedador por un tiempo más largo, durante el cual podría evitar el que se desencadene un proceso inflamatorio. En este mismo sentido, se ha comprobado que las especies de *Eimeria* que desarrollan merogonias en el interior de las células endoteliales tienden a desencadenar una modulación compleja del transcriptoma y el proteoma para asegurar su desarrollo **(Taubert y cols., 2010)**. Además, el desarrollo de *E. bovis* parece que previene la apoptosis de la célula hospedadora endotelial mediante la regulación de la expresión génica de moléculas anti-apoptóticas, según describieron **Lang y cols., (2009)**. Por otro lado, se ha podido constatar que el crecimiento y desarrollo de los macroesquizontes de esta especie de *Eimeria* bovina demandan una gran cantidad de moléculas para la biogénesis de la membrana celular, como el colesterol y ácidos grasos **(Hamid y cols., 2014)**, de ahí que la transcripción génica de la biosíntesis y el metabolismo de los lípidos de la célula hospedadora endotelial infectada con *E. bovis* se encuentre incrementada **(Taubert y cols., 2010)**. Los coccidios están considerados autotróficos en la síntesis del colesterol, por lo que necesitan utilizar el colesterol de la célula hospedadora para la biogénesis de su membrana durante el desarrollo de la fase de replicación intracelular, como se ha demostrado en *T. gondii* y *C. parvum* **(Coppens y cols., 2000; Ehrenman y cols., 2013)**. En las especies patógenas de *Eimeria* en rumiantes, que producen un número mayor de merozoítos, los requisitos de colesterol son, incluso, más altos **(Hamid y cols., 2014)**.

Recientemente, se ha encontrado un nuevo mecanismo relacionado con la respuesta inmune innata que consiste en la formación de trampas extracelulares (ETs). Este mecanismo fue Inicialmente descrito en PMN, por lo que recibió el nombre de NETs **(Brinkmann y cols., 2004; Brinkmann y Zychlinsky, 2007; Hellenbrand y cols., 2013; Jenne, 2013)**, pero actualmente también se ha observado en otros tipos celulares, tales como eosinófilos **(Yousefi y cols., 2008)**, mastocitos **(von köckritz-Bliclwede y cols., 2008)**, monocitos **(Muñoz-Caro y cols., 2014; Pérez y cols., 2015)** y macrófagos **(Aulik y cols., 2012; Bonne-Année y cols., 2014)** frente a diferentes patógenos. La liberación de ETs puede promover la muerte extracelular y/o la inmovilización de agentes patógenos virales, bacterianos o fúngicos, levaduras de distintos géneros y determinados parásitos. La formación de ETs se ha observado como respuesta innata en numerosos vertebrados e invertebrados **(Hermosilla y cols., 2014; Silva y cols., 2014)**, ya no sólo asociadas a situaciones patológicas de origen infeccioso. Tales estructuras están formadas por redes de matrices de ADN mitocondrial y proteínas celulares que resultan de un complejo proceso en el que parece cobrar un importante protagonismo el sistema NADPH oxidasa como mecanismo iniciador; aparte, la mieloperoxidasa (MPO) y la elastasa de los neutrófilos (NE) también parecen contribuir a regular la liberación adecuada de ETs **(Brinkmann y cols., 2004; Brinkmann y Zychlinsky, 2012)**.

Behrendt y cols. (2010) describieron por primera vez la formación de ETs como un mecanismo efector adicional de los PMN en respuesta a la coccidiosis producida por el género *Eimeria*, concretamente en *E. bovis;* posteriormente, se demostró que también *E. arloingi* era capaz de estimular el desarrollo de ETs en neutrófilos **(Silva y cols., 2014a)**. En ambos casos, las denominadas "trampas extracelulares" se demostraron mediante la visualización, a través de ensayos de fluorescencia y microscopia electrónica de barrido (SEM), de delicadas fibras procedentes de la red extracelular, originadas a partir de PMN, capaces de atrapar esporozoítos y otros estadios de ambas especies de *Eimeria*. Posteriormente, también se ha demostrado que los monocitos pueden sufrir ETosis en respuesta a la infección por esporozoítos de *E. bovis* y taquizoítos de *B. besnoiti* siguiendo un mecanismo similar **(Muñoz-Caro y cols., 2014)**. La formación de ETs se ha puesto de manifiesto, igualmente, en otros protozoos del phylum Apicomplexa, entre ellos *Plasmodium falciparum* y *Toxoplasma gondii* **(Baker y cols., 2008; Abi Abdllah y cols., 2012)**.

3.2.3. Respuesta inmune específica en rumiantes

La respuesta inmune específica se caracteriza porque es efectiva ante aquellos antígenos frente a los cuales se ha iniciado y desarrollado. Este tipo de respuesta es mediada por los linfocitos, que pueden ser de dos tipos: linfocitos B y linfocitos T. Los linfocitos T, a su vez, se diferencian en linfocitos T colaboradores (Th), linfocitos T citotóxicos (Tc) y linfocitos T supresores/reguladores (Ts).

La respuesta inmune específica puede ser de dos tipos, humoral y celular, aunque esta separación es más de tipo didáctico que real. En general, se considera que la respuesta es de tipo humoral cuando el elemento efector final son las inmunoglobulinas formadas por los linfocitos B, mientras que cuando participan los linfocitos T, tanto colaboradores (Th) como citotóxicos (Tc), la respuesta inmune suele catalogarse como de tipo celular. Ambos tipos de respuesta se han descrito implicadas en la coccidiosis de rumiantes **(Daugschies and Nadjdrowski 2005)**.

3.2.3.1. *Respuesta inmune humoral frente a la coccidiosis en rumiantes*

Aunque, tradicionalmente, se ha considerado que la respuesta inmune celular tiene más relevancia en la coccidiosis, cada vez hay más trabajos centrados en el estudio de la respuesta inmune humoral y su papel en la inmunidad protectora frente a infecciones por diferentes especies de *Eimeria*.

En el estudio de la respuesta inmune innata se comentó que los mastocitos presentan en su membrana receptores de alta afinidad para la IgE, por lo que, en animales previamente sensibilizados al antígeno, la unión de la inmunoglobulina puede provocar su degranulación **(Miller, 1996)**. Por este motivo, se ha sugerido que los mastocitos podrían ejercer de células puente entre la respuesta inmune innata y adaptativa **(Salinas y cols., 2007; Metcalfe, 2008)**. Las células **NK** también disponen de receptores de inmunoglobulinas (FCR) capaces de reconocer objetivos recubiertos con anticuerpos; cuando esto ocurre, la célula inicia su actividad como mecanismo de citotoxicidad dependiente de anticuerpos. Este tipo celular también produce citoquinas inflamatorias que influyen de manera decisiva en el desarrollo de respuestas inmunes adaptativas **(Vivier y cols., 2008)**.

La respuesta inmune humoral frente a la eimeriosis caprina no ha sido tratada en profundidad. En los escasos estudios realizados en este sentido destacan los trabajos de **Kanyari (1994)**, quien analizó mediante inmunohistoquímica la antigenicidad de diversas formas evolutivas de *E. apsheronica* en la cabra y el perfil de anticuerpos en dos razas de caprino sujetas a infecciones multiespecíficas de *Eimeria* que incluían principalmente *E. christenseni* (49%), *E. apsheronica* (29%), y menores proporciones de *E. arloingi, E. hirci, E. ninakohlyakimovae* y *E. alijevi* **(Kanyari, 1988)**. Para el análisis de anticuerpos desarrollaron un ELISA indirecto utilizando como antígeno homogenizados de ooquistes esporulados de estas mismas especies de *Eimeria.*

Relativamente más numerosos son los estudios inmunológicos realizados sobre eimeriosis bovina y ovina. Así, en bovinos, se ha observado que la respuesta inmune humoral en animales infectados por coccidiosis se desarrolla rápidamente, llegándose a observar un alto título de anticuerpos en el suero. Inicialmente, se produce un incremento de la IgM y, más tarde, de la IgG, pudiendo aparecer, además, otro tipo de anticuerpos específicos como la IgA **(Hughes, 1985)**. En la mayoría de los casos, la respuesta humoral se ve potenciada cuando los animales se

ven expuestos continuamente a los ooquistes. Del mismo modo, en infecciones experimentales con *E. faurei* y *E. ovinoidalis* en ovejas se demostró la presencia de anticuerpos, tanto en infecciones primarias como secundarias **(Nolan y cols., 1987)**. En dichos trabajos se demostró que existían considerables reacciones cruzadas entre las especies y que, en general, el incremento sérico de las inmunoglobulinas aparecía demasiado tarde como para ser de utilidad para un diagnóstico individual, aunque sí podría serlo para un diagnóstico a nivel de rebaño.

El estudio de la respuesta humoral y su importancia para el control de la enfermedad se ha abordado más ampliamente en la coccidiosis aviar, donde se ha demostrado la capacidad de los anticuerpos para bloquear la invasión, el desarrollo y la trasmisión del parásito, así como la existencia de una inmunidad pasiva **(Wallach, 2010)**. En otras coccidiosis, como las producidas por *Cryptosporidium* y *Toxoplasma gondii* en cabras, también se ha señalado la importancia y el papel que juegan los anticuerpos, no sólo a nivel periférico, sino también en la mucosa intestinal de los animales infectados **(Gomez Morales y cols., 2022; Conde y cols., 2001)**. Por ejemplo, el análisis de la respuesta de IgG en cabras infectadas experimentalmente con *Toxoplasma gondii* utilizando un ELISA indirecto reveló que los anticuerpos podían detectarse a los 14 días después de la inoculación (p.i.), alcanzando un pico al día 35 p.i. y mostrando fluctuaciones ligeras hasta el final del experimento (91 p.i.) **(Conde y cols., 2001)**. En el mismo estudio se identificaron mediante Western-blot un panel de péptidos reconocidos específicamente por la IgG.

En general, en las infecciones por coccidios se ha demostrado que los principales isotipos de inmunoglobulinas implicados son la IgG_2, la IgM y la IgA **(Faber y cols., 2002)**. Aunque la IgG_2 se considera la fracción principal en la respuesta humoral en la eimeriosis, el tipo de la respuesta serológica puede variar dependiendo del nivel infección **(Hughes, 1985)**. Los tres isotipos se han correlacionado positivamente con la eliminación de ooquistes, siendo dependiente esta correlación de la dosis infectante que el ternero haya ingerido durante la primoinfección y de la severidad de la enfermedad. A pesar de ello, se sabe que la inmunidad humoral no es suficiente como para controlar una reinfección por *Eimeria* **(Daugschies y Najdrowski, 2005)** y que, aunque los anticuerpos reflejan la exposición al parásito, la protección que confieren no es absoluta **(Fiege y cols., 1992)**.

Los anticuerpos pueden transferirse por el calostro, lo que podría constituir un mecanismo de inmunidad pasiva frente a la coccidiosis en rumiantes. Este hecho fue publicado por **Gregory y cols. (1989)** y **Fiege y cols. (1992)**, quienes, tras administrar calostro con un alto contenido en IgG a corderos infectados por *E. crandallis*, observaron un mayor título de anticuerpos en los animales alimentados con calostro que en aquellos que no lo recibieron. En las infecciones experimentales se observó un aumento considerable en los niveles de anticuerpos IgG_1 e IgG_2, mientras que los valores de IgM aumentaron sólo ligeramente. Profundizando en el estudio de la influencia del calostro sobre la transmisión pasiva de anticuerpos en la

coccidiosis, **Fayer y cols. (1992)** concluyeron que la administración de calostro procedente de vacas inmunizadas con diferentes antígenos de *Eimeria acervulina* (esporozoítos, merozoítos y antígeno recombinante de merozoíto) reducía el desarrollo *in vivo* e *in vitro* del parásito. Como resultado, con excepción de los inmunizados con antígeno recombinante, todos los pollos inmunizados eliminaron menos ooquistes por las heces y presentaron menos etapas de desarrollo parasitario en las secciones histológicas.

Posteriormente, **Faber y cols. (2002)**, tras una infección experimental con *E. bovis* en terneros, no lograron demostrar inmunoprotección mediante la administración de anticuerpos del calostro materno, por lo que ha sido motivo de controversia el si los anticuerpos maternos tienen efectos inmunoprotectores o no en la coccidiosis de rumiantes **(Catchpole y Devonshire, 1989 y Gregory y Catchpole, 1989; Gregory y cols., 1989b)**. Sí se considera más aceptado, como se ha descrito en ovinos **(Reeg y cols., 2005)**, el que los niveles de anticuerpos maternos presentan un marcado descenso después del nacimiento y un posterior aumento debido a nuevos anticuerpos específicos sintetizados. Dado que la vida media de la IgG_1, el isotipo predominante transmitido en las ovejas con el calostro **(Reynolds y Griffin, 1990)**, alcanza un pico máximo a los 11-13 días **(Klobasa y Werhahn, 1989; Watson, 1992; Domínguez y cols., 2001**) el período de tiempo en torno a 40 días después del nacimiento podría representar un período en el que los anticuerpos maternos se superponen a los producidos por los corderos. De hecho, a esta edad se han observado correlaciones directas entre anticuerpos y el patrón de excreción de ooquistes, lo que sugiere que los corderos habrían desarrollado ya una respuesta inmune frente a las infecciones por coccidios. Sin embargo, **Reeg y cols., (2005)** no pudieron confirmar hipótesis previas según las cuales los animales con bajos niveles de anticuerpos iniciales frente a los antígenos de *Eimeria* tienden a alcanzar finalmente títulos altos y viceversa **Gregory y Catchpole (1989)**, aunque parece lógico desde el punto de vista inmune **(Morein y cols., 2002)**. De cualquier forma, la respuesta de anticuerpos en corderos ha de considerarse el resultado de la interacción entre la inmunidad maternal pasiva y activa (resultado de la infección natural), el tipo de antígeno y otros factores, por lo que las características y la intensidad de la respuesta no siempre son predecibles **(Watson y Gill, 1991; Morein y cols., 2002).**

3.2.3.2. Respuesta inmune celular en la coccidiosis

La respuesta inmune de tipo celular es compleja en sus efectos y acciones finales, así como en su iniciación y desarrollo. Además, ha de tenerse en cuenta que, durante todo el ciclo del parásito dentro del tracto gastrointestinal, los distintos estados parasitarios pueden generar una respuesta específica, lo cual complica aún más la respuesta inmune celular que se desarrolla frente a los coccidios. De hecho, cualquier factor que afecte negativamente la capacidad de respuesta inmunológica

del hospedador puede favorecer que el parásito exprese todo su potencial de multiplicación, llegando a la etapa de gametogonia y, por tanto, incrementando el número de células afectadas y la gravedad de la enfermedad. Sin embargo, lo habitual es que la exposición natural al coccidio asegure un contacto continuo que permita ir desarrollando una inmunidad específica frente a las distintas formas parasitarias del ciclo endógeno del parásito, y así evitar casos clínicos en contactos posteriores **(Daugschies y Najdrowski, 2005)**. En condiciones normales, tras el pico de eliminación de ooquistes, la enfermedad es autolimitante en los terneros y, a partir de ese momento, se reduce la prevalencia y los niveles de excreción.

Existen claras diferencias en la biología de coccidios de rumiantes y la especies *Eimeria* en roedores, lo que arroja dudas sobre la posibilidad de establecer comparaciones directas entre unas especies y otras, por ejemplo, a la hora de abordar las diferentes estrategias de evasión de la respuesta inmune del huésped en el curso de una infección primaria. Así, los coccidios de roedores parecen evadir los ataques del sistema inmune repitiendo antigénicamente diferentes generaciones de merozoítos de forma rápida hasta que se desarrollan las etapas sexuales **(Long, 1978)**, mientras que, al menos algunos coccidios en rumiantes, incluyendo *E. bovis* en el ganado vacuno y *E. ninakohlyakimovae* en caprinos, producen los llamados macroesquizontes, capaces de liberar grandes cantidades de merozoítos. Además, el ciclo endógeno en rumiantes suele ser mucho más largo. Por ejemplo, en *E. bovis*, la maduración de los merozoítos necesita de 14-18 días **(Hammond y cols., 1964)**; posteriormente, las etapas sexuales se desarrollan rápidamente y los ooquistes se liberan después de 18-21 días p.i. **(Hammond y cols., 1964)**. Estas diferencias en cuanto a estrategias de evasión y duración del desarrollo serían el resultado de reacciones particulares del sistema inmune del huésped, posiblemente relacionadas con la respuesta inmune celular, pero los estudios realizados hasta el momento en rumiantes son escasos **(Speer y cols., 1985; Hughes y cols., 1989; Aleksandersen y cols., 1995)**.

La inmunidad celular es considerada la más importante frente a la eimeriosis, dado que las formas intracelulares del parásito (esquizontes, macrogamentos, microgametos), teóricamente, no pueden ser alcanzados por los anticuerpos. Las células parasitadas expresan en su superficie antígenos que son reconocidos por el sistema inmune, implicando a diferentes poblaciones celulares de linfocitos T, algunas de ellas capaces de desarrollar mecanismos de citotoxicidad **(Rose, 1987)**. Numerosos estudios han demostrado que, probablemente, se produzca una polarización hacia una respuesta Th1 frente a las primeras etapas del desarrollo parasitario, por ejemplo, los esporozoítos o los esquizontes inmaduros (de < 8 días), lo cual se considera de gran importancia para el control del parásito después de la infección **(Rose y cols., 1992b; Shi y cols., 2001a)**.

Por otro lado, al analizar la participación de las subpoblaciones de células T en el curso de infecciones primarias y en reinfecciones en ratones se ha observado que la población de linfocitos T $CD4^+$ se involucra, particularmente, en resolver las

infecciones primarias, regular su duración y el nivel de la eliminación de ooquistes, mientras que los T CD8+ estarían más implicados en las reinfecciones **(Rose y cols., 1992a; Findley y cols., 1993; Ovington y cols., 1995; Smith y Hayday, 2000; Shi y cols., 2001a)**.

Del mismo modo, **Hermosilla y cols. (1999)** observaron que en terneros inoculados con ooquistes de *E.bovis,* las células T CD4+ circulantes aumentaban durante la prepatencia de la infección primaria decayendo tras el período patente (a los 25 días p.i.). Coincidiendo con esta observación, las necropsias realizadas el día 35 p.i. demostraron un aumento de tamaño en todos los nódulos linfático, incluso de los esplénicos y de áreas más lejanas. Durante la postpatencia, el recuento de células T CD4+ en órganos linfáticos fue mayor que el de células T CD8+, lo que confirmaría que las células CD4+ están implicadas en la terminación de una infección primaria, mientras que los CD8+ median la inmunidad en reinfecciones **(Rose y cols., 1992)**.

Los linfocitos T CD8+ tienen propiedades citotóxicas y pueden actuar sobre células con Ag fijados al Complejo Mayor de Histocompatibilidad (MHC). Este tipo celular se ha observado que aumenta en la circulación periférica al comienzo de la infección con *E. bovis* pero luego decae, incluso durante el periodo de patencia, lo que sugiere que su consolidación en la respuesta efectiva surge tras la primo-infección. En reinfecciones (posteriores al día 35 p.i.) aumenta la presencia local de células CD8+ a nivel de la mucosa intestinal, además de en otros órganos como bazo y ganglios; tal incremento coincidiría en el tiempo con un descenso en la circulación periférica **(Hermosilla y cols., 1999)**. También en aves parece ser esta línea de linfocitos la asociada a la respuesta en reinfecciones, lo cual se ha comprobado al comparar diferentes líneas genéticas de pollos con mayor y menor capacidad de respuesta inmune frente a la coccidiosis **(Lillehoj, H. y cols., 2004).**

Un aumento en la expresión de CD2+ en células T se ha relacionado con la activación y el aumento de la capacidad de reconocimiento antigénico de estas células **(Davis y Van der Merwe, 1996)**, un proceso en el que, tanto las células T CD8+ como las T CD4+ pueden estar implicadas **(Baldwin y cols., 1988)**. Se sabe poco sobre la participación de las células T CD2+ en las infecciones de *Eimeria*. Sin embargo, su incremento porcentual, de forma persistente en sangre periférica durante la infección experimental con *E. bovis,* sugiere un intenso estímulo antigénico, probablemente, debido al desarrollo de la primera esquizogonia **(Hermosilla y cols., 1999)**.

En estudios recientes se ha investigado el posible papel de los receptores celulares αβ+TCR y γδ+TCR, y se ha observado que estas últimas células también pueden reconocer antígenos de manera independiente de MHC **(De Libero, 1997)** y que se encuentran predominantemente en tejidos epiteliales **(Stingl y cols., 1987; Bonneville y cols., 1988; Goodman y Lefrançois, 1988)**, el área principal donde las especies de *Eimeria* se están desarrollando. Son especialmente numerosos en el ganado bovino y ovino **(Mackay y Hein, 1989; Hein y Mackay, 1991)**, pero su papel

en la protección frente a *E. vermiformis* en ratones parece ser, poco significativo **(Rose y cols., 1996)**. Sin embargo, probablemente debido a su capacidad inmunomoduladora **(Mc Menamin y cols., 1994, 1995)**, las células T γδ⁺ parecen ser capaces de reducir las consecuencias patológicas de la infección intestinal por coccidios **(Roberts y cols., 1996)**.

La participación de las células T γδ⁺ fue estudiada por **Hermosilla y cols. (1999)** tras inocular terneros con *E. bovis.* Al analizar las muestras tomadas en la necropsia el día 35 p.i. (postpatencia) se observó que los linfocitos γδ⁺ aumentaban en los tejidos intestinales, pero no variaban en la circulación. El aumento de las células γδ+TCR también se ha observado en el intestino de ratones infectados por *E. vermiformis* **(Findley y cols., 1993)**, en ratas parasitadas por *E. separata* **(Meiching, Huther y Zahner, datos no publicados)** y por diferentes especies de *Eimeria* en pollos **(Lillehoj, 1994; Rothwell y cols., 1995)**. De acuerdo con lo observado por **Roberts y cols. (1996)**, las células γδ+TCR podrían tener un efecto antiinflamatorio, ya que las infecciones por *E. vermiformis* entre ratones normales y ratones con una depleción de los γδ+TCR encontraron claramente alteraciones más graves en este último grupo. Teniendo en cuenta que, ocasionalmente, se produce una exacerbación del daño tisular en la mucosa en infecciones por *E. bovis* **(Fitzgerald y Mansfield, 1972; Daugschies y cols., 1986)**, esta capacidad de las células γδ+TCR sería de especial importancia.

El papel de las citoquinas se considera crucial en la inducción de la resistencia protectora (inmune) o la exacerbación de las infecciones parasitarias. Se reconocen dos perfiles de citoquinas dependientes del tipo de respuesta inmune: Th1 y Th2 **(Meeusen y cols., 2005)**. La respuesta Th1 se asocia a la producción de las citocinas IFN-γ e IL-2 **(Khan y Collins, 2004)**. Por el contrario, la respuesta Th2 se relaciona con la producción de las citocinas IL-3, IL-4 e IL-5 **(Grencis, 1997)**, las cuales amplifican y regulan el reclutamiento, proliferación y diferenciación de células efectoras tales como eosinófilos, mastocitos celulares, leucocitos globulares y células secretoras de anticuerpos.

Tanto los linfocitos T CD4⁺ como los T CD8⁺ median en la acción de citoquinas como el INF□b la IL1, reclutando células fagocíticas que actúan *per se* o liberando nuevas citoquinas que promueven inflamación y lisis celular (IL-2, IL-a e IL-10). En general, durante la infección primaria de especies de *Eimeria* en rumiantes se observa un aumento de la producción de INF□ lo que sugiere el establecimiento de una respuesta inmune celular Th1 **(Taubert y cols., 2008)**. Por otro lado, en terneros primoifectados con *E. bovis*, se observa un predominio de la expresión génica de IL2 (Th1) sobre la expresión de IL4 (Th2) en relación a los controles, lo que sugiere un aumento de la respuesta celular citotóxica sobre células que presentan antígenos (fijados al MHC). Además, el incremento de INF-□y de IL2 estimularía la activación de los linfocitos y potenciarían la actividad fagocítica de los macrófagos a través de un aumento en la producción de nitritos **(Hermosilla y cols., 1999)**.

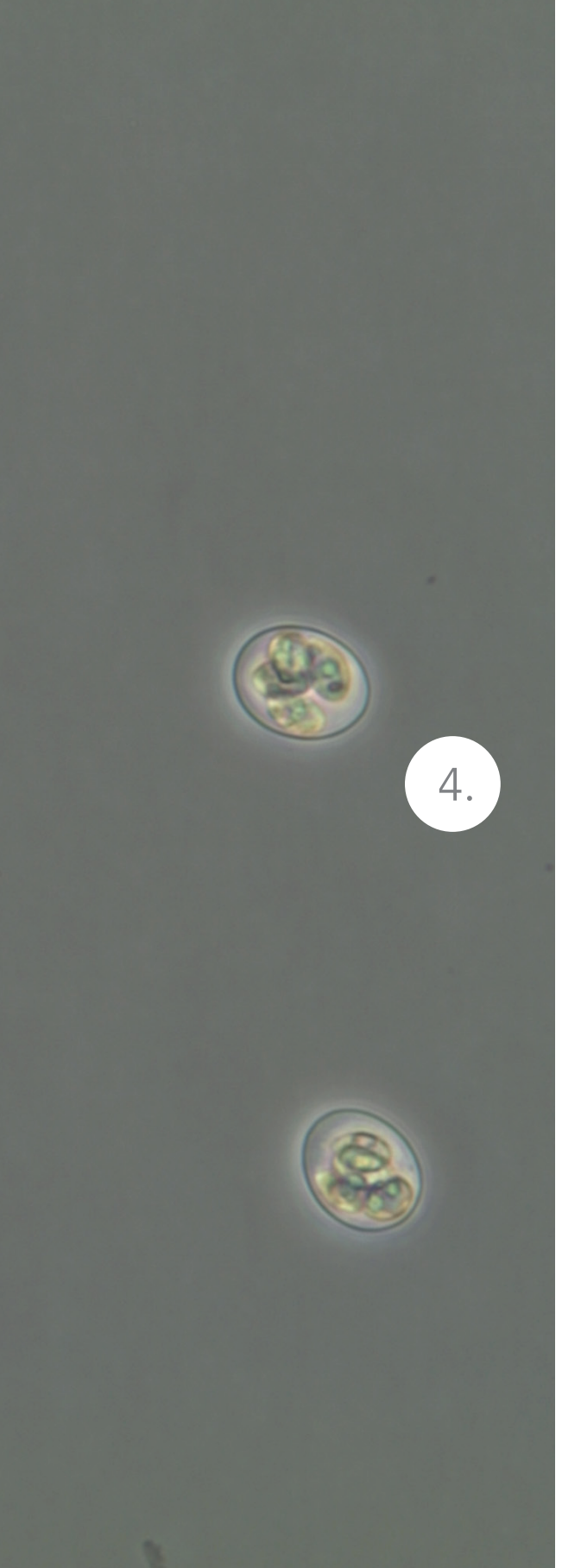
Además de los linfocitos, la inmunidad celular es expresada en otros tipos celulares propiamente relacionadas con el sistema inmune como por ejemplo, los eosinófilos, las células dendríticas y las células NK, y también en células en las que se desarrollan determinados estados del desarrollo endógeno de algunas especies de *Eimeria,* por ejemplo, las células endoteliales.

Los eosinófilos, a los cuales se hizo referencia anteriormente al abordar la respuesta inmune innata, también se han descrito como importantes componentes de la respuesta adquirida en la infecciones por protozoos de phylum Apicomplexa. Así, en pavos primoinfectados y reinfectados por *E. adenoeides* **(Gadde y cols, 2009**), en los que se evaluó la respuesta de los leucocitos frente a la infección, se observó que con la excepción de los eosinófilos en día 11 post-infección, las proporciones de los otros leucocitos no era diferente entre pavos infectados y no infectados. El aumento de la concentración y las proporciones de eosinófilos en la sangre sugirieron el reclutamiento y la participación de estas células en la fase efectora de la respuesta inmune. Debido a que los eosinófilos son conocidos, en general, por estar involucrados en la eliminación de parásitos extracelulares, su reclutamiento en la respuesta a la infección por *Eimeria* no es sorprendente, siendo un reflejo de la actividad inmune inflamatoria antiparasitaria **(Abbas y cols., 2007)**. Como ya se mencionó, los eosinófilos son células mieloides diferenciadas, que se desarrollan en la médula ósea bajo la influencia de un número variado de factores de transcripción, incluyendo el factor 1 de unión al factor de transcripción eritroide y citoquinas como la IL3, IL-5 y el factor estimulantes de colonias de granulocitos y macrófagos **(Khoury y cols., 2014)**. Atendiendo a la respuesta adquirida, la Il-5 es la citoquina clave que media la liberación de los eosinófilos al torrente sanguíneo y datos recientes sugieren que la secreción local de IL-5 podría tener un papel importante en su acumulación en los tejidos **(Nussbaum y cols., 2013)**.

Por otro lado, también se ha demostrado que las **células NK** tienen la capacidad de proteger al hospedador mediante la producción de citoquinas inflamatorias, que influyen de manera decisiva en el desarrollo de respuestas inmunes adaptativas **(Vivier y cols., 2008)**. Además, en las infecciones causadas por parásitos intracelulares aumenta la producción de INF, lo que confiere un estado de alerta a las células próximas, a la vez que se activan las células NK por mediación de la IL-12. Como se indicó previamente, las células NK son un componente importante de la inmunidad innata frente a especies de *Eimeria* aviares **(Lillehoj, 1994)**, así como frente a otros Apicomplexa como *C. parvum* **(Barakat y cols., 2009)**, pero su papel en la respuesta adquirida frente a la coccidiosis no está completamente aclarado

Por último, las **células endoteliales** se consideran células inmunorreactivas, es decir, capaces de participar activamente tanto en las respuestas inmune innatas como en las adquiridas. En las células endoteliales se sintetizan moléculas inmunorreguladoras, entre ellas factores del crecimiento, citoquinas, quimioquinas, moléculas de adhesión, factores del complemento, etc. **(Taubert y cols., 2006)**. La

expresión endotelial de las moléculas de adhesión y de las quimioquinas cambia cuando la inflamación aguda progresa a crónica, lo que da lugar a la extravasación de diferentes células efectoras y el paso de respuesta inmune hacia una inmunidad específica. Algunas de estas moléculas, como las citoquinas TNF-α y IL-1, son eficaces en la estimulación de la expresión de genes pro-inflamatorios en diversas células del sistema inmune, especialmente en reinfecciones **(Dinarello, 2000**). Además de las citoquinas pro-inflamatorias, las células endoteliales son capaces de producir citoquinas anti-inflamatorias como (IL-1ra), IL-10, IL-13 y TGF-β **(Kofler y cols., 2005)**, que tienen la capacidad de bloquear el inicio del proceso desencadenado por las citoquinas pro-inflamatorias, o bien la progresión de la cascada inflamatoria.



4.

PRESENTACIÓN DE ARTICULOS

ARTÍCULO Nº 1

Aislamiento e infectividad en cabritos de una cepa de *Eimeria ninakohlyakimovae* en Gran Canaria (España)

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▪ RESUMEN▪

La coccidiosis caprina puede afectar al 100% de los cabritos entre las 4 y las 10 semanas, y causar graves pérdidas económicas al afectar la salud animal y la rentabilidad de la industria caprina. Estudios realizados en las Islas Canarias, donde la industria caprina representa un recurso importante para los ganaderos, indican que, dentro de las especies más frecuentes de *Eimeria* en caprinos, *E. ninakohlyakimovae* se encuentra entra las de la mayor patogenicidad. *E. ninakohlyakimovae* infecta las células endoteliales en la primera esquizogonia y es capaz de producir una enfermedad intestinal grave caracterizada por diarrea catarral, pérdida de peso, deshidratación y retraso en el crecimiento. La alta prevalencia y la severa patogenicidad de esta particular especie de *Eimeria* hacen de ella un modelo ideal para el estudio de la respuesta inmune del huésped y los mecanismos de patogenicidad que se desencadenan en el sistema caprino.

El presente estudio tuvo como objetivo el aislamiento de una cepa de origen caprino de *E. ninakohlyakimovae* definida y la realización de infecciones experimentales para valorar su grado infectividad, patogenicidad y capacidad de desarrollar una respuesta inmune en los hospedadores. La disponibilidad de una especie de *Eimeria* con estas características podría permitir la realización de estudios experimentales de investigación tanto básica como aplicada sobre coccidiosis en el ganado caprino, que a su vez podrían constituir la base de futuras estrategias de profilaxis y control de la enfermedad.

Los ooquistes se obtuvieron inicialmente a partir de animales infectados de forma natural con *E. ninakholyakimovae*. Una vez que se confirmó que las heces eran positivas para esta especie, las muestras se mezclaron con dicromato potásico ($K_2Cr_2O_7$), se filtraron para eliminar los residuos de mayor tamaño, se dispensaron en placas de Petri formando una capa fina y se mantuvieron a temperatura ambiente (25°C) durante una semana hasta la esporulación. A partir de flotaciones con ClNa de los cultivos se recogieron los ooquistes esporulados con una micropipeta utilizando un microscopio de micromanipulación y posterior confirmación de la especie mediante un microscopio óptico.

Estos ooquistes se emplearon para infectar experimentalmente cabritos que fueron adquiridos con 1-4 días y mantenidos bajo condiciones libres de parásitos. Los cabritos fueron separados en tres grupos (n = 3). En dos ensayos diferentes se evaluó la inmunoprotección conferida por primoinfecciones (Grupo I: S4PI) y posterior reinfección con ooquistes de *E. ninakohlyakimovae* (Grupo II: S7RI). Como control de infección se utilizaron animales no infectados (Grupo III: C). El nivel de inmunoprotección se evaluó en base a parámetros productivos (peso corporal), clínicos, parasitológicos (recuentos de ooquistes en heces) e histopatológicos.

El periodo prepatente, tanto en animales primo- como reinfectados, osciló entre 14-15 días, siendo mayor la proporción de cabritos que comenzaron a liberar ooquistes en heces a los 15 días post-infección. En su conjunto, el número de ooquistes por gramo de heces fue menor en los animales reinfectados (grupo II) que en el correspondiente control de reinfección (Grupo I). Con respecto a los parámetros de producción, el grupo I mostró tasas de reducción de crecimiento entre las 5 y 9 semanas en comparación con controles, mientras que el grupo II tales diferencias se encontraron entre las 8 y 9 semanas. La tasa de crecimiento global fue significativamente mayor en el grupo control III en comparación con cualquiera de los animales infectados.

A pesar de la gravedad de los signos clínicos, las alteraciones hematológicas no fueron tan sorprendentes como sería de esperar, sino relativamente moderadas. Los recuentos de leucocitos sanguíneos demostraron indicios claros de eosinofilia, así como un mayor número de monocitos en cabritos durante la infección primaria, en concreto a las 7 semanas. Sin embargo, las alteraciones leucocitarias no fueron significativas. A nivel anatomopatológico, se encontraron lesiones inflamatorias con extensas de infiltración eosinofílica en la mucosa intestinal e infiltración difusa de otras poblaciones celulares.

La reducción significativa de los recuentos de OPG en los animales reinfectados por *E. ninakohlyakimovae* en comparación con los controles de infección, así como de la gravedad clínica de la enfermedad proporcionan claras evidencias de que la cepa GC es apta para inducir respuestas inmunes protectoras, un fenómeno bien documentado para otras especies de *Eimeria*. Estos datos indicarían que la cepa específica de *E. ninakohlyakimovae* aislada en Gran Canaria (GC) podría ser empleada para futuros estudios terapéuticos o inmunológicos en la coccidiosis de caprina.

Isolation of an *Eimeria ninakohlyakimovae* field strain (Canary Islands) and analysis of its infection characteristics in goat kids

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Abstract

The current study was conducted to isolate a field strain of *Eimeria ninakohlyakimovae*, characterize its infectivity and the response to challenge under experimental conditions. The isolated strain (GC) induced a prepatent period of 14-15 days p. i., a patency of 7 ± 2 days and a noticeable pathogenicity in infected goat kids. Challenge trials resulting in a decrease of oocysts per gram counts as well as a milder intensity of clinical signs in re-infected animals indicated the capacity of this strain to induce protective immune response. Altogether, the data reported in the present study suggest that the strain *E. ninakohlyakimovae* GC is a useful tool for the investigation of mechanisms of pathogenicity as well as host protective immune response in caprine coccidiosis, representing a valuable prerequisite for the development of future strategies in prophylaxis and control of this important parasitic disease in goat.

Key words: *Eimeria ninakohlyakimovae* - Apicomplexa - Coccidiosis - Caprine

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1. Introduction

Infections with different subspecies of the apicomplexan genus *Eimeria* represent one of the most common parasitoses in goat productive systems worldwide (Koudela and Boková, 1998). Depending of the mode of management, caprine coccidiosis may affect 100% of goat kids within the age range of 4-10 weeks, and cause severe economic losses by affecting animal health and profitability of the goat industry (Koudela and Boková, 1998; Ruiz et al., 2010). Reports for the Canary Islands (Spain), where the goat industry represents an important resource for farmers (Fresno et al., 1994), indicate the most frequent species of *Eimeria* in caprine flocks are *Eimeria arloingi* Marotel 1905, *E. ninakohlyakimovae* Yakimoff and Rastegaieff 1930 emend Levine 1961, *E. alijevi* Musaev 1970 and *E. caprina* Lima 1979 (Ruiz et al., 2006). Within this spectrum, *E. ninakohlyakimovae* showed the highest pathogenicity in parasitized animals, especially in goat kids (Ruiz et al., 2006). Analogous to the bovine system, where the most pathogenic Eimeria species infect endothelial cells and develop into macromeronts (Vieira et al., 1997), E. ninakohlyakimovae also resides in this peculiar location and shows similar developmental features (Ruiz et al., 2010) leading to severe intestinal disease characterized by catarrhal diarrhoea, weight loss, dehydration and stunted growth (Koudela and Bokova, 1998).

In contrast to other well characterized eimerian infections in the murine (Shi et al., 2001; Al-Quraishy et al., 2011), avian (Rothwell et al., 2004) and bovine (Hermosilla et al., 1999, 2008; Taubert et al., 2008, Sühwold et al., 2010) system, caprine eimerian infections have been neglected in the past and only limited numbers of studies were conducted on this topic. These reports are mainly restricted on some investigations concerning the biology or the pathogenic effects of *E. ninakohlyakimovae* in countries such as Brasil (Vieira et al., 1997) or China (Dai et al., 2006). The high prevalence and the severe pathogenicity of this particular *Eimeria* species in the Canary Islands suggest *E. ninakohlyakimovae* as an ideal model for the study of the host immune response and the mechanisms of pathogenicity in the caprine system.

In general, the termination of *Eimeria* spp. primary infections as well as the control of homologous challenge infections is based on host cellular adaptive immune reactions (Sühwold et al., 2010). Several investigations dealing with rodent model systems suggest that responses to primary infections are predominantly controlled by $CD4^+$ T cells with Th1-associated T cell reactions being key to the control of primary infection, whilst cytotoxic $CD8^+$ T cells seem to be the major effector cell type against challenge infections (Rose et al., 1992; Findly et al., 1993; Ovington et al., 1995; Smith and Hayday 2000; Shi et al., 2001). However, data generated in rodent models may only be of limited value for the ruminant system, as most of the pathogenic *Eimeria* species in ruminants (e. g. *E. bovis* Zublin 1908, *E. zuernii* Rivolta 1878, *E. bakuensis* Musaev 1970, *E. arloingi*, *E. ninakohlyakimovae*) develop differently from the rodent ones with respect to primary host cells, the formation of macromeronts and duration of replication, i. e., features that will potentially influence developing immune responses. In addition, cellular adaptive immunity against *Eimeria* spp. is reported as a species specific (Rose 1987) or even strain specific reaction (Shirley and Bellatti 1988; Fitz-Coy 1992; Martin et al., 1997; Smith et al., 2002). In consequence, protective cross immunity is rare giving reasons for immunological studies performed on defined individual *Eimeria* species in the respective host. Owing to the current lack of investigations on cellular immune responses against *Eimeria* spp. affecting goats, there is an urgent need for defined caprine eimerian strains for basic research and for the development of strategies on prophylaxis and control of the disease.

The present study aims to isolate a defined strain of *E. ninakohlyakimovae* from the field and to evaluate its infectivity, pathogenicity as well as the development of protective immunity in the caprine host.

2. Material and methods

2. 1. Animals

Majorera breed goat kids were purchased from a local farmer at the age of 1-4 days, treated with a single dosis of 1mg/kg b.w. diclazuril (Vecoxan®, Janssen-Cilag) and halofuginone (Halocur®, Intervet) during 5 consecutive days at dosis of, assessed for parasitic infections and, when deemed parasite free, maintained under parasite-free conditions in autoclaved stainless steel cages until exposed to experimental *E. ninakohlyakimovae* infections. Goat kids were fed with substitute (Bacilactol®, Capisa) and commercial concentrate pellets (Starting Concentrate®, Capisa). Water and sterilized hay were given *ad libitum.* A total of eight goat kids were employed for the isolation of the *E. ninakohlyakimovae* strain and additional 9 goat kids were used for the experimental studies on infectivity, pathogenicity and immune response of the new strain.

2. 2. Parasite

For the isolation of a defined *E. ninakohlyakimovae* strain, oocysts from naturally infected animals were collected according to the previously described protocol by Silva and Lima (2000), with minor modifications. Given that faecal samples contained *E. ninakohlyakimovae* oocysts at > 90% purity, samples were subjected to a flotation process (using saturated sodiumchloride solution (Panreac), 1.19 g/l density, 40 min RT). Oocysts were collected by flotation and suspended in 2% potassium dichromate (w/v) solution (Merck) according to Hermosilla et al. (2002). Sporulation of oocysts was achieved by leaving this suspension at room temperature (25 °C) and stirring the oocyst suspension daily to infuse oxygen. Thereafter, individual sporulated oocysts, diagnosed microscopically as *E. ninakohlyakimovae* (Levine and Ivens, 1986, Alyousif et al., 1992, Soe and Pomroy, 1992), were collected using a micromanipulator (Olympus ITM-2). A total of 2 x 10^4 of sporulated *E. ninakohlyakimovae* oocysts were isolated by this technique and resuspended in 2% potassium dichromate (w/v). All oocysts were administrated orally to one goat kid at the age of four weeks. Throughout patency faecal samples were collected and respective oocysts were isolated as described below. In total, three consecutive goat kid infections were performed in order to achieve a 100% pure *E. ninakohlyakimovae* strain.

For harbouring oocysts, goat kids were orally infected at the age of 4 weeks with 2 x 10^5 sporulated *E. ninakohlyakimovae* oocysts. Excreted oocysts from experimentally infected animals were obtained from faeces collected after two weeks post infection according to the method of Jackson (1964). Briefly, the faeces were washed with tap water using decreasing pore size sieves for elimination of debris. For flotation, the resulting oocyst suspension was mixed 1:1 with saturated sugar solution (1.5 g/l density), transferred to rectangular plastic bowls and incubated for 2h at RT. Oocysts were collected by applying glass plates (25 x 25 cm) to the surface of the sugar/oocyst suspension. Oocysts attached to the glass plates were washed from the plates by rinsing with Aqua dest, concentrated by centrifugation (1,100 x *g*, 10 min), and treated to induce sporulation as described above. The resulting sporulated *E. ninakohlyakimovae* oocysts were stored at 4 °C in 2% potassium dichromate (w/v) until further use.

2.3 Experimental design of the challenge infection trial

For experimental infections, goat kids were separated into three groups (n=3) and kept under parasite-free conditions. In two independent experiments, the

immunoprotection induced by a primary infection was evaluated by the challenge infection with the same dose of *E. ninakohlyakimovae* oocysts. The level of immunoprotection was evaluated by productive (body weight), clinical (presence of clinical signs of coccidiosis, e. g. diarrhoea, variations within blood parameters), parasitic (oocyst counts in faeces) and histological (macro- and micro-lesions in the intestine at the necropsy) parameters. Four week-old goat kids were primary infected by the oral inoculation of 2 x 10^5 sporulated *E. ninakohlyakimovae* oocysts per animal (W4PI) and challenge infected three weeks later (W7RI) by applying the same dose of oocysts (group I = challenge infected animals). Goat kids primary-infected at 7 weeks of age (W7PI) served as challenge-infection controls (group II = primary infected animals), and non-infected animals were used as negative controls (group III). Measurement of the body weight was performed weekly. At 10 weeks of age, all animals were euthanized by using sodium pentobarbital and subjected to further pathological analyses.

2.4. *Coprological, pathological and histopathological analyses*

For coprological analyses, faecal samples were obtained daily on 13-21 days p. i. (in both primary- and challenge-infections). The non-infected controls were also subjected to coprological analysis in order to verify the absence of infection. The counts of oocysts per gram (OPG) in the faeces were determined in all groups of primary- and challenge-infected animals by the modified McMaster technique (Maff, 1989). Faecal samples were obtained rectally and subjected immediately to OPG analyses.

For haematological analyses, blood samples were taken weekly by puncture of the *Vena jugularis*. Some additional blood samples were taken on days 16-17 p. i., coinciding with the highest peak of oocysts being shed. Total leukocyte counts and analysis of haemoglobin concentration were determined by using the haematological Lasercyte® (IDEXX). The values of packed cell volume (PCV) were calculated according to standard procedures by centrifugation of capillary tubes in a microhaematocrit centrifuge. Differential blood counts were performed manually by analysing 200 leukocytes in blood smears stained by Diff-Quick (QCA Labs.).

After euthanasia of the goat kids, all animals were subjected to necropsy. All macroscopic lesions were annotated and tissue samples were collected from the intestinal mucosa (ileum, colon) and from the mesenteric lymph nodes. The tissue samples were fixed in 10% formalin and embedded in paraffin. Cross sections of 4-5 µm were stained by haematoxylin and eosin (H&E) according to standard staining procedures and the samples were analysed microscopically.

2.5. *Statistical analyses*

Faecal oocyst counts were logarithmically transformed and added by one (log [OPG + 1]). For the estimation of bodyweight improvements, the data were expressed as growth rate (*ln weight 2 – ln weight 1)/t*100*), with "t" representing the number of days between the sampling time points 1 and 2. The data were analyzed using the Mann-Whitney rank sum test (SigmaStat 2.03).

3. Results

3.1. Morphological characteristics of *Eimeria ninakohlyakimovae* GC strain.

The oocysts of *E. ninakohlyakimovae* were a medium size of 22.5 x 16.7 µm with values varying from 16.3 x 28.4 µm (longitudinal diameter) and 12 x 20.8 µm (transversal diameter). The morphology of the oocysts was round to slightly oval; the micropyle was manifested weakly and the micropyle capsule was absent (Fig. 1). The duration of the sporulation process at room temperature (22-25 °C) varied from 48-96 h. The sporocysts showed an elongated morphology, with a mean size of 11.6 x 6.3 µm. Within the *E. ninakohlyakimovae*-sporocyst a Stieda body was detected. In addition, residual bodies and

polar granules were observed within sporocysts. The inner layer of the oocyst wall had a shiny red-orange colour and dispersed granulation was frequently observed in between the sporocysts (Fig. 1).

Figure 1. Sporulated oocysts of *Eimeria ninakohlyakimovae* GC after incubation at room temperature for one week in 2% sodium dichromate (A, B, C). Details of the Stieda body and dispersed granulation in between the sporocysts are marked by arrows in images B and C, respectively.

Primary infected animals (group II) scarcely had parasitic stages in the examined tissue samples of ileum and colon, probably because necropsy was performed at the post-patent period, when most of the parasites had already finished their endogenous development; only some sexual stages of the parasite could be found as depicted in Fig. 2A and 2B. The same was observed in challenged goat kids, although in this case several macroschizonts were encountered in the ileum mucosa, some of them forming

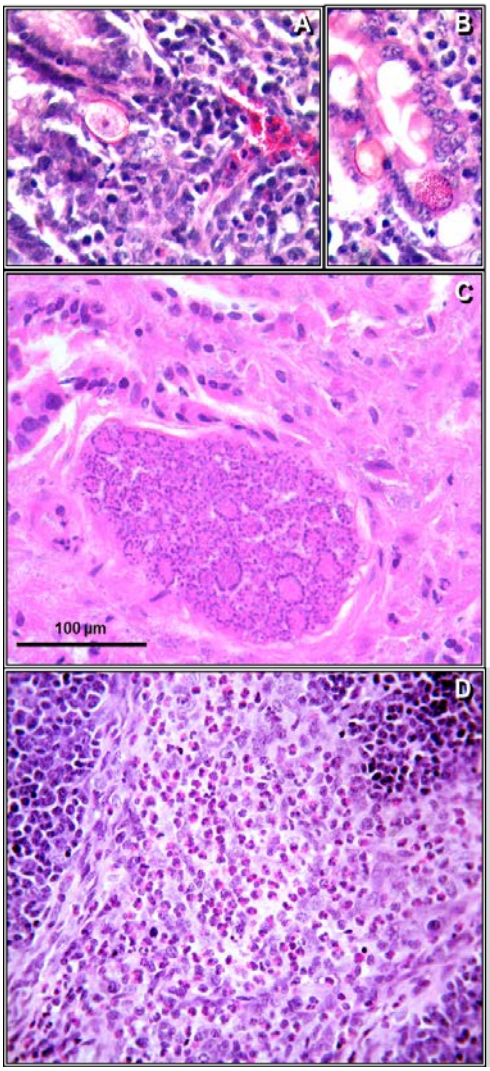


Figure 2. Histological sections of goat kids orally infected with 2 x 10^5 sporulated oocysts *Eimeria ninakohlyakimovae* GC and sacrifized 3 weeks later showing sexual stages of the parasite (oocysts and macrogametes) in the colon (A, B) and a segmented macroschizont in ileon mucosa (C). Fig. 2D represents a profuse infiltration of eosinophils at the ileum interstitium in one of the challenge infected animals.

compartments in the interior of the macromeront (Fig. 2C). Most of them were mature macroschizonts, measured 186.2 (117.3-320.1) x 132.6 (61.6-176.9) µm and contained a large number of merozoites.

3.2. Characterization of *E. ninakohlyakimovae* (GC strain) in primary and challenge infections

The prepatent period in both primary- and challenged-infected animals varied from 14-16 days, with most of the animals (68.4%) beginning to shed oocysts at 14 days p. i. Irrespective of the age of goat kids, no significant differences were observed between primary- and challenge-infected animals referring to the time point of patency onset.

Patency of *E. ninakohlyakimovae* infection lasted from 4 to 10 days in primary infected animals and from 1 to 8 days in challenged ones. The oocyst shedding of primary-infected animals at 4 weeks of age (group I, W4PI) started at 14 days p. i. and reached highest levels at 17-18 days p. i. Thereafter, oocyst shedding gradually decreased and ceased between days 19 and 22 p. i. In challenge infected animals (group I, W7RI), the faecal oocysts counts were significantly lower than in the challenge control group II (W7PI) between days 14 to 18 p. i. ($P < 0.05$), with an overall reduction of the OPG counts of 99.9% ($P < 0.05$) during the whole experiment (Fig. 3). Approximately the same reduction was achieved when OPG from reinfected goat kids of group I (W7RI) were compared with those recorded during the corresponding primary infection (W4PI) (99.8%, $P < 0.05$) (Fig. 3). The maximum faecal count in primary infected animals was 10.1 x 10^6 OPG, whilst in challenged goat kids a maximum of 1.9 x 10^4 OPG was detected. No oocyst shedding was observed in the negative control animals (group III).

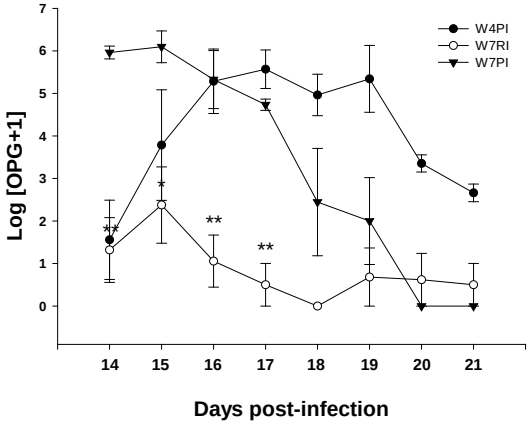
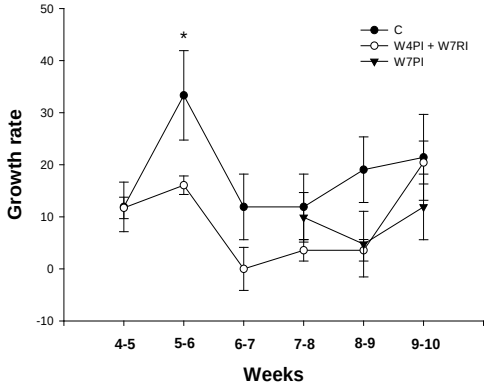


Figure 3. OPG (oocysts per gram of faeces) counts of goat kids orally infected at week 4 of life (W4PI) with 2 x 10^5 sporulated oocysts *Eimeria ninakohlyakimovae* GC and challenged three weeks later with the same dose (W7RI). Triangles represent the OPG counts of animals primary infected with 2 x 10^5 sporulated oocysts at week 7 of life (W7PI). OPG counts are depicted as the logarithm of the OPG plus one (log [OPG + 1]) and represent the mean ± SEM in all the experimental groups. (*) *P<0.05* and (**) *P<0.01* represent significant differences between primary (W7PI) and challenge infection (W7RI).

With respect to production parameters, group I (primary plus challenge infected goat kids, W4PI+W7RI) showed reduced growth rates between weeks 5 and 9 when compared to negative controls, whilst group II (challenge control animals) showed decreased growth rates exclusively between weeks 8 and 9 of the experiment (Fig. 4A). The overall growth rate was significantly higher in control group III compared to either primary or challenged infected animals ($P < 0.05$) (Fig. 4B). The highest differences in growth rates were detected one week p. i. in both group I and II with respect to control group III, indicating a reduction of body weight of approximately 15% in both cases (Fig. 4A).



B

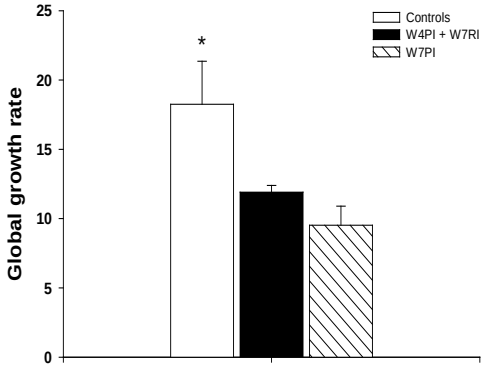
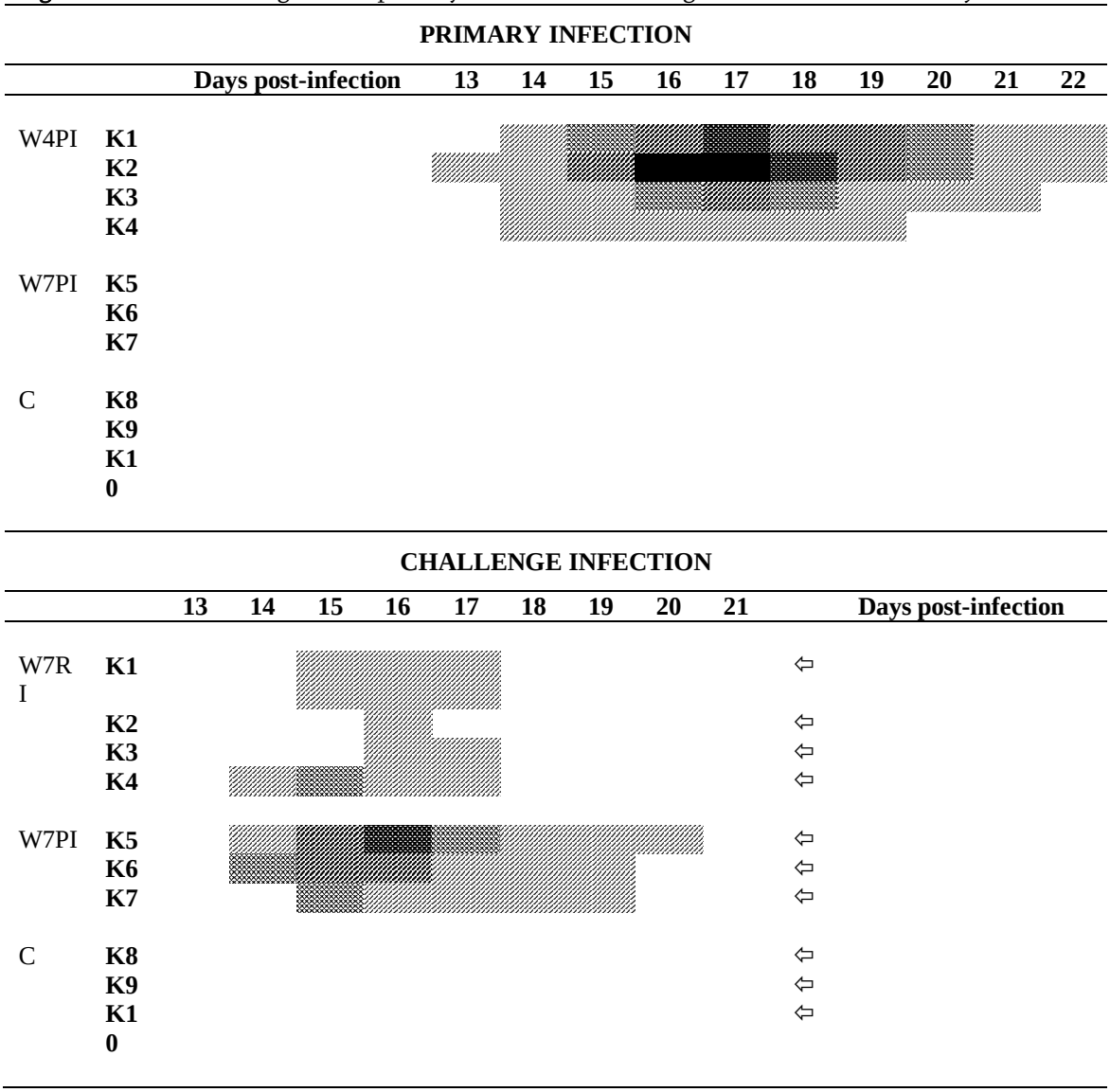


Figure 4. Growth rates (*ln weight 2 – ln weight 1)/t*100*) in goat kids orally infected at week 4 of life (W4PI) with 2 x 10^5 sporulated oocysts *Eimeria ninakohlyakimovae* GC and challenged three weeks later with the same dose (W7RI). Goat kids primary infected with 2 x 10^5 sporulated oocysts at week 7 of life (W7PI) were used as challenge controls and group C as uninfected controls. The rate of growth rates amongst different sampling times and global growth are depicted on Fig. 2A and 2B, respectively. Data represent the mean ± SEM in all the experimental groups. (*) *P<0.05* represents significant differences between infected *vs* uninfected control animals.

Although individual variations of clinical signs were apparent, all 4-weeks old primary infected animals showed clinical signs compatible with coccidiosis such as diarrhoea, dehydration, deterioration of the general animal condition, anorexia and, occasionally, prostration. Different degrees of faecal texture were observed, ranging from paste-like to completely liquid and sometimes explosive diarrhoea containing blood and pieces of mucosa (Table 1). More severely affected animals during primary infection with *E. ninakohlyakimovae* needed rehydration, which was performed by oral or subcutaneous saline application (Ringer Lactate, BRAUN), and vitamin treatment (Amicen's Plus, CENASA). Clinical signs were observed in parallel to the onset of oocyst shedding from 13-14 days p. i. which continued for 6-10 days. After challenge infection, the clinical signs were significantly less pronounced (Table 1), in some cases not even apparant. However, when comparing the 4-weeks-old primary-infected animals (group I – W4PI) with 7-weeks-old primary-infected animals (group II– W7PI), it appeared that older *E. ninakohlyakimovae*-infected animals showed milder clinical signs of coccidiosis (Table 1).

As to blood parameters, the total protein serum level were moderately reduced in primary-infected goat kids (groups I and II) within days 14-17 p. i. (Table 2). No significant changes were observed in the red blood cell fraction, although a moderate increase of PCV was detected 14-17 days p. i. in primary infected animals (group I and II), coinciding with the patency of primary-infected animals. Blood haemoglobin content and total leukocyte counts did not reveal any significant change during *E. ninakohlyakimovae* infection (Table 2). However, in animals primary infected at 7 weeks of age (group II – W7PI) a sustained eosinophilia was detected from 7 days p. i. onwards (Table 2). Counts of monocytes and

Fig. 1 Faecal scores of goat kids primary infected and challenged with *Eimeria ninakohlyakimovae*.



Individual faecal scores of goat kids (K) primary infected at week four of life with 2 x 10^5 sporulated oocysts of *E. ninakohlyakimovae* (W4PI) and challenged (W4RI) three weeks latter with the same dose are represented. Three animals primary infected at week 7 of life were used as challenge controls and three uninfected kids (C) served as controls of infection. The arrow (⇦) point to the day of sacrifice and autopsy of the animals. The clinical evaluation of the diarrhoea was determined using the following score:

- Clean normal
- Dried soiling normal
- Wet soiling diarrhoea
- Diarrhoea down legs
- Explosive diarrhoea
- Fluid, watery or bloody diarrhoea

lymphocytes varied irregularly and PMN counts appeared unaffected, although neutrophil bands were occasionally observed in some of the infected animals (Table 2). Transient increase in eosinophil counts were also recorded for challenged animals, whilst almost no changes were recorded in goat kids primary infected at 4 weeks of age. In general, the reactions observed both in the red and white series were weak or moderate, and the differences were not statistically significant.

At necropsy, the intestines of infected animals showed no relevant macroscopic alterations. Histology revealed a moderate hyperplasia of the intestinal epithelium and hypertrophy of the mesenteric lymph nodes and Peyer's patches (PP). Futhermore, a clear eosinophilic enteritis (Fig. 2D) and a diffuse infiltration of mast cells, lymphocytes and PMN was detected. Parasitic stages found in the large intestine of affected animals corresponded mainly to the sexual stages of *Eimeria*, and were mainly represented by immature and mature oocysts. Control animals did not show significant morphological changes.

4. Discussion

In this investigation we isolated a field strain of *E. ninakohlyakimovae* from the Canary Islands (Spain) that exhibited morphological and biological features previously described for this caprine *Eimeria* species (Alyousif et al., 1992; Levine and Ivens, 1986, Vieira et al., 1997). Furthermore, we demonstrated that the *E. ninakohlyakimovae* GC strain is capable to cause coccidiosis in goat kids and to induce protective immune responses against challenge infections.

The pathogenicity of *E. ninakohlyakimovae* GC was clearly demonstrated by the intensity of clinical signs. One of the most evident clinical effects was the diminished body weight gain in kids. Overall, the growth rates over time in primary infected and challenged animals were 9.5 ± 1.4 and 11.9 ± 0.5, respectively, in relation to uninfected controls (18.3 ± 3.1), which means a reduction of about 10% and, in consequence, a substantial economical loss for the farmers. Reduced body weights and a retarded physical development of infected animals are common features observed in coccidiosis (Fox, 1985; Alyousif et al., 1992; Daugschies et al., 2007) and are of economic importance for farmers worldwide. This clinical consequence of caprine coccidiosis may affect goat production in arid/semiarid areas even more profoundly than elsewhere, as goats are the only and ultimate source for milk and meat available. It is assumed that even subclinical ruminant coccidiosis can alter the conversion indices and, because of the frequency of subclinical coccidiosis, the economic losses might be higher than the ones caused by clinical coccidiosis (Fox, 1985). It can be easily be assumed that the impact of *E. ninakohlyakimovae* coccidiosis on productive parameters could be even more important under field conditions, especially in caprine farms with low hygienic and management standards, as shown previously for other ruminant *Eimeria* spp. (Daugschies and Najdrowski, 2005, Daugschies et al., 2007).

The clinical signs generally corresponded to those previously reported in naturally infected goat kids (Koudela and Bokova, 1998), as well as in experimental infections with different inoculum sizes (Dai et al., 2006). In the later study, infection doses higher than 1 x 10^6 sporulated oocysts per animal resulted in a more severe clinical signs of infected animals, nevertheless, no deaths were reported. This observation is somewhat contradictory to our findings, as – using only 1/5 of the dosage – some of the *E. ninakohlyakimovae*-infected animals developed such a severe clinical disease that an immediate emergency treatment was necessary for survival. These findings may indicate differences in the pathogenicity of strains as the *E. ninakohlyakimovae* GC strain appears more pathogenic than the Chinese strain used by Dai et al. (2006). Similar features have previously been reported for diverse strains of avian *Eimeria* species (Li et al., 2004, Loo et al., 2010).

The onset of clinical manifestations coincided with patency and was most severe between days 16-18 p. i. in primary-infected animals which may reflect the massive host epithelial cell destruction in the caecum and colon (Vieria et al., 1997). The patency of the GC strain was rather short when compared to other experimental *E. ninakohlyakimovae* infections, where patency and clinical signs were observed for a minimum of 10 days when using an infection dose of 1×10^5 (Dai et al., 2006).

Despite the severity of the clinical signs, haematological alterations were not as striking as would be expected, but were also found rather moderate in other *E. ninakohlyakimovae* experimental infections (Dai et al., 2006). Similar observations were also made in the ovine system (infections with *E. ovinoidalis* and *E. faurei,* Chapman, 1974; Shumard, 1957). However, it has to be kept in mind, that effects on haematological parameters may have been distorted by the rehydration therapy conducted in this study for the benefit of the animals. In accordance, the total serum protein levels also did not change significantly when compared to uninfected controls and may additionally be based on the fact that the decrease of serum albumin was compensated by the increase of plasma globulin levels (Chapman, 1974). The slight increase of PCV values observed in primary *E. ninakohlyakimovae*-infected animals corresponds well with previous reports on ruminant coccidiosis (Shumard, 1957; Chapman, 1974; Dai et al., 2006), and was explained as a decrease of the total blood volume in circulation as a result of blood losses during the haemorrhagic phase of diarrhoea.

As regards blood leukocyte counts, we found clear indications of an infection-induced eosinophilia, as well as increased numbers of monocytes in goat kids experiencing primary infection at the age of 7 weeks. However, the fact that these alterations of leukocyte numbers were not significant in younger goat kids (infected at the age of 4 weeks) may indicate the relevance of the developing immune competence and may consequently be linked to less severe clinical disease and a reduced phase of oocyst shedding detected in the older goat kids primary-infected group. In accordance with other reports (Dai et al., 2006), eosinophilia - in addition to hyperplasia of epithelial cells and hyperplasia of reactive mesenteric lymph nodes - was reflected in inflammatory lesions showing eosinophilic infiltration of the gut mucosa and confirmed common assumptions in parasitic infections.

Primary infections with the GC strain of *E. ninakohlyakimovae* led to protection of goat kids. Thus, the significant reduction of OPG counts in *E. ninakohlyakimovae*-challenged infected goat kids compared to infection controls, as well as the amelioration of clinical disease provides clear evidence that the GC strain is capable to induce protective immune responses, a phenomenon well documented for other closely related *Eimeria* species in cattle (Hermosilla et al., 1999; Taubert et al., 2008), chicken (Rothwell et al., 2004) and rodents (Shi et al., 2001). However, immunoprotection induced by *E. ninakohlyakimovae* GC strain was

Table 2. Haematological parameters in *Eimeria ninakohlyakimovae* primary- and challenged-infected goat kids.

	PCV %	HGB g/dl	WBC cells/µl	NEU cells/µl	BAN cells/µl	LYM cells/µl	MONO cells/µl	EOS cells/µl	TP g/dl
***Group I* – W4PI (primary infection at 4 weeks of age)**									
0 dpi / W4	26.3	8.4	11752.5	6177.5	0.0	5363.2	101.5	110.3	4.8
	(1.5)	(0.4)	(2030.3)	(1007.8)	(0.0)	(1168.2)	(87.0)	(76.0)	(0.1)
7 dpi / W5	23.8	7.2	8540.0	4691.3	0.0	3561.4	222.8	64.8	5.0
	(2.0)	(0.5)	(968.2)	(1223.5)	(0.0)	(914.6)	(71.6)	(53.6)	(0.1)
14 dpi / W6	25.0	7.8	8535.0	3691.7	0.0	4772.3	0.0	71.1	4.7
	(2.7)	(0.8)	(1260.0)	(1060.1)	(0.0)	(1108.6)	(0.0)	(49.1)	(0.3)
17dpi / W6	28.0	8.2	11952.5	4416.8	617.0	6665.8	253.0	0.0	4.6
	(3.8)	(1.3)	(5252.3)	(2344.2)	(457.8)	(2621.2)	(138.0)	(0.0)	(0.3)
21 dpi / W7	24.5	7.5	13872.5	7753.9	0.0	6045.8	0.0	72.8	4.6
	(2.5)	(0.7)	(2780.7)	(1838.1)	(0.0)	(1300.9)	(0.0)	(49.5)	(0.4)
***Group I* – W7RI (challenge infection at 7 weeks of age)**									
7 dpri / W8	24.3	7.4	10487.5	5436.3	76.0	4458.3	92.5	424.0	4.7
	(2.8)	(0.9)	(1400.3)	(1423.2)	(51.8)	(938.92)	(41.0)	(141.2)	(0.2)
14 dpri / W9	27.3	8.9	10180.0	3783.4	0.0	6069.4	0.0	327.3	4.90
	(3.6)	(0.6)	(1005.4)	547.8)	(0.0)	(878.3)	(0.0)	(108.2)	(0.2)
17 dpri / W9	27.0	8.7	9125.0	4079.2	0.0	4856.0	24.8	165.3	4.9
	(3.0)	(0.9)	(1104.1)	(294.7)	(0.0)	(908.5)	(28.6)	(58.4)	(0.2)
21 dpri / W10	27.0	9.2	9477.5	3875.5	0.0	5296.5	93.5	212.5	4.9
	(3.5)	(0.9)	(1573.4)	(946.8)	(0.0)	(1240.0)	(50.6)	(81.0)	(0.1)
***Group II* – W4PI (primary infection at 4 weeks of age: challenge controls)**									
0 dpi / W7	30.3	8.6	12796. 7	7227.8	0.0	5345.2	37.7	186.0	5.2
	(1.1)	(0.5)	(2961. 7)	(2201.8)	(0.0)	(1094.9)	(46.1)	(135.6)	(0.2)
7 dpi / W8	31.3	9.7	11096. 7	6032.7	30.0	4312.1	134.7	587.7	5.0
	(2.7)	(0.6)	(1767.5	(1908.0)	(36.7)	(630.7)	(114.3)	(250.9)	(0.14)
14 dpi / W9	37.7	10.5	10703.3	4674.3	22.0	5477.4	121.3	408.0	4.8
	(4.1)	(0.8)	(2630.3)	(1978.6)	(26.9)	(555.9)	(82.0)	(225.2)	(0.3)
17 dpi / W9	35.3	10.1	12490.0	5502.0	38.8	6141.4	108.8	699.1	4.5
	(3.9)	(0.9)	(1535.6)	(1729.9)	(47.4)	(809.2)	(133.1)	(305.2)	(0.4)
21 dpi / W10	32.0	10.2	12766.6	6354.9	0.0	5803.7	90.3	517.8	4.94
	(3.2)	(0.9)	(964.2)	(1272.9)	(0.0)	(1666.7)	(55.3)	(118.6)	(0.4)
***Group III* (uninfected controls)**									
W4	30.0	8.8	13636.6	6455.4	0.0	6585.3	0.0	295.7	4.8
	(0.7)	(0.4)	(594.7)	(1198.8)	(0.0)	(659.7)	(0.0)	(145.9)	(0.2)
W5	28.0	9.20	13396.7	6986.3	0.0	6066.7	41.0	302.3	4.8
	(2.8)	(0.5)	(1936.5)	(1706.5)	(0.0)	(1587.2)	(50.2)	(118.3)	(0.2)
W6	32.3	9.3	11510.0	5697.0	0.0	5490.7	127.3	241.0	5.1
	(1.1)	(0.4)	(1416.3)	(1635.5)	(0.0)	(555.7)	(98.5)	(111.4)	(0.11)
W7	36.3	10.9	14513.3	5410.1	0.0	8853.9	136.0	113.3	5.2
	(2.2)	(0.4)	(1790.7)	(1930.8)	(0.0)	(854.7)	(86.7)	(138.8)	(0.3)
W8	32.3	9.6	13013.3	4492.6	0.0	8257.7	0.0	263.3	5.2
	(0.8)	(0.6)	(1875.6)	(1345.6)	(0.0)	(485.6)	(0.0)	(115.6)	(0.2)
W9	31.3	9.7	13680.0	3726.1	0.0	9686.9	136.3	130.7	5.0
	(1.63)	(0.1)	(785.9)	(339.0)	(0.0)	(879.9)	(92.0)	(92.6)	(0.1)
W10	32.7	9.8	12940.0	2847.2	0.0	9780.7	0.0	312.0	5.2
	(1.8)	(0.4)	(1197.8)	(536.7)	(0.0)	(1051.4)	(0.0)	(191.7)	(0.1)

Goat kids (K) were primary infected at week four of life with 2 x 10^5 sporulated oocysts of *E. ninakohlyakimovae* (W4PI) and challenged (W4RI) three weeks latter with the same dose. Three animals primary infected at week 7 of life were used as challenge controls and three uninfected kids (C) served as controls of infection. TP: total proteins; PCV: packed cell volume; HGB: hemoglobine; WBC: while blood cells; NEU: neutrophils; BAN; band neutrophils; LYM: lymphocytes; MONO: monocytes; EOS: eosinophils. All he results are expressed as the mean ± (SEM) of different weeks of life (W), days post-infection (dpi) or days post-reinfection (dpri).

only partial, as some clinical signs - although in a clearly reduced manner - still occurred after challenge-infections, especially when higher re-infection doses were used (A. Ruiz, unpublished data). Comparable observations were made by Dai et al. (2006), who could also only achieve partial protection applying high infection and challenge doses.

Overall, we here present data on the isolation and characterization of a specific strain of *E. ninakohlyakimovae*, indicating the usefulness of this strain for future therapeutic or immunological studies in goat coccidiosis.

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ARTÍCULO Nº 2

La infección primaria en cabras por *Eimeria ninakohlyakimovae* no proporciona inmunidad protectora frente a las reinfecciones

Small Ruminant Research113 (2013) 258–266

▪ RESUMEN▪

La coccidiosis se ha considerado como una causa importante de enfermedad entérica en cabras que es potencialmente patógena para animales de todas las edades, aunque las manifestaciones clínicas son sobre todo importantes en cabritos. Afecta gravemente la rentabilidad de la industria caprina, sobre todo en zonas rurales semiáridas, regiones que dependen económicamente de la cría de cabras en muchas ocasiones.

Las coccidiosis producida por especies de *Eimeria* en rumiantes, y en otras especies hospedadoras, generalmente inducen fuertes respuestas inmunes protectoras que impiden la enfermedad clínica en reinfecciones homólogas. Sin embargo, éste y otros aspectos de la respuesta inmune desarrollada frente a especies de *Eimeria* caprinas han sido poco estudiados hasta el momento.

Los cabritos a la edad de 10-12 semanas son particularmente susceptibles a la coccidiosis por *E. ninakohlyakimovae*, lo que reflejaría una falta de inmunidad protectora, ya que la mayoría de los cabritos previamente han sufrido infecciones primarias a esa edad. La existencia de diferentes cepas virulentas con *E. ninakohlyakimovae* en combinación con una falta de inmunidad cruzada, también podría dar una explicación plausible de la falta de inmunidad protectora en las reinfecciones. Adicionalmente, esta circunstancia podría estar influenciada por otros factores tales como la intensificación de la producción, la falta de higiene en algunas granjas e incluso las condiciones climáticas.

En el presente estudio se han simulado infecciones experimentales en caprinos con altas dosis con el fin de investigar el desarrollo de reacciones inmunitarias celulares en asociación con alteraciones histopatológicas en la mucosa del intestino parasitado. Para ello, se utilizaron cabritos de edad de 1-5 días de aptitud lechera de raza Majorera que se mantuvieron libres de parásitos hasta el comienzo del estudio. Los animales se distribuyeron en tres grupos; grupo 1, infectado experimentalmente por vía oral con 2×10^5 ooquistes esporulados y reinfectados el día 21 pi con 1×10^6 ooquistes esporulados. Grupo 2, infectados en el día 0 y reinfectados en el día 21 con 2×10^5 ooquistes esporulados y Grupo 3 controles.

La excreción de ooquistes se monitorizó desde el día 14 p.i. en adelante mediante un examen fecal diario. Las muestras de sangre para hematología se obtuvieron en los días 8 y 16 después de la infección primaria y en los días 8 y 16 después de reinfección. La necropsia se llevó a cabo tanto en los animales que murieron durante la experiencia como al finalizar el estudio: 17 después de la infección. Se tomaron muestras de diferentes áreas del tracto gastrointestinal y ganglios linfáticos regionales para el análisis histopatológico.

Los recuentos de OPG fueron ligeramente más altos en los animales reinfectados con 2 x 10^5 ooquistes esporulados. Sin embargo, el cuadro clínico sufrido por los animales reinfectados con la dosis mayor fue mucho más severo, e incluso condujo a la muerte de algunos de los animales. En el análisis histopatológico se evidenció que los animales infectados presentaban una importante infiltración de eosinófilos y linfocitos en la mucosa del íleon con recuentos significativamente mayores en los animales reinfectados con 1×10^6 ooquistes esporulados. En el tejido intestinal de animales parasitados, además de infiltración celular inflamatoria, se observaron zonas de necrosis focal rodeando a las criptas y alteraciones histopatológicas severas en otros órganos (con zonas de infiltración por eosinófilos y necrosis). Se encontraron formas parasitarias en los animales infectados, desde ooquistes hasta macroesquizontes en un animal que murió durante el periodo prepatente.

En conclusión, los datos del presente trabajo demuestran que la infección primaria con *E. ninakohlyakimavae* en cabritos reinfectados posteriormente con una dosis de infección alta pueden padecer una coccidiosis aguda fatal y, en consecuencia, mostrar graves alteraciones intestinales. La excesiva respuesta inmune desarrollada frente a las etapas intracelulares de *E. ninakohlyakimovae* (esporozoitos/meronts I), tal como demuestra la elevada infiltración leucocitaria a nivel del intestino, podría haber contribuido al desenlace fatal durante la reinfección con la dosis infectante más elevada. Estos resultados enfatizan la necesidad de establecer medidas de gestión que prevengan la contaminación excesiva del ambiente con ooquistes. Por otra parte, nuestros resultados indican la importancia del fondo genético individual asociado a la capacidad de superar re-infecciones por *E. ninakohlyakimovae*. En todo caso, queda por determinar si los mecanismos de patogenicidad responsables del cuadro agudo desarrollado son consecuencia directa de las lesiones mucosas inducidas por el parásito, de una exacerbación de la respuesta inflamatoria o ambas circunstancias.

Primary infection of goats with *Eimeria ninakohlyakimovae* does not provide protective immunity against high challenge infections.

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Abstract

Coccidiosis caused by the apicomplexan protozoa *Eimeria ninakohlyakimovae* has a high impact on animal health and profitability of the goat industry worldwide. Primary *E. ninakohlyakimovae* infections induce clinical disease mainly in goat kids, as homologous re-infections are commonly under immunological control. Nevertheless, there is evidence of fatal acute *E. ninakohlyakimovae* challenge-infections to occur in goat kids. So far, the nature of the immune response promoting protection or failure in *E. ninakohlyakimovae* challenge-infected goat kids has not been investigated. Therefore, we here analyzed cellular immune responses and histopathological alterations in the gut mucosa of fatal *E. ninakohlyakimovae* challenge-infected goat kids. Overall, a severe eosinophilic enteritis was observed in affected animals. Hyperplasia of epithelial cells, hypertrophy of globet cells and marked hyperplasia of Peyer's patches and mesenteric lymph nodes draining affected areas were also relevant findings. The majority of goat kids suffering from acute *E. ninakohlyakimovae* challenge-infections showed severe diarrhea and some of them even died within 11-16 days post challenge, indicating that the mechanisms of pathogenicity might be related to the first generation macromeronts. Interestingly, infiltration of eosinophils and, in less extent, of intraepithelial lymphocytes and neutrophils was observed even within degraded *E. ninakohlyakimovae* first generation macromeronts. The excessive immune response mounted against intracellular *E. ninakohlyakimovae* stages (sporozoites/meronts I) as measured by intestinal leukocyte infiltration might result in severe pro-inflammatory reactions and contribute to the fatal outcome of the challenge infection.

Keywords: *Eimeria ninakohlyakimovae*; Challenge infection; Fatal coccidiosis; Caprine

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1. Introduction

The global goat population, which was estimated 879 million heads in 2009, experienced a percentual increase of 17.5% during the last ten years (2000-2009, FAOSTAT). This feature was particularly relevant in less developed and in developing countries with the former showing an increase of even 55.4%. Easy handling and effective conversion of limited food resources into meat and milk are key factors favouring the goat as a stock animal for farmers in poor socio-economic areas worldwide (Harper and Penzdorn, 1999). Caprine coccidiosis severely affects the profitability of goat industry particularly in rural semi-arid geographic regions depending economically on goat rearing, such as the Mediterranean Basin (Ruiz et al. 2006), Africa (Harper and Penzhorn, 1999; Kimbita et al., 2009), Asia (Faizal and Rajapakse, 2001) and Latin America (Cavalcante et al., 2011). Up to date, coccidiosis is considered as a major cause of enteric disease in goats and potentially pathogenic for animals of all ages, although clinical manifestations are mostly seen in goat kids (Koudela and Bokova, 1998; Ruiz et al., 2006).

Caprine coccidiosis is caused by monoxenic apicomplexan parasites of the genus *Eimeria*, which parasitize different parts of the small and large intestine of goats. *E. ninakohlyakimovae* is considered highly pathogenic especially in goat kids (Harper and Pentzhorn, 1999; Ruiz et al., 2006). To a lesser extent, *E. caprina* and *E. christenseni* have also been reported to induce clinical goat coccidiosis (Taylor and Catchpole, 1994). *E. ninakohlyakimovae* coccidiosis is characterized by a severe non-haemorrhagic typhlocolitis with up to 30% mortality rates in goat kids (Koudela and Bokova, 1998). In some geographic areas, more than 96% of goat kids might be affected (Ruiz et al., 2006). Goat kids at the age of 10-12 weeks are particularly susceptible to clinical *E. ninakohlyakimovae* coccidiosis, which rather reflects a lack of protective immunity than age resistance (Ruiz et al., 2006), as most of the kids had previously suffered from primary infections. The existence of different virulent *E. ninakohlyakimovae* strains in combination with a lack of cross immunity, as reported for avian *Eimeria* spp. (Abu-Akkada and Awad, 2010; Loo et al., 2010), might also give a plausible explanation for the lack of protective immunity in challenge infections. Additionally, fatal *E. ninakohlyakimovae* challenge infections might be influenced by other factors such as the intensification of production, the poor hygiene in some farms and even the climate conditions (Ruiz et al., 2006), i. e., circumstances that promote massive *Eimeria* infections (> 10^6 OPG/day) in the weaning period (personal field observations).

Coccidiosis produced by *Eimeria* spp. in ruminants (Catchpole et al., 1993) and in other host species (Shi et al., 2000) generally induces strong protective immune responses which prevent clinical disease derived from homologous challenge infections. The immune reactions developed against caprine *Eimeria* spp. have poorly been investigated, but studies conducted in cattle *Eimeria* infection indicate that both humoral (B-cell) and cellular (T-cell) responses are involved (Daugschies and Najdrowsky, 2005). In the bovine system, serum IgM, IgA and IgG2 levels negatively correlate with excretion of oocysts of *E. bovis* (Faber et al., 2002). However, T cell reactions markedly induced in *Eimeria*-infected animals are generally considered more effective than humoral responses for the development of protective immunity. In the ruminant system, the expansion of both CD4+ and CD8+ T cell subsets was demonstrated during primary *E. bovis* infection (Hermosilla et al. 1999) and recent data showing enhanced antigen-specific IFN-γ production in *E. bovis* infections in calves suggest Th1-dominated immune responses in prepatency (Taubert et al. 2008). It has also been reported that antigen-specific T cells proliferate effectively during a restricted time span during prepatency of primary infection (Hermosilla et al., 1999) but fail to do so after challenge infection suggesting early abrogation of re-

infection (Sühwold et al., 2010). Accordingly, peripheral $CD4^+$ T cells expanded during primary *E. bovis* infection, whilst after challenge neither the proportions of $CD4^+$ or $CD8^+$ T cell subsets nor those of $\gamma\delta TCR^+$ T cells were influenced. Overall, analyses of T cell infiltration into parasitized gut mucosa suggest a major involvement of $CD4^+$ T cells in the termination of primary infection and a role of both $CD4^+$ and $CD8^+$ T cells in the control of re-infections (Sühwold et al., 2010). Additionally, macrophage-, monocyte- and PMN-mediated innate immune reactions play an important role in the early immune response of coccidiosis in calves (Behrendt et al., 2008; Taubert et al., 2009).

In the present study we simulated high dose challenge infections in goat kids in order to investigate the development of cellular immune reactions in association with histopathological alterations in the parasitized gut mucosa. For this purpose, primary *E. ninakohlyakimovae* infected goat kids challenged with either a moderate or high infective dose of homologous oocysts were monitored for clinical signs, oocyst shedding and changes of leukocyte populations in the parasitized gut mucosa. The results indicate that primary-infected goat kids reared in environments with excessive infection pressure might indeed suffer from severe acute *E. ninakohlyakimovae* challenge infections. The mechanisms causing the failure of protective immune responses need further investigations but the data rather suggest an exacerbation of the cellular immune response than direct mucosal lesions caused by the parasite as cause of the fatal outcome.

2. Material and methods

2.1. Animals

Goat kids were purchased from a local farmer at the age of 1-5 days, treated with Vecoxan® (Janssen-Cilag) and Halocur® (Intervet), assessed for parasitic infections and, when deemed parasite-free, maintained under parasite-free conditions in autoclaved stainless metabolic steel cages until experimental *E. ninakohlyakimovae* infections were performed. The goat kids were fed with milk substitute Bacilactol® (Capisa) and commercial pellet concentrates (Starting Concentrate®, Capisa). Water and sterilized hay were given *ad libitum.*

2.2. Parasite maintenance

The *E. ninakohlyakimovae* strain GC used in the present study was initially isolated in 2006 from naturally infected goats in Gran Canary Island (Spain) and maintained by passages in goat kids for oocyst production according to Ruiz *et al.* (2010). Briefly, *E. ninakohlyakimovae* oocysts were isolated from the faeces by the use of a micromanipulator (Olympus IMT-2) and then suspended in 2% potassium dichromate (w/v) according to Yim *et al.* (2011). Sporulation was achieved by leaving the oocyst suspension at room temperature (RT). Sporulated oocysts were collected and stored at 4 ºC until further use. For oocyst production, goat kids were orally infected at the age of 4 weeks with 2 x 10^5 sporulated *E. ninakohlyakimovae* oocysts which were less than six-month old. Oocysts were isolated from faeces beginning 14 days p. i. according to the method proposed by Hermosilla et al. (2002).

2.3. Experimental design

Three groups of 4 weeks-old goat kids of the Majoreta milk aptitude breed were used in the *E. ninakohlyakimovae* challenge infection trial. Group 1, composed of eight goat kids, was orally infected with 2 x 10^5 sporulated oocysts/animal and challenged on day 21 p. i. with 1 x 10^6 sporulated oocysts/animal. Group 2, composed of four goat kids, was orally infected on day 0 as described above and challenged on day 21 with 2 x 10^5 sporulated oocysts/animal. Group 3 consisted of three uninfected goat kids and served as negative control. Shedding of oocysts was monitored from day 14 p. i. onwards by daily faecal examination. Clinical examination of all experimental animals was performed on a daily base. Blood samples for haematology

were obtained on days 0, 8, and 16 after primary infection and on days 8 and 16 after challenge infection.

Complete necropsy was carried out both in animals which died during *E. ninakohlyakimovae* challenge-infection and all the other animals from groups 1, 2 and 3 were humanely euthanized on day 17 post challenge. Samples from different areas of the gastrointestinal tract and regional lymph nodes, in addition to specimens from the rest of the organs, were taken for histopathological analysis.

2.4. Parasitological, biochemical and haemathological determinations

The shedding of oocysts was quantified by faecal examination using the modified McMaster technique (Bangoura and Daugschies, 2007) and expressed as oocysts per gram (OPG) of faeces. Blood samples were obtained by venipuncture from the jugular vein. For haemathological determinations, samples were collected in VetCollect® tubes (IDEXX) and immediately processed by using the haemathology analyzer LaserCyte® (IDEXX). Packed cell volume (PCV) was determined by centrifugation with a standard capillary microhaematocrit centrifuge. Additionally, blood smears were stained with panoptic staining (Diff-Quick) to perform differential leukocyte counts.

2.5. *Histopathological analysis*

During necropsies, tissue specimens were collected and fixed in 4% formaldehyde (Merck) in phosphate-buffered saline (PBS) for 24 h, dehydrated and embedded in paraffin according to standard procedures. Cross sections of 4 µm were stained with haematoxylin-eosin (H&E; Merck) and examined by light microscopy (Laborlux X Wild® microscope; Leitz). Quantification of leukocyte populations (lymphocytes, eosinophils and neutrophils) in tissue sections was performed on ileum and colon samples of uninfected and *E. ninakohlyakimovae* challenge-infected goat kids. Cells were counted using a 10x eye piece containing a calibrated graticule and 40x objective lens viewing an area of 0.05265 mm^2. The counts were randomly taken on 20 graticule fields within the mucosal surface. The counts were expressed as number of cells per mm^2 of mucosa according to Amarante *et al.* (2005).

2.6. Statistical analysis

For the statistical analyses OPG counts, cell counts at the intestinal mucosa and haemathological parameters (all dependent variables) were evaluated under different *Eimeria* infection regimes (independent variables: uninfection, primary infection with 2 x 10^5, challenge infection with 2 x 10^5 and challenge infection with 1 x 10^6). Faecal oocyst counts were logarithmically transformed and added by one (log [OPG + 1]) to obtain normal distributions (Kolmogorov-Smirnov's Normality Test). Normalization of the data was not necessary either for cell counts at the intestinal mucosa or the different haemathological parameters. One way factorial analysis of variance ANOVA and Tukey Multiple Comparison Test were used to compared OPG counts. Additionally, Student's t test was employed to analyze cell counts and haemathological paremeters. All the statistical analysis were carried out using the SigmaStat 2.03 software. For all the variable analyzed, comparisons were considered significantly different at $P < 0.05$.

3. Results

3.1. Parasitological, biochemical and haemathological data

The oocyst shedding of primary infected animals started at 14 days p. i. and reached highest levels at 16 days p. i. Thereafter, oocyst shedding gradually decreased up to day 21 p. i. In challenge infected animals, either with 2 x 10^5 or 1 x 10^6 sporulated oocysts, faecal oocyst counts were significantly lower than during primary infection between days 16 to 17 p. i. ($P < 0.05$ (Fig. 1). Animals challenged with 2 x 10^5 sporulated oocysts showed slightly higher OPG counts than

those challenged with the higher dose, although differences were not significant.

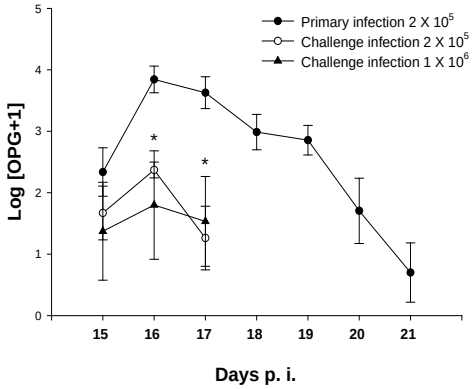


Figure 1. OPG (oocysts per gram of faeces) counts of goat kids orally infected with 2 x 10^5 sporulated oocysts *Eimeria ninakohlyakimovae* (closed circles) and challenged on day 21 p. i. with either 1 x 10^6 (closed triangles) or 2 x 10^5 sporulated oocysts (open circles). The OPG counts are depicted as the logarithm of the OPG plus one (log [OPG + 1]) and represent the mean ± SEM in all the experimental groups. (*): $P < 0.01$ between primary and challenge infections.

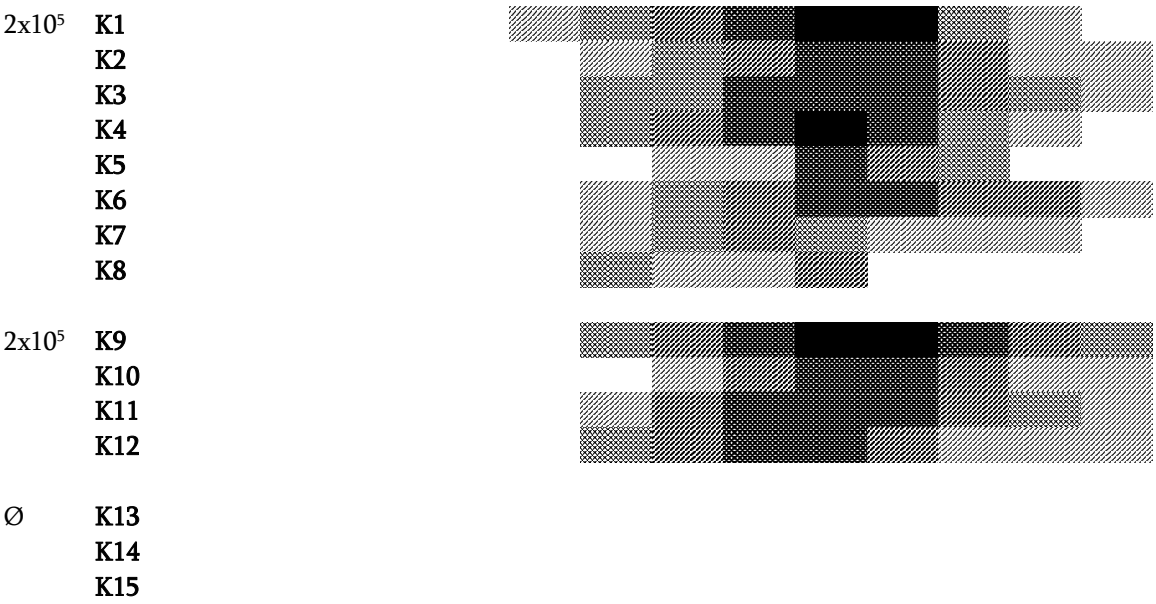
All goat kids challenged with 2 x 105 E. ninakohlyakimovae oocysts hardly showed clinical signs, whilst individual kids challenged with 1 x 106 sporulated oocysts succumbed from an acute fatal coccidiosis. Within this challenge trial, 62.5% of goat kids developed severe clinical disease and 60% of them died before the end of the investigation period (Table 1). Furthermore, 12.5% of the animals showed moderate signs of disease whilst only 25% remained unaffected (Table 1). In addition, there were also differences in the intensity of clinical signs already during the primary infection (Table 1), but no individual correlations were observed between the primary- and re-infection clinical phases. The signs of diarrhoea were accompained with dehydration, anorexia and recumbency in more severely affected goat kids. Besides, haemathological analyses revealed a moderate non-significant decrease in PCV and haemoglobin values especially in re-infected animals (Table 2). Although without significant differences, leukocytosis with neutrophilia and eosinophilia were also observed in re-infected animals, particularly in those challenged with 1 x 10^6 sporulated oocysts (Table 2).

3.2. Histopathological analysis

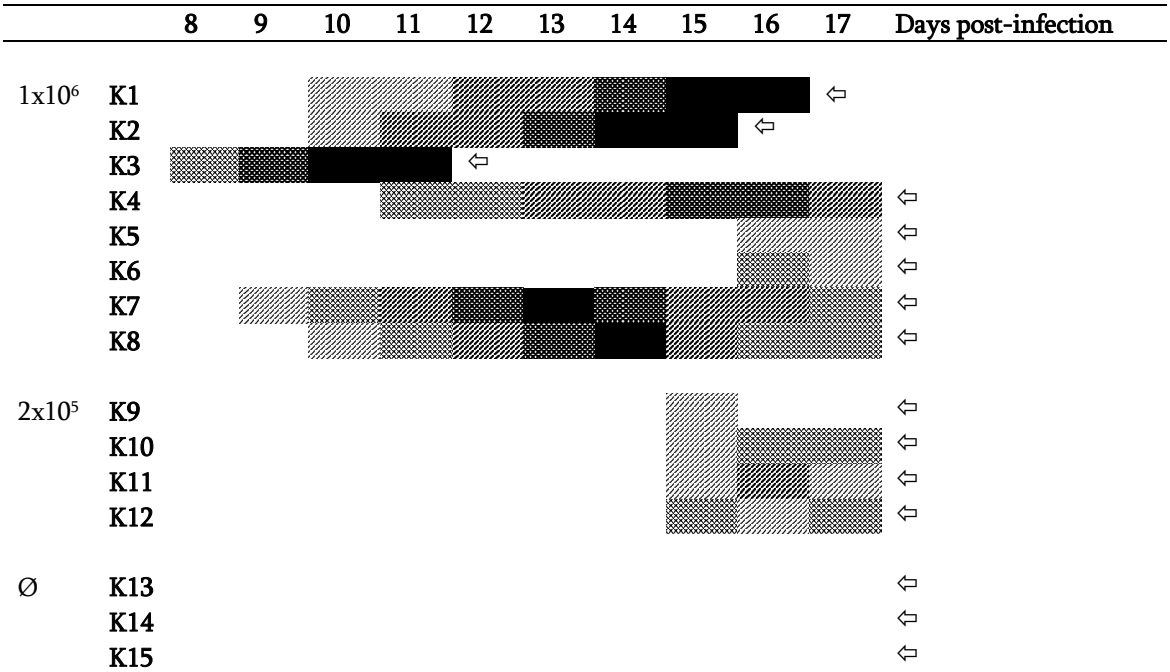
In this investigation both eosinophils and lymphocytes significantly infiltrated the mucosa of the ileum in challenged animals (2 x 10^5: $P<0.05$; 1 x 10^6: $P < 0.01$, Fig. 2), with significantly higher counts in animals challenged with 1 x 10^6 sporulated oocysts ($P < 0.05$, Fig. 2B, C). Similar data were determined for the colon mucosa, although in this case differences between the challenge groups were not significant for lymphocyte counts (Fig. 2). For illustration, Fig. 3B and 3E show exemplary images of eosinophil and lymphocyte infiltration into the ileum mucosa of animals suffering clinical disease, respectively. Interestingly, infiltration of mainly eosinophils could even be observed within degraded macromeronts. Moderate increased counts of neutrophils ($P < 0.05$) were recorded in animals challenged with 1 x 10^6 sporulated oocysts, whilst in those challenged with 2 x 10^5 sporulated oocysts counts were similar to values found in the control tissue samples. Besides these two gut sections, increased counts of eosinophils and in a lesser extent of lymphocytes could also be observed in the duodenum, the jejunum and the caecum of animals suffering from clinical disease. In addition to inflammatory cellular infiltration, other pathological findings such as hyperplasia of the globet cells, hyperplasia of the Peyer's patches (PP) in the ileum mucosa, areas of focal necrosis surrounded by hypertrophic crypts were observed in parasitized gut tissue. Intestinal lesions were also accompanied by severe histopathological alterations in other organs. Thus, mesenteric lymph nodes and the spleen showed a marked infiltration of eosinophils and reactive hyperplasia. Lungs were congestive and presented variable degrees of alveolar and interstitial oedema and liver samples displayed focal degeneration and necrosis. Signs of

Table 1. Faecal scores of goat kids primary infected and challenged with *Eimeria ninakohlyakimovae* oocysts.

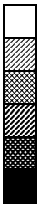
PRIMARY INFECTION



CHALLENGE INFECTION



Individual faecal scores of goat kids (K) primary infected on day 0 with 2 x 10^5 sporulated oocysts and challenged at day 21 with either 1 x 10^6 or 2 x 10^5 sporulated oocysts of *E. ninakohlyakimovae* are represented. Three uninfected kids (Ø) served as controls of infection. The arrow (⇦) point to the day of death and/or sacrifice and necropsy of the animals. The clinical evaluation of the diarrhoea was determined using the following score:



Clean normal
Dried soiling normal
Wet soiling diarrhoea
Diarrhoea down legs
Explosive diarrhoea
Fluid, watery or bloody diarrhoea

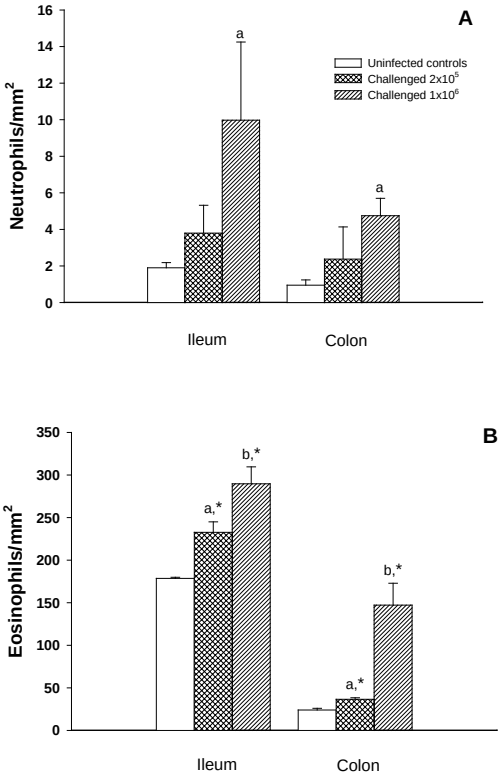
Table 2. Haematological parameters in *Eimeria ninakohlyakimovae* primary- and challenge-infected goat kids.

	PCV %	HGB g/dl	WBC cells/µl	NEU cells/µl	BAN cells/µl	LYM cells/µl	MONO cells/µl	EOS cells/µl	TP g/dl
Primary infection (2 x 10^5 sporulated oocysts)									
W4	29.5	9.2	8732.5	3329.0	0.0	5131.5	86.3	185.8	5.5
(0 dpi)	(1.4)	(0.7)	(490.0)	(951.0)	(0.0)	(548.8)	(37.2)	(117.7)	(0.3)
W5	27.3	8.8	9915.0	4822.3	0.0	4380.8	254.5	429.8	5.4
8 dpi	(2.1)	(0.7)	(806.6)	(324.1)	(0.0)	(845.2)	(102.4)	(312.7)	(0.2)
W6	27.0	8.4	8682.5	2884.3	0.0	4064.0	373.8	528.8	5.0
(16 dpi)	(3.7)	(1.0)	(1705.7)	(350.1)	(0.0)	(634.7)	(205.7)	(299.6)	(0.4)
Challenge infection (2 x 10^5 sporulated oocysts)									
W8	24.3	7.4	10487.5	5436.3	76.0	4458.3	92.5	424.0	4.7
(8 dpri)	(2.8)	(0.9)	(1400.3)	(1423.2)	(51.4)	(938.9)	(41.0)	(141.2)	(0.2)
W9	27.0	8.7	9125.0	4079.2	0.0	4856.0	24.8	465.3	4.9
(16 dpri)	(3.0)	(0.9)	(1104.1)	(294.7)	(0.0)	(908.5)	(28.6)	(158.4)	(0.2)
Challenge infection (1 x 10^6 sporulated oocysts)									
W8	23.3	7.3	15287.5	6295.0	22.5	6410.8	389.0	2056.3	4.9
(8 dpri)	(1.9)	(0.6)	(2883.5)	(679.4)	(26.0)	(879.3)	(186.3)	(431.2)	(0.2)
W9	28.5	7.9	34662.0	17033.5	1141.5	11663.5	3827.5	1618.3	5.1
(16 dpri)	(14.8)	(2.6)	(4098.4)	(290.6)	(1614.3)	(9337.3)	(5080.6)	(369.6)	(0.8)
Uninfected controls									
W4	29.3	9.0	13582.7	6046.4	0.0	6434.7	0.0	258.3	4.9
	(2.3)	(0.1)	(697.2)	(229.9)	(0.0)	(552.3)	(0.0)	(82.7)	(0.1)
W5	27.7	9.2	14314.0	7057.3	0.0	6313.5	25.7	331.7	(4.9
	(2.2)	(0.4)	(1024.0)	(883.3)	(0.0)	(1484.9)	(20.0)	(75.6)	(0.1)
W6	30.7	9.3	10834.0)	6148.6	0.0	5626.9	230.0	248.3	5.0
	(1.1)	(0.3)	(947.1)	(1274.1)	(0.0)	(832.7)	(160.5)	(74.5)	(0.1)
W8	29.7	9.6	12846.7	4785.0	0.0	7988.8	0.0	290.3	5.1
	(2.2)	(0.5)	(1900.7)	(1017.8)	(0.0)	(379.1)	(0.0)	(84.4)	(0.2)
W9	30.3	9.8	13680.0	3938.3	0.0	9855.4	121.7	162.0	5.1
	(1.1)	(0.2)	(785.9)	(59.0)	(0.0)	(594.4)	(75.4)	(96.5)	(0.1)

Goat kids (K) were primary infected at week four of life with 2 x 10^5 sporulated oocysts of *Eimeria ninakohlyakimovae* and challenged three weeks latter with either 1 x 10^6 or 2 x 10^5 sporulated oocysts. Three uninfected kids (Ø) served as controls of infection. TP: total proteins; PCV: packed cell volume; HGB: haemoglobin; WBC: while blood cells; NEU: neutrophils; BAN; band neutrophils; LYM: lymphocytes; MONO: monocytes; EOS: eosinophils. All he results are expressed as the mean ± (SEM) of different weeks of life (W), days postinfection (dpi) or days postre-infection (dpri).

congestion and degeneration of muscle fiber could be found in heart samples and kidney sections revealed mild glomerular and interstitial infiltration of mononuclear cells, calcium deposits and protein accumulation in tubules. As a common histopathological finding, leukocytosis and vascular stasis were also observed.

In accordance to the sloughing of the mucosa and haemorrhagies observed in clinical cases, histopathology of the corresponding animal samples showed clear signs of denudation of the mucosal surface. Groups of merozoites I could be detected within the denudated mucosa in the lumen of some ileum samples (Fig. 3D). Remaining intact mucosa of ileum sections from the goat kid which died during the prepatent period (day 11 p. i.) presented a high number of macromeronts of *E. ninakohlyakimovae* (Fig. 3A, 3C), whilst in animals necropsied during the patent period (from day 15 to 17 p. i.) exclusively microgamonts, macrogamonts and oocysts were observed in samples from the colon mucosa.



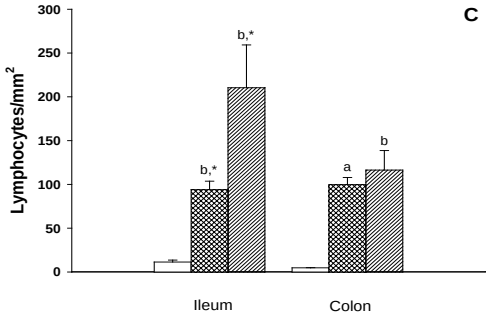


Figure 2. Differencial leukocyte counts, expressed as number of cells per mm², in the mucosa of the ileum and colon of goat kids primary- and challenged-infected with two infective doses of *Eimeria ninakohlyakimovae* oocysts (2 x 10^5 and 1 x 10^6). (a): $P < 0.05$ between control and challenge-infected animals; (b): $P < 0.01$ between control and challenge-infected animals; (*): $P < 0.05$ between goat kids challenged with 1 x 10^6 and 2 x 10^5 sporulated oocysts.

4. Discussion

In general, protective immunity developed by ruminant hosts against *Eimeria* spp. does not prevent subsequent homologous challenge infections but will restrain the clinical signs of disease. Continuous exposure to homologous *Eimeria* oocysts will maintain host protective immunity with the exception of animals showing a compromised immune status based on 1) nutritional deficiences, 2) stress produced by transport, sudden changes in feeding, weaning etc., or 3) concomitant diseases (Lucas et al., 2006; Rausch et al., 2010). This appears particularly important in young animals, although a failure of immunity against *Eimeria* infections has also been reported in adult goats during the periparturient period (Faber et al., 2002). The results of the present study give additional evidence that *E. ninakohlyakimovae* primary infected goat kids subjected to homologous challenge infections may also develop clinical disease under high infection pressure.

Goat kids challenged with 2 x 10^5 *E. ninakohlyakimovae* oocysts hardly showed clinical signs. In contrast, individual goat kids challenged with 1 x 10^6 sporulated oocysts

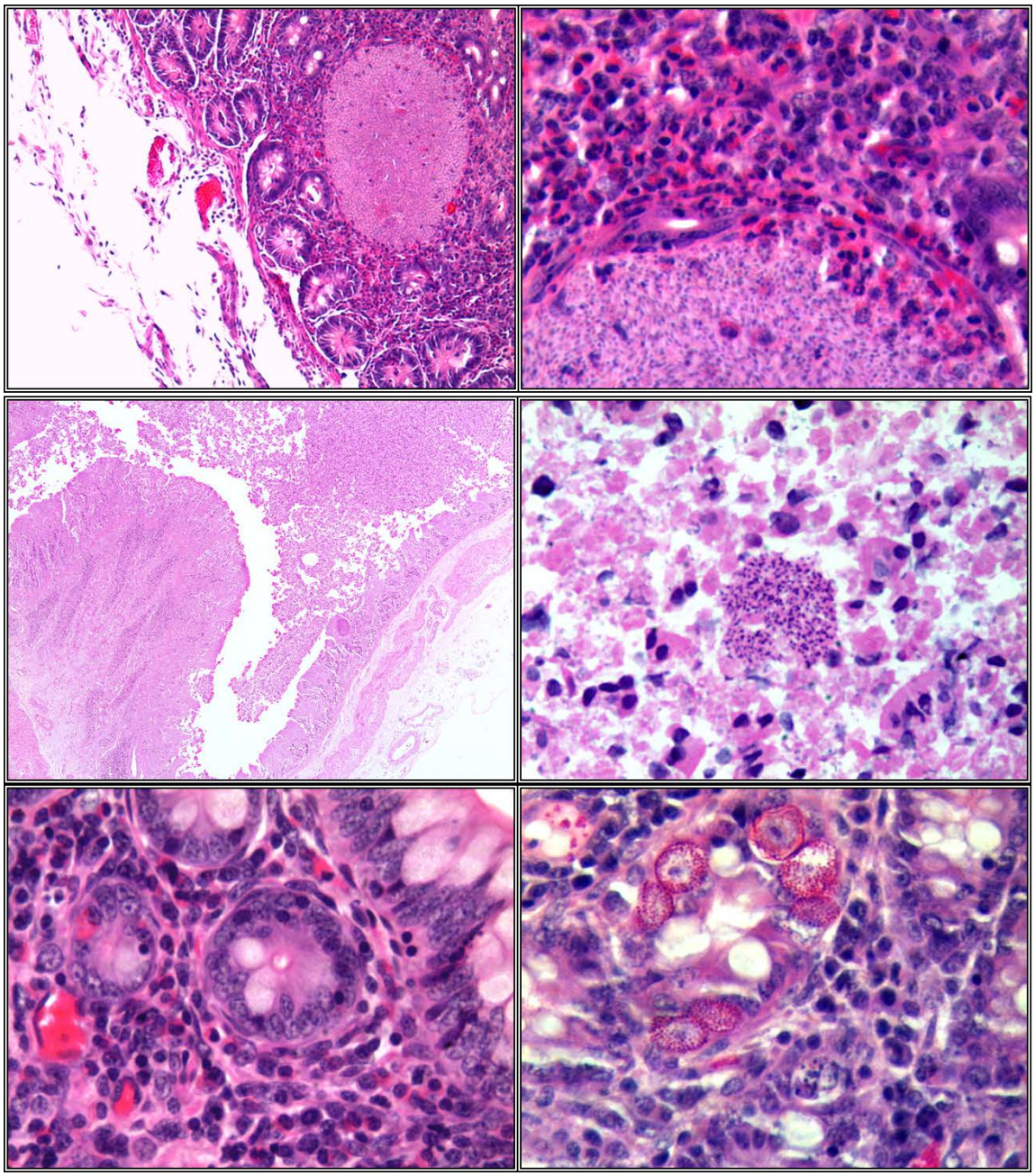


Figure 3. Histopathological finding in *Eimeria ninakohlyakimovae* primary infected goat kids and subsequently challenged with 1x10^{6} *Eimeria ninakohlyakimovae* sporulated oocysts. (A) First generation macromeront at 11th day postinfection within the ileum mucosa (H&E, 100X). (B) Detail of a macromeront surrounded by an intense infiltration of eosinophils, some of them inside the meront (H&E, 400X). (C) Low magnification detail showing lesions in the mucosa of the ileum at day 11th postinfection; the arrow points a macromeront in the surface of the mucosa (H&E, 40X). (D) Bulk of merozoites I within partially degraded mucosa inside the lumen of the ileum (H&E, 400X). (E) Infiltration of eosinophils and lymphocytes (arrow) in the ileum mucosa.

(higher infection dose) succumbed from an acute fatal coccidiosis, indicating that individual factors and/or the genetic background may be involved. The development of clinical disease at a high percentage of challenged goat kids has a clear impact on economical and epidemiological aspects of coccisiosis, as susceptible challenged animals will have increased losses attributable to deaths and delayed growth performance and further contribute to the environmental oocyst contamination. Thus, improvement of the hygienic and management conditions is a crucial strategy to minimize excessive contaminated environments to prevent massive infections with *Eimeria* spp. in caprine husbandry (Ruiz et al., 2006). In addition, as all animals included in this study were kept under identical experimental conditions to minimize external factors influencing the infection outcome, the high individual variation of clinical responses to *E. ninakohlyakimovae* infections suggests a strong genetic component to be involved in the development of protective immunity against this *Eimeria* species. In agreement, the importance of genetic influence on protective immune responses has been reported in Merino lambs and in goat breeds experimentally infected with *Eimeria* spp. (Reeg et al, 2005; Kanyari, 1988). In gastrointestinal nematode infections, individual variations have also been described with respect to both acquired (Nejsum et al. 2009; Kanobana et al., 2004) and innate (Jackson et al., 2005) immune responsiveness. The individual ability to deal with primary- and challenge-infections with coccidian parasites may be exploited in future for genetic selection breeding purposes, as it has been done for nematode infections of livestock (Díez-Tascón et al., 2004; Kadarmideen et al., 2010).

According to other reports (Bangoura and Daugschies, 2007), the data of both primary and challenge infected goat kids showed a positive association between intensity of the clinical signs and oocyst output. As expected, challenge infected kids shed significantly lower amounts of oocysts than primary infected ones. Interestingly, goat kids re-infected with the higher infection dose (1 x 10^6 oocysts/animal) showed slightly lower OPG values than those re-infected with 2 x 10^5 oocysts/animal. It is not clear if this reflects the true *in vivo* situation and could thus be attributed to a "crowding" phenomenon (Daugschies et al., 2002) or whether the results are falsified by either reduction of epithelial cell surface via infection-induced destruction of the mucosa (Bürger, 1983) or by extensive clotting of oocysts within fibrin particles or discharged mucosal tissue as observed in severe bovine coccidiosis (Taubert and Hermosilla, personal communication). Overall, the clinical and haemathological findings of this investigation are in agreement with the common symptomatology of coccidiosis caused by direct effects of the sexual and asexual multiplication of the *Eimeria* parasites (Koubdela and Bokova, 1998; Daugschies and Najdrowski, 2005), which clearly occurred in challenge-infected animals as exemplary shown in Fig. 3. Besides, in agreement to histopathological features found in the present study, Dai et al. (2006) also reported on lymphoid hyperplasia in the mesenteric lymph nodes and focal degeneration and necrosis in livers. However, they additionally described signs of chronic cholecystitis in gallbladders, probably related to the ability of *E. ninakohlyakimovae* to develop in epithelial cells of this organ and of bile ducts as previously published (Schafer et al., 1995; Oruc, 2007). In contrast, we could not find any evidence of either parasite development or tissue damage of the gallbladder/bile ducts in the present study. Deaths are a consequence of maintained inappetance and dehydration caused by the limited reabsorbing capacity of the damaged musosa and the continuous losses of fluids related to diarrhoea, as reported for acute coccidiosis in other ruminant species (Taylor and Catchpole, 1994; Daugschies and Najdrowski, 2005). So far, it remains to be elucidated if the fatal outcome of *E. ninakohlyakimovae* challenge infections results as a direct effect of parasite replication or if immunopathological effects

driven by the excessive cellular immune reactions are responsible for clinical disease.

The intestinal homeostasis depends on the interaction of several compartments, including mesenteric lymph nodes, PP and intraepithelial lymphocytes (IEL). The enterocytes play a critical role in mucosal immunophysiology which in part consists of a paracrine association to the underlying immune and pro-inflammatory cells (Kasper and Buzoni-Gatel, 2001). Analyses on leukocyte gut infiltration after challenge infection revealed that cells of both innate and acquired immune system were recruited to the site of infection (Fig. 3). These findings in principle correspond with other reports on *E. ninakohlyakimovae* infected goats (Dai et al., 2006) and with data on experimental *E. crandalis* infection in sheep (Taylor et al., 2003). However, in this investigation both eosinophils and IEL were significantly increased in highly challenged animals (1 x 10^6 sporulated oocysts) when compared to those challenged with 2 x 10^5 sporulated oocysts. In addition to eosinophils and IEL, significantly increased counts of neutrophils, a immune cell type that was previously reported to be crucially involved in *E. bovis*-triggered early innate immune reactions (Behrendt et al., 2008, 2010), were recorded on a more moderate level only in animals experiencing the high challenge dose. Besides these two gut sections (ileum and colon), increased counts of eosinophils and in a lesser extent of lymphocytes could also be observed in duodenum, jejunum and caecum of animals suffering from clinical disease, emphasizing the overall pathological finding of a severe eosinophilic enteritis. Especially eosinophils can cause tissue damage by a number of different mechanisms, including direct cellular cytotoxicity, physical damage caused by tissue infiltration (space occupying), and thromboembolic phenomena caused by eosinophil-induced hypercoagulability. However, the main effector mechanism of eosinophils is the release of eosinophil granule proteins and other cytotoxic molecules which, for example, have been correlated with helminth-induced tissue damage (see Klion et al., 2004 for review). Importantly, as clinical signs of coccidiosis and the corresponding histopathological finding were recorded during the prepatent period, compared to primary infections, this hypothetical hyper-inflammatory response would be mainly mounted against the first asexual stages namely macromeronts I of the endogenous development of *E. ninakohlyakimovae.*

In conclusion, the data presented here demonstrate that *E. ninakohlyakimavae* primary infected goat kids subsequently challenged with a homologous high infection dose indeed may suffer from fatal acute coccidiosis and, in consequence, show severe pathological intestinal alterations. The results emphasize the need for management measures preventing excessive environmental oocyst contamination. Moreover, our results indicate the importance of the individual genetic background associated with the ability to overcome *E. ninakohlyakimovae* challenge infections. Whether the mechanisms of pathogenicity are based on direct parasite-induced mucosal lesions or on an exacerbation of inflammatory response or both, remain to be investigated.

5. Acknowledgements

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ARTÍCULO Nº 3

Caracterización de la respuesta de anticuerpos en cabritos por una infección experimental por *Eimeria ninakholyakimovae*.

In preparation to: *Experimental Parasitology*

▪ RESUMEN▪

La mayoría de los animales de un rebaño pueden estar infectados con diferentes especies de *Eimeria*, pero la enfermedad afecta principalmente a cabritos entre 4 a 20 semanas. *E. ninakholyakimovae* se considera como uno de las especies más patógenas en cabras, capaz de producir un daño masivo de la mucosa intestinal, lo que resulta en un síndrome de malabsorción y diarrea severa, a menudo acompañada de sangre y mucus. La clínica está asociada a pérdida de peso, deshidratación y las tasas de retraso en el crecimiento, lo que conlleva pérdidas productivas importantes.

El control de la coccidiosis en rumiantes se basa principalmente en la combinación de prácticas de manejo y el uso de anticoccidiósicos; sin embargo, ya se han descrito resistencias frente a este tipo de fármacos, lo cual, junto con la creciente demanda de los consumidores de productos pecuarios orgánicos libres de residuos, ha incrementado el interés por nuevas alternativas de control, como el desarrollo de vacunas, para lo cual, un requisito previo es una mejor comprensión de la inmunología básica y la interacción huésped-parásito.

En la coccidiosis en rumiantes, aunque tanto la respuesta inmune celular como la humoral se activan después del primer contacto con el antígeno, muchos estudios indican que el componente celular mediado por linfocitos T es el principal responsable del desarrollo de respuestas inmunitarias protectoras frente a *Eimeria*. La respuesta humoral frente al coccidio caprino *E. ninakholyakimovae* no ha sido estudiada con anterioridad y, en general, hay pocos datos sobre la respuesta inmune en la coccidiosis caprina. El objetivo principal de este trabajo ha sido estudiar la respuesta inmune humoral de esta especie de *Eimeria* mediante ELISA, incluyendo el análisis de IgG e IgM séricas y de IgA a nivel local, tanto en primo-como en reinfecciones. Además, se llevó a cabo una primera aproximación hacia reconocimiento mediado por anticuerpos (IgG) específicos de péptidos mediante SDS-PAGE e *immunoblotting*.

Se empleó una cepa de *E. ninakholyakimovae* aislada con anterioridad en nuestro laboratorio y mantenida mediante pases en animales donantes. Los cabritos empleados en el estudio fueron adquiridos con 1-4 días y se mantuvieron bajo condiciones libres de parásitos hasta comenzar el estudio, momento en el cual se distribuyeron en tres grupos (n = 6-9): (i) animales infectados a las 5 semanas de edad

y reinfectados tres semanas más tarde (W5PI + W8RI); (ii) animales primoinfectados a las 8 semanas o controles de reinfección (W8PI), y (iii) animales no infectados (C). Las infecciones se realizaron en todos los casos vía oral con 2 x 10^5 ooquistes esporulados de *E. ninakohyakimave.*

Se observó que los animales primoinfectados presentaban signos clínicos graves y altas tasas de excreción de ooquistes, mientras que los reinfectados casi no mostraron signos de enfermedad y los recuentos de OPG fueron significativamente menores. Los niveles de IgG en suero aumentaron significativamente después de las 3 semanas pi, ligeramente después registrar los niveles máximos de ooquistes, lo que sugieren que el sistema inmune necesita un impulso para la producción de anticuerpos específicos. Los valores de IgG específicos se mantuvieron altos y aumentaron progresivamente durante el muestreo, probablemente relacionado con una mayor estimulación inducida por la reinfección en la semana 8. Los niveles de IgM también se vieron incrementados tras las infección por *E. ninakholyakimovae* pero, en comparación con el perfil de IgG, los valores más altos ya se encontraron a las dos semanas p.i. Del mismo modo, los niveles de IgA local en el mucus del ileon se incrementó significativamente en los animales infectados en comparación con los controles. Teniendo en cuenta que todos los animales que se adquirieron tenían 1-5 días de vida en el momento de comenzar el estudio, los niveles de anticuerpos encontrados durante todo el experimento se deben considerar como de nueva síntesis y no anticuerpos maternos.

Aunque las diferentes inmunoglobulinas realmente reflejan la exposición a *E. ninakholyakimovae*, al relacionar los anticuerpos con los recuentos de OPG no pudo demostrarse una correlación inequívoca con el nivel de protección, tal como han indicado otros autores en ovino. De acuerdo con esto, no se observaron claras diferencias entre los niveles de ninguna de las inmunoglobulinas analizadas al comparar animales re-infectados con los controles de reinfección.

Utilizando como antígeno ooquistes esporulados de *E. ninakholyakimovae*, la IgG sérica de los animales infectados logró reconocer un panel de péptidos específicos cuyos pesos moleculares oscilaron entre 134 y 16 kDa, apoyando el valor del *electroimmunobotting* como una técnica para detectar proteínas inmunorreactivas en coccidiosis caprina. Sin embargo, tampoco en esta ocasión se encontraron bandas de reconocimiento específico en los animales reinfectados.

En conjunto, los resultados de este estudio contribuyen a entender la complejidad de la respuesta de anticuerpos frente a la coccidiosis en cabras y proporcionan un valioso punto de partida para promover investigaciones sobre la identificación de antígenos protectores que puedan ser de utilidad para futuros estrategias de inmunoprofilaxis.

Characterization of antibody response to experimental infection with *Eimeria ninakholyakimovae* in goat kids

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Abstract

Infections produced by different *Eimeria* species, which are some of most important parasitic diseases affecting profitability of most production system worldwide in ruminants, are under immunological control. Although cellular immune response seems to be crucial for the development of protective immune response, there are also evidences showing an active involvement of the humoral counterpart. In the present study, we have analyzed the antibody response to an experimental infection with *Eimeria ninakholyakimovae* in goat kids. Therefore a total of 22 kids were divided into three groups: (i) primary infected animals at 5 weeks of age, and reinfected three weeks later (W5PI + W8RI), (ii) primary infected animals at 8 weeks or challenge controls (W8PI) and (iii) uninfected animals (C). For infection, sporulated oocysts of a local (GC) strain of *Eimeria ninakohlyakimovae* were used. The levels of specific IgG and IgM antibodies were determined in serum samples taken weekly throughout the experiments; additionally, IgA levels were estimated in the ileal mucus applying indirect ELISA tests based on homogenized oocyst antigen. Infected kids produced significant increase of IgG levels that was consistently observed from 3 weeks post infection onwards. Furthermore, an increase of specific IgM and secretory IgA levels were observed in infected animals. A wide range of peptides from sporulated oocyst antigen (SOA) were recognized by specific IgG using SDS-PAGE and subsequent immunoenzimatic botting, however, as for the indirect ELISA results, no correlations were found with OPG counts. Altogether, these results suggest that antibody analysis of experimental infection with *E. ninakohlyakimovae* in goat kids certainly reflect the exposure to the parasite but is not directly associated to the protection conferred after challenge. Further studies should be addressed to clarify if the lack of correlation might be associated to the type of antigen used for the immunoenzimatic assays, the age of the animals or other factors.

Key words: *Eimeria ninakohlyakimovae*, goat kids, IgG, IgM, IgA

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1. Introduction

The caprine coccidiosis caused by *Eimeria* spp. is one of the most common parasitic disease affecting goats in production systems worldwide (Faizal and Rajapakse, 2001; Donkin and Boyazoglu, 2004; Cavalcante et al., 2012; Silva et al. 2014). Although most of the animals of a herd may be infected with different *Eimeria* species, the disease mainly affect kids between 4 to 20 weeks, sometimes with high mortality rates (Koudela and Boková 1998, Ruiz et al. 2006). Among most frequent *Eimeria* species in goats, *E. ninakholyakimovae* is considered as one of the most pathogenic, able to produce massive damage of the intestinal mucosa, which results in a malabsorption syndrome and severe diarrhea, often accompanied by blood and mucus. The clinic involves weight loss, dehydration and stunted growth rates, which results in important production losses (Koudela and Boková 1998).

Control of ruminant coccidiosis is mainly based on the combination of management practices and the use of anticoccidials, such as sulfonamides (Svensson 1998), toltrazuril (Mund et al. 2003) and diclazuril (Ruiz et al. 2012). However, because of the indiscriminate use of these products, mainly in poultry, anticoccidial drug resistance has already been described (Peek and Landman 2005). This circumstance, together with the growing demand of consumers for organic livestock products free of residues, has increased the interest for new alterative control methods. One of these alternatives is the development of vaccines, for which a prerequisite is a better understanding of basic immunology and host-parasite interactions.

Although both cellular and humoral immune responses are triggered against ruminant coccidiosis after the first contact with the antigen (Daugschies and Nadjdrowski 2005), many studies indicate that the cellular counterpart, mediated by T-cells, is the main responsible for the development of protective immune responses against *Eimeria* spp. infections and other Apicomplexa, such as *Toxoplasma*, *Neospora* or *Cryptosporidium* (Abrahansem, 1998; Yap and Sher, 1999; Hermosilla et al., 1999; Correia et al., 2013). Accordingly, T-cells response was associated with lower excretion of oocysts in feces before reinfection with *E. bovis* in cattle (Hermosilla et al., 1999; Taubert et al., 2008; Sühwold et al., 2010). Besides, it has been demonstrated that T-cells involved in the primary immune response in cows are mainly $CD4^+$ (Findley et al., 1993; Hermosilla 1999; Taubert et al., 2010) whereas cytotoxic $CD8^+$ T cells may represent the major effector cell type against the parasites in case of reinfections. In addition, *E. bovis*-mediated T-cells activation may induce a molecular regulation of a network associated with the movement and trafficking of immune cells, probably through the production of certain cytokines such as IL2, IL4 and INFγ (Taubert et al., 2010).

In contrast, humoral immune reactions of the host are not sufficient for the termination of *Eimeria* spp. primary infections (Daugschies y Najdrowski, 2005) and, even when antibodies may reflect the exposure to the parasite, the protection which they confer is not (Fiege y cols., 1992). The humoral response develops rapidly and involves high titers of antibodies in the serum of infected animals. Usually, there is an initial increase of IgM, followed by IgG and other specific antibodies such as IgA (Hughes 1985). Experimental infections with *E. ovinoidalis* and *E. faurei* in sheep demonstrate that specific antibodies increased, not only in the primary infections, but also in secondary infections (Nolan et al. 1987). Like $CD4^+$ T cells and probably other lymphoid cells, antibodies can be transferred in colostrum to the calf resulting as a mechanism of passive immunity against coccidiosis (Fiege et al., 1992). Actually, lambs and calves fed with colostrum with a high content of IgG had higher levels of specific antibodies against *Eimeria* than those which did not received it. Studies on the role of humoral immune response in avian coccidiosis have also demonstrated the ability of antibodies to block the invasion, development and transmission of the parasite and, like in ruminants, the existence of passive immunity (Wallach 2010). In other Apicomplexa parasites, such *Toxoplasma gondii* and

Crytosporidium, the involvement of both, systemic and local, antibody responses in parasitized goats has also been reported (Conde et al. 2001, Gomez Morales et al. 2002).

The antibody response during *E. ninakholyakimovae* infection has not been studied before and, in general, there are few data on the immune response in caprine coccidiosis. The main objective of this work was to evaluate the humoral immune response of this *Eimeria* species by ELISA sequential analysis of peripheral IgG and IgM and local IgA in primary and challenge infected animals. Additionally, a preliminary approach for antibody-mediated recognition (IgG) of specific peptides was conducted by SDS-PAGE and immunoblotting methodology.

2. Material and methods

2.1. Parasites and parasitic antigen

The *E. ninakholyakimovae* strain GC used in the present study was initially isolated in 2006 from the field of naturally infected goats in Gran Canaria Island (Spain) and maintained by passages in goat kids for oocysts production (Ruiz et al. 2014). To grow oocysts, goat kids were orally infected at the age of 4 week with 2 x 10^5 sporulated *E. ninakholyakimovae* oocysts. Oocysts were isolated and purified from feces after 2 week post infection according to the methods of Jackson (1964) and then concentrated. Sporulation of oocysts was achieved in a week by leaving this suspension at room temperature and stirring the oocysts suspension daily to infuse oxygen into the suspension. Finally, sporulated oocysts were stored in a 2% potassium dichromate solution at 4°C until further use.

For the preparation of the antigen used in ELISA and immunoblotting determinations, a solution containing approximately 10^6 sporulated *E. ninakholyakimovae* oocysts was employed. The oocysts were mixed and crushed with glass beads in a 15ml tube (Nunc) ten times for 60s on a vortex at maximum speed. When the oocysts were partially broken, the glass beads were withdrawn and the solution was subjected to ultrasounds (Vibra Cell TM) at 80Hz until they were completely crushed. The ultrasound treatment was carried out ten times for 60s each, maintaining the solution in water ice for 1 min between each ultrasound pulse. Thereafter, the final solution was transferred to Eppedorf tubes and centrifuged during 30 min at 11000 g and 4°C (MPW 65-R Centrifugue). The supernatant was then transferred to sterilized tubes to calculate the antigen concentration by using the BCA method. Briefly, 2ml of BCA solution (22ml Bicinchoninic acid solution + 0.44 ml Coopper sulfate) (Sigma-Aldrich) were added to a tube containing 100 µl of sporulated oocyst antigen (SOA), different dilutions of a reference stock protein (Bio-Rad) and negative controls (distilled water). All the samples were incubated 1h at 60°C before reading the absorbance values in a SmartSpec™Plus spectrophotomemter (Bio-Rad). Different aliquots of the resulting SOA antigen having a 103.678µg/ml concentration were stored at -20°C until the ELISA (Enzyme-linked immunosorbent assay) and Western immunoblotting tests were carried out.

2.2. Animals, experimental design and sample processing

A total of (21) goat kids of the Majorera breed was purchased from a local farmer at the age of 1-5 days and maintained under parasite-free conditions in autoclaved stainless steel cages in a restricted stable (Faculty of Veterinary Medicine, University of Las Palmas de Gran Canaria, Spain). The kids were treated with Vecoxan® (Janssen-Cilag) and Halocur® (Intervet) just at their arrival. Goat kids were fed with the milk substitute Bacilactol® (Capisa) and commercial concentrate pellets for weaning goat kids (Starting Concentrate, Capisa). Water and sterilized hay were always given *ad libitum*. All animal procedures were conducted in strict accordance with national ethics and by institutional review board-approved protocols. For experimental purposes goat kids were divided into the following three groups: group 1 (n=9, W5PI+W8RI: primary infected at week 5 of life with *E. ninakholyakimovae*

sporulated oocysts and reinfected at week 8); group 2 (n=6, W8PI: animals infected with *E. ninakholyakimovae* sporulated oocysts at week 8 of age; group 3 (n=7, CONTROL: non infected animals). All infections were performed orally with a gastro-ruminal tube using an infection dose of 2 x 10^5 sporulated *E. ninakholyakimovae* oocysts. The animals were weekly bled from the jugular vein and the corresponding serum individual samples were stored at -20°C until the serological studies were performed.

In order to have a monitoring of the parasitological and clinical course of the experimental infection, individual fecal samples were collected from 14 to 21 days post-infection (p.i.), both during primary and challenge infections, and the presence of clinical signs were daily inspected. The estimation of the faecal release of oocysts was determined by a modified McMaster technique (Thienpont et al., 1979) and expressed as OPG (oocyst per gram of faeces) counts. The animals were humanely euthanized at the end of the experiment and mucus samples, for the analysis of specific IgA, were collected from the ileum. For their preservation, the samples were suspended in a pH 7.1 buffer containing proteinases inhibitors (all compounds from Sigma-Aldrich): 0.1 M Na_2HPO_4, 0.05 M NaCl, 3 mM NaN3, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM ethylenediaminetetraacetic acid (EDTA). Mix solutions containing mucus were centrifuged at 5000 x *g* (Eppendorf Centrifuge 5804R) for 1h at 4°C and the resulting supernatant was conserved at -20°C for further analyses.

2.3. Enzyme – linked immunosorbent assay (ELISA)

A total of 100µl of a SOA solution in carbonated buffer at a 5µg/ml concentration was dispensed in 96 wells ELISA plates (Corning Glass Works) and incubated overnight at 4°C. After three washes with 200µl of PBS-0.05 % Tween 20, 200µl of PBS-3% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) was added and the plates were incubated for 45 min at 37°C. After each new step a similar washing procedure was addressed. Following treatment with BSA, individual serums, positive and negative controls were analyzed in duplicate using a 1/25 dilution in PBS-Tween 20 0.02% sodium acid. For each sample, a volume of 100µl was added to the plates and a new incubation was performed at 37°C for 1h. The plates were then incubated with 100µl per well of 1/2000 anti-Goat IgG (anti-goat IgG peroxidase conjugate, IgG fraction of antiserum, Sigma-Aldrich) diluted in 0.01M PBS and the incubation was performed in the same conditions. Thereafter, a total of 100µl of substrate was added to each well, a mix of compounds that includes citric-phosphate buffer, 0.04% (w/v) dihydrochloride ortho-phenyleneadiamine (OPD, Sigma-Aldrich) and 30% hydrogen peroxide (Panreac) at a final concentration of 0.1% (v/v). The incubation with the substrate was performed at room temperature in darkness for approximately 10 min. Finally, the reactions was stopped by adding 35 µl per well of a 2M solution of sulfuric acid (Panreac) and the optical density (O.D.) measured at a wavelength of 492 nm (Termo Labsystems, Multiskan Ascent).

For specific IgM and IgA indirect ELISA tests, a similar protocol was employed but using 1/3000 anti-goat IgM peroxidase and 1/7000 anti-goat IgA peroxidase conjugates, respectively (Sigma-Aldrich). Similarly, dilutions 1/25 of the different serum or mucus samples were employed as primary antibody.

2.4. SDS-PAGE and Western immunoblotting

A 86.65 µg/ml antigen solution (SOA) and low molecular weight standards (Pierce Blue Prestained Molecular Weight Marker, Thermo Scientific) ranging from 215 to 18 KDa were run on 12% SDS-polyacrylamide gels in a cuvette (Mini-Protean® Tetra Cell BioRad) at 100V constant voltage at 4°C. After electrophoresis, proteins were either revealed with silver stain (Sigma-Aldrich) or transferred to a nitrocellulose membrane (blotting Pure Nitrocellulose Membrane, Bio Trace® NT, Life Sciences) by applying an electric field perpendicular to the membrane

and gel. The transfer was performed in the same bucket than electrophoresis at 100V constant voltage for 1h at 4°C. After the transference was finished, the membrane was sliced and the resulting strips were firstly washed with PBS-0.05 % Tween 20 and then incubated with PBS containing 3% (w/v) BSA for 1 h at 37°C. After a new washing step, the membranes were incubated overnight at 4°C with different pool sera from the different experimental groups, previously diluted 1/25 in PBS-0.05 % Tween 20 containing 0.02% NaN_3 (w/v) (Sigma-Aldrich). Next to a new washing, the strips were incubated for 1 h at 37°C with anti-Goat IgG diluted 1/1000 in PBS. Finally, the membranes were washed again with PBS-0.05 % Tween 20 and revealed using an AEC Chromogen Kit (AEC Staining Kit, Sigma- Aldrich).

The determination of the molecular weight of the different polypeptide subunits present, both in the acrylamide gels that were stained with silver after electrophoresis and in the nitrocellulose membrane strips from the Western-blot immunoenzymatic reaction, was carried out by a regression analysis (Milton, 1994; Gardiner, 1997) using the migration of proteins of molecular weight marker as a reference.

2.4. Statistical analysis of data

Faecal OPG were logarithmically transformed and added by 1 [log (OPG+1)] in order to obtain normal distributions according to the Kolmogorov-Smirnov's normality test. Levels of the different immunoglobulins analysed in the study were expressed as the relative percentage of the optical density (O.D.) of a positive control pool in order to avoid inter-assay differences (Relative O.D.). One factorial analysis of variance, Tukey's multiple comparison test and Student's t-test were used to analyse the data between different experimental groups and the Pearson correlation test for the evaluation of the association between OPG counts and the antibody levels. For that purpose, an statistical software (SigmaPlot 12.0) was employed and the differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. OPG counts and clinical signs

After primary infection, goat kids primary infected at week 5 of age (W5PI) with 2 x 10^5 *E. ninakohlyakimovae* sporulated oocysts showed a prepatent period ranged from 14 to 19 days p.i., and had a maximum average value of 4.197 at day 16 p.i., which corresponds to 157,245 OPG counts (Table 1). A similar variation for the prepatent period (15 to 18 days) was found in the challenge control group (W8PI), although the maximum average in this case was much higher, reaching peak values of 5,393 (approximately 2,5 x 10^6 OPG) also at 16 days p.i.. Faecal counts were consistently lower in challenged kids (W5PI+W8RI) with differences being significant when comparing to results of both primary infections (W5PI and W8PI) ($p < 0.05$ to $p < 0{,}001$) (Table 1).

There were no differences between clinical signs when comparing animals primary infected either at week 5 (W5PI) or at week 8 of age (W8PI). With variable intensity depending on the goat kid, different degrees of diarrhea, dehydration and anorexia were observed. In some cases, the diarrhea was profuse, bloody and contained small debris of mucosa. By contrast, less obvious or even imperceptible clinical signs were recorded for challenged animals (W5PI+W8RI). During the whole experiment, uninfected control animals showed no oocysts in their feces nor clinical signs.

3.2. Enzyme-linked immunosorbent assay (ELISA)

Mean values for specific IgG against SOA (sporulated oocyst antigen) for the different experimental groups are depicted in Figure 1.

dpi	W5PI	W5PI+W8RI	W8PI
14	0,701 ± 0,468 (a)	2,152 ± 0,535 (a)	2,813 ± 0,977
15	3,270 ± 0,991	2,525 ± 0,804 (*)	4,467 ± 0,684 (*)
16	4,197 ± 0,986	2,511 ± 0,744 (**)	5,393 ± 0,558 (**)
17	3,863 ± 0,904	2,204 ± 0,650 (**)	4,783 ± 0,458 (**)
18	3,455 ± 0,775	2,109 ± 0,697 (*)	4,005 ± 0,615 (*)
19	3,097 ± 0,588 (a)	1,337 ± 0,536 (a)	2,451 ± 0,994
20	2,689 ± 0,627 (a)	0,674 ± 0,460 (a)	1,276 ± 0,893
21	0,985 ± 0,493	0,000 ± 0,000	0,000 ± 0,000

Table 1. OPG (oocysts per gram of faeces) counts of goat kids orally infected at week 5 of life (W5PI) with 2 x 10^5 sporulated oocysts of the GC strain of *Eimeria ninakohlyakimovae* and challenged three weeks later with the same dose (W5PI+W8RI). Animals primary infected with 2 x 10^5 sporulated oocysts at week 8 of life (W7PI) were used as challenge controls. OPG counts are depicted as the logarithm of the OPG plus one (log [OPG + 1]) and represent the mean ± SEM in all the experimental groups. (*) $P < 0.05$ and (**) $P < 0.01$ represent significant differences between re-infected animals (W5PI+W8RI) and challenge controls (W8PI), while (a) $P < 0.05$ indicates differences between primary (W5PI) and challenge infection (W5PI+W8RI).

The analysis of the results show that increased IgG levels from primary infected goat kids at week 5 of age progressively increased during the next three weeks p.i. with significant differences to control animals being observed at week 8 p.i. The same animals, but challenged at week 8 (W5PI+W8PI), had sustained and increasing levels of IgG during the following weeks with peak values at the last sampling point (week 11). Significant different IgG values ranging from $p < 0.05$ to $p < 0.001$ were observed in challenged goat kids with respect to control group after reinfection. A similar IgG profile was found in animals which were primary infected at week 8 (W8PI, challenge control group), although in this occasion the levels of this immunoglobulin reached significant increased values ($p < 0.05$) at two weeks p.i. and slightly decreased afterwards. There were no differences between IgG levels between the re-infected animals and the corresponding challenge control group.

Significant increased levels of specific IgM were first recorded at 7 weeks p.i. in goat kids primary infected at week 5 ($p < 0.05$) and remained high up to the end of the experiment (Figure 2). However, one week after challenge infection, serum samples of this group of animals (W5PI+W8PI) slightly decreased before showing new peak values at week 10. IgM levels gradually increased also in goat kids primary infected at week 8 (W8PI), with significant differences ($p < 0.05$) being observed at the last sampling point compared to control group. No differences were found between re-infected goat kids and the corresponding challenge controls.

Finally, the analysis of the immunoglobulin isotype A (IgA) levels showed that mucus samples from both re-infected (W5PI+W8PI) and challenge control animals (W8PI) had significant higher levels of specific IgA than controls ($p < 0,05$), but there were no differences between these two groups.

The analysis of correlations between oocyst excretion and antibody levels showed an inverse relationship between cumulative OPG counts (14 to 21 days p.i.) and specific serum IgM values in primary infected animals 3 weeks after infection (W8) but differences could not be proven statistically ($p = 0.0213$); no correlations were observed to either IgG or IgA levels during primary infections. Considering antibody levels of challenged animals (W5PI+W8RI), only slight non-significant correlations to OPG counts were recorded. In this occasion, negative correlation coefficients were also found for IgG ($p= 0.127$), while for local IgA the correlation was positive ($p= 0.158$).

3.2. SDS–PAGE and Western blotting

SDS–PAGE analysis of different concentration of sporulated oocyst antigen (SOA) employed showed that a number of polypeptides were present in the homogenate with high, medium and low molecular weights (Fig. 4). Most of the bands were concentrated in the range between 54 and 108 kDa, but numerous peptides of smaller molecular weights were also identified between 16 and 38 kDa. The strongest bands corresponded to peptides of 74, 54, 23 and 20 kDa.

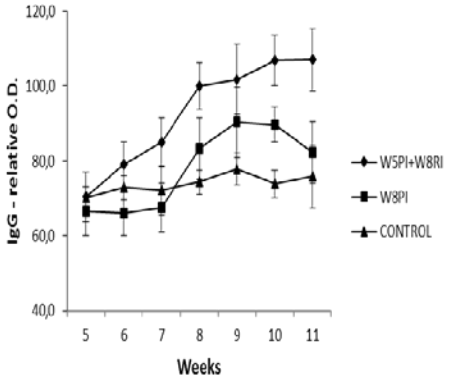

Figure 1. Evolution of mean IgG anti-SOA levels (±standard error) expressed as relative optical densities (O.D.) in sera from all the experimental groups: W5PI+W8RI = primary infected with 2 x 10^5 *Eimeria ninakholyakimovae* sporulated oocysts at week 5 of age and reinfected at week 8; W8PI= primary infected at week 8 of age or challenge controls; C= uninfected animals. (*) *P<0.05* and (**) *P<0.01* represent significant differences between infected animals and control group.

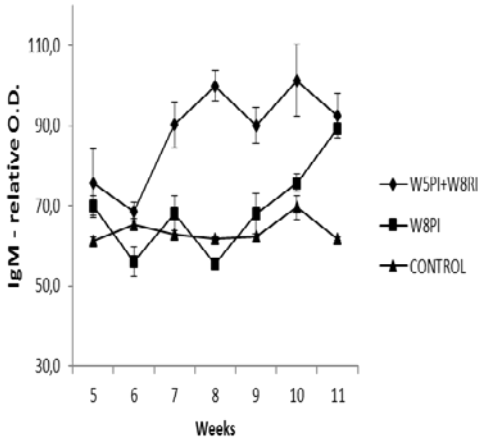

Figure 2. Evolution of mean IgM anti-SOA levels (±standard error) expressed as relative optical densities (O.D.) in sera from all the experimental groups: W5PI+W8RI = primary infected with 2 x 10^5 *Eimeria ninakholyakimovae* sporulated oocysts at week 5 of age and reinfected at week 8; W8PI= primary infected at week 8 of age or challenge controls; C= uninfected animals. (*) *P<0.05* and (**) *P<0.01* represent significant differences between infected animals and control group.

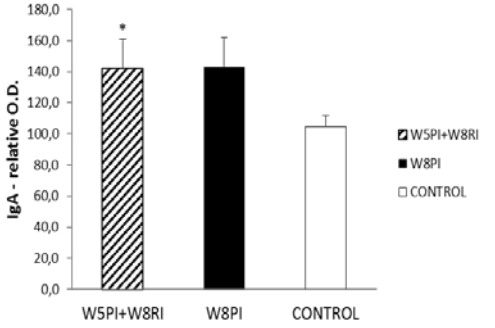

Figure 3. Levels of IgA anti-SOA levels ± SD in ileal mucus expressed as relative optical densities (O.D.) in sera from all the experimental groups: W5PI+W8RI = primary infected with 2 x 10^5 *Eimeria ninakholyakimovae* sporulated oocysts at week 5 of age and reinfected at week 8; W8PI= primary infected at week 8 of age or challenge controls; C= uninfected animals. (*) *P<0.05* and (**) *P<0.01* represent significant differences between infected animals and control group.

For the representation of the Western blotting results, a selection of the clearest and most exemplary weeks was made (Figure 5). The staining of the nitrocellulose membrane with Ponceau-S confirmed a good transference of proteins, as shown for the molecular weight marker (MWM). IgG from primary infected animals at week 5 firstly recognized specific bands after 1 weeks p.i., both in kids primary infected at 5 (W6/W5PI+W8PI) and 8 weeks of age (W9/W8PI); the intensity of the bands was bigger the following weeks though. In contrast to the pool of all week negative control sera (C), those of infected goat kids reacted with polypeptides of different molecular weights, ranging from 108 to 21 kDa. Peptides of 108 kDa and 28 kDa were the most strongly recognized. No differences either on the band profile or the intensity of the reactions were found between sera from re-infected (W5PI+W8RI) animals and the corresponding challenge controls (W8PI).

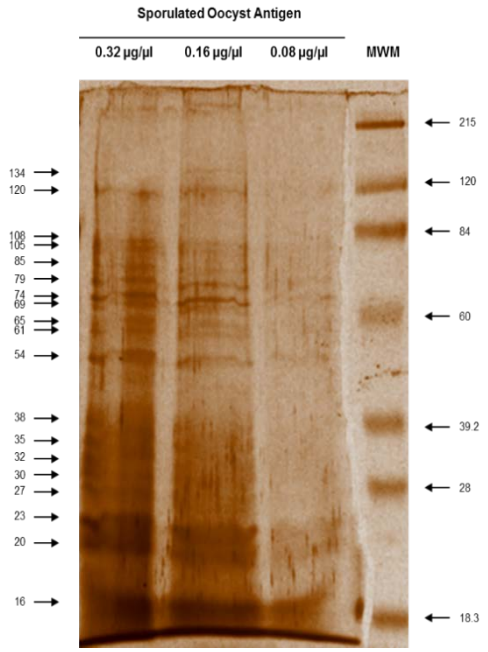

Figure 4. Protein bands separated by SDS–PAGE analysis using different concentration of sporulated oocyst antigen (SOA). MWM: molecular weight marker in kDa.

4. Discussion

Based on the data obtained in the present study, the experimental infection of goat kids with 2 x 10^6 *E. ninakohlyakimovae* sporulated oocysts results in a patent infection with moderate so severe clinical signs and high rates of oocyst excretion in primary infected animals, while challenged goat kids only displayed mild signs of illness or no symptoms at all and the OPG counts were significantly lower. This is in accordance to previous studies performed with *E. bovis* in cattle (Hermosilla et al.,, 1999; Taubert et al., 2008; Sühwold et al., 2010) and also to those found by our group in goats (Ruiz et al., 2013; 2014), which reinforce the conviction that this *Eimeria* species is able to develop protective immune responses in goats. Probably related to this response, increased serum levels of specific IgG and IgM and local IgA were found to be increased in all the infected animals. Additionally, peripheral IgG could recognize a panel of different antibodies against the SOA (sporozoite oocyst antigen) used for immunoenzimatic reactions.

More than 20 years ago, Kanyari (1988) demonstrated significantly increased antibody titres post-infection in two breeds of goats (Saanen and Anglonubians) after an experimental infection with 200,000 sporulated oocysts comprising mainly *E. christenseni* (49%), *E. apsheronica* (29%) and lesser proportions of *E. arloingi*, *E. hirci*, *E. ninakohlyakimovae* and *E. alijevi*. At our knowledge, no further studies on the humoral immune response in goats have been performed afterwards. Relatively more information concerning antibody response against coccidiosis in sheep and cattle is available in literature (Nolan et al., 1987; Hughes et al., 1989; Fiege et al., 1992; Dominguez et al., 2001; Faber et al., 2002). The results of the study published by Dominguez et al. (2001) showed that the half-life time of IgG1, by far the predominant isotype transmitted with colostrum in sheep (Reynolds and Griffin, 1990), comes up to 11–13 days in the lamb (Klobasa and Werhahn, 1989; Watson, 1992; Dominguez et al., 2001), which suggests that this, and probably other maternal antibodies, would not be longer present in serum samples from goat kids used in the present study, taking into account that all the animals were purchased having 1-5 days of life. So, the levels of antibodies founds throughout the experiment should be considered as *de novo* synthesis. Significantly increased levels of serum IgG were already observed after 3 weeks p.i., slightly after the oocyst shedding reached peak values, which suggest that the immune system needs a boosting for the production of specific antibodies. Specific IgG values remained high and progressively increased during the next sampling times, probably related to further stimulation induced by re-infection made at week 8. In accordance, IgG levels recorded in challenge control group did not last increased but declined after the three week peak. Similarly, serum IgG against sporozoites reached a peak of activity between 10 and 20 days p.i., coinciding with oocyst shedding on

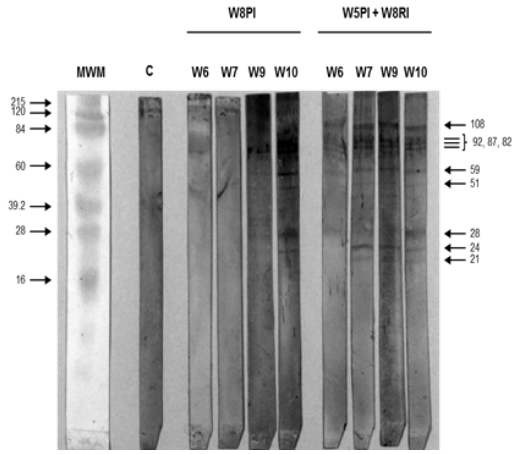

Figure 5. Immunorecognition using Western blotting of *Eimeria ninakholyakimovae* sporulated oocysts antigens (SOA) by pool sera from goat kids infected with 2 x 10^5 oocysts at different weeks p.i. (Lane 1) molecular weight markers (MWM) in kDa.; (Lane 2) negative control; (lines 3-6) W8PI= primary infected at week 8 of age or challenge controls; (lines 7-10) W5PI+W8RI = primary infected at week 5 of age and re-infected at week 8.

days 17 to 24, and the antibody titers dropped to base levels by 40 days p.i. (Hughes et al., 1989). Faber et al. (2002) also found IgG_2 as the main isotype in the humoral response, in association to Th2 responses against *E. bovis*. Apart from IgG_2, the IgG_1 subfraction and specific IgM can be transferred to calves via colostrum (Fiege et al., 1992), and serum IgM, IgA and IgG2 have been correlate significantly with excretion of oocysts of *E. bovis,* whereas IgG1 is not directly related to the course of the disease (Faber et al., 2002). In our study, IgM levels were equally boosted by *E. ninakholyakimovae* infection but, compared to IgG profile, significant high values were already demonstrated after two weeks p.i. However, for this immunoglobulin, differences between re-infected animals and challenge controls could not be found. Similarly, local IgA from ileal mucus was significantly increased in infected animals compared to controls, but without differences between primary and re-infected animals. Altogether, the analysis of the antibody response suggests that, although the different immunoglobulins actually reflect exposure to *E. ninakholyakimovae* an unambiguous correlation to the level of protection could not be demonstrated, as previously referred (Fiege et al., 1992). Accordingly, although occasional relationships between OPG counts and the levels of either IgG, IgM or IgA were found, correlations could not be proven statistically.

Specific peptide fractions recognized by serum IgG of animals using sporulated oocysts antigens (SOA), with molecular weights ranging between 134 to 16 kDa, support the value of electroimmunoblotting as a technique to detect immunoreactive proteins in goat coccidiosis. This approach, which had not been used before in caprines against *Eimeria* infections, has been extensively employed in poultry coccidiosis (Reduker et al., 1986; Jenking and Dame, 1987; Karkhanis et al., 1991; Xie et al., 1992; Krücken et al., 2008. Likewise), could demonstrated a panel of both common and unique protein bands in extracts of merozoites and sporozoites *E. bovis*, ranging in molecular weight from 15,000 to 215,000. The authors showed that of 3 types of immune sera used to probe immunoblots, serum taken from a calf that had been inoculated with oocysts of *E. bovis* and boosted 10 weeks later consistently identified and reacted more intensely with more antigens of merozoites and sporozoites than the other immune sera tested. This observation however could not be demonstrated in our study. By contrast, the pattern of peptide of SOA recognition did not differ between primary infected and challenged animals, indicating that there was no correlation between natural protection by previous exposure to the parasite and the specific antibody response, as also discussed for the ELISA results. This circumstance might be related to the type of antigen used in the ELISA and EIB determinations, as different *Eimeria* antigens have been demonstrated to exhibit specific immunogenicity (Fayer et al., 1992). It should be also considered that the age of the animals could strongly affect the development of protective immune response, both cellular and humoral (Smith et al., 1985; unpublished personal observations). Nevertheless, Lillehoj and Ruff (1987), found similar antibody

response y chickens with different susceptibility to coccidiosis. Besides, sublines of chickens selected for high (HA) or low (LA) antibody response that differed at the major histocompatibility complex (MHC) had no differences in response to *E. tenella* infections (Dunnington et al., 1992). Fiege et al. (1992) also found no correlations between the levels of any specific antibody or the recognition patterns and the status of immunity to a severe challenge in experiments performed on colostral transfer of *E. bovis* antibodies in cattle.

Classical research studies carried out worldwide to try to elucidate the mechanism of protective immunity against coccidiosis usually conclude that cellular immunity is the key to protection against *Eimeria*, whereas humoral immunity plays a very minor role in resistance against infection. By contrast, other studies have pointed towards the ability of antibody to block parasite invasion, development and transmission and to provide passive and maternal immunity against challenge infection (Wallach et al., 2010). Altogether, the results of the present study contribute to understand the complexity of the antibody response against caprine coccidiosis. Further investigations on the immunorecognition of other antigen preparations would be valuable for the immunoprophylactic approach for coccidiosis in livestock.

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ARTÍCULO Nº 4

Influencia de la edad en la respuesta inmune a la infección experimental por *Eimeria ninakholyakimovae* en los cabritos.

In preparation to: *Parasitology Research*

▪ RESUMEN ▪

La coccidiosis de los pequeños rumiantes es de especial relevancia clínica y productiva en el animal joven, particularmente alrededor del destete. *Eimeria ninakohlyakimovae* es considerado como uno de los más patógenos para la cabra, capaz de producir altas tasas de mortalidad y considerable retraso de peso. Al igual que en la coccidiosis en otras especies hospedadoras, frente a las especies de *Eimeria* que afectan a la cabra se produce una fuerte respuesta inmune tras la infección primaria, que induce protección frente a reinfecciones futuras, al menos parcialmente. Tanto la respuesta inmune desarrollada como la capacidad de albergar infecciones patentes por *Eimeria* spp. son circunstancias que pueden depender de la edad del huésped. Con el objetivo de evaluar la influencia de la edad en el desarrollo de la respuesta inmune protectora frente a *E. ninakholyakimovae*, cabritos de 3, 4 y 5 semanas de edad se inocularon vía oral con 2 x 10^5 ooquistes esporulados y 3 semanas después se sometieron a una reinfección homóloga (animales reinfectados). Cabritos primo-infectados a las 6, 7 y 8 semanas de edad con la misma dosis sirvieron como controles de la reinfección. Por último, se emplearon animales no infectados como controles de la infección. Los tres grupos de edad se denominaron A, B y C, respectivamente. La inmunidad protectora después de la primo-infección y la reinfección posterior en los diferentes grupos de edad se evaluó en base a parámetros clínicos, productivos, hematológicos, parasitológicos, inmunológicos (respuesta inmune celular y humoral) y patológicos (lesiones macroscópicas y microscópicas en colon e íleon). Para ello, se tomaron muestras fecales de cada individuo y muestras de sangre. El peso corporal y condición clínica de los animales también se examinaron y, al final del experimento, todos los cabritos fueron sacrificados y sometidos a una necropsia y posterior análisis histopatológico. En conjunto, los resultados del presente estudio demuestran que los cabritos de 3, 4 o 5 semanas de edad son capaces de desarrollar infecciones patentes y respuestas inmunoprotectoras frente a *E. ninakholyakimovae*: (i) en todos los grupos de edad el recuento ooquistes fue significativamente menor después de la reinfección, y se

asoció a signos clínicos más leves en comparación con los controles de reinfección; (ii) todos los grupos de edad mostraron una respuesta proliferativa a nivel local con aumento significativo en la mayoría de las poblaciones de células analizadas (eosinófilos, linfocitos, neutrófilos, leucocitos globulares y mastocitos); y (iii) aunque la respuesta de anticuerpos no fue tan evidente como la celular, se encontraron evidencias de que el aumento de los niveles de IgG e IgM y, principalmente, de IgA local, podrían estar asociados a la infección por *E. ninakholyakimovae*. Sin embargo, cuando se realizó un análisis más detallado de los datos, se observaron algunas diferencias entre los tres grupos de edad, relacionadas con el resultado de la infección por *Eimeria* y la respuesta inmune resultante. Estas diferencias sugieren que los cabritos más jóvenes pueden no ser completamente inmunocompetentes. Este hallazgo puede ser de interés para el diseño de estrategias inmunoprofilácticas o, incluso, de pautas de tratamiento metafiláctico/profiláctico frente a la coccidiosis caprina.

Age-related immune responses to experimental infection with *Eimeria ninakholyakimovae* in goat kids

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Abstract

Coccidiosis of small ruminants is of special clinical and productive relevance in young animal, particularly around weaning. *Eimeria ninakohlyakimovae* is considered as one of the most pathogenic for goat, able to produce high rates of mortality and considerable weight delay. As for coccidiosis in other host species, a strong immune response is mounted against Eimeria infections in goats, which induces protection against future re-infection, at least partially. Both the immune response developed against the parasite and the ability to bear patent Eimeria spp. infections are circumstances that may depend on the age of the host. With the aim to evaluate the influence of the age in the development of protective immune responses against E. ninakholyakimovae, goat kids of 3, 4 and 5 weeks of age were infected with 2 x 10^5 sporulated oocysts and subjected to a homologous challenge 3 weeks latter (challenge infected animals). Goat kids primary infected at 6, 7 and 8 weeks of age with the same dose served as challenge controls. Finally, uninfected animals were used as controls for the infection. The three age groups were named A, B and C, respectively. The protective immunity after primary infection and subsequent reinfection in the different age groups was assessed by clinical, productive, hematological, parasitological, immunological (antibody and cellular immune responses) and pathological parameters (gross and microscopic lesions in colon and ileum). For this purpose, individual faecal and blood samples were taken. The body weight and clinical condition of all the animals were also examined and, at the end of the experiment, all the goat kids were euthanized and further subjected to necropsy. Altogether, the results of the present study demonstrated that goat kids of either 3, 4 or 5 weeks of age are able to develop patent infections and immunoprotective responses against *E. ninakholyakimovae* oocysts: (i) all age groups released significantly less oocysts per faeces after challenged, which was associated to milder clinical signs compared to challenge controls; (ii) all age groups displayed a proliferative immune response at the local levels with significant increase in most of the cell populations analysed in the study (eosinophils, lymphocytes, neutrophils, globular leucocytes and mast cells); and (iii) although the antibody response was not so evident than the cellular counterpart, there were evidences showing that light increases in IgG and IgM, and mainly in local IgA, could be associated to E. ninakholyakimovae infection. Nevertheless, when a more detailed analysis of the data was performed, some differences were observed between the three age groups, related both to the Eimeria infection outcome and the resulting immune response, all of them suggesting that the youngest kids may be not fully immunocompetent. This finding may be of interest for the design of immunoprophylactic approaches or even prophylactic/methaphylactic treatments against goat coccidiosis.

Key words: *Eimeria ninakohlyakimovae*, immune response, age, goat kids

1. Introduction:

The infection caused by most pathogenic *Eimeria* species affecting goats, including *Eimeria ninakholyakimovae,* may produce severe clinical disease and important production losses, particularly in intensive production systems (Dai et al., 2006; Ruiz et al., 2006; 2014). Within a herd, most of the animals are usually infected irrespectively of the age, but those which suffer from a more severe disease are young animals between 2-4 weeks and 4 months (Koudela and Boková, 1998; Ruiz et al., 2006). Beyond this age range, the disease becomes self-limiting and aged animals transform in silent carriers able to contaminate the environment through the release of continuous amounts of oocyts with faeces, as it has been shown for cattle (Daugschies y Najdrowski, 2005). This outcome is the consequence of a complex interaction between both innate (Rosenberg et al, 2005; Boysen et al., 2006; Taubert et al., 2009; Behrendt et al, 2010; Min et al, 2013) and acquired immune reactions (Fiege et al., 1992; Rose et al, 1992; Hermosilla et al., 1999; Shi et al. 2001; Wallach, 2010). Besides, the specific immune response in ruminant coccidiosis involves a variety of reactions that includes both cellular and humoral components (Daugschies and Najdrowski, 2005).

In the *Eimeria* species that affect higher mammals, especially during the process of cell invasion, the sporozoites are exposed to different immunocompetent cells, i.e. PMN, which could constitute an important opportunity of the innate immune system early to eliminate or reduce the level of infection, as demonstrated in different species from cattle and goats (Hermosilla et al., 2006; Behrendt et al., 2010; Ruiz et al., 2014). The role of monocytes is less known, although there is evidence that this cell type is capable to induce degeneration of macroesquizontes both in animals infected with *E. bovis* and *in vitro* (Friend and Stockdale, 1980; Hughes et al., 1987). It has also been shown that bovine monocytes increase the oxidative and phagocytic activity in presence of sporozoites of *E. bovis* and produce different cytokines and chemokines when they are infected with merozoites I (Taubert et al., 2009). Both cell populations have been proven to elicit extracellular traps upon activation with different *E. bovis* stages/antigens (Behrendt et al, 2010; Muñoz- Caro et al., 2014; Silva et al., 2014). It has also pointed out the importance of mast cells in the innate immune response against coccidia, mainly in birds, where it has been shown an increase in the number and activity of these cells after infection by different species of the genus *Eimeria* (Morris et al., 2004). On another hand, many studies indicate and confirm the important role played by lymphocytes in immunity type T against coccidian parasites, e.g. in the re-infection by *Eimeria* sp. and *Toxoplasma gondii*. In particular, the T-cell-mediated response has been associated with a lower excretion of oocysts in animals re-infected with *E. bovis* (Hermosilla et al., 1999; Taubert et al., 2008; Suhwold et al., 2010), with CD4 + and CD8 + lymphocytes being the main population involved. Both CD4 + and CD8 + T cells lymphocytes mediate the action of cytokines, such as the INFγ or IL-1, i.e. by recruiting phagocytic cells that further promote inflammation and cell lysis. In general, during the primary infection with *Eimeria* species an increase in the production of INFγ is observed, which suggest the establishment of a Th1 cellular immune response (Taubert et al., 2008).) On the other hand, in calves primary infected with *E. bovis*, there is a predominance of IL2 (Th1) gene expression compared to IL4 expression (Th2) in relation to the controls, suggesting an increase in the cytotoxic cellular activity (Hermosilla y cols., 1999). Finally, eosinophils have also been described as important components of both the innate and acquired response in Apicomplexa protozoan infections. Thus, in primary infected with *E. adenoeides*, eosinophils were the only leukocyte population increased in relation to non-infected turkeys (Gadde et al, 2009).

In addition to the cellular response in the acquired immunity against *Eimeria* infections, increasingly studies highlight the importance of the humoral response. This response develops quickly and is characterized by the appearance of high antibody titters in the

serum of the infected animals, with an initial increase of IgM, followed by IgG; also other specific immunoglobulins such as IgA, may also appear. In general, the amount of antibodies increases if the animals are continually exposed to the oocysts (Reeg et al., 2005; Daugschies and Najdrowski, 2005). The antibodies can even transmitted through the colostrum, as shown by Gregory et al (1989) and Fiege et al. (1992), sometimes in correlation with the intensity of the infection of the offspring (Faber et al., 2002).

The age of the animals influences the immune response to infection, not only in coccidiosis, but also in other common parasitic diseases of ruminants. For instance, studies conducted in sheep, from two weeks to 10 months of age, infected with *Teladorsagia circumcincta* showed that younger animals were more susceptible to reinfections and displayed a poor local immune response against gastrointestinal nematodes (Smith et al., 1985). Also in vaccination approaches, age-related immunoprotection seems to be a perspective to be considered. Thus, experimental challenge infection by immunization with excretory/secretory products (ES) from *Haemonchus contortus*, containing predominantly proteins of 15 and 24 kDa, depends on the age of the sheep; i.e. vaccinated sheep 9 and 6 months of age had reduced final worm burdens of 82 and 77, respectively, whereas no reduction in worm burden was found in 3-month-old lambs. This circumstance should be particularly considered for the development of immunoprophylactic and therapeutic strategies against ruminant coccidiosis, as vaccine-induced protection has to elicited within the first weeks of age, before the a clinical impairment of the disease occurs. Taking all these considerations into account, the objective of the present study has been to investigate the influence of age in the development of protective immune responses in experimental goat coccidiosis by *Eimeria ninakohlyakimovae*.

2. Material al methods

2.1. *Parasites and animals*

Experimental infections were performed by using the *Eimeria ninakholyakimovae* strain GC isolated from our laboratory from field samples and maintained by passages in goat kids for oocysts production (Ruiz et al. 2013). For harboring oocysts, goat kids were orally infected at the age of 4 week with 2 x 105 sporulated *E. ninakholyakimovae* oocysts, and the oocysts were isolated and purified from faeces after 2 week post infection according as described previously (Jackson 1964; Ruiz et al., 2013). The purified oocysts were maintained at 4 °C in a 2% potassium dichromate solution to further use.

A total of 24 goat kids of the Majorera breed purchased from local farmers (Gran Canaria) were used. Newborn kids of 1-5 days were washed with a diluted solution of sodium hypochlorite in warm water in order to eliminate oocysts that may be attached to the hair, then dried and distributed in separated boxes into metabolic cages equipped with heat sources. The day of the arrival and a week later, all animals were treated with Vecoxan® (Janssen-Cilag) and, for a week with Halocur® (Intervet), following the manufacturer's recommendations in both cases. They were fed with milk substitute (Bacilacto®, Capisa) and starter (Capisa) and, in all cases, sterile hay, minerals and water were available ad libitum. The animals were treated according to the guidelines adopted in the European Communities Council of 24 November 1986 (86/609 / EEX) and the current Spanish legislation for the use and care of animals RD 1201/2005 (BOE 252 / 34367-91, 2005).

2.2. Experimental design

For experimental purposes, the animals were divided into three experimental age groups, each consisting of three subgroups: (PI+RI) primary infected animals

at 3, 4 and 5 weeks of age, and three weeks later; (PI) primary infected animals at 6, 7 and 8 weeks or controls of re-infection; and (C) uninfected animals. Age groups were arbitrarily named as A (W3PI+W6RI; W6PI), B (W4PI+W7RI; W7PI) and C (W5PI+W8RI; W8PI). Both primary and challenge infection were performed orally with 2 x106 *Eimeria ninakholyakimovae* sporulated oocysts by using a gastric feeding tube. The protective immunity after primary infection and subsequent reinfection in the different age groups was assessed clinical parameters (signs of eimeriosis, i.e. diarrhea), production parameters (body weight), hematological, parasitological parameters (faecal oocyst counts), immunological parameters (antibody and cellular immune responses) and pathological parameters (gross and microscopic lesions in colon and ileum). For this purpose, individual faecal and blood samples were. The body weight and clinical condition of all the animals were also examined and, at the end of the experiment, all the goat kids were euthanized and further subjected to necropsy.

2.3. *Clinical, coprological and haematological determinations*

Throughout the whole the experience the clinical signs of the animals was evaluated by inspecting the hydration state, food intake and the presence of diarrhea, evaluated according to their consistence, color, presence of blood or mucus. In addition the weight of the animals was weekly monitored.

For coprological analyses, faecal samples were daily taken from day 14 postinfection (p.i.); non-infected controls were also subjected to coprological analysis in order to verify the absence of infection. For the quantification of the oocysts excretion a modified McMaster technique was used (Thienpont et al., 1979) and the results were expressed as oocysts per gram of faeces (OPG). In cases of very high OPG values, dilutions of the faecal suspension by 10 or 100 times were performed to enable accurate counting according to Bangoura and Daugschies (2007).
For hematological analysis, blood samples were taken weekly by jugular vein puncture. The total leukocyte count and the hemoglobin concentration were determined in IDEXX VetConnect® 1-mL tubes by using a LaserCyte® hematology analyzer (Idexx). The hematocrit value was calculated by centrifugation using standard centrifuge capillaries. Differential white blood cell (WBC) counting was performed manually; for this purpose, 200 leukocytes were counted in stained blood smears (Diff-Quick).

2.4. *Pathological and histopathological analysis*

At weeks 9, 10 and 11 of the experiment, animals of different age groups were sacrificed in order to perform histopathology. Goat kids primary infected at week 3 of age and challenged at week 6 (W3PI+W6RI) and the corresponding challenge control subgroup (W6PI) (age group A) were euthanized at week 9 of the experiment, while weeks 10 and 11 of the experience were reserved animals from age groups B and C, respectively. During the necropsy, all macroscopic lesions were annotated and tissue samples were collected from the intestinal mucosa (ileum, colon). Additionally, mucus samples were taken from the ileum of the animals for the analysis of specific IgA levels. For their preservation, the samples were suspended in a pH 7.1 buffer containing proteinases inhibitors (all compounds from Sigma-Aldrich): 0.1M Na2HPO4, 0.05 M NaCl, 3 mM NaN3, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM ethylenediaminetetraacetic acid (EDTA). Mix solutions containing mucus were centrifuged at 5000 x g (Eppendorf Centrifuge 5804R) for 1 h at 4°C and the resulting supernatant was conserved at -20ºC up to further analyses.
The tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Cross sections of 4–5µm were stained by haematoxylin and eosin (H&E) and Giemsa according to standard staining procedures. Quantification of leucocyte populations (neutrophils, lymphocytes, eosinophils, globular leukocyte and mast cells) in tissue sections was performed on the ileum and colon samples of uninfected (C), challenge

controls (PI) and challenge infected goat kids (RI). Cells were counted using a 10× eyepiece containing a calibrated graticule and 40× objective lens viewing an area of 0.05265 mm2. The counts were randomly taken on 20 graticule fields within the mucosal surface. The counts were expressed as number of cells per mm2 of mucosa (Amarante et al., 2005).

2.5. Enzyme – linked immunosorbent assay (ELISA)

To determine the levels of specific IgG and IgM antibodies, sera samples weekly collected were kept at -20°C until the appropriate tests performed. For the IgG indirect ELISA test, a total of 100 µl of a SOA solution in carbonated buffer at a concentration of 5µg/ml was dispensed in 96 wells ELISA plates (CORNING Dispensable Sterile ELISA Plates, Corning Glass Works) and incubated overnight at 4°C. After three washes with 200 µl of PBS-0.05 % Tween 20, 200 µl of PBS-3% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) was added and the plates were incubated for 45 min at 37°C. After each new step a similar washing procedure was addressed. Following treatment with BSA, individual serums, positive and negative controls were analyzed in duplicate using a 1/25dilution in PBS-Tween 20 0.02% sodium acid. For each sample, a volume of 100 µl was added to the plates and a new incubation was performed at 37°C for 1h. The plates were then incubated with 100 µl per well of 1/2000 anti-Goat IgG (anti-goat IgG peroxidase conjugate, IgG fraction of antiserum, Sigma-Aldrich) diluted in 0.01 M PBS and the incubation was performed in the same conditions. Thereafter, a total of 100 µl of substrate was added to each well, a mix of compounds that includes citric-phosphate buffer, 0.04% (w/v) dihydrochloride ortho-phenyleneadiamine (OPD, Sigma-Aldrich) and 30% hydrogen peroxide (Panreac) at a final concentration of 0.1% (v/v). The incubation with the substrate was performed at room temperature in darkness for approximately10 minutes. Finally, the reactions was stopped by adding 35 µl per well of a 2M solution of sulfuric acid (Panreac) and the optical density (O.D.) measured at a wavelength of 492 nm (Termo Labsystems, Multiskan Ascent).

For specific IgM and IgA indirect ELISA tests a similar protocol was employed but using 1/3000 anti-goat IgM peroxidase and 1/7000 anti-goat IgA peroxidase conjugates, respectively (both from Sigma-Aldrich). Similarly, dilutions 1/25 of the different serum or mucus samples were employed as primary antibody.

2.6. Statistical analysis of data

Faecal OPG were logarithmically transformed and added by 1 [log (OPG+1)] in order to obtain normal distributions according to the Kolmogorov-Smirnov's normality test. Levels of the different immunoglobulins analysed in the study were expressed as the relative percentage of the optical density (O.D.) of a positive control pool in order to avoid inter-assay differences (Relative O.D.). One factorial analysis of variance, Tukey's multiple comparison test and Student's t-test were used to analyse the data between different experimental groups and the Pearson correlation test for the evaluation of the association between different parameters assessed in this study. For that purpose, a statistical software (SigmaPlot 12.0) was employed and the differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. OPG counts, clinical signs and body weights

In all age groups of kids re-infected with *E. ninakohlyakimovae* sporulated oocyst (A, B and C) oocyst counts per gram of feces (OPG) were consistently lower than in animals from the corresponding challenge controls (Fig. 1). Similarly, after primary infection, goat kids subsequently challenged 3 weeks later had significantly reduced OPG counts ($p < 0.05$ to $p < 0.001$). In general, the prepatent period ranged from 14 to 17 days p.i., with slightly longer records found in goat kids primary infected at younger age, mainly W3PI group.

On the contrary, some of the re-infected animals (i.e. from W5PI+W8PI) extended their prepatent period up to day 18 p.i. Peak values were not certainly related with duration of the prepatent periods of the different animal groups, except for kids from primary infected at week 3 (W3PI) and subsequently challenged, in which the highest OPG counts were recorded slightly latter (day 17 p.i.) in both groups.

All inoculated animals developed a patent infection, but the severity of the disease was less apparent, and even imperceptible, in re-infected animals in all age groups (A, B and C) (Fig. 2). Clinical signs, which included anorexia, weakness, impairment of general condition and different degrees of diarrhoea, were particularly intense in some primary infected animals at week 5 and 4, moderate in those infected at week 6, 7 and 8 and moderate to mild in 3 week infected ones. In terms of production, both the challenge infected (PI+RI) and challenge controls groups of animals (PI) had lower mean body weights that the corresponding non-infected animals. Statistical significance ($p < 0.05$ to $p < 0.01$) was particularly evident when comparing the weight progress of W4PI+W7PI, although in this case the differences between re-infected animals and the corresponding challenge control (W7PI) could not be proven statistically. By contrast, slight significant differences ($p < 0.05$) were found when comparing body weights of re-infected animals at week 6 (W3PI+W6RI) to those primary infected at week 6 (W6PI), at least in certain weeks.

Hematologic changes were very mild in all groups and only a moderate increase in the total number of leukocytes, neutrophilia with left shift and temporary monocytosis was observed (data not shown). Finally, in the last weeks of the experiment, a moderate eosinophilia was observed, which was particularly evident in the group primary infected at 5 weeks and then challenged (W5PI+W8RI).

3.2. Pathological and histopathological analysis

At necropsy, all intestinal organs of the infected animals were apparently normal, except for some evidence of congestion and thickening of the intestinal mucosa, mainly affecting colon caecum, but also some portions of the ileum. Histological examination showed a moderate hyperplasia and hypertrophy of the mesenteric lymph nodes, and Peyer's patches, eosinophilic enteritis and mastocytosis with diffuse infiltration of lymphocytes, neutrophils and globular leukocytes. Uninfected animals did not show any alteration on gross morphology and microscopical examination did not reveal any obvious change either.

With some differences depending on the age group, both the ileum and colon mucosa of the infected goat kids were significantly infiltrated by different inflammatory cells, including neutrophils, mast cells, eosinophils, globular leukocytes and lymphocytes when compared to tissue samples from uninfected control animals. Eosinophils in ileum were higher than in colon for all animals (Fig. 3). For ileum samples, the higher counts corresponded to challenged groups (W3PI+W6RI, W4PI+W7RI and W5PI+W8RI), which, except for group W5PI+W8RI, had significantly increased values compared to their corresponding challenge controls (W6PI and W7PI) ($p < 0.05$ to $p < 0.001$). By contrast, approximately the same counts were recorded for colonic samples both in challenge and the corresponding challenge control groups. Significant differences with respect to controls were found for both challenged and control challenged goat kids in all age groups (Fig. 3). A similar pattern was found for lymphocyte counts, although in this occasion differences between ileum and colon were not so evident (Fig. 4). Like for eosinophils, lymphocyte counts were significantly higher in all the infected animals when compared to controls ($p < 0.001$ to $p < 0.0001$) and, in this case,

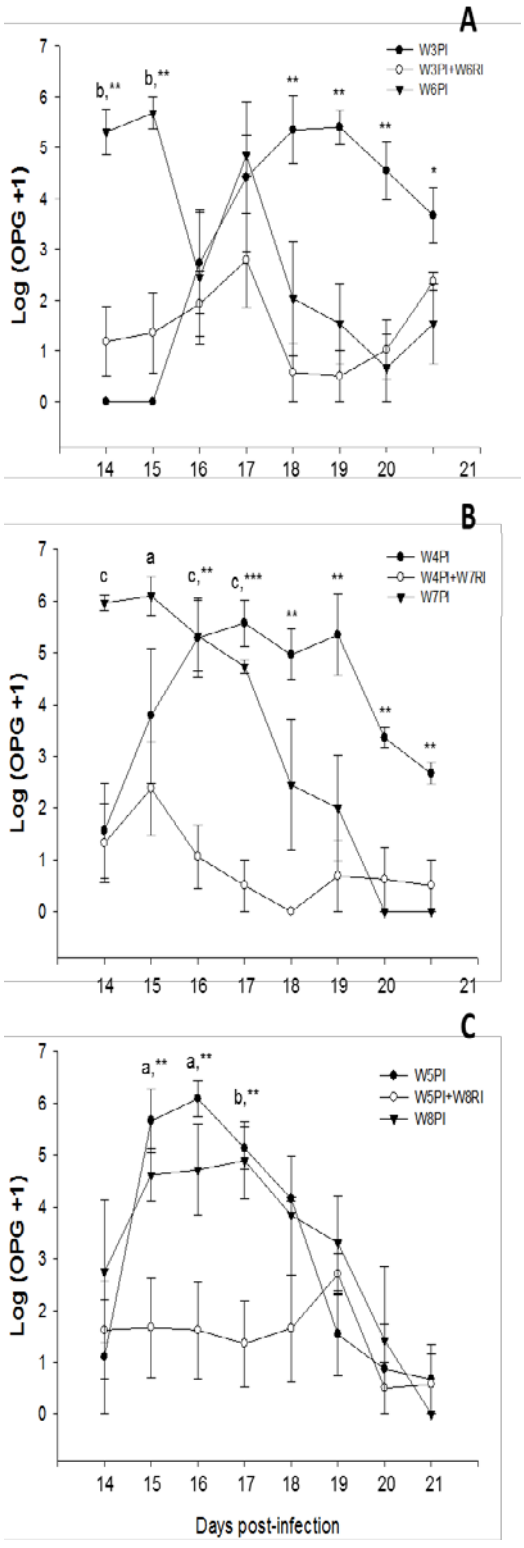


Figure 1. OPG (oocysts per gram of faeces) counts from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). OPG counts are depicted as the logarithm of the OPG plus one (log [OPG + 1]) and represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ represent significant differences between primary and challenge infections between a same age group; (a) $p < 0.05$, (b) $p < 0.01$ and (c) $p < 0.001$ 001 represent significant differences between challenge infected and challenge control subgroups.

statistical differences between challenge and control challenge groups could not be proven in group WPI3+W6RI. Most of neutrophil counts were found to be increased as well in comparison to uninfected controls, with significant levels ranging from $p < 0.05$ to $p < 0.001$ (Fig. 5). However, in this cell population, higher counts corresponded to primary infected challenge controls, with differences ranging from $p < 0.05$ to $p < 0.001$ in relation to challenged groups. As for neutrophil counts, the number of globular leukocytes at the intestinal mucosa of primary infected or challenge kids were, in general, higher than counts recorded for challenge groups (Fig. 6) ($p < 0.05$ to $p < 0.01$) and no many differences were found between ileal and colonic samples; all the infected animals had increased globular leukocyte counts though when compared to control samples ($p < 0.05$ to $p < 0.001$). Finally, in samples from ileum, mast cells counts were significantly increased in groups W3PI+W6RI and W6PI ($p < 0.001$ and $p < 0.05$, respectively) and also in group W7PI ($p < 0.05$), while in colonic samples the three challenge control groups had increased records for this cell population ($p < 0.01$ to $p < 0.001$) (Fig. 7). In colon, counts from challenge animals were generally lower than in the corresponding challenge groups, with significant differences ranging from ($p < 0.05$ to $p < 0.001$) The global cell counts for lymphocytes and neutrophils were approximately the same among the three age groups considered, whereas younger groups, particularly those primary infected at week 3

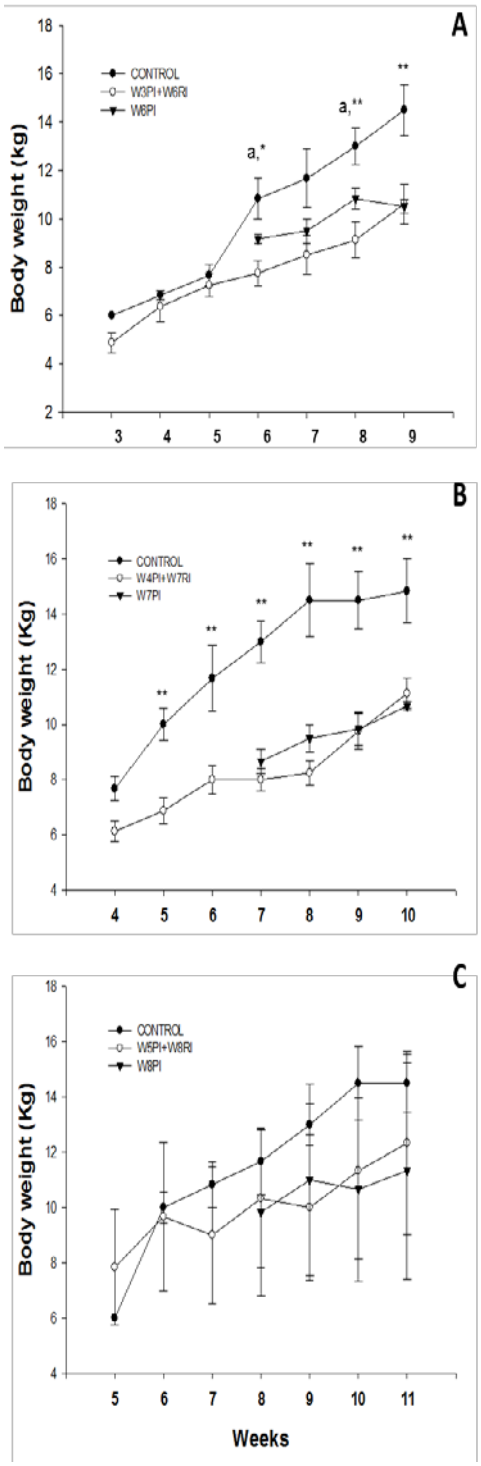


Figure 2. Body weights of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences between infected animals and CONTROL; (a) $p < 0.05$ represents significant differences between challenge infected and challenge control subgroups.

and challenged week latter (W3PI+W6RI) and the corresponding challenge control (W6PI), had higher records for the remaining cell populations analyzed in the study. Correlations between the different cell scores and their respective parasitological and clinical data were not proven statistically.

3.3. Enzyme-linked immunosorbent assay (ELISA)

The specific levels of IgG did not differ from uninfected controls profile in age groups A and B, while in those primary infected at week 5 and subsequently challenged at week 8 (group C) a gradual increase was observed up to the end of the experiment; the differences were not statistically significant though (Fig. 8). Peack values for specific IgM were recorded three week after the primary infection in group C and then gradually decreased up to the end of the experiment. By contrast, IgM relative O.D. for groups A and B slightly increased week to week from the beginning to the end of the experiment without a clear peak being detected. With some fluctuations, challenge controls of groups A, B and C slightly increased after infection and had weak peak values around 2 week p.i. No significant differences could be detected in any of the age groups (Fig. 9). At last, local IgA levels were proven to be significantly higher in challenge control animals of all age groups ($p < 0.05$ to $p < 0.01$), while only challenged kids from age group A had increased relative O.D. for this immunoglobulin ($p < 0.05$) (Fig. 10). Probably due to fluctuations of optical densities among animals and the scarce number of animals per group, as for cell counts, no correlations could be proven to parasitological or clinical data.

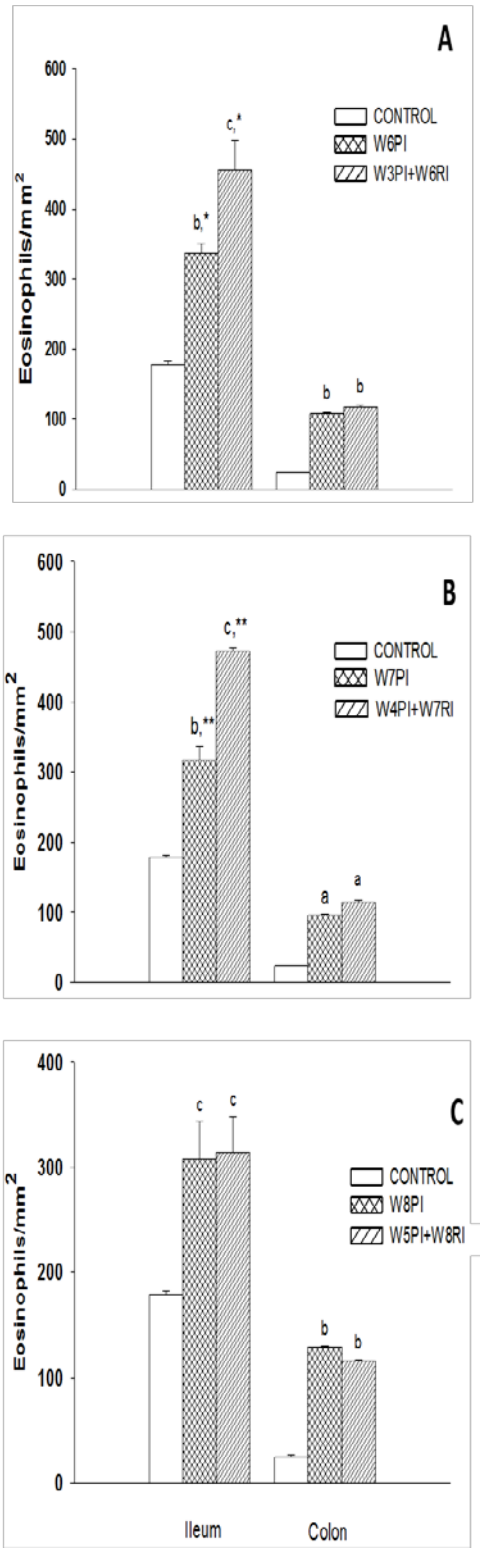


Figure 3. Eosinophils counts, expressed as number of cells per mm^2, in the mucosa of the ileum and colon of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences between infected animals and CONTROL; (b) $p < 0.01$ and (c) $p < 0.001$ represent significant differences between challenge infected and challenge control subgroups.

4. Discussion

There is enough evidence showing that vaccination approaches at early age have numerous advantages for many diseases and may be the only alternative for others. For instance, in chicken salmonellosis, which can occur by both vertical and horizontal transmission (Cox et al., 2000), initial infections of broilers usually takes place early post-hatch and the infected young chicks led to high levels of environmental contamination and rapid transmission of pathogens as a result of litter contamination (van Immerseel et al., 2005). Although high titres of specific maternal antibodies are transferred to new born chicks (Methner et a., 2002), they lasts no more than a few weeks, so there is a need control strategies that confer resistance just after hatching and maintain long-term protective effects. Vaccination of young birds themselves has the disadvantage that the very young bird is immunologically immature (Friedman et al., 2003).

Accordingly, a number of studies have investigated the response of chickens to immunisation against a variety of antigens, with results showing varying humoral and T cell responses in birds of different ages. T cell responsiveness to mitogens, including ConA, does not fully develop in birds until 1 week of age (Lowenthal et al., 1994), whilst variation in the humoral response to infection with *M. gallisepticum* and antigenic stimulation by BSA has been seen up until 2 weeks of age (Mast et al., 1999). However, a number of vaccines are able to provide protection when administered in ovo (Rick et al., 1999; Lillehoj et al., 2005). In general, age-related immunity

Fig. 4

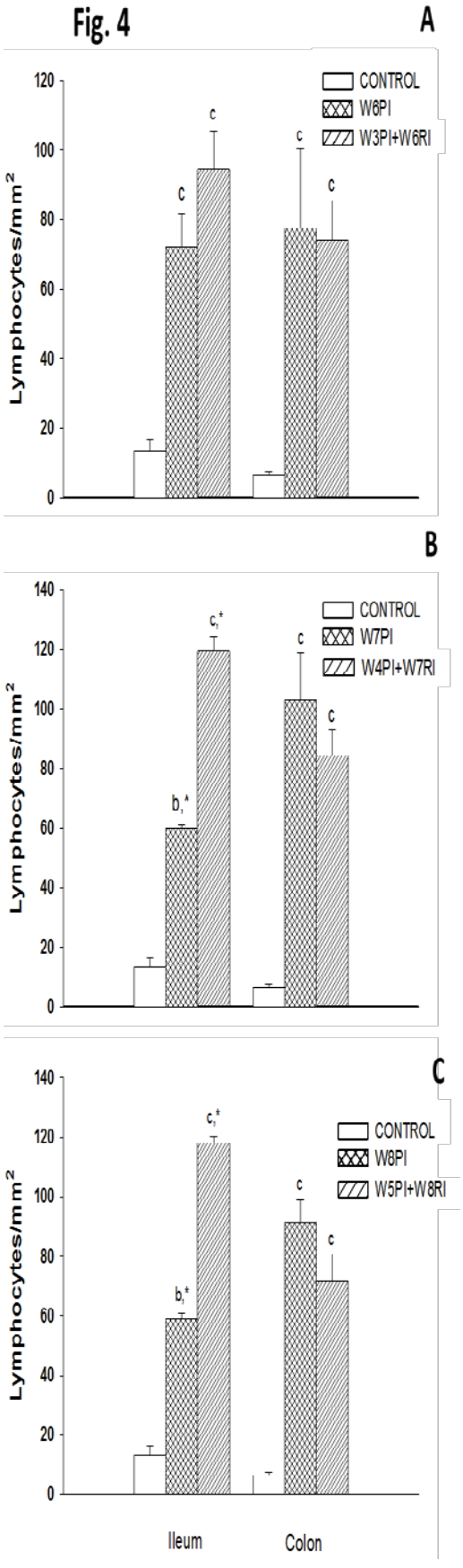
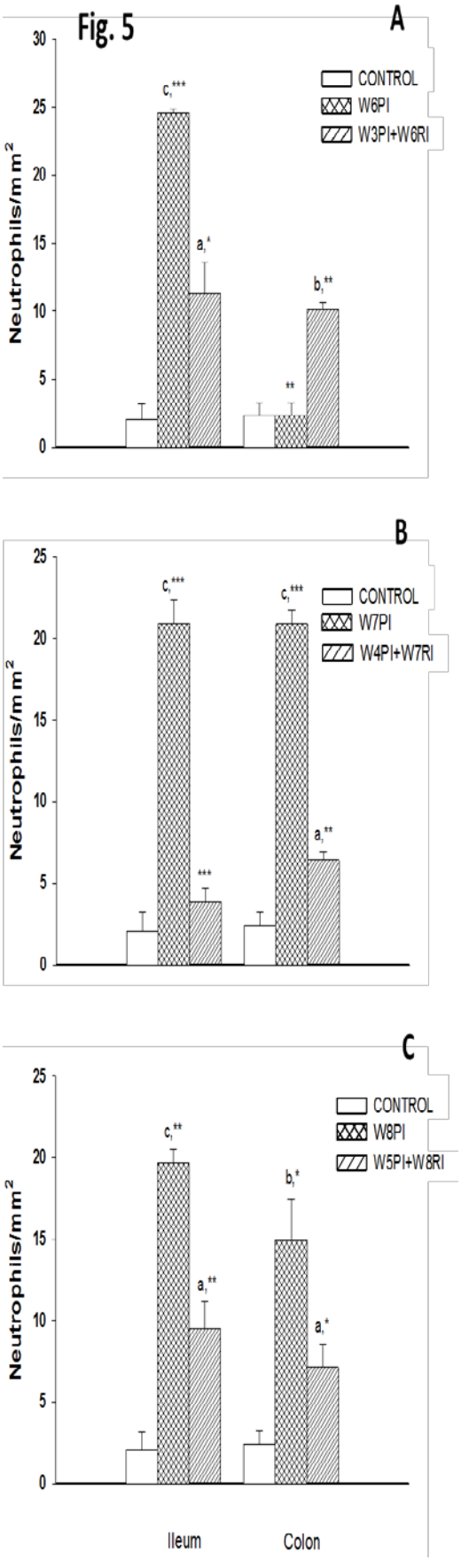

A
CONTROL
W6PI
W3PI+W6RI
Lymphocytes/mm²
B
CONTROL
W7PI
W4PI+W7RI
Lymphocytes/mm²
C
CONTROL
W8PI
W5PI+W8RI
Lymphocytes/mm²
Ileum
Colon

Fig. 5


A
CONTROL
W6PI
W3PI+W6RI
Neutrophils/mm²
B
CONTROL
W7PI
W4PI+W7RI
Neutrophils/mm²
C
CONTROL
W8PI
W5PI+W8RI
Neutrophils/mm²
Ileum
Colon

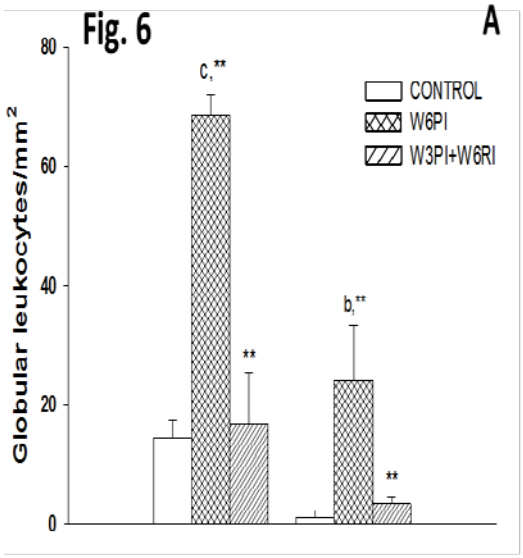
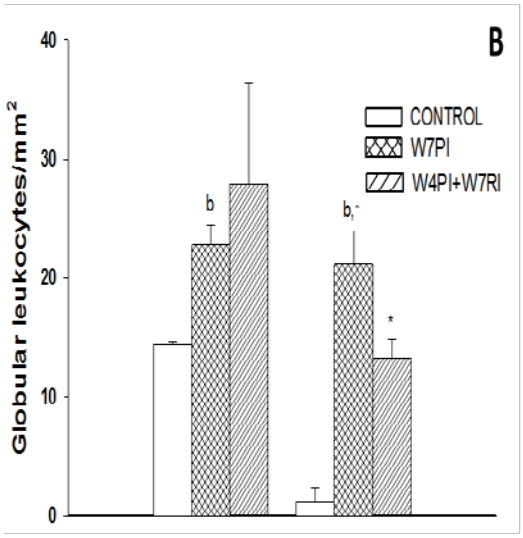
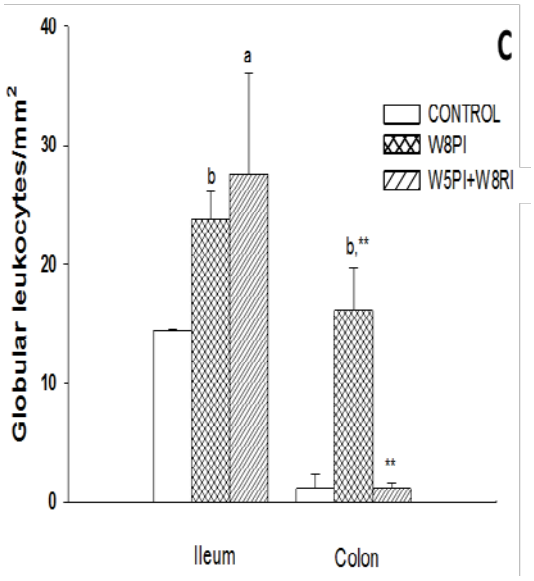


Fig. 6
A
Globular leukocytes/mm2
CONTROL
W6PI
W3PI+W6RI


B
Globular leukocytes/mm2
CONTROL
W7PI
W4PI+W7RI


C
Globular leukocytes/mm2
CONTROL
W8PI
W5PI+W8RI
Ileum
Colon

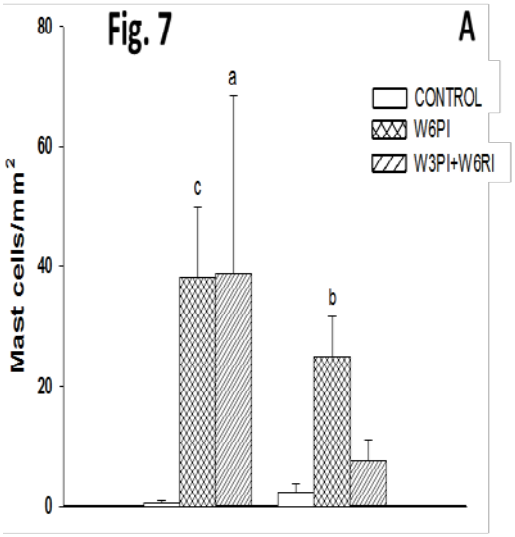
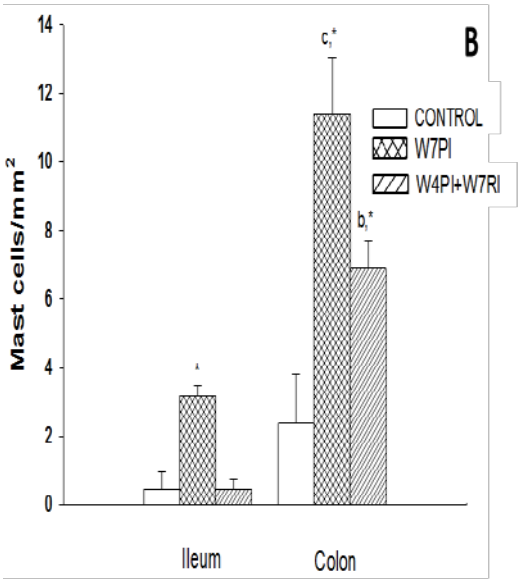


Fig. 7
A
Mast cells/mm2
CONTROL
W6PI
W3PI+W6RI


B
Mast cells/mm2
CONTROL
W7PI
W4PI+W7RI
Ileum
Colon

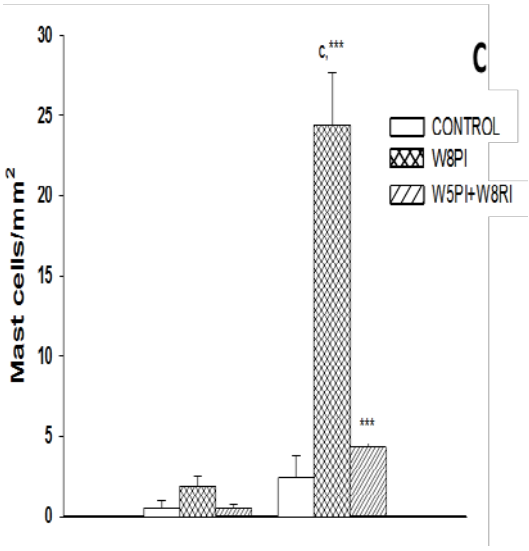

C
Mast cells/mm2
CONTROL
W8PI
W5PI+W8RI

Figure 4. Lymphocyte counts, expressed as number of cells per mm^2, in the mucosa of the ileum and colon of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ represents significant differences between infected animals and CONTROL; (b) $p < 0.01$ and (c) $p < 0.001$ represent significant differences between challenge infected and challenge control subgroups.

Figure 5. Neuthophils counts, expressed as number of cells per mm^2, in the mucosa of the ileum and colon of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.01$ represent significant differences between infected animals and CONTROL; (a) $p < 0.05$, (b) $p < 0.01$ and $p < 0.001$ represent significant differences between challenge infected and challenge control subgroups.

Figure 6. Globular leukocyte counts, expressed as number of cells per mm^2, in the mucosa of the ileum and colon of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences between infected animals and CONTROL; (a) $p < 0.05$, (b) $p < 0.01$ and $p < 0.001$ represent significant differences between challenge infected and challenge control subgroups.

Figure 7. Mast cell counts, expressed as number of cells per mm^2, in the mucosa of the ileum and colon of goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ and (***) $p < 0.01$ represent significant differences between infected animals and CONTROL; (a) $p < 0.05$, (b) $p < 0.01$ and $p < 0.001$ represent significant differences between challenge infected and challenge control subgroups.

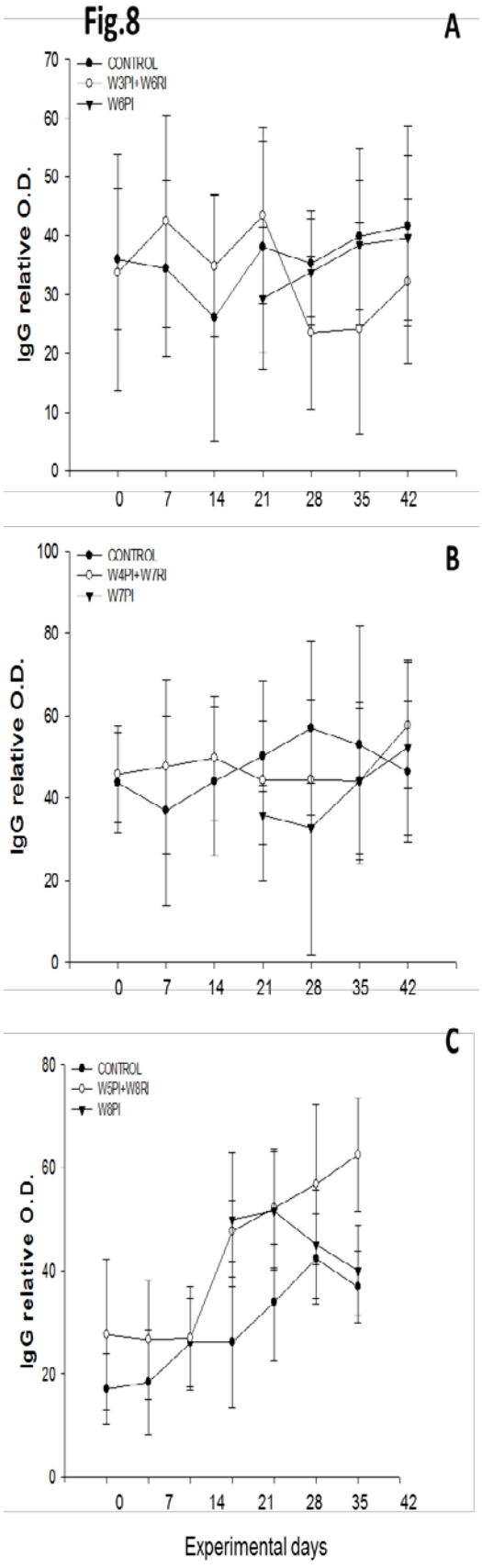


Fig.8
A
CONTROL
W3PI+W6RI
W6PI
IgG relative O.D.
B
CONTROL
W4PI+W7RI
W7PI
IgG relative O.D.
C
CONTROL
W5PI+W8RI
W8PI
IgG relative O.D.
Experimental days

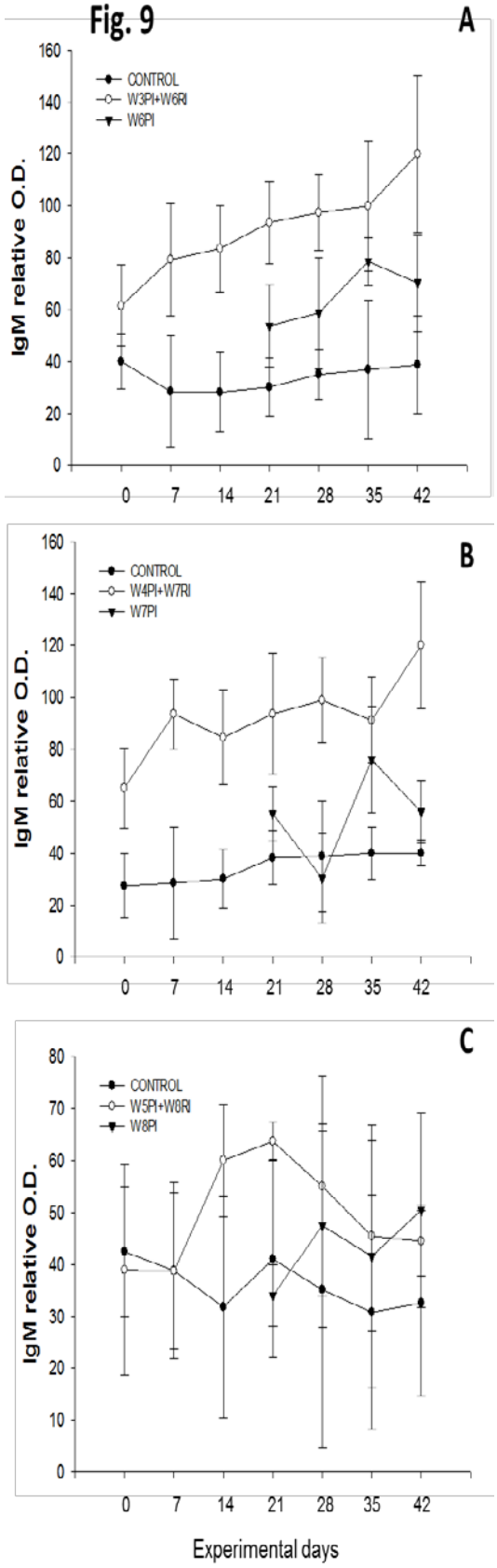


Fig. 9
A
CONTROL
W3PI+W6RI
W6PI
IgM relative O.D.
B
CONTROL
W4PI+W7RI
W7PI
IgM relative O.D.
C
CONTROL
W5PI+W8RI
W8PI
IgM relative O.D.
Experimental days

Figure 8. Evolution of mean IgG anti-SOA levels (±standard error) expressed as relative optical densities (O.D.) in sera from goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups.

Figure 9. Evolution of mean IgM anti-SOA levels (±standard error) expressed as relative optical densities (O.D.) in sera from goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups.

to different pathogens has been reported; i.e., age-related variations in the efficacy of immune responses to *Mycoplasma gallisepticum* (Gauson et al., 2006), *Teladorsagia circumcincta* (Vervelde et al., 2001), *Trypanosoma cruzi* (Pascutti et al., 2003) and *Cryptosporidium* spp. (Sréter et al., 1995; Rhee et al., 1999), among others, have been documented.

Classical studies published in poultry coccidiosis (Lillehoj, 1988) also demonstrated that the susceptibility to the disease and the development of resistance to *E. tenella* infections were influenced by the age of the chickens. Although there are obvious differences between the immune system of poultry and ruminants, it also admitted that both calves (Jäge et al., 2005), lambs (Reeg et al., 2005) and goat kids (Ruiz et al., 2006) are more susceptible than adults to suffer from clinical coccidiosis, which suggest the young ruminants are immunologically immature. This is particularly important when they are reared in highly contaminated environments, but also when considering immunoprophylactic approaches for the control of the disease.

The ontogenesis of the immune response in ruminants already starts during early embryogenesis. Actually, Schultz et al. (1973) proved that mesenteric lymph nodes were structurally present by 100 days of gestation, and lymphoid tissue of the gastrointestinal tract, particularly the lower ileum, was observed in histologic sections of a 175- day foetus with a bacterial infection (<40 days) and 106 foetuses of various ages. Besides, there also studies in lambs on the ontogeny of T cell recirculation during foetal life (Cahill et al., 1999) and on the development of B cells in the gut-associated lymphoid tissue of mid-gestational foetus (Alitheen et al., 2003). Further development of the immune system is probably prolonged after birth during weeks or even months, but not much is known about that, mainly in goat kids. Altogether, the results of the present study demonstrated that goat kids of either 3, 4 or 5 weeks of age are able to develop immunoprotective responses against a subsequent homologous challenge made 3 week latter with 2 x 10^6 *E. ninakholyakimovae* oocysts. Firstly, all age groups released significantly less oocysts per faeces after challenged, which was associated to milder clinical signs compared to challenge controls. However, differences on growth rates between challenge goat kids and the corresponding challenge controls could not be proven statistically, probably because the period of evaluation of body weights was too short. Secondly, all age groups displayed a proliferative immune response at the local levels with significant increase in most of the cell populations analysed in the study (eosinophils, lypmocytes, neutrophils, globular leucocytes and mast cells). From those, eosinophils and, particularly, lymphocytes were significantly more increased in challenge groups, which confirm the role of T cell response in the immune

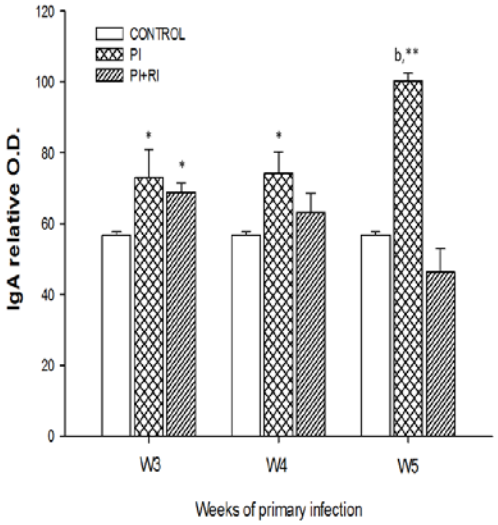


Figure 10. Evolution of mean IgA anti-SOA levels (±standard error) expressed as relative optical densities (O.D.) in ileal mucus from goat kids from different age group (A, B and C) after primary or challenge infections with *Eimeria ninakohlyakimovae* sporulated oocysts. (A) primary infected at week 3 and challenge at week 6 (W3PI+W6RI) and primary infected at week 6 or challenge control (W6PI); (B) primary infected at week 4 and challenge at week 7 (W4PI+W7RI) and primary infected at week 7 or challenge control (W7PI); (C) primary infected at week 5 and challenge at week 8 (W5PI+W8RI) and primary infected at week 8 or challenge control (W8PI). CONTROL represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$ represents significant differences between infected animals and CONTROL; (b) $p < 0.01$ represents significant differences between challenge infected and challenge control subgroups.

protection conferred against *Eimeria* infection in ruminants (Hermosilla et al, 1999; Taubert et al., 2008; Ruiz et al., 2013a). By contrast, as discussed by these same authors, cell related with the innate immune system were mainly, but not exclusively, increased in primary infected goat kids (challenge controls), particularly neutrophils (Behrendt et al., 2010). Finally, although the antibody response was not so evident than the cellular counterpart, as shown by other authors (Daugschies et al., 2005), there were evidences showing that light increases in IgG and IgM, and mainly in local IgA, could be associated to *E. ninakholyakimovae* infection. In spite of all these evidences, statistical differences between immunological results and the parasitological parameters (basically, OPG counts) could not be demonstrated, for what some explanations should be taking into consideration: (i) the difficulties to have a number of animals per group high enough to get significant differences in this kind of experiments; (ii) the huge individual variability of immune response against parasites in goats (Molina et al., 2012); and (iii) the complication of having a good synchronization between oocyst release and the moment when immunological parameters are recorded.

Nevertheless, when a more detailed analysis of the data was performed, some differences were observed between the three age groups, related both to the *Eimeria* infection outcome and the resulting immune response. For instance, the prepatent period were slightly longer in goat kids primary infected at week 3 and 4 of life, and their corresponding OPG peaks were delayed 1-2 days when compared to those of primary infected at week 5. Conversely, younger kids, particularly 3 old week primary infected kids, showed milder clinical signs compared to older groups. This finding could be explained considering that special host age-related requirements are needed for the infection, i.e. a partial development of the rumen, although there are data showing that *E. bovis* sporozoites are released even if the oocysts are injected directly to the abomasum (Kheysin and Todd, 1972). In agreement to our results, even though goat kids may become infected just after birth by sucking from contaminated breasts, something frequent in intensive production systems, signs compatible with clinical coccidiosis are rare in animals less than on month old in field conditions (personal observation).

Apart from biological and clinical parameters, differences were also observed in terms of immune response among the three age group analysed in this study. Thus, as referred before, the youngest group A was the

only in which no statistical differences were observed on lymphocytes counts between challenge animals and the corresponding challenge control in ileal samples, which is important taking into account that lymphocytes are the main effector cells for acquired immune responses (Hermosilla et al., 1999; Taubert et al., 2008; Sühwolf et al., 2010). By contract, group A was the one which had the highest increase of mast cells and globular leuckocytes, two cell populations which are mainly involved on innate immune responses (Petrone et al., 2007; Metcalfe, 2008).

In summary, this study proves that *E. ninakholyakimovae* induce patent infections and protective immune reactions in young goat kids of different ages subsequently subjected to a homologous challenge. Some evidences indicate that the youngest kids may be not fully immunocompetent though; therefore, further immunological studies focused to similar age range kids or even younger animals (1 or 2 weeks old) would be of high interest for the design of immunoprophylactic approaches or even prophylactic/methaphylactic treatments against ruminant coccidiosis, in particular goat coccidiosis.

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ARTÍCULO Nº 5

Estudio de la respuesta inmunitaria protectora durante el periodo prepatente en cabritos experimentalmente infectados con *Eimeria ninakholyakimovae*

In preparation to: Veterinary Parasitology

▪ RESUMEN ▪

Sin descartar la importancia de la inmunidad humoral en la eimeriosis en rumiantes, las respuestas inmunes celulares parecen jugar un papel crucial en la resistencia adquirida después de reinfecciones con el protozoo Apicomplexa *Eimeria ninakohlyakimovae*. En las cabras, las respuestas inmunes celulares frente a la coccidiosis han sido poco investigadas, especialmente en la fase temprana de la infección. En este estudio hemos evaluado los cambios en las poblaciones de células inmunes en la mucosa intestinal en el período prepatente tras infecciones y posteriores reinfecciones con esta especie de *Eimeria*. Para ello, un total de 15 cabritos se dividieron en tres grupos: (i) cabritos primo-infectados a las 4 semanas de edad y reinfectados tres semanas más tarde (Grupo RI); (ii) animales primo-infectados a las 8 semanas de edad (= controles de re-infección, Grupo PI); y (iii) cabritos no infectados (Grupo C). Todos los grupos fueron sacrificados una semana después de la reinfección, es decir, dentro del plazo de prepatencia. Los animales se inocularon con ooquistes esporulados de la cepa GC de *Eimeria ninakholyakimovae*. La inmunidad protectora en los diferentes grupos se evaluó en base a parámetros clínicos, productivos, hematológicos, parasitológicos, inmunológicos (anticuerpos y respuestas inmunes celulares) y parámetros patológicos (lesiones macroscópicas y microscópicas en el colon y el íleon). Con este propósito, se tomaron muestras fecales y de sangre individuales. También se examinaron el peso corporal y la condición clínica de todos los animales y, al final del experimento, todos los cabritos fueron sacrificados y se realizó la necropsia de todos ellos. Se tomaron muestras de las diferentes secciones de íleon, colon y nódulos linfáticos mesentéricos (MLN). Así mismo, se identificaron las lesiones histopatológicas y se realizaron recuentos celulares de linfocitos, eosinófilos, neutrófilos y leucocitos globulares (identificados por hematoxilina y eosina) y de mastocitos (mediante tinción con azul de Giemsa). Además, se llevó a cabo un análisis de expresión génica de IL-2, IL-4, IL-10 e INFγ en íleon, colon y MLN, así como la caracterización de células inmunes por inmunohistoquímica en secciones de íleon y colon. La infección por *E. ninakohlyakimovae* fue de moderada a severa, se observaron diversos grados de diarrea y se acompañó de altos recuentos de OPG durante la infección primaria.

También se observó un aumento en el recuento de casi todos los tipos de células inmunes analizados en comparación con los animales no infectados.

Por otra parte, los recuentos de eosinófilos, linfocitos, leucocitos globulares y mastocitos fueron significativamente más elevados en los animales reinfectados que en los primoinfectados, mientras que ocurrió lo contrario para los recuentos de neutrófilos. La primoinfección también se asoció a un aumento moderados/leve de los niveles séricos de IgG e IgM e IgA local. Sorprendentemente, el número de esquizontes inmaduros encontrados en la mucosa del íleon fue estadísticamente mayor en el grupo de primoinfectados. Además de la reducción del número de esquizontes inmaduros, en los animales reinfectados se observó un aumento de los linfocitos y otras poblaciones celulares; en concreto, los recuentos de T CD4 + y T CD8 + se encontraron aumentados, lo que indica que las células T podrían estar relacionada con el desarrollo de la respuesta protectora. La respuesta inmune desarrollada fue, sin embargo, muy compleja, ya que células presentadoras de antígeno y otras poblaciones de células efectoras del sistema inmune innato, así como ciertas citoquinas, también estuvieron involucradas. En su conjunto, los resultados de este estudio contribuyen a comprender la complejidad de la respuesta inmune celular y humoral caprina frente a la coccidiosis, en particular durante la fase de prepatencia, lo cual puede ser de utilidad para el desarrollo de estrategias para la modulación de la respuesta inmune y para la identificación de compuestos anticoccidiales o vacunas.

Study of protective immune responses during prepatency in goat kids experimentally infected with *Eimeria ninakholyakimovae*

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Abstract

Without dismissing the importance of humoral immunity in ruminant eimeriosis, cellular immune responses seem to play a crucial role in acquired resistance after reinfections with the apicomplexan protozoa *Eimeria ninakohlyakimovae*. In goats, the cellular immune responses against coccidiosis have poorly been investigated, especially in the early phase of infection. In this study we have evaluated the changes of immune cell populations in intestinal mucosa in the prepatent period of infections as well as after challenge infection with this *Eimeria* species. Therefore, a total of 15 goat kids were divided into three groups: (i) goat kids primary infected at 4 weeks of age and re-infected three weeks later (Group RI); (ii) primary infected animals at 8 weeks of age (= controls of re-infection, Group PI); and (iii) uninfected goat kids (Group C). All groups were sacrificed one week after challenge infection, that is, within the period of prepatency. For infection, sporulated oocysts of the GC strain of *Eimeria ninakholyakimovae* were used. The protective immunity after primary infection and subsequent reinfection in the different groups was assessed by clinical, productive, hematological, parasitological, immunological (antibody and cellular immune responses) and pathological parameters (gross and microscopic lesions in colon and ileum). For this purpose, individual faecal and blood samples were taken. The body weight and clinical condition of all the animals were also examined and, at the end of the experiment, all the goat kids were euthanized and further subjected to necropsy. Samples were taken from different sections of the ileum, colon and mesenteric lymph nodes (MLN). Histopathological lesions were identified and cell counts of lymphocytes, eosinophils, neutrophils and globular leukocytes (identified by hematoxylin and eosin staining) and mast cells (identified by Giemsa blue staining) were recorded. Additionally, gene expression analysis of IL-2, IL-4, IL-10 and INFγ of ileal, colonic and MLN were performed, as well as the characterization of immune cells by immunohistochemistry in sections of ileum and colon. The *E. ninakohlyakimovae* infection resulted in moderate to severe enteritis and different degrees of diarrhoea and was accompanied by high OPG counts during the primary infection and an increase of almost all immune cell types analyzed compared to uninfected control animals. Furthermore, the counts of eosinophils, lymphocytes, globular leukocytes and mast cells were significantly higher in reinfected than in primary infected animals, whilst the opposite was true for neutrophils counts. Challenge infection was also associated to moderate/mild increased levels of serum IgG and IgM and local IgA. Interestingly, the number of immature schizonts found at the ileal mucosa was statistically higher in challenge group compared to challenge control animals. Apart from the association of

reduced number of immmature schizonts in reinfected animals to mean values for lymphocyte and other cell population, in this group of animals a greater number of $CD4^+$ and $CD8^+$ cells were observed, all indicating that T cell response could be related to the development of the protective response. The immune response developed was, however, very complex, as antigen presenting cells and other effector cell populations of the innate immune system, as well as certain cytokines, are involved. As a whole, the results of this study contribute to understand the complexity of the cellular and humoral immune response of the goat hosts against coccidiosis, in particular during the prepatency, which can be used for the development of strategies for modulation of the immune response and for the identification of anticoccidial compounds or vaccines.

Key words: *Eimeria ninakholyakimovae; prepatency; immune response; goats*

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1. Introduction

In arid and semi-arid areas (Ruiz et al., 2006; Khodakaram-Tafti et al., 2013), but also in other different geographical areas having diverse climatic conditions (Balicka-Ramisz et al., 2012; Zainalabidin et al., 2013), coccidian infections are considered one of the most important parasitic disease affecting goat production. The clinical signs and pathological changes associated to this parasitic disease (Dai et al., 2006; Kheirandish et al., 2014) mainly affect young kids, leading to high economic losses in terms of production, sometimes difficult to be determined (Koudela and Boková 1998). Sporozoites of *Eimeria ninakholyakimovae,* one of the most pathogenic species of goats (Dai et al., 2006), primary invades endothelial cells of lacteal ducts of the villi of the distal ileum where they develop first generation schizonts (up to 166 μm x 124 μm in size) within 10-12 days post-infection (p. i.), which finally release thousands of merozoites I (Vieira et al, 1997; Behrendt et al., 2010). The development of the schizonts, their rupture and the subsequent merozoite release lead to an extensive destruction of the intestinal mucosa of the infected animals, which may result in a malabsorption syndrome, even before the start of the faecal oocyst release and typical clinical signs such as the diarrhea appear. Actually, when high doses of oocysts are ingested, the magnitude of the tissue damaged may conduct to the death of the animal. This may explain why, under certain conditions, coccidiosis can be associated with sudden death without previous digestive signs, especially in young animals of between 2 and 4 months old (Chartier et al., 1994).

The control of goat coccidiosis is principally based on the combination of management practices with the use of coccidiostats and anticoccidials, as sulfonamides (Svensson, 1998) or toltrazuril (Mund et al., 2003). Although new drug have been proven to be effective for goat coccidiosis, i.e. diclazuril (Ruiz et al., 2012) and ponazuril (Love et al., 2015), up to date there are no drugs registered for goats in the E.U. This, together with the increasing appearance of anticoccidial drug resistance in different geographic areas (Kawazoe and di Fabio, 1994; Williams, 2006) has stimulated the search of new alternatives for control, such as the use of vaccines. Recently, Ruiz et al. (2014) demonstrated that X-rad attenuated oocysts induce an immunoprotective response against a homologous challenge with *E. ninakholyakimovae* oocysts, and there is extensive literature in poultry showing that both sexual and asexual derived antigens may be useful for the design of recombinant vaccines (Vermeulen, 1998; Tewari and Maharana, 2011). Both for using live, attenuated or recombinant vaccines a deep knowledge of the immune response against *Eimeria* infections are a prerequisite.

Coccidiosis produced by *Eimeria* spp. in ruminants (Catchpole et al., 1993) and in other host species (Shi et al., 2000) generally induces strong protective immune responses which prevent clinical disease derived from homologous challenge infections. The immune reactions developed against caprine *Eimeria* spp. have poorly been investigated, but recent experimental studies conducted in *E. ninakholyakimovae* demonstrate that previous exposure to the parasite induce strong protective immune responses which involves both innate and acquired components (Ruiz et al., 2013a). However, the same authors also found that protection can be truncated in case of high challenge infections (Ruiz et al., 2013b), suggesting that the immune response against the parasite is certainly complex. In the bovine system, different studies performed in *Eimeria* infections indicate that both humoral (B-cell) and cellular (T cell) responses are involved (Daugschies and Najdrowsky, 2005). Actually, levels of IgM, IgA and IgG_2 transferred through the colostrum to calves have been found to negatively correlate with excretion of oocysts of *E.bovis* (Faber et al., 2002). Besides, several investigations dealing with cellular immune response demonstrate that both CD4+ and CD8+ T cell subsets are involved during primary *E. bovis* infection (Hermosilla et al.,1999) and that enhanced antigen-specific IFN-production in *E.bovis* infections indicate a Th1 immune response in prepatency in calves (Taubert et al.,2008). It

has also been reported that antigen-specific T cells proliferated effectively during an strict time span during prepatency of primary infection (Hermosilla et al.,1999) but fail to do so after challenge infection, suggesting early abrogation of re-infection (Sühwold et al., 2010). Targets for the innate immune response against ruminant *Eimeria* species involve both sporozoites and merozoites, as shown by numerous *in vitro* studies performed in *E. bovis* (Hermosilla et al., 2006; Taubert et al., 2009; Behrendt et al., 2010; Muñoz-Caro et al., 2014), *E. arloingi* (Silva et al., 2014) and *E. ninakholyakimovae* (Pérez et al., 2015). The specific mechanisms involved in the development of acquired immune responses *in vivo* against ruminant coccidiosis are much less investigated. In this regards, apart from their pathological implications, first generation schizonts developed during prepatency are of great interest in various aspects. For one, the length of the maturation of schizonts suggests that *E. ninakholyakimovae* is influencing and / or modifying the host cell to allow the persistence of the parasite. Moreover, there is abundant evidence that first generation schizonts represent an important target for immune protective reactions (Taubert et al., 2006).

In the present study we have investigated both innate and acquired immune reactions during the prepatent period of the disease in experimentally infected goat kids by *E. ninakohlyakimovae*. For this purpose, parasitological, haematological, clinical and pathological parameters were evaluated. Additionally, the antibody response, the characterization of immune cells by immunohistochemistry and the gene expression of different cytoquines were assessed.

2. Material and methods

2.1. Parasites and animals

The *Eimeria ninakholyakimovae* strain GC used in the present study was initially isolated from the field of naturally infected goats in Gran Canaria Island (Spain) and maintained by passages in goat kids for oocysts production (Ruiz et al., 2013a). For harboring oocysts, goat kids were orally infected at the age of 4 week with 2 x 10^5 *E. ninakholyakimovae* sporulated oocysts. Newly produced and purified were concentrated and stored in a 2% potassium dichromate solution at 4°C until further use after they fulfill the sporulation proces.

A total of 15 goat kids of the Majorera breed was purchased from a local farmer at the age of 1-5 days and maintained under parasite-free conditions in autoclaved stainless steel cages in a restricted stable (Faculty of Veterinary Medicine, University of Las Palmas de Gran Canaria, Spain). The kids were treated with Vecoxan® (Janssen-Cilag) and Halocur® (Intervet) just at their arrival to the Faculty of Veterinary Medicine. Goat kids were fed with the milk substitute Bacilactol® (Capisa) and commercial concentrate pellets for weaning goat kids (Starting Concentrate, Capisa). Water and sterilized hay were always given ad libitum. All animal procedures were conducted in strict accordance with national ethics and by institutional review board-approved protocols.

2.2. Experimental design

For experimental purposes goat kids were divided into the following three groups: group 1 (n=6; re-infected or challenged animals): animals infected with *E. ninakholyakimovae* sporulated oocysts at day 1 of the experiment and challenged at day 21; group 2 (n=5; animals primary infected or challenge controls): animals inoculated with *E. ninakholyakimovae* sporulated oocysts at day 21; group 3 (n=4; uninfected control): no infected animals. Day 1 of the experiment corresponds to 4th week of age in all groups.

All infections were performed orally via gastro-ruminal tube using an infection dose of 2 x 10^5 *E. ninakholyakimovae* sporulated oocysts. The animals were weekly bled from the jugular vein and with the same interval their body weight was recorded. During the primary infection, faecal samples were individually taken for coprological analysis and the presence of clinical signs

were daily monitored. The animals from all experimental groups were euthanized at day 28th of the study (one week after the challenge infection) and subsequently subjected to necropsy. Apart from a gross morphological examination, tissue samples from mesenteric lymph nodes, ileum and colon, as well as ileal mucus, were taken during the necropsy.

2.3. Parasitological, clinical and haemathological analysis

The number of oocysts per gram of feces (OPG) for all the animals from group 1 during the primary infection was determined from day 14 to day 21 of the experiment by using a modified McMaster technique (MAFF, 1989). During the whole experiment, the weight of the animals was weekly taken in order to evaluate production parameters. The presence of clinical signs compatible with coccidiosis was also daily monitored, paying special attention to characteristic of the diarrhea.

Individual blood samples were assessed by using a hematology analyzer (LaserCyte® hematology analyzer, Idexx) in order to determine total leukocyte counts and the hemoglobin concentration. The hematocrit value was calculated by centrifugation using standard centrifuge capillaries and differential white blood cell (WBC) counting was performed manually; for this purpose, 200 leukocytes were counted in stained blood smears (Diff-Quick).

2.4. Pathobiological and histolopathogical analysis

All gross lesions were recorded during the necropsy and, afterwards, tissue samples from the ileal and colonic mucosa, as well as mesenteric lymph nodes (MLN), were taken and fixed in 4% buffered formalin. Next, tissue samples were included in paraffin blocks and 4-5 µm sections were cut and stained by hematoxylin and eosin (H&E) for the histopathological study and quantification of leucocyte populations (neutrophils, lymphocytes, eosinophils and globular leukocyte). A Giemsa staining was performed in order to determine mast cell counts. Finally, for the demonstration immature parasitic forms of *E. ninakholyakimovae* during the early prepatency, a PAS staining (*Periodic Acid Schiff*) was employed according to previously described methods (Barnhill et al., 2010). In all cases, cells or parasitic forms were counted using a 10× eyepiece containing a calibrated graticule and 40× objective lens by viewing an area of 0.05265mm^2. The counts were randomly taken on 20 graticule fields within the mucosal surface. The counts were expressed as number of cells per mm^2 of mucosa (Amarante et al., 2005).

Mucus samples were taken from the ileum of the animals for the analysis of specific IgA levels. For their preservation, the samples were suspended in a pH 7.1 buffer containing proteinases inhibitors (all compouds from Sigma-Aldrich): 0.1 M Na_2HPO_4, 0.05M NaCl, 3mM NaN3, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5mM ethylenediaminetetraacetic acid (EDTA). Mix solutions containing mucus were centrifuged at 5000 x *g* (Eppendorf Centrifuge 5804R) for 1h at 4°C and the resulting supernatant was conserved at -20 ºC up to further analyses.

2.5. Enzyme – linked immunosorbent assay (ELISA)

To determine the levels of specific IgG and IgM antibodies, sera samples collected weekly from the different groups were pooled (one pool per group and week), aliquoted and kept at -20°C until the appropriate tests was performed. For the IgG indirect ELISA test, a total of 100 µl of a SOA solution in carbonated buffer at a concentration of 5µg/ml was dispensed in 96 wells ELISA plates (CORNING Dispensable Sterile ELISA Plates, Corning Glass Works) and incubated overnight at 4°C. After three washes with 200 µl of PBS-0.05 % Tween 20, 200 µl of PBS-3% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) were added and the plates were incubated for 45 min at 37°C. After each new step a similar washing procedure was done. Following treatment with BSA, individual

serums, positive and negative controls were analyzed in duplicate using a 1/25dilution in PBS-Tween 20 0.02% sodium acid. For each sample, a volume of 100 µl was added to the plates and a new incubation was performed at 37°C for 1h. The plates were then incubated in the same conditions after adding 100 µl per well of 1/2000 anti-Goat IgG (anti-goat IgG peroxidase conjugate, IgG fraction of antiserum, Sigma-Aldrich) diluted in 0.01 M PBS. Thereafter, a total of 100 µl of substrate was incorporated to each well, a mix of compounds that includes citric-phosphate buffer, 0.04% (w/v) dihydrochloride ortho-phenyleneadiamine (OPD, Sigma-Aldrich) and 30% hydrogen peroxide (Panreac) at a final concentration of 0.1% (v/v). The incubation with the substrate was performed at room temperature in darkness for approximately10 minutes. Finally, the reactions was stopped by adding 35 µl per well of a 2M solution of sulfuric acid (Panreac) and the optical density (O.D.) measured at a wavelength of 492 nm (Termo Labsystems, Multiskan Ascent).

For specific IgM and IgA indirect ELISA tests a similar protocol was employed but using 1/3000 anti-goat IgM peroxidase and 1/7000 anti-goat IgA peroxidase conjugates, respectively (both from Sigma-Aldrich). Similarly, dilutions 1/25 of the different serum or mucus samples were employed as primary antibody.

2.6. Immunohistochemistry

For the characterization of the immune cell populations, additional samples of mesenteric lymph nodes (NLM) and intestinal mucosa from ileum and colon were taken at necropsy on each of the proposed experimental groups. Depending on the primary antibody used (Table 1), tissue samples of approximately 1 cm thick were preserved either in paraffin after fixation with 4% buffered formalin during 24 h or at - 80°C previous collection in liquid nitrogen. Before freezing, samples were included in OCT (Optimal Cutting Temperature, Tissue-Tek, Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and then immersed in a container with 2-methylbutane (Merck, Darmstadt, Germany). For rapid freezing of tissue, the samples were subsequently submerged in liquid nitrogen and finally stored at -80 ° C until they were cut. Tissue samples 4 µm thick were cut by a cryostat (Reichert-Jung, 2800 Frigocut N, Germany) at -24°C, dried at room temperature for 40-60 min and fixed in acetone (Panreac) at 4°C for 5 min. Afterwards, tissue sections were dried again at room temperature for 30 min and routinely stained with H&E. Other cuts were stored wrapped in aluminium foil at -80°C up to IHC analysis were performed.

The avidin-biotin-peroxidase (ABC) method (Navarro *et al.*, 1996), with some modifications, was used on sections of both formalin-fixed paraffin wax-embedded tissues and frozen tissues. The former were de-waxed and rehydrated, and endogenous peroxidase activity was blocked by incubation of the sections with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were then treated with pronase (Sigma-Aldrich) 0.1% in Tris-buffered saline (TSB), pH 7.2 for 4 min at room temperature. When snap-froze tissue samples were used, endogenous peroxidase was blocked by incubation with phenyl-hydrazine (Sigma-Aldrich) 0.055 in PBS, without subsequent pronase treatment.

After rehydration, all sections were incubated with 10% normal serum (Vector Laboratories) for 30 min at room temperature. The primary antibodies (Table 1) were then applied overnight at 4°C (formalin-fixed tissue) or for 2 h at 37°C (frozen tissue). Preliminary experiments were carried out to determine the optimal dilution for the different antibodies. All monoclonal (mAbs) and polyclonal antibodies (pAbs) were raised against immune cells of bovine origin but had been previously shown to cross-react with caprine antigens (Navarro et al., 1996; Rodríguez et al., 2000).

Biotinated goat or rabbit anti-mouse IgG (for mAbs) and anti-rabbit IgG (for pAbs), both obtained from Vector Laboratories and diluted in 1 200, were used as the secondary reagents. An ABC complex (Vector) diluted 1 in 50 was applied as the third reagent. The sections were then incubated for 1 min with

3, 3'-diaminobenzidine tetrahydrochloride (Sigma) 0.035% in TBS containing hydrogen peroxide 0.1%. After rinsing in tap water, they were lightly counterstained with Harri's haematoxilin, and mounted under DPX for microscopy. Sections in which the specific primary antibodies were replaces by TBS, normal goat or rabbit serum or inappropriate antibodies were included as negative controls. Sections from the lymph nodes of controls animals were used as positive control for all the primary antibodies.

Positive labeled cells were counted in 20 selected fields (x 400 magnification) in each of the tissue sections (ileum or colon) from each goat kid.

2.7. Isolation of total mRNA and DNase treatment

Total mRNA was isolated with the kit QuickPrep Total RNA (Amersham Pharmacia Biotech). The mRNA obtained was analyzed in a spectrophotometer Nanodrop 1000 (Thermo Scientific) in order to determine the concentration and purity of the sample. The purity of the mRNA was estimated by the A260/A280 relation and the alcoholic contamination agents by the A260/A230 relation (only samples with values between 1.8 – 2.1 and 2.0- 2.1, respectively, were here employed). In addition, 1.2 % (*w/v*) MOPS (N-morpholine-propanesulfonic acid-3, Fluka Analytical) agar gels were used in order to prove the integrity and quality (Maniatis et al., 1982). Once checked, the mRNA sample was stored at -80 ° C until use. To further purify mRNA samples, genomic DNA was neutralized by using the kit RQ1 RNase-Free DNase (Promega) according to manufacturer's instructions.

2.8. Reverse transcription of total RNA and Real-time qPCR for the relative quantification of caprine IL-2, IL-4, INFγ and IL-10 gene transcripts

The cDNA amplification process was performed in the iCycler (BioRad) thermocycler using a MyiQ™ Single Color Real Time PCR Detection System (BioRad) and SYBR Green® I as fluorophore. To monitor the process, the iQ™ 5 Optical System Software Version 2.0 for Windows 2000 and XP (BioRad) was used. For the amplifications, the GoTaq polymerase included in qPCR Master Mix® (Promega) at concentration 1X per sample was employed. The molecules analyzed in the present study were INF-γ, IL-2, IL-4 and IL-10. The β-actin gene was used as housekeeping, so the results were normalized according to the amplified copies of β-actin in each one of the samples assessed by using the software iQTM5 Optical System Software Version 2.0 for Windows 2000 and XP operating systems (BioRad).

The primer pairs for each gene were designed using the corresponding goat or sheep sequences published in the Genbank (NCBI). The primer design was performed using the program Gene runner V. 3.0 and primer sequences were purchased from Invitrogen (Table 2). Based on the theoretical Tm of each primer and preliminary tests carried out at different temperatures, the optimal temperature (Tm) for all the primer pairs was set at 61°C. Thus, the amplification cycles and dissociation (melting-curve) for all genes were established as follows: denaturation 1 cycle at 95°C for 2 min, DNA replication 45 cycles at 94°C for 15 sec, 61°C for 20 sec and 72°C for 15 sec, and finally, one final elongation cycle at 72°C for 2 min.

As referred before, the kit GoTaq® qPCR Master Mix (Promega) used for amplifications was provided as a simple-to-use stabilized 2X master mix which includes all components for quantitative PCR except sample DNA, primers and water. This formulation, which includes a proprietary dsDNA-binding dye, a low level of carboxy-X-rhodamine (CXR) reference dye, GoTaq® Hot Start Polymerase, $MgCl_2$, dNTPs and a proprietary reaction buffer, produces optimal results in qPCR experiments. For β-actin, IL-4, IL-2 and IL-10 primers, $MgCl_2$ had to be increased up to a final concentration of 50 mM using a commercial $MgCl_2$ solution (BioRad). For β-actin and IL-4, primer sets were used at a final concentration of 2 μM, while for IL-2, IL-10 and INF-γ a 4 μM final concentration of the corresponding primer sets were employed. For all the genes, 1:3 cDNA dilutions were added to each PCR

reaction, 2.5 µl for β-actin and 5 µl for the remaining genes. Amplifications were performed in triplicates in a total volume of 25 µl using 10 µl of GoTaq® qPCR Master Mix. The reaction mixtures were always performed at 4 °C under sterile conditions using 96-well optical plates and each sample was analyzed in triplicates.

To determine the linear range and amplification efficiencies and to confirm the specificity of the primer pairs of the β-actin and problem genes, DNA samples from lymphocytes isolated from caprine lymph nodes were here employed. In order to stimulate the gene transcription of IL-4, caprine lymphocytes were incubated with phorbol myristate acetate (PMA) (Sigma-Aldrich) at a 5 ng/ml final concentration (Gohin et al., 1997), whereas lymphocytes stimulated with ConA (Sigma-Aldrich) were utilized as positive controls for INF-γ, IL-2 and IL-10 gene amplifications. PCR reactions of these positive samples were run in duplicate in order to obtain standard curves. For the calibration of the curve, 6 serial dilutions of 1:4 from the cDNA were obtained in a pool out of all the samples used. The differences of the slopes between standard curves obtained for actin-β and the problem molecules (which should be <0.1 for reliable quantification) were plotted against the logarithm input of total RNA and a corresponding regression line was calculated. The data were visualized by the program iQTM5 Optical System Software Version 2.0 for Windows 2000 and XP operating systems.

2.10. Statistical analysis of data

Faecal OPG were logarithmically transformed and added by 1 [log (OPG+1)] in order to obtain normal distributions according to the Kolmogorov-Smirnov's normality test. Levels of the different immunoglobulins analysed in the study were expressed as the relative percentage of the optical density (O.D.) of a positive control pool in order to avoid inter-assay differences (Relative O.D.). One factorial analysis of variance, Tukey's multiple comparison test and Student's t-test were used to analyse the data between different experimental groups and the Pearson correlation test for the evaluation of the association between different parameters assessed in this study. For that purpose, the statistical software SigmaPlot 12.0 was employed and the differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. OPG counts, clinical signs and body weights

Primary infected animals at 4 weeks of age already had high OPG counts at day 14 post infection (p.i.) with maximum values being observed at day 15 p.i., which corresponded to mean OPG counts of approximately 5.5 x 10^5 (Fig. 1). Thereafter, the oocyst excretion gradually decreased up to day 21st p.i. and, at day 22 p.i., negative counts were recorded in all animals. Challenge controls (group 2) and uninfected animals (group 3) had negative counts through the whole study.

From day 14 p.i. up to day 22 p.i., faecal consistence was altered in all the five primary infected animals, ranging from slightly soft faeces to watery diarrhea with blood and small portions of gut mucosa. Clinical signs also included anorexia, weakness, dehydration and growth delay as shown by the weekly determination of body weights. In general, the severity of the disease was more evident between days 14-18 p.i., and, except for the presence of slightly soft faeces, clinical sings had already disappeared by day 22 p.i. In spite of treatment with fluidotherapy and vitamins, one of the goat kids died at day 20 p.i. Apart from some alterations due to dehydration, haematological determinations were not relevant. Any evidence of clinical signs compatible with coccidiosis was observed in the remaining animals during the whole experiment.

3.2. Pathologically and histological analysis

Gross morphological examination of the animals from challenge group 1 did not revealed other changes than those observed in the intestinal mucosa, particularly of ileum,

colon and caecum, where different degrees of congestion and thickening were found. Histological examination showed a moderate hyperplasia and hypertrophy of the mesenteric lymph nodes and Peyer's patches, eosinophilic enteritis and mastocytosis with diffuse infiltration of lymphocytes, neutrophils and globular leukocytes. Challenge control and uninfected animals (group 2 and 3, respectively) did not show any alteration on gross morphology, while microscopical examinations in group 2 exhibited a mild inflammatory cell infiltration involving different immune cells.

According to histopathological observations, cell counts analysis revealed increased scores for most of the cell populations examined. Ileal osinophils were significantly increased both in challenged animals (group 1) and challenge controls (group 2) with respect to uninfected controls ($p < 0.001$ and $p < 0.05$, respectively), while in colonic samples this cell population was only increased ($p < 0.05$) in group 1 (Fig. 2A). The number of eosinophils in challenge group 1 was significantly higher than those of challenge controls ($p < 0.05$). A similar tendency was observed for lymphocyte counts, although in this occasion no differences between groups 1 and 2 could be proven statistically (Fig. 2B). By contrast, neutrophils were predominantly increased in challenge controls (group 2) both in ileum and colon samples when compared to re-infected animals, with significant differences being recorded for ileal counting ($p <0.001$) (Fig. 2C). Only neutrophil counts from the small intestine samples were not different from those of uninfected controls. Globular leukocytes at ileal mucosa followed the same profile described above for eosinophils, with differences between groups 1 and 2 being significant ($p < 0.01$), but almost no counts were recorded for this cell population in colon (Fig. 2D). Finally, mast cells were significantly increased in ileal and colonic mucosa of challenge controls animals ($p < 0.001$ and $p < 0.05$, respectively), whereas in challenged kids the scores were similar to those of uninfected control group (Fig 2E).

The histopathological analysis of ileal samples from challenge control animals, sacrificed 7 days p.i., also include the identification and further counting of immature schizonts, which should be the parasitic forms at this stage of the endogenous lifecycle of *E. ninakholyakimovae.* When compared to H&E and Giemsa, PAS staining allowed an easier localization of the parasite with the interstitial tissue of the villi of distal ileum samples. The immature schizonts were approximately 10-15 µm in diameter and contained small semi-refractive granules with a strong fuchsia colour. The counting of immature schizonts revealed that challenge control group 1 had significantly higher scores than group 2 ($p < 0.001$), and negative counts were recorded for uninfected controls (Fig. 2F).

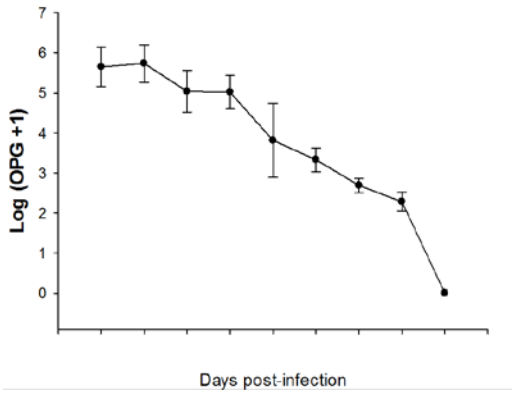

Figure 1. OPG (oocysts per gram of faeces) counts of goat kids orally infected at week 4 of life with 2 x 10^5 sporulated oocysts *Eimeria ninakohlyakimovae* GC. OPG counts are depicted as the logarithm of the OPG plus one (log [OPG + 1]) and represent the mean ± SEM.

3.3. Enzyme-linked immunosorbent assay (ELISA)

Relative values for specific antibodies IgG, IgM and IgA against *E. ninakholyakimovae* sporulated oocyst antigen (SOA) are represented in Fig. 3. For serum levels of IgG and IgM from uninfected controls, mean values of all sampling points

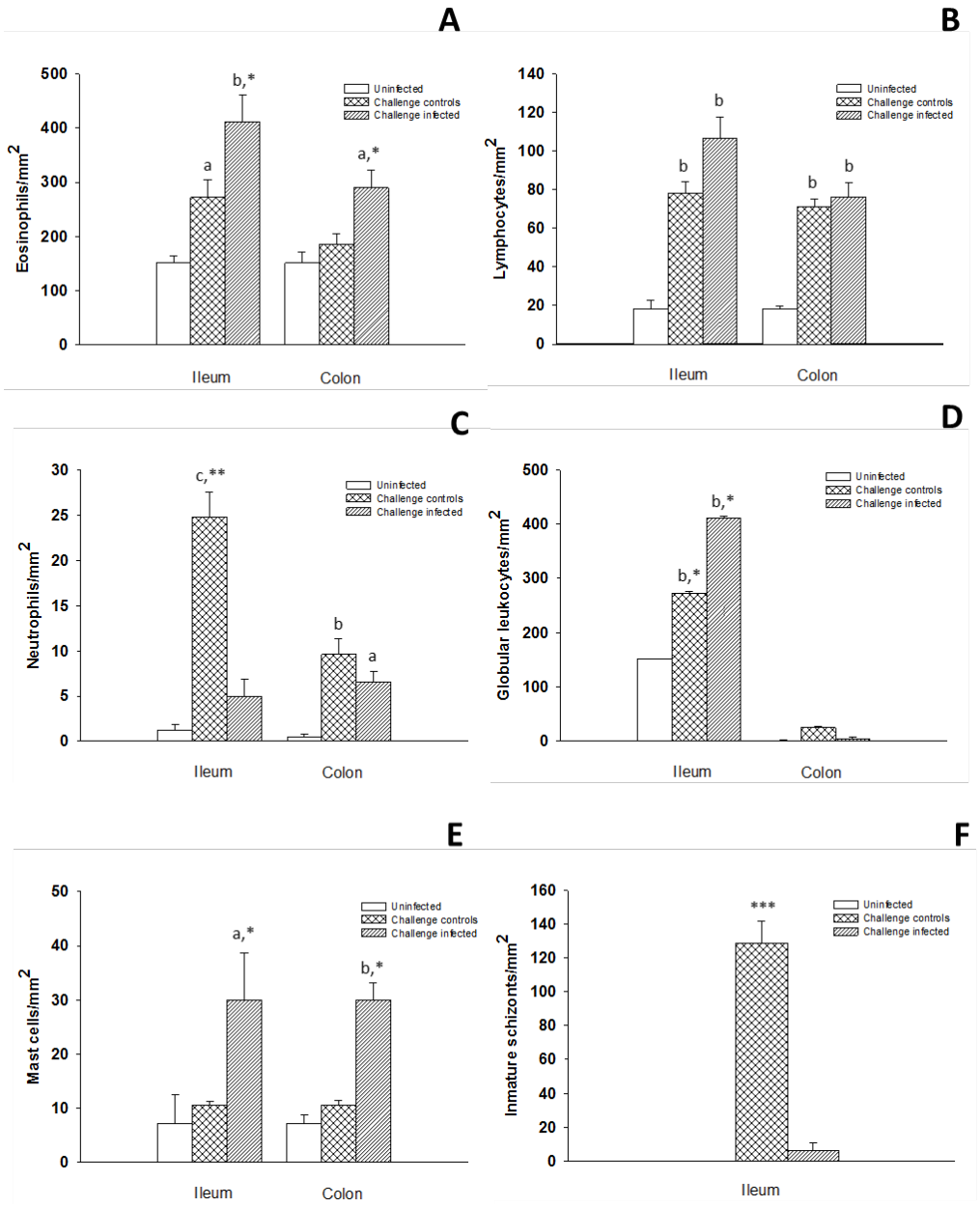

Figure 2. Differential leukocyte counts, expressed as number of cells per mm², in the mucosa of the ileum and colon of goat kids after primary (group 1, challenge controls) or challenge infections (group 2, challenge infected) with *Eimeria ninakohlyakimovae* sporulated oocysts. Control group 3 represents uninfected animals. (A) eosinophil counts; (B) lymphocyte counts; (C) neutrophil counts; (D) globular leukocyte counts; (E) mast cell counts. The number of immature schizonts stained by PAS at ileum is also represented (F). Data represent the mean ± SEM in all the experimental groups. (*) $p < 0.05$, (*) $p < 0.01$ and (*) $p < 0.001$ represent significant differences between infected animals and control group 3; (a) $p < 0.05$, (b) $p < 0.01$ and (c) $p < 0.001$ represent significant differences between challenge infected and challenge control groups.

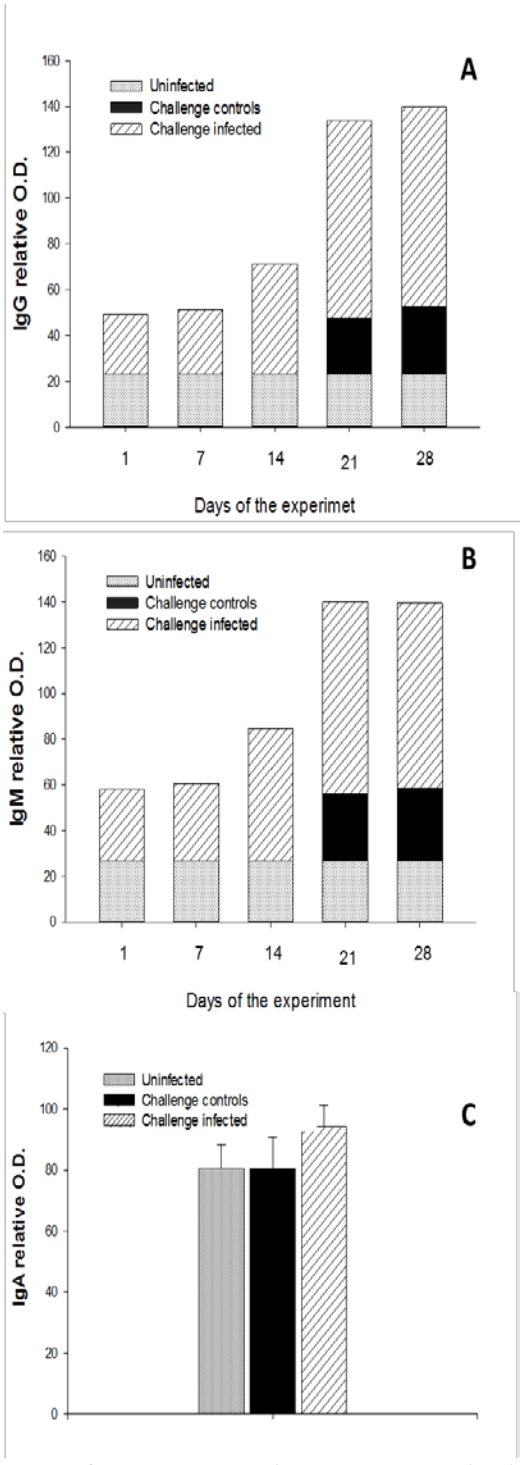

Figure 3. IgG, IgM and IgA anti-SOA levels expressed as relative optical densities (O.D.) in pool sera or individual mucus samples from goat kids after primary (group 1, challenge controls) or challenge infections (group 2, challenge infected) with *Eimeria ninakohlyakimovae* sporulated oocysts. Control group 3 represents uninfected animals. Data for IgA represent the mean ± SEM in all the experimental groups.

(1-28 days p.i.) are represented (Fig. 3A and 3B). Goat kids from group 1 gradually increased their IgG and IgM values from day 14 p.i. onwards, and in the next two sampling points the level were approximately three time higher than mean values recorded for uninfected controls. By contrast, the relative O.D. for both isotypes was almost the same in group 2 than in non-infected goat kids. By last, levels of immunoglobulin isotype A (IgA), depicted in Fig. 3C, were slightly increased in the ileal mucus of challenged animals compared to uninfected controls, although differences were not significant; by contrast similar IgA relative levels were found between challenge control kids and the uninfected group.

3.4. Effects of E. ninakholyakimovae infection on gene transcription of IL-2, IL-4, IL-10 and INFγ

The relative expression levels of the cytokines IL-2, IL-4, IL-10 and INFγ are represented in Fig. 4. There were certain changes on gene transcription in most of these four cytokines but, probably due to high variability between individual samples, only limited significant differences were recorded; it was only possible to determine some trends in the elicited responses. Relative expression for IL-2 showed that both group 1 and 2 had slightly increased gene transcription compared to uninfected controls (Fig. 4A; these differences were also observed in mesenteric lymph nodes (MLN) and proven stastistically (p <0.01). Significant increased levels of expression were also recorded in colonic and MLN tissue samples from challenge infected animals (p < 0.01), whereas for the remaining samples taken from infected animals only slightly higher values than those of control group 3 were found (Fig. 4B). Colonic samples from both challenge controls and challenge infected animals (group 1 and 2, respectively) were the only that showed significantly increased gene expression compared to uninfected controls (p < 0.05) (Fig. 4C). On the contrary, most of the tissue samples had a variable increase of the INFγ

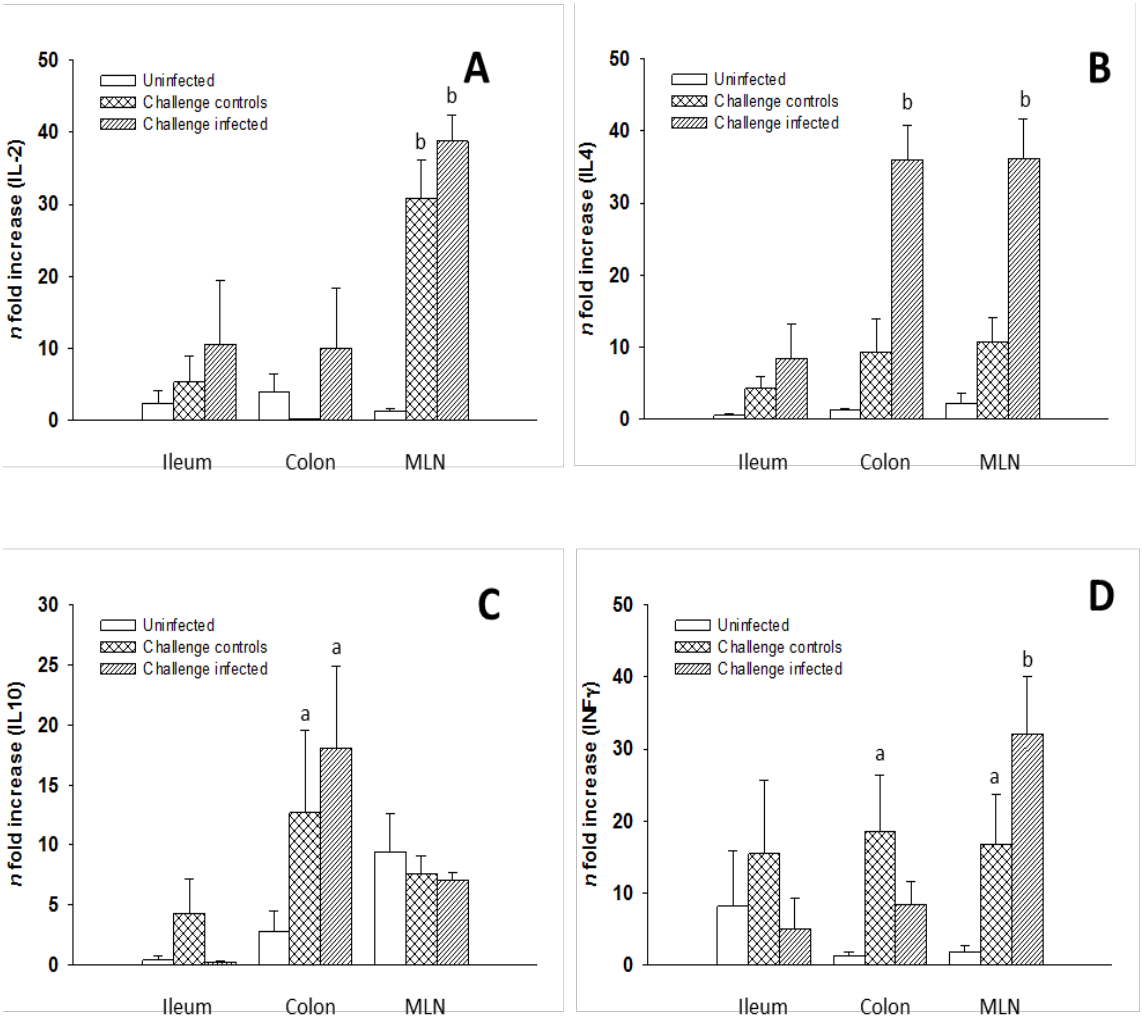

Figure 4. Gene transcription of IL-2 (A), IL-4 (B), IL-10(C) and INF-γ (D) from goat kids after primary (group 1, challenge controls) or challenge infections (group 2, challenge infected) with *Eimeria ninakohlyakimovae* sporulated oocysts. Control group 3 represents uninfected animals. Data represent the mean ± SEM in all the experimental groups. (a) $p < 0.05$ and (b) $p < 0.01$ represent significant differences between infected animals and control of infection.

gene transcription, with significant differences being recorded for MLN samples in group 1 and 2 ($p < 0.05$ and $p < 0.01$, respectively) and for colonic tissue of challenge controls ($p < 0.05$).

3.5. Immunohistochemical analysis

Mean values for counts of the different immune cell populations characterized by IHQ are represented in Table 3. Scores for $CD4^+$, $CD8^+$ and CD45 T lymphocytes were higher in challenge animals, both in ileal and colonic samples ($p < 0.05$ to $p < 0.01$), while a light no significant increase were also recorded at ileum of challenge control group 2. A similar tendency was observed for $CD45^+$ T cells, although in this occasion only differences from ileal mucosa of challenge infected goat kids were proven statistically. Accordingly, counts for pan T CD3 was also increased, although mayor differences for this antibody were detected on challenge controls in this case ($p < 0.05$).

In contrast to most of the T cell population analysed, the staining with antibodies against MHCII and the myeloid/histocyte marker MAC387 resulted in increased counts primarily in challenge controls; in particular, scores from ileum using these two markers resulted in significant increases when compared to uninfected controls ($p < 0.05$ and $p < 0.01$, respectively).

Table 1. Primary antibodies used in immunohistochemical technique

Antibody	*Specificity*	*Fixation method*	*Dilution (1 in)*	*Source*
H42A (mAb)	MHC II	Frozen	1:10000	VMRD
GC50A1 (mAb)	CD4	Frozen	1:50	VMRD
CACT80C (mAb)	CD8	Frozen	1:25	VMRD
CD45 (mAb)	CD45	Frozen	1:25	Dako
Mac 387 (mAb)	Myeloid/histiocyte Ag	Formalin	1:200	Dako
Lysozyme (pAb)	Lysozyme	Formalin	1:250	Dako
S-100	S-100	Formalin	1:500	Dako
CD3 (pAb)	CD3	Formalin	1:200	Dako

Table 2. Sequences and accession number of the primers used for amplifications

PCR Primers	Sequence	Accession number
β-Actine (ca)	CCAACCGTAGAGAAGATGACCC CCCAGAGTCCATGACAATGCC	AF481159
INF-γ (ca)	AGATAACCAGGTCATTCAAAGGAG GGCACAGGTATTCATCAC	U34232
IL-2 (ov)	GTGAAGTCATTGTGTGCTGGA TGTTCAGGTTTTGCTTGGA	Craig et al; 2007
IL-4 (ca)	GCTGGTCTGCTTACTGGTATG CGATGTGAGGATGTTCAGC	FJ936316
IL-10 (ca)	GTGGAGCAGGTGAAGAGAGTC TGGGTCGGATTTCAGAGG	AJ458378

(ca) caprine; (ov) ovine

Table3: Media counts and standard error of cell counts in ileum and colon by immunohistochemistry for each group of animals; controls, primary infection and reinfected.

	Uninfected		Challange controls		Challange infected	
	Ileum	*Colon*	*Ileum*	*Colon*	*Ileum*	*Colon*
MHCII	68 ±8.7	57.5±9.2	139±10.4[a]	79.8±5.2	80.4±11.2	68.8±16
CD4+	22.5±2.2	21.3±0.9	24.8±2.7	20.6±0.4	63.6±0.41[b]	46.8±6.13[a]
CD8+	31.2±3	23.6±2.4	54.6±9.2	27.4±2.3	93.6±12.9[b]	49.4±9[a]
CD45	62.6±8.2	52±8.90	69.8±3.8	52±5.70	108.6±7.6[b]	73.6±12.6
MAC387	13.5±2.7	8.25±1.7	32.8±7.1[b]	11.8±1.3	22.2±6.3	7±0.94
LYSOZYME	16±2.0	9.5±1.04	21.4±1.1	11.8±1.3	20.0±1.8	10.2±0.9
S-100	8.0±0.4	6.25±0.5	11.8±2.5	10±0.70	13.8±0.9	7.0±0.31
CD3	83±4.8	54±3.40	98.6±6.9	75±2.71[a]	80.6±3.23	66.2±2.7

4. Discussion

The present study aimed to investigate whether young goat kids are able to mount protective immune response against early stages of the endogenous development of the caprine coccidia *E. ninakholyakimovae,* for what an experimental infection with the parasite and subsequent challenge was performed. Previous studies *in vivo* performed by Vieira et al. (1997) showed that first-generation meronts could be found 7 days p.i. in endothelial cells of the lacteals of the intestinal villi, in the lamina propria, and with less frequency in the lymphatic vessels of the ileal submucosa. The authors illustrated one meront, detected 7 days p.i., which measured 58.2 X 49.9 μm and contained well defined nuclei, with a residuum of 4.5 X 3.3 pm, and were surrounded by a 4.1-Rm-thick, capsule-like structure. Although, we took into consideration these data when selecting the time for sacrifice of the animals, these observations are only partially in agreement with our results, as the schizonts we could find at this stage were approximately half size and they were not surrounded by a clear capsule-like structure. The schizont appearance was more similar to parasitic stages of *E. ninakholyakimovae in vitro* described by Ruiz et al. (2010) at the same time p.i. when comparing the development of the parasite in different culture systems, particularly in cells of ruminant origin. Actually, phase contrast daily examination of the *in vitro* culture could demonstrated the presence of refractive granules, which would correspond to the fuchsia stained ones that we observed by PAS in the interstitial of the villi of parasitized animals. Surprisingly, when the counts for these schizont-like structures were analysed, significant lower scores were found in challenged group compared to challenge control or primary infected animals, which indicates that protective immune response was effective against early stages of merogony I and, therefore, might abrogate further development of the parasite inside the host. The mechanism by which the immune system has interacted with early schizogony may not be deduced from the experiments included in the present study. However, there is evidence for the presence of early parasite-specific antigens on the surface of *E. bovis*-infected host cells carrying first generation meronts, which was suggested to have a potential role in the development of protective immunity (Bradawy et al., 2010). The authors demonstrated the presence of *E. bovis* host cell surface antigens (EbHCSAg) on the surface membrane of *in vitro* infected bovine host cells. EbHCSAg were earliest detected on day 7 p. i., i. e., their occurrence coincided with the morphologically detectable onset of the first merogony (Hermosilla et al. 2002; Behrendt et al. 2008). Correspondingly, EbHCSAg were detectable in all host cell types permissive for the development of *E. bovis* first generation meronts *in vitro*. Since EbHCSAg were recognized by both, hyperimmune sera (HIS) and rat immune sera (rIS) to *E. bovis* merozoite I stages, it appeared likely that these antigens are of parasite origin. This was confirmed by the binding pattern of the affinity purified sera on *E. bovis* meront I infected BFGC (affHIS). Whether the same or a similar type of antigen is expressed in the surface and/or the immature schizonts identified at prepatency in our study deserves futures investigations.

Because at the moment of euthanasia the onset of the oocyst excretion had not started yet and no clinical signs are commonly observed during the first week post infection, as shown in previous *E. ninakholyakimovae* experimental incoulations in goat kids performed by our group (Ruiz et al., 2013a; 2013b), no more data related to immunoprotection are available at this stage apart from the number of immature schizonts. Nevertheless, the evaluation of the antibody response offered some evidence which reflects the exposure to the parasite both at peripheral and local level. Accordingly, the levels of the three isotypes analysed (IgG, IgM and IgA) were increased in re-infected animals. The magnitude of the humoral response was not particularly high, so no correlations could be demonstrated with the number of immature schizonts.

Although there are many data showing that sexual stages of *Eimeria* spp are the main target for immune system in order to develop

acquired immune responses, classical studies already demonstrated the immunogenicity of the first schizogony in poultry (McDonald et al., 1986). Many other publications also sustain the hypothesis that adaptive cellular immune responses occur *in vivo* already during the prepatency of *E. bovis* infections (Hughes et al. 1988, 1989; Fiege et al. 1992; Hermosilla et al. 1999; Taubert et al. 2007, 2008). Since sporozoites of this *Eimeria* species rapidly invade also endothelial host cells and then are situated intracellular, developing immune reactions should be directed against infected host cells as well. Given that endothelial cells generally have the capacity of antigen presentation (Wagner et al. 1984; Bosse et al. 1993; Knolle 2006; Behling-Kelly and Czuprynski 2007), it appears likely that T cells may act against early stages, such as intracellular sporozoites or meronts I, rather than meronts II and gamonts. Accordingly, mean values for lymphocyte counts recorded in the present study were increased in re-infected animals, like other cell populations such as eosinophils, mast cells and globular leukocytes. Likewise, in this group of animals a greater number of $CD4^+$ and $CD8^+$ cells were observed, all indicating that T cell response could be related to the development of the protective response. Contrarily to our data, Hermosilla et al. (1999) observed that calves inoculated with oocysts *E. bovis* had $CD4^+$ T cells increased during prepatency in primary infections decaying after the patent period. The population of $CD4^+$ T cells are actually admitted to be particularly involved in resolving primary infections by regulating the duration and level of oocyst shedding. On another hand, in agreement to our data, CD8 + T cells have been involved in *Eimeria* re-infections (Rose et al., 1992a; Findly et al., 1993; Ovington et al., 1995; Smith and Hayday, 2000; Shi et al, 2001a). The analysis of the T cells populations also showed that $CD45^+$ T cells were increased in ileum of re-infected animals, which could be related to the mild increase of IgA in mucus samples. In contrast to T cell populations, except pan T $CD3^+$, cell counts of other immune cell types did not changed or increased only in primary infected goat kids (challenge controls). For instance, $MHII^+$ cells had significantly higher counts in ileal mucosa, as well as MAC387, which suggests an active recognition of antibodies and a dynamic participation of myeloid/histiocytic cells, probably involved in innate immune reactions.

Finally, when the involvement of certain cytokines was evaluated we found some evidences that both Th1 and Th2 responses could be mounted against *E. ninakohlyakimovae* infections, as shown by increased relative expression of IL-2, IL-4, IL-10 and INFγ. In general, during primary infection in ruminants, *Eimeria* species levels of INFγ and IL-2 gene expression are elevated in comparison to IL-4 transcription levels, which suggest an increased cytotoxic cellular response to parasite cells (Taubert et al., 2008). Furthermore, increased IFN and IL2 could stimulate lymphocyte activation and enhance the phagocytic activity of macrophages via an increase in nitrite production (Hermosilla et al., 1999).

As a whole, the results of this study give evidence that protective immune responses may be addressed to early developmental stages of *E. ninakohlyakimovae* during the first schizogony, which probably results from a complex framework of mechanisms, effector cells and cytokines that involves both innate, humoral and cellular immune responses. The present data contribute to understand the complexity of the cellular and humoral immune response of the goat hosts against coccidiosis, which can be used for the development of strategies for modulation of the immune response and for the identification of anticoccidial compounds or vaccines.

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5. CONCLUSIONES

- **PRIMERA.-** La cepa GC de *Eimeria ninakohlyakimovae* aislada en nuestro laboratorio presenta no sólo una gran capacidad infectante sino un elevado poder patógeno, capaz de producir cuadros clínicos severos.

- **SEGUNDA.-** Además, dicha cepa puede desarrollar respuestas inmunes protectoras, por lo cual constituiría un modelo idóneo para estudiar la respuesta inmune y los mecanismos de patogenicidad en las coccidiosis caprina.

- **TERCERA.-** Aunque *Eimeria ninakohlyakimovae* induce una importante immunoprotección frente a reinfecciones homólogas con dosis infectantes moderadas similares a las empleadas en la primoinfección, la inmunidad puede ser sólo parcial cuando se utilizan dosis de infección masivas. En tales casos, el desenlace fatal durante la reinfección no está claro si se produce por la acción directa del parásito o por una exhacerbación de la respuesta inmune asociada.

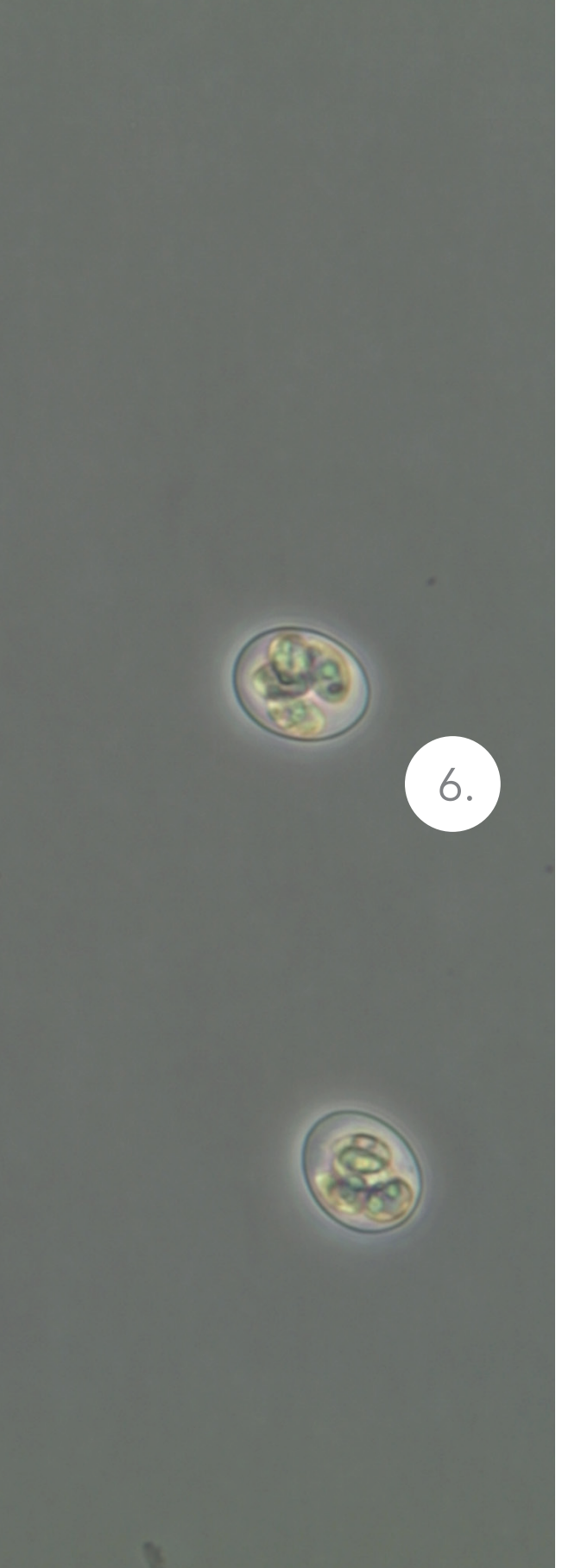
- **CUARTA.-** La infección y posterior reinfección con *Eimeria ninakohlyakimovae* estimula la producción de IgM e IgG séricas, así como la producción de IgA en el mucus del íleon. Estos niveles no se correlacionan con la producción de ooquistes y no se observan diferencias claras entre animales reinfectados y controles de reinfección, por lo que, aunque la respuesta de anticuerpos es reflejo de la exposición al parásito, parece no haber una asociación clara con el nivel de protección.

  QUINTA.- Utilizando como antígeno ooquistes esporulados de *Eimeria ninakholyakimovae*, la IgG sérica de los animales infectados logró reconocer un panel de péptidos específicos cuyos pesos moleculares oscilaron entre 134 y 16 kDa, apoyando el valor del *electroimmunobotting* como una técnica para detectar proteínas inmunorreactivas en coccidiosis caprina. Sin embargo, tampoco en esta ocasión se encontraron bandas de reconocimiento específico en los animales reinfectados.

- **SEXTA.-** La infección de cabritos a las 3, 4 y 5 semanas de edad con *Eimeria ninakohlyakimovae* desencadena en todos los casos respuestas inmunes protectoras frente a reinfecciones homólogas realizadas 3 semanas más tarde. La inmunoprotección se tradujo en una reducción significativa de la producción de ooquistes y de la intensidad del cuadro clínico y estuvo asociada a componentes celulares, tanto innatos como adquiridos, de la respuesta inmune, no siendo tan destacada la implicación de la respuesta inmune humoral.

- SÉPTIMA.- Sin embargo, cuando se realizó un análisis más detallado de los datos, se observaron algunas diferencias entre los tres grupos de edad, relacionado con el resultado de infección por *Eimeria* y la respuesta inmune resultante; tales resultados sugieren que los cabritos más jóvenes pueden no ser completamente inmunocompetentes, lo cual podría ser de interés para el diseño de estrategias inmunoprofilácticas para el control de la coccidiosis caprina.

- OCTAVA.- La respuesta inmune adquirida frente a *Eimeria ninakohlyakimovae* puede estar dirigida hacia los estados parasitarios que se desarrollan durante la fase de prepatencia de la enfermedad, en concreto frente a los esquizontes inmaduros. Dicha respuesta resulta ser compleja, en tanto que intervienen diversas poblaciones celulares y mediadores químicos (por ejemplo citoquinas), además de células presentadoras de antígenos y células propias de respuestas inmunes innatas.



6. RESUMEN

6.1. RESUMEN

La coccidiosis caprina puede afectar al 100% de los cabritos en el rango de edad de 4 a 10 semanas, y causar graves pérdidas económicas en la industria caprina. En ocasiones, puede dar lugar a una clínica grave con un desenlace fatal, especialmente, en cabritos sometidos a situaciones de estrés, como cambios bruscos de manejo o de alimentación, o en condiciones de hacinamiento y ambientes excesivamente contaminados con ooquistes. Entre las especies más frecuentes de *Eimeria* en caprinos, *E. ninakohlyakimovae* se considera una de las más patógenas y frecuentes en el Archipiélago Canario. Infecta células endoteliales en primera instancia, pero su ciclo endógeno completo también compromete a células epiteliales, todo lo cual conduce a una enfermedad intestinal grave caracterizada por diarrea catarral, pérdida de peso, deshidratación y retraso en el crecimiento. El objetivo general del presente trabajo ha sido analizar la respuesta inmune frente al coccidio caprino *Eimeria ninakohlyakimovae* mediante infecciones experimentales y su relación con los mecanismos de patogenicidad del parásito y las posibles implicaciones en la profilaxis y control de la enfermedad. Para ello, se aisló una cepa de origen caprino de *E. ninakohlyakimovae* y se realizaron infecciones experimentales para valorar su grado de infectividad, patogenicidad y capacidad de desarrollar una respuesta inmune en los hospedadores. La cepa aislada resultó tener, no sólo una gran capacidad infectante sino un elevado poder patógeno, capaz de producir cuadros clínicos severos. Además, dicha cepa pudo desarrollar respuestas inmunes protectoras, por lo que en los siguientes experimentos que se plantearon se empleó como modelo experimental. Con el siguiente objetivo planteado se pretendieron simular las infecciones masivas por coccidios que tienen lugar en determinadas situaciones de campo. Para ello, se sensibilizaron cabritos con una dosis moderada-alta de ooquistes de *E. ninakohlyakimovae* y tres semanas más tarde se reinfectaron con una dosis elevada equivalente a 2×10^6 ooquistes. Tras investigar diversos parasitológicos, clínicos e inmunológicos se comprobó que la infección primaria con *E. ninakohlyakimavae* en cabritos reinfectados posteriormente con una dosis de infección alta pueden padecer una coccidiosis aguda fatal y, en consecuencia, mostrar graves alteraciones intestinales e, incluso, muerte. La excesiva respuesta inmune desarrollada frente a las etapas intracelulares de *E. ninakohlyakimovae*, tal como demuestra la elevada infiltración leucocitaria a nivel del intestino, podría haber contribuido al desenlace fatal durante la reinfección con la dosis infectante más elevada, aunque no se descarta que el desenlace pudiera haber sido debido directamente a la acción del parásito. Estos resultados enfatizan la necesidad de establecer medidas de gestión que prevengan la contaminación excesiva del ambiente con ooquistes. En otro grupo de experimentos se pretendió evaluar el papel de la respuesta de anticuerpos frente a las infecciones por *E. ninakohlyakimovae.* Con tal fin se analizó la respuesta de IgG e IgM periféricas, así como de IgA local en el curso de infecciones y reinfecciones por dicha especie

parásita. Además, se realizó una primera aproximación al reconocimiento mediado por anticuerpos (IgG) específicos de péptidos mediante SDS-PAGE e *immunoblotting*. De este estudio se concluyó que los niveles de IgG, IgM e IgA no se correlacionan con la producción de ooquistes y no se observan diferencias claras entre animales reinfectados y controles de reinfección, por lo que, aunque la respuesta de anticuerpos es reflejo de la exposición al parásito, parece no haber una asociación clara con el nivel de protección. Por otro lado, puesto que el efecto de la edad parece ser decisivo en general, en la respuesta inmune frente diferentes agentes parasitarios, además de en estrategias inmunoprofilácticas, en el presente proyecto de tesis doctoral se evaluó la influencia de la edad en el desarrollo de respuestas inmunes protectoras en la coccidiosis experimental caprina por *Eimeria ninakohlyakimovae*. Los dados parasitológicos, biopatológicos y productivos de este estudio demuestran que la respuesta inmune frente a la coccidiosis producida por *E. ninakohlyakimovae* se desarrolla en cabritos, mínimo a partir de la tercera semana de vida. La inmunoprotección se traduce en una reducción significativa de la producción de ooquistes y de la intensidad del cuadro clínico y se asocia a componentes celulares tanto innatos como adquiridos. Sin embargo, cuando se realizó un análisis más detallado de los datos, se observaron algunas diferencias entre los tres grupos de edad, relacionado con el resultado de infección por *Eimeria* y la respuesta inmune resultante; tales resultados sugieren que los cabritos más jóvenes pueden no ser completamente inmunocompetentes, lo cual podría ser de interés para el diseño de estrategias inmunoprofilácticas para el control de la coccidiosis caprina. Finalmente, se pretendió analizar la respuesta inmune humoral y celular en la fase prepatente de la enfermedad producida por *E. ninakohlyakimovae* en cabritos y su relación con respuestas inmunoprotectoras frente a las formas inmaduras del parásito (esquizontes inmaduros) que se desarrollan durante esta fase del ciclo endógeno. Mediante tinción con PAS se evidenciaron formas parasitarias en la mucosa del íleon, compatibles con esquizontes inmaduros, en número significativamente mayor en los animales primoinfectados que en los reinfectados. En asociación con esta respuesta, los recuentos medios de linfocitos T estuvieron elevados en los animales reinfectados, al igual que otros tipos celulares como eosinófilos, mastocitos y leucocitos globulares. Así mismo, en este grupo de animales se observó un mayor número de células $CD4^+$ y $CD8^+$, todo lo cual indica que la respuesta tipo celular tipo T podría estar relacionada con el desarrollo de la respuesta protectora. La respuesta es, no obstante, muy compleja, en el sentido que intervienen diversas poblaciones celulares y mediadores químicos (por ejemplo citoquinas), además de células presentadoras de antígenos y células propias de respuestas inmunes innatas. En su conjunto, los resultados del presente estudio constituyen aportaciones novedosas que podrían ser útiles para comprender la complejidad de la respuesta inmune celular y humoral de los hospedadores caprinos frente a la coccidiosis, todo ello enfocado al desarrollo de estrategias de modulación de la respuesta inmune y a la identificación de compuestos anticoccidiósicos o vacunas.

6.2. SUMMARY

Caprine coccidiosis may affect 100% of kids in the age range of 4 to 10 weeks, and cause serious economic losses in the goat industry. Occasionally, this parasitic disease may lead to serious clinical problems with a fatal outcome, especially in kids subjected to stress situations, such as abrupt changes in management or feeding, or in crowded conditions and environments too contaminated with oocysts. Among the most frequent species of *Eimeria* in goats, *E. ninakohlyakimovae* is considered one of the most pathogenic and frequent in the Canary Islands. It infects the endothelial cells in the first instance, but its full endogenous cycle also commits to epithelial cells, all of which leads to a severe intestinal illness characterized by catarrhal diarrhea, weight loss, dehydration and growth delay. The general objective of the present work was analyze the immune response against goat coccidia *Eimeria ninakohlyakimovae* through experimental infections and their relationship with the mechanisms of pathogenicity of the parasite and the possible implications in the prevention and control of the disease. With this aim, an *E. ninakohlyakimovae* strain of caprine origin was isolated from field samples and experimental infections were conducted to assess their degree of infectivity, pathogenicity and ability to develop an immune response in the hosts. The isolate had, not only a great infective capacity but a high pathogenicity, able to produce severe clinical signs. In addition, this strain could develop protective immune responses, so that in the following experiments this *Eimeria* species was used as an experimental model. The following objective aimed to simulate massive infections from coccidia that take place in some field situations. For this purpose, goat kids were infected with a moderate dose of oocysts of *E. ninakohlyakimovae* and three weeks later they re-infected with high dose equivalent to 2 x 10^6 oocysts. After the investigation of several parasitological, clinical and immunological parameters we found that animales primary infected with *E. ninakohlyakimavae* and then challenged with a high infection dose can suffer from a fatal acute coccidiosis with severe intestinal disorders and even death. An exacerbate immune responses developed against the intracellular stages of *E. ninakohlyakimovae*, as evidenced by elevated leukocyte infiltration at the gut level, could have contributed to the fatal outcome during re-infection with the highest infective dose, though it is also possible that the outcome could have been due directly to the action of the parasite. These results emphasize the need to establish management measures that prevent excessive contamination of the environment with oocysts. In another set of experiments the role of the antibody response against infections by *E. ninakohlyakimovae* was evaluated. For that purpose, we analyzed the response of peripheral IgG and IgM as well as local IgA in the course of infections and reinfections by this parasitic species. In addition, a preliminary approach for the recognition by antibodies (IgG) of peptides by SDS-PAGE and *immunoblotting* was addressed. This study concluded that IgG, IgM and IgA levels do not correlate with the production of oocysts and there are no clear differences

between reinfected animals and controls of reinfection, so, although the antibody response is a reflection of exposure to the parasite, it seems that there is no clear relationship with the level of protection. On the other hand, since the effect of age seems to be decisive, in general, in the immune response to different parasitic agents, in the present PhD project the influence of the age on the development of protective immune responses in goat experimental coccidiosis by *E. ninakohlyakimovae* was assessed. Based on parasitological, biopathological and productive parameters, this study shows that the immune response against coccidiosis produced by this *Eimeria* speies in kids develops, at least, from the third week of life onwards. The immunoprotection resulted in a significant reduction in the production of oocysts and the intensity of the clinical signs and involved both innate and acquired cellular components. However, when a more detailed analysis of the data was conducted, some differences among the three age groups were observed. These differences were related to the magnitude and characteristics of the *Eimeria* infection and the resulting immune response, suggesting that young kids may not be fully immunocompetent, which could be of interest for the design of immunoprophyfilactic strategies for the control of caprine coccidiosis. Finally, we intended to analyze the humoral and cellular immune response in the prepatent phase of the disease produced by *E. ninakohlyakimovae* in kids and their relationship with immunoprotective response against immature forms of the parasite (immature schizonts) that develop during this phase of the endogenous lifecycle. By staining with PAS, parasitic forms in the mucosa of the ileum compatible with immature schizonts were observed, with significantly increased scores being recorded in primary infected animals when compared to challenge ones. In association with this response, mean values for lymphocyte counts were elevated in reinfected animals, like other cell populations such as eosinophils, mast cells and globular leukocytes. Likewise, in this group of animals a greater number of $CD4^+$ and $CD8^+$ cells were observed, all indicating that T cell response could be related to the development of the protective response. The immune response is, however, very complex, as antigen presenting cells and other effector cell populations of the innate immune system, as well as certain cytokines, are involved. As a whole, the results of this study contribute to understand the complexity of the cellular and humoral immune response of the goat hosts against coccidiosis, which can be used for the development of strategies for modulation of the immune response and for the identification of anticoccidial compounds or vaccines.

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