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Universidad del País Vasco
Euskal Herriko Unibertsitatea

Mycobacteria in rabbits: Searching for an efficient paratuberculosis infection model

PhD Thesis

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2016

Mycobacteria in rabbits:

Searching for an efficient paratuberculosis infection model

Memoria del trabajo realizado en el departamento de Sanidad Animal de NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, para optar al grado de Doctor por la Universidad del País Vasco.

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2016

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EUSKO JAURLARITZA GOBIERNO VASCO

PRESENTACIÓN DE LA TESIS

El presente trabajo de Tesis Doctoral ha sido realizado en el departamento de Sanidad animal de NEIKER-Instituto Vasco de Investigación y Desarrollo agrario, empresa pública del Departamento de Desarrollo Económico y Competitividad del Gobierno Vasco. Su desarrollo ha sido posible gracias al Ministerio de Economía y Competitividad (MINECO) a través de la financiación del proyecto titulado: "Desarrollo de modelos de patogenia e inmunización en paratuberculosis" (AGL2012-39818-C02-02) y a la financiación de la beca predoctoral del Departamento de Educación, Política Lingüística y Cultura del Gobierno Vasco de la que ha disfrutado el doctorando (BFI-2012-237).

La presente Tesis Doctoral ha sido redactada en la modalidad de compendio de publicaciones. A continuación, se presentan las referencias bibliográficas de los cinco artículos publicados:

1- **Mycobacterial Infections in Rabbits: From the Wild to the Laboratory**. R Arrazuria, RA Juste, N Elguezabal. Transbound Emerg Dis. 2016 Jan 22.
2- **Detection of *Mycobacterium avium* subspecies in the gut associated lymphoid tissue of slaughtered rabbits.** R Arrazuria, IA Sevilla, E Molina, V Pérez, JM Garrido, RA Juste, N Elguezabal. BMC Vet Res. 2015 Jun 11; 11:130.
3- **Effect of various dietary regimens on oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* in a rabbit model**. R Arrazuria, E Molina, M Mateo-Abad, I Arostegui, JM Garrido, RA Juste, N Elguezabal. Res Vet Sci. 2015 Aug; 101:80-3.
4- ***Mycobacterium avium* subspecies *paratuberculosis* infection modifies gut microbiota under different dietary conditions in a rabbit model.** R Arrazuria, N Elguezabal, RA Juste, H Derakhshani, E Khafipour. Front Microbiol. 2016 Mar 31; 7:446.
5- **Vaccination sequence effects on immunological response and tissue bacterial burden in paratuberculosis infection in a rabbit model.** R Arrazuria, E Molina, JM Garrido, V Pérez, RA Juste, N Elguezabal. Vet Res. 2016 Aug 5; 47(1):77.

Agradecimientos / Acknowledgments

Me gustaría manifestar mi más sincero agradecimiento a todas las personas que durante todo este tiempo me han apoyado en el desarrollo de este trabajo.

En primer lugar me gustaría agradecer a mis directores, Natalia Elguezabal y Ramón A. Juste por su enorme dedicación y compromiso, sin el que indudablemente este trabajo no hubiera culminado. Gracias a Ramón por aportar su punto de vista critico en todo momento y por esas discusiones tan enriquecedoras. Natalia muchísimas gracias por tu compromiso, esfuerzo e implicación durante todos estos años, y por haberme enseñado tantas cosas, no solo en el ámbito profesional, sino que también en el personal.

Otra persona de la que no me puedo olvidar con la que me inicie en este camino es Joseba M. Garrido. Muchas gracias por ser como eres, y haber estado ahí para cualquier cosa que he necesitado.

Llegado este momento, no puedo evitar que me asalten un sinfín de buenos recuerdos, de los que mucha gente ha formado parte. No me gustaría convertir esta parte tan personal en una lista sin fin de nombres, en la cual se diluya la relevancia que cada uno ha tenido en la consecución de este objetivo.

Quiero agradecer y agradezco a todos los becarios con los que he compartido esta experiencia, los que ya son doctores y los que sin duda alguna lo serán en el futuro. Además tampoco me puedo olvidar del gran equipo humano del departamento de Sanidad Animal de Neiker, formado por tan buenas personas tanto en lo profesional como en lo personal. Gracias a todos los investigadores, técnicos y auxiliares de diagnóstico e investigación así como al personal de administración, mantenimiento y limpieza. Muchas gracias por escucharme, ayudarme pero sobre todo por acompañarme día a día en este camino.

Quisiera agradecer y hacer una mención especial a mis compañeros de mesa (Elena, Naiara, Felix, Miriam y Maitane), que tanto en los buenos como en los malos momentos han sabido sacarme una sonrisa, haciendo que cada día sea especial. Muchas gracias por estar ahí siempre.

Por otro lado, me gustaría agradecer la colaboración de todo el personal del matadero cunícola Untxi, pero especialmente a la veterinaria oficial Susana Rodríguez, por su implicación y dedicación sin los cuales parte de este trabajo no hubiera sido posible.

I would like to express my gratitude to Professor Ehsan Khafipour for giving me the opportunity to join his group and to enjoy such a rewarding personal and professional experience. I would like to thank all the members of the gut microbiome laboratory of the University of Manitoba with whom I had the pleasure to share my stay in Canada. They were really friendly and made me feel a part of the team since the very first day. Thank you for those amazing burgers, those passionate conversations and especially for the kindness I received. In particular, I would like to thank Hooman, for everything he has taught me. You always believed in me and I really appreciate the support you showed me in Canada and also in the distance when I returned to Spain. I will never forget your delicious home-made beer and the great weekend we spent at Caddy Lake. I hope to see you again in a near future.

De igual modo quisiera agradecer a Valentín Perez y Julio Benavides la oportunidad de haber pasado este último verano en su compañía. Gracias por sus ánimos en esta última etapa a todo el personal del Instituto de Ganadería de Montaña (CSIC) y el departamento de Anatomía Patológica de la Universidad de León con los que he tenido el placer de coincidir. Especialmente gracias a Marcos, mi fugaz compañero de piso, y a Maikel por enseñarme a desarrollar y poner a punto las técnicas inmunohistoquímicas.

Finalmente quería agradecer a todas las "Arrazuriatarrak", especialmente a ti, amatxo, por estar a mi lado en la cercanía y en la distancia, por confiar en mí y porque todo lo que soy os lo debo a vosotras. Por ultimo pero no por ello menos importante, quería agradecer a Iván su paciencia y su apoyo durante todos estos años. Espero poder recompensártelo algún día.

¡Muchas Gracias! Mila esker! Thank you so much!

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16S rRNA	16S ribosomal ribonucleic acid
ADN	Ácido desoxirribonucleico
APPCC	Análisis de peligros y puntos de control críticos
ARNr 16S	Subunidad 16S del ácido ribonucleico ribosómico
ASA	Aminosalicílico
BAAR	Bacilo ácido-alcohol resistente
C	Carbono
CARD15	Dominio reclutador de caspasa 15/ Caspase Associated Recruiment Domain 15
CD	Cluster de diferenciación/ Cluster of differentiation
CE	Comisión Europea
CMH	Complejo Mayor de Histocompatibilidad
CpG	Citosina fosfato Guanina
CR	Receptor del complemento / Complement Receptor
DDS	Dapsona
DNA	Deoxyribonucleic acid
EC	Enfermedad de Crohn
EEI	Enfermedades inflamatoria intestinal
EEUU	Estados Unidos de América
ELISA	Ensayo por inmunoadsorción ligado a enzimas/ Enzyme Linked Immunosorbent Assay
FAP	Proteinas de acoplamiento de fibronectina/ Fibronectin Attachment Proteins
FcR	Receptor Fc/ Fc Receptor
FoxP3	Forkhead Caja P3/ Forkhead box P3
HCP	Hexadecylpyridinium chloride

HEYM	Medio de Herrold con yema de huevo/ Herrold Egg Yolk Medium
HIS	Hibridación *in situ*
HIV	Human immunodeficiency virus
IDGA	Inmunodifusión en gel de agar
IDR	Intradermorreacción
IFN-γ	Interferón Gamma
Ig	Inmunoglobulina
IHQ	Inmunohistoquímica
IS	Secuencia de inserción / Insertion sequence
ITS	Espaciador transcribible interno/ Internal Transcribed Spacer
IV	Intravenoso
LAM	Lipoarabinomananos
Maa	*Mycobacterium avium* subspecies *avium*
MAC	*Mycobacterium avium* comlex
Mah	*Mycobacterium avium* subspecies *hominisuis*
Map	*Mycobacterium avium* subspecies *paratuberculosis*
Mas	*Mycobacterium avium* subspecies *silvaticum*
miRNA	micro ácido ribonucléico/ micro Ribonucleic acid
MP	Mercaptopurina
MTC	Complejo *Mycobacterium tuberculosis*/ *Mycobacterium tuberculosis* complex
NLR	Receptores tipo NOD/ NOD-Like Receptors
NaOH	Hidroxido sódico/ Sodium hydroxide
NOD2	Dominio de oligomerización por unión de nucleótidos que contiene la proteína 2/ Nucleotide Binding Oligomerization Domain 2
Nramp 1	Proteina1 de macrófago asociado a resistencia natural/ Natural resistance associated macrophage protein 1,
OADC	Oleico Albúmina Dextrosa Catalasa

PAMP	Patrones moleculares asociados a patógenos/ Pathogen-Associated Molecular Patterns
PCR	Reacción en cadena de la polimerasa / Polymerase Chain Reaction
PCR-REA	Reacción en cadena de la polimerasa- análisis con endonucleasas de restricción/ Polymerase Chain Reaction - Restriction Endonuclease Analysis
PFGE	Electroforesis en gel de campo pulsado/ Pulsed-Field Gel Electrophoresis
PPD	Derivado proteico purificado/ Purified Protein Derivative
PPA-3	Antígeno protoplasmatico-3/ Protoplasmic Antigen-3
PRR	Receptores de reconocimiento de patrón/ Pathogen Recognition Receptor
qPCR	Reacción en cadena de la polimerasa cuantitativa/ quantitative Polymerase Chain Reaction
RFLP	Polimorfismos en la longitud de los fragmentos de restricción/ Restriction Fragment Length Polymorphism
SC	Subcutáneo
SCID	Inmunodeficiencia combinada severa/ Severe Combined Immunodeficiency
SNPs	Polimorfismos de un solo nucleótido/ Single Nucleotide Polymorphisms
TB	Tuberculosis
T_{reg}	Células T reguladoras/ Regulatory T cells
Tc	Células T citotóxicas/ Cytotoxic T cells
Th	Células T colaboradoras/ T helper cells
TLR	Receptores de tipo Toll/ Toll Like Receptors
TNF-α	Factor de necrosis tumoral alfa/ Tumor necrosis factor alpha
Tr1	Células T reguladoras tipo 1/ Type 1 regulatory T cells
TGF-β	Factor de crecimiento transformante beta/ Transforming growth factor beta
UFC	Unidad formadora de colonias
ZN	Ziehl-Neelsen
VIH	Virus de la inmunodeficiencia humana

I. ANTECEDENTES Y OBJETIVOS

La paratuberculosis o enfermedad de Johne es una enfermedad infecciosa producida por *Mycobacterium avium* subsp *paratuberculosis (Map)* que afecta principalmente a rumiantes, tanto domésticos como salvajes (Nielsen y Toft, 2009), aunque la infección por *Map* también se ha descrito en numerosas especies animales monogástricas (Beard *et al.*, 2001a) entre ellas, el conejo (Angus, 1990; Beard *et al.*, 2001b; Fuentes y Cebrian, 1988; Greig *et al.*, 1997, 1999; Maio *et al.*, 2011). *Map* produce una enteritis y linfadenitis granulomatosa de curso crónico que ocasiona adelgazamiento progresivo, diarrea, y finalmente la muerte de los individuos afectados. La principal vía de transmisión del agente etiológico es fecal-oral, al ingerir los animales, micobacterias eliminadas en heces por individuos infectados, las cuales pueden contaminar el agua, leche, calostro o cualquier otro alimento (Sweeney, 1996).

La paratuberculosis se encuentra distribuida ampliamente por todo el mundo. Su importancia radica en las grandes pérdidas económicas que ocasiona en los rebaños afectados, tanto por la eliminación o muerte de animales (Arrazuria *et al.*, 2014; Johnson-Ifearulundu *et al.*, 1999; Ott *et al.*, 1999) como por el descenso en la producción (Garcia-Ispierto and López-Gatius, 2016; Groenendaal *et al.*, 2002; Ott *et al.*, 1999; Stabel, 1998). Estas pérdidas a menudo resultan muy difíciles de cuantificar, debido a la existencia de un gran número de animales infectados de forma subclínica y que por tanto, pueden pasar desapercibidos. Además, la paratuberculosis es motivo de creciente preocupación por su posible implicación en la salud pública, al existir cada vez más indicios de que *Map* pudiera estar relacionado con la enfermedad de Crohn en la especie humana, debido a que esta patología presenta similitudes clínico-patológicas con la paratuberculosis y *Map* ha sido aislado de tejidos (Bull *et al.*, 2003; Mendoza *et al.*, 2010), sangre (Naser *et al.*, 2004) y heces (Sohal *et al.*, 2008) de pacientes con dicha enfermedad. Por estas razones, en los sistemas productivos más avanzados, se ha hecho indispensable la implantación de programas de control de la paratuberculosis de diferente índole, especialmente teniendo en cuenta las consecuencias que pudiera tener *Map* en el ámbito de la seguridad alimentaria.

Se ha demostrado la presencia de *Map* en diversos productos ganaderos que forman parte de la cadena alimentaria, como son leche y derivados lácteos (Ellingson *et al.*, 2005; Faria *et al.*, 2014; Ikonomopoulos *et al.*, 2005; O'Reilly *et al.*, 2004), y la canal de ganado vacuno (Alonso-Hearn *et al.*, 2009; Meadus *et al.*, 2008) lo que pone de

manifiesto que los humanos pueden entrar en contacto con *Map* mediante el consumo de estos productos crudos o poco cocinados. Hay que tener en cuenta que además de los rumiantes domésticos existen otros animales susceptibles a la infección por *Map*, que también pueden ser consumidos por los humanos.

Los conejos silvestres han demostrado ser susceptibles a *Map* (Angus, 1990; Fuentes y Cebrian, 1988; Greig *et al.*, 1997; Maio *et al.*, 2011) y a otras micobacterias en condiciones naturales (Gill and Jackson, 1993; Kelleher, 2008; Ludwig *et al.*, 2009). Sin embargo, a pesar de que otros estudios han descrito la presencia de diferentes micobacterias en bovino (Alonso-Hearn *et al.*, 2009; Klanicova *et al.*, 2011), porcino (van Ingen *et al.*, 2010; Muwonge *et al.*, 2012), corderos y pollos (Klanicova *et al.*, 2011) de matadero, no se ha realizado ningún estudio en conejos de producción destinados a consumo humano. España es uno de los principales países productores de carne de conejo de la Unión Europea, con 64.300 toneladas anuales y además el consumo de carne de conejo se ha visto aumentado en un 6,3% en el año 2013 (MAGRAMA, 2015). Teniendo en cuenta el impacto de esta industria en nuestro país **el primer objetivo de la presente Tesis Doctoral ha sido el estudio de la posible presencia e identificación de micobacterias en el tracto gastrointestinal y tejido linfoide asociado, de conejos producidos para el consumo humano.**

La paratuberculosis es una enfermedad compleja, y aunque se ha dedicado mucho esfuerzo a su estudio, la patogenia se conoce muy escasamente. Esto es debido, entre otras causas, al amplio periodo de incubación de la propia enfermedad y a la falta de un modelo animal de laboratorio que permita reproducir experimentalmente todos los síntomas clínicos en menor tiempo y con un menor coste económico del que supone el uso de los rumiantes domésticos que son los hospedadores naturales.

Los modelos animales de laboratorio más estudiados han sido los ratones y los conejos. A pesar de que el modelo murino ha sido el más utilizado, el conejo es un modelo animal atractivo debido a su susceptibilidad a la infección por *Map* en condiciones naturales (Angus, 1990; Beard *et al.*, 2001b; Fuentes y Cebrian, 1988; Greig *et al.*, 1997, 1999; Maio *et al.*, 2011), y, contrariamente a lo que ocurre en ratones, permite observar lesiones histopatológicas producidas por la infección a nivel digestivo tras la infección por vía oral (Mokresh y Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*,

2005). Además, el conejo reproduce algunos de los síntomas clínicos de la paratuberculosis en rumiantes como son la diarrea (Mokresh y Butler, 1990; Mokresh *et al.*, 1989) aunque para observar sintomatología clínica evidente se requieren de 5 a 20 meses (Mokresh y Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005). No obstante, se ha sugerido que factores estresantes como la inmunosupresión o los cambios en la dieta podrían acelerar la aparición de la sintomatología clínica (Vaughan *et al.*, 2005). De hecho, la dieta tiene un papel relevante en la inmunidad habiéndose demostrado en ratones que pequeños cambios en la dieta pueden modular las enfermedades inmunomediadas (Ooi *et al.*, 2014). **Por ello, el segundo objetivo de este estudio ha sido estudiar el efecto de cambios en la dieta en la infección experimental con *Map* en conejo y ver si estos cambios aceleran la progresión de la enfermedad**.

Por otra parte, la dieta puede modificar la microbiota intestinal (Graf *et al.*, 2015; Xu and Knight, 2014), debido a que los nutrientes ingeridos son la base alimentaria de los microrganismos que residen en el sistema digestivo. Actualmente se desconoce el papel de la microbiota intestinal en el establecimiento y progresión de la infección paratuberculosa, aunque se ha demostrado que animales infectados experimentalmente muestran patrones bacterianos diferentes de los no infectados (Derakhshani *et al.*, 2014). De esta manera, la dieta podría inducir un cambio en la microbiota con efecto en la modulación de la infección por *Map*. Así, **el tercer objetivo de la presente Tesis Doctoral comprende el estudio de la microbiota intestinal en conejos infectados experimentalmente con *Map* alimentados con diferentes dietas.**

Actualmente, la herramienta más eficaz para el control de la paratuberculosis es la vacunación. Son numerosos los estudios en los que se describe una reducción en la sintomatología asociada a la enfermedad en rebaños donde se procede a la vacunación (Alonso-Hearn *et al.*, 2012; Bastida y Juste, 2011; Griffin *et al.*, 2009; Harris y Barletta, 2001; Santema *et al.*, 2013; Singh *et al.*, 2013a). Además, se ha observado una disminución en la excreción de *Map* a través de las heces (Alonso-Hearn *et al.*, 2012; Dhand *et al.*, 2013; Eppleston *et al.*, 2011; Griffin *et al.*, 2009; Juste *et al.*, 2009a; Knust *et al.*, 2013; Reddacliff *et al.*, 2006; Singh *et al.*, 2007, 2013a; Tewari *et al.*, 2014; Toribio y Sergeant, 2007; Windsor *et al.*, 2014), una disminución en la colonización de tejidos intestinales tras su exposición a *Map* (Alonso-Hearn *et al.*, 2012; Hines *et al.*,

2007; Juste *et al.*, 1994; Kathaperumal *et al.*, 2008, 2009; Sweeney *et al.*, 2009) y una disminución de la frecuencia de presentación de lesiones histopatológicas graves (Corpa *et al.*, 2000a; Reddacliff *et al.*, 2006).

Las vacunas basadas en células enteras han demostrado que incrementan tanto la inmunidad humoral como la celular en bovino (Kohler *et al.*, 2001; María Muñoz de Frutos, 2014; Muskens *et al.*, 2002; Stabel *et al.*, 2011), ovino (Begg and Griffin, 2005; Corpa *et al.*, 2000a) y caprino (Hines *et al.*, 2014). En consecuencia, la vacunación se ha asociado con una reducción de la mortalidad (Corpa *et al.*, 2000b; Eppleston *et al.*, 2004; Reddacliff *et al.*, 2006) y se ha utilizado como herramienta en el control de la enfermedad a nivel de rebaño.

Generalmente la vacunación se aconseja en animales jóvenes, basándose en el hecho de que de esta manera los animales desarrollarán una respuesta inmune protectora al entrar en contacto por primera vez con la micobacteria (Bastida y Juste, 2011; Larsen *et al.*, 1964; Saxegaard y Fodstad, 1985). Sin embargo, en diversos estudios se ha visto que los efectos beneficiosos pueden observarse también vacunando a animales adultos (Alonso-Hearn *et al.*, 2012; Corpa *et al.*, 2000b; Santema *et al.*, 2013; Singh *et al.*, 2010, 2013), por lo que se sospecha un efecto terapéutico que no ha sido posible confirmar en modelos observacionales y que tendría un enorme potencial para el tratamiento de las distintas formas de enfermedad inflamatoria intestinal y, en particular, de las humanas. Por todo ello, el **cuarto objetivo** ha sido **estudiar el efecto de la vacunación antes y después de la infección experimental con *Map* en el modelo de conejo.**

I. BACKGROUND AND OBJECTIVES

Paratuberculosis or Johne's disease is an infectious disease caused by *Mycobacterium avium* subsp *paratuberculosis* (*Map*), that mainly affects ruminants, both domestic and wild (Nielsen and Toft, 2009). *Map* infection has also been reported in a wide range of monogastric animal species (Beard *et al.*, 2001a) including rabbits (Angus, 1990; Beard *et al.*, 2001b; Fuentes and Cebrian, 1988; Greig *et al.*, 1997, 1999; Maio *et al.*, 2011). *Map* produces a chronic granulomatous enteritis and lymphadenitis resulting in progressive weight loss, diarrhea, and eventual death of affected animals. The infectious agent is transmitted mainly through the fecal-oral route, by ingestion of mycobacteria shed in the feces of infected animals, which could contaminate water, milk, colostrum or any other food (Sweeney, 1996).

Paratuberculosis is widely spread all over the world. The disease produces a premature removal or death of affected animals (Arrazuría *et al.*, 2014; Johnson-Ifearulundu *et al.*, 1999; Ott *et al.*, 1999) as well as a decline in production (Garcia-Ispierto and López-Gatius, 2016; Groenendaal *et al.*, 2002; Ott *et al.*, 1999; Stabel, 1998) resulting in a large economic impact. The economic losses are often difficult to quantify due to the existence of a huge number of subclinically infected animals, which may be overlooked. Moreover, paratuberculosis is becoming a concern because of its possible implication in public health. There is increasing evidence that *Map* could be related to Crohn's disease in humans due to the clinical and pathological similarities between both diseases and because *Map* has been isolated from tissues (Bull *et al.*, 2003; Mendoza *et al.*, 2010), blood (Naser *et al.*, 2004) and feces (Sohal *et al.*, 2008) of Crohn disease patients. For these reasons, the implementation of different types of paratuberculosis control programs has become essential, especially considering the possible implications of *Map* in the field of food security.

Map presence has been demonstrated in livestock products that are part of the food chain, such as milk, dairy products (Ellingson *et al.*, 2005; Faria *et al.*, 2014; Ikonomopoulos *et al.*, 2005; O'Reilly *et al.*, 2004) and bovine carcasses (Alonso-Hearn *et al.*, 2009; Meadus *et al.*, 2008). This would mean that humans could be exposed to *Map* by consuming these products raw or undercooked. It should be taken into account that besides ruminants, other non-ruminant species are susceptible to *Map* infection and they can also be consumed by humans.

It has been proved that wild rabbits are susceptible to infection by *Map* (Angus, 1990; Greig *et al.*, 1997; Maio *et al.*, 2011) and other mycobacteria (Gill and Jackson, 1993; Kelleher, 2008; Ludwig *et al.*, 2009) under natural conditions. Moreover, although several studies have reported the presence of different mycobacteria in cattle (Alonso-Hearn *et al.*, 2009; Klanicova *et al.*, 2011), pigs (van Ingen *et al.*, 2010; Muwonge *et al.*, 2012), lambs and chickens (Klanicova *et al.*, 2011) at the slaughterhouse, there are no previous studies in rabbits produced for human consumption. Spain is one of the leading producer countries of rabbit meat in the European Union, producing 64,300 tons per year. In addition, in Spain the consumption of rabbit meat has increased by 6.3% in 2013 (MAGRAMA, 2015). Given the impact of this industry in our country, **the first objective of this Doctoral Thesis has been to study the possible presence of mycobacteria in the gastrointestinal tract and associated lymphoid tissue of rabbits produced for human consumption.**

Paratuberculosis is a complex disease and although much effort has been devoted to its study, pathogenesis is not well understood. This is due, among other reasons, to the long incubation period of the disease and the lack of an appropriate laboratory animal model, which allows reproducing clinical symptomatology in less time and at a lower economic cost than using the natural hosts, the domestic ruminants.

Mice and rabbits have been the most studied laboratory animal models. Although the murine model has been the one most frequently used, the rabbit model is attractive because of its susceptibility to *Map* infection under natural conditions (Angus, 1990; Beard *et al.*, 2001b; Fuentes and Cebrian, 1988; Greig *et al.*, 1997, 1999; Maio *et al.*, 2011). In addition, contrary to what happens in mice, rabbits have shown histopathological lesions at the gastrointestinal level after oral challenge with *Map* (Mokresh and Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005). Furthermore, rabbits reproduce some of the paratuberculosis clinical symptoms described in ruminants such as diarrhea (Mokresh and Butler, 1990; Mokresh *et al.*, 1989). However, 5 to 20 months are required to observe evident clinical symptomatology (Mokresh and Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005) and it has been suggested that stressors as immunosuppression or dietary changes could accelerate the onset of clinical symptomatology (Vaughan *et al.*, 2005). Actually, diet plays an important role in immunity, and, in mice, for instance, it has been shown that small changes in diet can

modulate immune-mediated diseases (Ooi *et al.*, 2014). **Therefore, the second objective of this work was to study the effect of dietary changes in experimental *Map* infection in rabbits and see if these changes accelerate disease progression.**

Diet has also the capability to modify the intestinal microbiota (Graf *et al.*, 2015; Xu and Knight, 2014), because the consumed food is the main nutrient source for the digestive system microbiota. Currently, the role of intestinal microbiota in the establishment and progression of *Map* infection is unknown, although it has been reported that experimentally infected calves showed different bacterial population patterns than uninfected ones (Derakhshani *et al.*, 2014). Thereby, diet could induce changes in the microbiota resulting in *Map* infection modulation. **For this reason, the third objective of the present Doctoral Thesis focuses on the study of intestinal microbiota in rabbits experimentally infected with *Map* and fed with different diets.**

Currently, vaccination is the most effective tool to control paratuberculosis. Many studies have reported a decrease in clinical symptomatology in vaccinated flocks (Alonso-Hearn *et al.*, 2012; Bastida y Juste, 2011; Griffin *et al.*, 2009; Harris y Barletta, 2001; Santema *et al.*, 2013; Singh *et al.*, 2013a). Moreover, vaccinated animals have shown a decrease of *Map* shedding in feces (Alonso-Hearn *et al.*, 2012; Dhand *et al.*, 2013; Eppleston *et al.*, 2011; Griffin *et al.*, 2009; Juste *et al.*, 2009a; Knust *et al.*, 2013; Reddacliff *et al.*, 2006; Singh *et al.*, 2007, 2013a; Tewari *et al.*, 2014; Toribio y Sergeant, 2007; Windsor *et al.*, 2014), reduction in intestinal tissue colonization after *Map* exposure (Alonso-Hearn *et al.*, 2012; Hines *et al.*, 2007; Juste *et al.*, 1994; Kathaperumal *et al.*, 2008, 2009; Sweeney *et al.*, 2009) and lower frequency of severe histopathological lesions (Corpa *et al.*, 2000a; Reddacliff *et al.*, 2006).

Whole cell vaccines increase both humoral and cellular immunity in cattle (Kohler *et al.*, 2001; María Muñoz de Frutos, 2014; Muskens *et al.*, 2002; Stabel *et al.*, 2011), sheep (Begg and Griffin, 2005; Corpa *et al.*, 2000a) and goats (Hines *et al.*, 2014). Consequently, vaccination has been associated with a reduction in mortality (Corpa *et al.*, 2000b; Eppleston *et al.*, 2004; Reddacliff *et al.*, 2006) and it has been used at herd level to control the disease.

Usually, vaccination is recommended in young animals, based on the fact that in this way animals will develop a protective immune response by the time they get in contact with the mycobacteria (Bastida and Juste, 2011; Larsen *et al.*, 1964; Saxegaard and Fodstad, 1985). However, several studies have shown that the beneficial effects can be observed also vaccinating adult animals (Alonso-Hearn *et al.*, 2012; Corpa *et al.*, 2000b; Santema *et al.*, 2013; Singh *et al.*, 2010, 2013). Therefore, it is suspected that vaccination may provide a therapeutic effect that has not been possible to confirm in observational models. This finding could have a tremendous potential for the treatment of the different forms of inflammatory bowel disease and particularly the ones which affect human's health. Accordingly, **the fourth objective has been to study the effect of vaccination before and after experimental infection with *Map* in the rabbit model.**

II. REVISIÓN BIBLIOGRÁFICA / LITERATURE REVIEW

II.1 PARATUBERCULOSIS

II.1.1 ETIOLOGÍA

II.1.1.1 Taxonomía

Mycobacterium avium subespecies *paratuberculosis* (*Map*), es el agente causal de la paratuberculosis también llamada enfermedad de Johne (Thorel *et al.*, 1990). La primera descripción de esta enfermedad la realizaron Johne y Frothingam en 1895 al observar bacilos ácido-alcohol resistentes (BAAR) en muestras de tejido intestinal de una vaca de la raza Frisona con sintomatología clínica de la enfermedad. *Map* es un bacilo del género *Mycobacterium*, único género de la familia Mycobacteriaceae perteneciente al suborden Corynebacterinae, orden Actinomycetales, subclase Actinobacteridae, clase Actinobacteria, división Firmicutes y superreino Bacteria (Stackebrandt *et al.*, 1997).

En base a las características filogenéticas, dentro del género *Mycobacterium*, destacan dos grandes grupos en los que se encuentran las principales micobacterias patógenas para humanos y animales. El árbol filogenético de las especies más relevantes de micobacterias se muestra en la Figura 1. El complejo *Mycobacterium tuberculosis* (MTC) incluye a las micobacterias causantes de tuberculosis en mamíferos. Dentro de este grupo se encuentra *Mycobacterium tuberculosis* y *Mycobacterium bovis* que son las principales especies causantes de tuberculosis en humanos y animales. A nivel genético, los miembros del MTC presentan más de un 99,95% de identidad en su secuencia de nucleótidos (Brosch *et al.*, 2002).

El complejo *Mycobacterium avium* (MAC), está formado por bacterias no tuberculosas que comparten la misma secuencia génica del ARNr 16S por lo que se requieren otras dianas genéticas como la región ITS (*Internal Transcribed Spacer*) para su clasificación (Turenne y Alexander, 2010). *Map* se encuentra dentro de este complejo, donde destacan otras subespecies como *Mycobacterium avium* subspecies *avium* (*Maa*), *Mycobacterium avium* subspecies *hominisuis* (*Mah*) y *Mycobacterium avium* subspecies *silvaticum* (*Mas*). La clasificación taxonómica de esta última subespecie (*Mas*) se ha cuestionado recientemente, sugiriendo que se trata de un tipo de cepa de *Maa* (Turenne *et al.*, 2007). Dentro de este complejo se incluyen micobacterias ambientales, otras intrínsecamente patógenas del ganado y de las aves, y subespecies que actúan como patógenos oportunistas del hombre (Biet *et al.*, 2005; Glawischnig *et al.*, 2006; Rindi y Garzelli, 2014; Turenne y Alexander, 2010). Por ello, este complejo adquiere gran

relevancia en medicina veterinaria (Thorel *et al.*, 2001) y humana (Primm *et al.*, 2004; Weiss and Glassroth, 2012; Whiley *et al.*, 2012; Kim *et al.*, 2013).

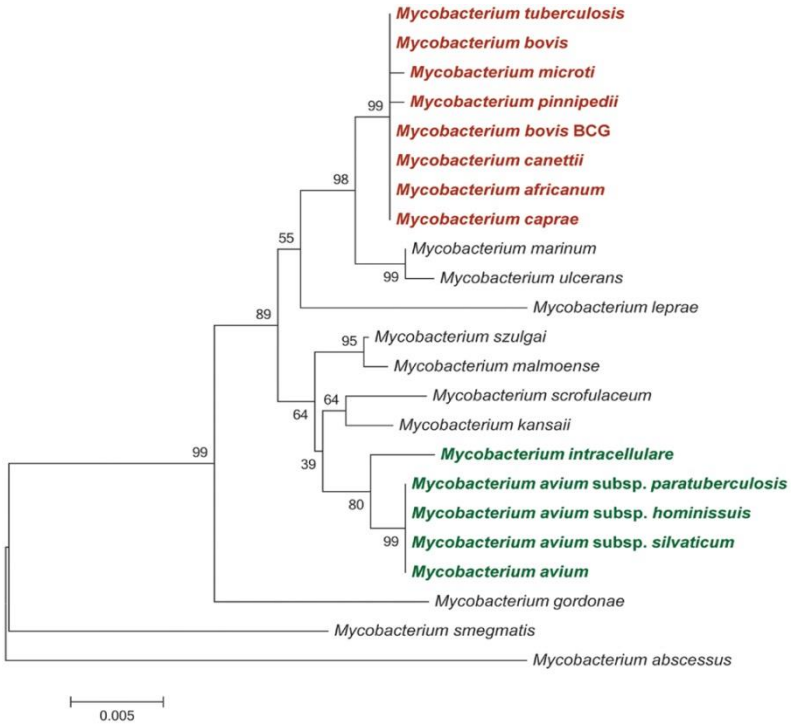

Figura 1. Árbol filogenético de diversas especies y subespecies de micobacterias. Las especies pertenecientes al complejo *Mycobacterium tuberculosis* se muestran en rojo y los miembros del complejo *Mycobacterium avium* se muestran en verde. Fuente: Rue-Albrecht *et al.*, 2014.

Los avances en biología molecular han contribuido al desarrollo de técnicas de tipificación, lo que ha permitido la identificación de diversas cepas de *Map.* Algunas de las técnicas más empleadas en este área han sido: el polimorfismo de la longitud de fragmentos de restricción (*Restriction Fragment Length Polymorphism*, RFLP) (Collins *et al.*, 1990; Pavlik *et al.*, 2000; Pavlík *et al.*, 1995; Saunders *et al.*, 2003), la reacción en cadena de la polimerasa y posterior análisis con endonucleasas de restricción (*Polymerase Chain Reaction - Restriction Endonuclease Analysis*, PCR-REA) (Marsh *et al.*, 1999; Sevilla *et al.*, 2005; Whittington *et al.*, 2000b) y la electroforesis en gel de

campo pulsado (*Pulsed-Field Gel Electrophoresis*, PFGE) (Feizabadi *et al.*, 1997; Sevilla *et al.*, 2008; Stevenson *et al.*, 2002). Gracias al uso de estas técnicas se han identificado los principales genotipos de *Map* denominados como "S" (*Sheep*) para las cepas de tipo ovino, y "C" (*Cattle*) para las de tipo bovino (Collins *et al.*, 1990; Whittington *et al.*, 2000b), debido a su aparente predominancia en esas especies, así como el "B" (*Bison*), aislado de bisontes y cabras (Sevilla *et al.*, 2007; Whittington *et al.*, 2001b). Además, se ha descrito otro tipo de cepa denominado "I" (intermedio) con patrones de hibridación intermedios entre los de las cepas C y S (Collins *et al.*, 1990). Mediante la restricción enzimática también se han definido los grupos o cepas "tipo I", comparable al tipo ovino o S, el "tipo II", semejante al tipo bovino o C (Stevenson *et al.*, 2002) y el "tipo III" (de Juan *et al.*, 2005), que sería comparable a las cepas intermedias descritas por Collins *et al.*, (1990).

II.1.1.2 Características morfológicas y bacteriológicas

Map es un microorganismo de forma cocobacilar de 1-2 x 0,5 micras de tamaño, que a pesar de ser teñido débilmente con la tinción Gram se considera como Gram-positivo (Smith, 1969). Al igual que otras micobacterias, la pared celular posee características únicas y distintivas del resto de bacterias. Posee un elevado porcentaje en lípidos (30-60% de la masa celular total) en comparación con otras bacterias (5% en bacterias Gram-positivas y 10% en Gram-negativas) (Goren and Brennan, 1979, Crellin *et al.*,2013). Esta característica, explica la tendencia a producir un crecimiento en forma de grumos o agregados (Thorel *et al.*, 1990), siendo además la disposición que adoptan las micobacterias al replicarse dentro de los macrófagos que infectan.

La pared celular micobacteriana es la responsable de las principales propiedades estructurales y antigénicas (Grange *et al.*, 1990; Guenin-Macé *et al.*, 2009). En la Figura 2 se puede observar una representación esquemática de la pared celular. Está formada internamente por una capa de peptidoglicanos llamada mureína, que es la responsable de dar rigidez, forma y poder adyuvante a la micobacteria. Más externamente, aparece la capa de arabinogalactano, a la que se unen ácidos micólicos como sulfolípidos y dimicolatos de trealosa (*Cord factors*) hacia el exterior, que proporcionan un carácter ceroso que dificulta la penetración y eliminación de colorantes (Wayne y Kubica, 1986). Los ácidos micólicos son los principales responsables de la ácido-alcohol resistencia

(Daffé y Reyrat, 2008), haciendo que el método de tinción Ziehl-Neelsen (ZN) sea uno de los más adecuados para su identificación, aunque se han descrito formas que no son resistentes a la decoloración con un alcohol ácido o que lo son parcialmente (Stavitsky y Beck, 1946). Dentro de la pared celular también se encuentran los lipoarabinomananos (LAM) que son los responsables de la supresión de la activación de los macrófagos y las células T, inhibiendo la respuesta inmune del hospedador (Briken *et al.*, 2004; Chan *et al.*, 1991).

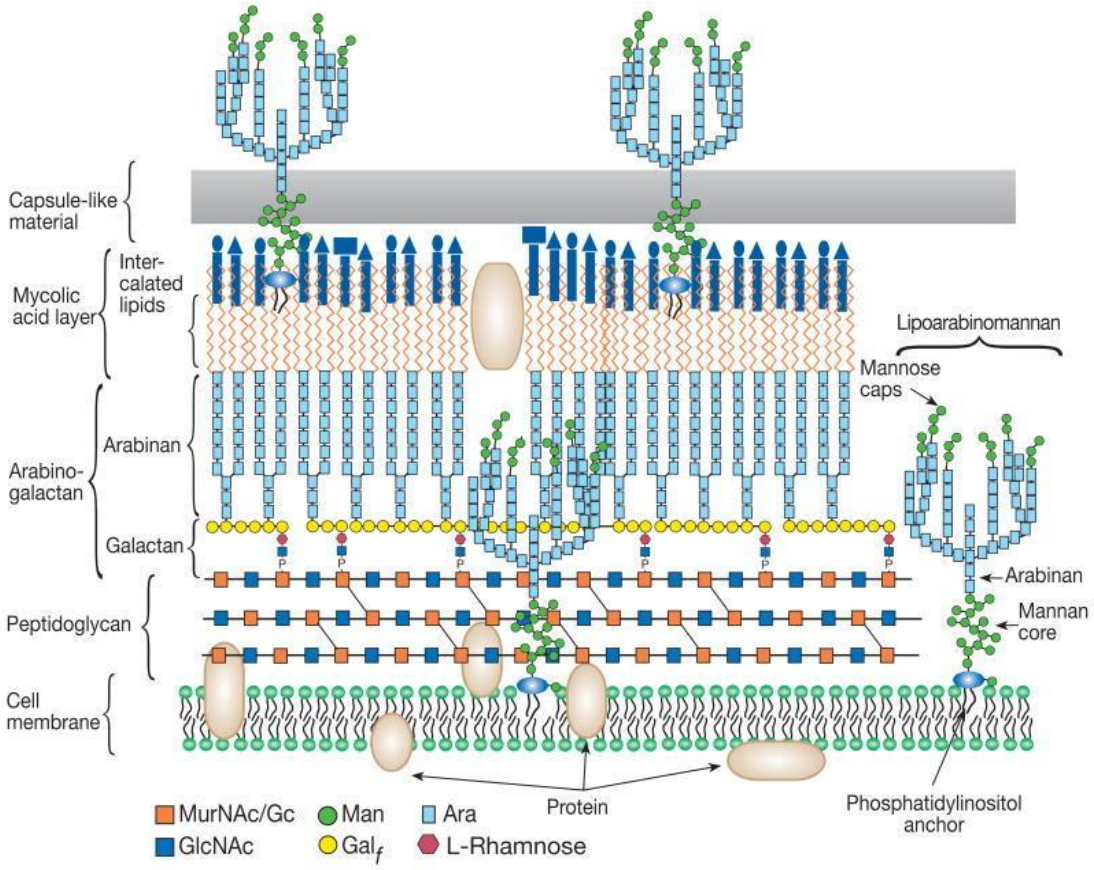

Figura 2. Diagrama de la composición de la pared celular micobacteriana. Fuente: Brennan y Crick, 2007.

Al igual que ocurre con otros géneros microbianos y otras especies de micobacterias, *Map* puede encontrarse en fases de protoplasto, esferoplasto o fase transicional (Beran *et al.*, 2006). Estas fases se caracterizan por la ausencia parcial o total de la pared celular. Hay que destacar que las formas defectivas de pared celular son relevantes, ya que se han encontrado asociadas a enfermedad clínica en rumiantes (El-Zaatari *et al.*, 2003) sin poder ser detectadas con la tinción de ZN, ni mediante cultivo. Además, en los pacientes de Crohn se ha observado la presencia de *Map* en fase de esferoplasto (Chiodini *et al.*, 1986; Wall *et al.*, 1993; Mendoza *et al.*, 2010).

En cuanto a sus necesidades de oxígeno, *Map* es un microorganismo aerobio o microaerobio (Chiodini *et al.*, 1984) cuya temperatura óptima de crecimiento es de 37 ºC, aunque puede crecer entre 30 ºC y 45 ºC (Lévy-Frébault y Portaels, 1992; Wayne y Kubica, 1986). Respecto a su velocidad de crecimiento, *Map* está clasificada dentro del grupo de micobacterias de crecimiento lento (Grange *et al.*, 1990), pudiendo requerir de 2 a 6 semanas para la obtención de un aislamiento primario y mostrando un tiempo de generación medio de 36 horas (Elguezabal *et al.*, 2011). Esta lenta velocidad de crecimiento, junto con el requerimiento de micobactina para su crecimiento son características muy útiles para su identificación (Chiodini *et al.*, 1984; Thorel *et al.*, 1990).

La micobactina es un agente quelante del hierro que producen las micobacterias en condiciones limitantes, para poder transportar este metal al interior de la célula. Está demostrado que *Map* puede crecer perfectamente sin aporte exógeno de micobactina si el medio contiene suficiente hierro y otros nutrientes disponibles (Juste *et al.*, 1993; Merkal y Curran, 1974). Por lo tanto, la incapacidad de crecer sin micobactina añadida que muestran algunas micobacterias (*Map*, ciertas cepas de *M. avium* y algunas especies no patógenas), no residiría en la imposibilidad real de ser sintetizada, sino en la represión de su producción, o en la presencia de sustancias quelantes en el medio (Merkal y Curran, 1974). Recientemente se ha observado que el gen responsable de la síntesis de la micobactina (mbtA) es más corto en *Map* que en *M. tuberculosis*, sugiriendo que esta podría ser la razón por la cual la producción de micobactina está atenuada en *Map* (Li *et al.*, 2005). En todo caso, existen evidencias de que *Map* sería capaz de bloquear los mecanismos de circulación del hierro a través de la inhibición de la síntesis de ferroportina en los macrófagos infectados (Landeros *et al.*, 2016), lo que

sugiere una adaptación muy sofisticada a un nicho de vida intracelular en la que dichos quelantes ya no serían necesarios.

II.1.1.3 Persistencia en el medio

A pesar de que *Map* se considera un patógeno intracelular obligado, presenta una gran resistencia y puede permanecer viable por largos periodos de tiempo en condiciones adversas (Lovell *et al.*, 1944) como la desecación, fluctuación de temperaturas, condiciones acidas o alcalinas, desinfectantes, e incluso es resistente a muchos antibióticos (Krishnan *et al.*, 2009a). Esto se debe en parte a la gruesa capa cérea que le rodea, y a la posibilidad de ralentizar su metabolismo en determinadas condiciones (Merkal y Curran, 1974).

Así, su viabilidad ha sido estimada en 163 días en ríos (Lovell *et al.*, 1944), 9 meses en aguas cenagosas (Whittington *et al.*, 2005), 12 meses en heces bovinas y pastos (Whittington *et al.*, 2004), pero únicamente 7 días en orina (Chiodini *et al.*, 1984). Debido a que la orina tiene un efecto bactericida (Larsen, 1956), se ha sugerido que mezclada con las heces, que tienen un efecto bacteriostático, es menos probable que permanezca viable por largos periodos de tiempo que si se encuentran en las heces sin mezclar (Chiodini *et al.*, 1984). Hay que tener en cuenta también, que si se utiliza abono contaminado con *Map* en los pastos, es posible que la micobacteria pueda sobrevivir al proceso de ensilado, debido a su gran resistencia a niveles bajos de pH y a la competición microbiana (Cook *et al.*, 2013). Además, la detección de la presencia de *Map* viable en el polvo de instalaciones ganaderas sugiere que esta bacteria tiene la capacidad de sobrevivir en el ambiente durante algún tiempo (Eisenberg *et al.*, 2010).

Se ha descrito que *Map* puede mantenerse a -14ºC durante al menos un año (Richards y Thoen, 1977). Sin embargo, recientemente se ha demostrado que tras almacenar durante una semana a -20ºC muestras de heces procedentes de 11 vacas con paratuberculosis, la viabilidad de *Map* en dichas muestras se vio reducida (Khare *et al.*, 2008). *Map* es sensible a la luz solar (Merkal y Whipple, 1982), pero puede permanecer al menos 55 semanas en ambientes secos y sombríos (Whittington *et al.*, 2004). Se trata de un microorganismo sensible a las altas temperaturas y a la elevada concentración de calcio (Whittington *et al.*, 2004, 2005). Sin embargo, se ha estimado que entre un 3% y 5% de

las micobacterias podrían sobrevivir a la pasteurización (Chiodini y Hermon-Taylor, 1993; Grant *et al.*, 2002; Rowan *et al.*, 2001) y a los tratamientos de esterilización aplicados al agua para consumo humano (Whan *et al.*, 2001). No obstante, la combinación de temperatura y la aplicación de campos eléctricos pulsátiles ha demostrado ser una buena estrategia para eliminar las micobacterias (Rowan *et al.*, 2001).

II.1.2. EPIDEMIOLOGÍA

II.1.2.1 Hospedadores susceptibles

A pesar de que la paratuberculosis afecta fundamentalmente a rumiantes domésticos, los rumiantes silvestres también pueden verse afectados, asumiéndose que puede afectar a todos los géneros incluidos en el suborden taxonómico "Ruminantia" además de a los camélidos (Manning, 2011). Tradicionalmente la paratuberculosis se ha estudiado en ovino, bovino y caprino, sin embargo debido a la dificultad de control de la enfermedad, se ha señalado que los rumiantes silvestres pueden jugar un papel importante en la epidemiologia de la enfermedad. Así, *Map* puede encontrarse en animales como ciervo (Mackintosh *et al.*, 2002; Pavlik *et al.*, 2000), gamo (Balseiro *et al.*, 2008; Pavlik *et al.*, 2000), corzo (Pavlik *et al.*, 2000), antílope (Dukes *et al.*, 1992), muflón (Weber *et al.*, 1992), llama (Miller *et al.*, 2000), alpaca (Miller *et al.*, 2000; Ridge *et al.*, 1995), bisonte (Buergelt *et al.*, 2000), búfalo (Sivakumar *et al.*, 2005) o camello (Kramsky, 2000).

La infección natural también se ha descrito en numerosas especies monogástricas, como cerdos (Miranda *et al.*, 2011), jabalíes (Alvarez *et al.*, 2005; Zanetti *et al.*, 2008), caballos (Cline *et al.*, 1991; Larsen *et al.*, 1972), burros (Dierckins *et al.*, 1990), comadrejas (Beard *et al.*, 2001a), tejones (Beard *et al.*, 2001a), lobos (Beard *et al.*, 2001a), coyotes (Anderson *et al.*, 2007), zorros (Beard *et al.*, 2001a), osos pardos (Kopecna *et al.*, 2006), mandriles (Zwick *et al.*, 2002), diferentes especies de aves, algunos tipos de roedores (Beard *et al.*, 2001a; Daniels *et al.*, 2003), liebres (Salgado *et al.*, 2011) y conejos (Beard *et al.*, 2001b; Fuentes y Cebrian, 1988; Greig *et al.*, 1997; Judge *et al.*, 2006; Maio *et al.*, 2011; Raizman *et al.*, 2005).

De las especies monogástricas, el conejo es el que mayor importancia epidemiológica podría tener o, al menos, la que más atención ha atraído por su amplia difusión compartiendo pastos con las especies primarias. Se ha descrito que los conejos pueden eliminar hasta $4x10^6$ UFC (unidades formadoras de colonias) por gramo de heces (Daniels *et al.*, 2001) y además se ha comprobado que micobacterias aisladas en estos animales son capaces de reproducir experimentalmente la enfermedad en bovinos (Beard *et al.*, 2001c). Por tanto, el papel de los conejos como reservorios de *Map* en regiones donde se dé el contacto entre las heces de conejos y bovinos en el pasto podría limitar la eficacia de las estrategias de control de la enfermedad (Shaughnessy *et al.*, 2013). Hay que tener en cuenta que en los pastos compartidos entre animales infectados y conejos la transmisión de *Map* se puede dar en la otra dirección, aumentando la prevalencia de la enfermedad entre los conejos. Los conejos son presas de otros animales carnívoros como los zorros y la depredación podría tener un gran impacto en la dispersión espacial de la enfermedad, ya que estos animales se mueven en áreas de hasta 4.000 hectáreas (Daniels *et al.*, 2001).

En un estudio a gran escala realizado en colaboración entre diversos grupos europeos, se identificaron mediante diversas técnicas moleculares los mismos genotipos de *Map* en hospedadores silvestres y domésticos que convivían en el mismo hábitat, lo que refuerza la teoría de transmisión interespecie (Stevenson *et al.*, 2009). Sin embargo, otros hallazgos cuestionan la transmisión de *Map* entre animales silvestres y domésticos. Recientemente, algunos autores no detectaron una asociación positiva entre la seropositividad en cérvidos y la exposición de éstos al ganado vacuno de carne, a pesar de detectar infecciones por *Map* en ambas especies (Pruvot *et al.*, 2014). A nivel de genética molecular, otros autores han descrito genotipos exclusivos de infecciones en el ganado vacuno no identificados en animales silvestres (Fritsch *et al.*, 2012; Whittington *et al.*, 2001b).

El impacto de la paratuberculosis en animales silvestres se ha revisado en profundidad y tras analizar las publicaciones en las que se documentaban infecciones en especies silvestres en cautividad y de vida libre se ha sugerido que la paratuberculosis presenta un considerable impacto en especies cinegéticas y no tanto así en las especies de vida libre (Carta *et al.*, 2013).

II.1.2.2 Transmisión

La principal vía de infección por *Map* es la fecal-oral (Clarke, 1997; Stehman, 1996; Sweeney, 1996), a través de la ingestión de bacilos en el agua, leche, calostro o cualquier alimento contaminado por las heces de los animales infectados (Dieguez *et al.*, 2008; Kudahl *et al.*, 2008; Lombard, 2011; Tiwari *et al.*, 2009). La infección tiene lugar predominantemente en animales lactantes, en sus primeros días de vida, cuando maman de las ubres manchadas por las heces infectadas de su propia madre o de otras (Barkema *et al.*, 2009; Chiodini *et al.*, 1984; Clarke, 1997). Sin embargo, teniendo en cuenta la posible existencia de animales "supereliminadores", que pueden llegar a excretar más de 10^4 UFCs de *Map* por gramo de heces (Whitlock *et al.*, 2006), cualquier terreno, pasto o suelo contaminado con *Map* se puede convertir en posible fuente de transmisión del bacilo.

Los animales también se pueden infectar por la ingestión de calostro o leche no contaminada con heces, debido a que se ha descrito la excreción de *Map* por esta vía (Sweeney *et al.*, 1992a; Stabel *et al.*, 2014) incluso en la fase subclínica de la enfermedad (Streeter *et al.*, 1995). A pesar de que la transmisión vertical es la principal vía de infección en los animales jóvenes, también se puede dar la transmisión horizontal, la cual podría estar vinculada a fómites (Marcé *et al.*, 2010; van Roermund *et al.*, 2007). Además, el hecho de que se haya aislado *Map* en el agua estancada, en sedimentos (Whittington *et al.*, 2005) y en polvo de explotaciones ganaderas (Eisenberg *et al.*, 2010), ha hecho que esta vía de transmisión cobre mayor relevancia.

Otra vía de transmisión de la infección es la vertical uterina. Se estima que aproximadamente el 10% de vacas en la etapa subclínica transmiten la infección a la descendencia, porcentaje que asciende al 50% en el caso de que éstas presenten enfermedad clínica (Kennedy y Benedictus, 2001; Sweeney, 1996). Sin embargo, aunque parece que esta vía de transmisión no juega un papel muy importante en los rumiantes domésticos (Adaska y Whitlock, 2012; Lambeth *et al.*, 2004; Sweeney *et al.*, 1992b), sí parece tener un papel epidemiológico significativo en los cérvidos de vida silvestre (van Kooten *et al.*, 2006).

Finalmente, otras posibles vías de transmisión estudiadas recientemente, con menor impacto epidemiológico son: los aerosoles (Corner *et al.*, 2004; Eisenberg *et al.*, 2011), vectores como insectos (Fischer *et al.*, 2001, 2003, 2004a, 2004b, 2005), nematodos (Lloyd *et al.*, 2001; Whittington *et al.*, 2001a) y amebas de vida libre (Mura *et al.*, 2006), la saliva (Sorge *et al.*, 2013) y la depredación de presas infectadas, la cual podría tener un papel más relevante entre los animales silvestres (Beard *et al.*, 2001a; Machackova *et al.*, 2004; Daniels *et al.*, 2001).

II.1.2.3 Distribución y prevalencia

La paratuberculosis está ampliamente distribuida por todo el mundo. No obstante, su prevalencia en los rumiantes domésticos varía entre países o regiones.

Hay que tener en cuenta que estimar la prevalencia real de la enfermedad plantea serias dificultades, debido a la falta de sensibilidad en las técnicas de diagnóstico, al planteamiento de los estudios de campo y a la falta de información de los casos reales. Es por ello que en ocasiones la utilización de modelos epidemiológicos puede ser de gran ayuda (Davidson *et al.*, 2012).

Debido al impacto económico que produce esta enfermedad en vacuno lechero, es en esta especie donde se han llevado a cabo el mayor número de estudios de prevalencia. Nielsen y Toft (2009) revisaron los trabajos realizados desde 1990 hasta 2007 con el fin de estimar la prevalencia de la paratuberculosis bovina, ovina y caprina en Europa, teniendo en cuenta que la prueba de diagnóstico de referencia para considerar un rebaño "positivo" podía variar desde un ELISA (*Enzyme Linked Immunosorbent Assay)*, cultivo de heces y de tejidos, o PCR (*Polymerase Chain Reaction*), según el estudio. Los resultados obtenidos en ese estudio se resumen en la Tabla I.

Tabla I. Prevalencia de paratuberculosis en animales domésticos. Fuente: Nielsen y Toft, 2009.

Especie	País	Años	Prevalencia	Referencias
Bovino	Alemania	2002-2004	42%	(Donat *et al.*, 2005)
Bovino	Bélgica	1997-1998	18%	(Boelaert, 2000)
Bovino	Dinamarca	1998	55%	(Nielsen *et al.*, 2000)
Bovino	Eslovenia	1997-2001	3-12%	(Ocepek *et al.*, 2002)
Bovino	España	Desconocido	8-10%	(Sevilla *et al.*, 2002)
Bovino	Francia	1998-1999	68%	(Petit, 2001)
Bovino	Holanda	1998	54%	(Muskens, 2000)
Bovino	Italia	1997-1998	7%	(Vicenzoni *et al.*, 1999)
Bovino	Italia	2000-2001	65%	(Robbi *et al.*, 2002)
Bovino	Italia	Desconocido	42%	(Lillini *et al.*, 2005)
Bovino	Reino Unido	1995	17%	(Cetinkaya *et al.*, 1998)
Bovino	Irlanda	2000-2001	13%	(O'Reilly *et al.*, 2004)
Bovino	Suecia	2000-2001	0%	(Sternberg y Viske, 2003)
Bovino	Suiza	2005	3%	(Bosshard *et al.*, 2006)
Caprino	España	1996	52%	(Reviriego *et al.*, 2000)
Caprino	Reino Unido	1998	1%	(Grant, 2001)

Especie	País	Años	Prevalencia	Referencias
Caprino	Suiza	2002	23%	(Muehlherr *et al.*, 2003)
Rebaño mixto Ovino y caprino	Eslovenia	2000-2001	12%	(Ocepek *et al.*, 2002)
Rebaño mixto Ovino y caprino	Portugal	Desconocido	27%	(Mendes *et al.*, 2004)
Ovino	España	1996	29%	(Reviriego *et al.*, 2000)
Ovino	Reino Unido	1998	0%	(Grant, 2001)
Ovino	Suiza	2002	24%	(Muehlherr *et al.*, 2003)

Hay que señalar que estudios llevados a cabo posteriormente o no tenidos en cuenta en el estudio de Nielsen y Toft (2009) y por lo tanto no recogidos en la Tabla I, cifran la prevalencia de la paratuberculosis bovina en el norte de España en valores más elevados. Así se ha descrito que la prevalencia del 18,7% al 20% en el País Vasco (Vazquez *et al.*, 2009), del 28,35% al 44,39% en Asturias (Balseiro *et al.*, 2004) y del 2,78% al 27,77% en Galicia (Diéguez *et al.*, 2007).

En España también se han reportado prevalencias en ovino mayores a las descritas en la Tabla I. Así, en Aragón se observó que el 46,7% de los más de 150 rebaños ovinos estudiados estaban infectados, tal y como indicó la combinación de técnicas bacteriológicas, anatomopatológicas y serológicas empleadas (Juste *et al.*, 1991a).

En EEUU se estima que la paratuberculosis bovina afecta al 68% de las explotaciones de vacuno de leche (USDA-APHIS-VS-CEAH, 2008). Sin embargo, Lombard *et al.* (2013) mediante análisis bayesianos estimaron que la prevalencia real ascendería hasta el 91%. En Canadá, la tasa de infección arroja resultados que van desde el 2,7% (VanLeeuwen *et al.*, 2005) al 7% (Sorensen *et al.*, 2003) en bovino. Un estudio reciente en rebaños lecheros de pequeños rumiantes señala prevalencias mucho mayores siendo del 35,2% y 48,3% en caprino y ovino respectivamente (Bauman *et al.*, 2016).

En Asia la seroprevalencia bovina individual se cifró entre el 6,7% y 7,1% de los animales examinados (Pak *et al.*, 2003). En el ganado bovino de Australia se observaron prevalencias más bajas, siendo del 0,43% (Vandegraff *et al.*, 1994) al 1,6% (Jubb, 2000).

Hay que tener en cuenta que la evaluación del estatus sanitario de los rebaños mediante el diagnóstico individualizado supone un alto costo económico. Recientemente, se ha sugerido que la utilización de muestras ambientales puede ser una alternativa útil y de bajo coste (Lombard *et al.*, 2006; Pillars *et al.*, 2009). De esta manera, dos de los métodos más eficaces como indicadores de infección en explotaciones son; la detección de ADN de *Map* en los filtros de leche en las explotaciones de aptitud láctea (Slana *et al.*, 2012) y el aislamiento de *Map* de las calzas de las zonas próximas al ganado, permitiendo identificar la presencia de *Map* en más del 90% de las explotaciones infectadas (Eisenberg *et al.*, 2013).

II.1.2.4 Impacto económico

La importancia económica de la paratuberculosis radica en las pérdidas que ocasiona en los rebaños afectados, tanto por la eliminación o muerte de animales como por el descenso en la producción, siendo una vez más la especie bovina donde estos aspectos han sido más ampliamente estudiados. Es importante destacar que el 70% de las pérdidas económicas se corresponden con el desvieje prematuro y el empeoramiento de los parámetros productivos, básicamente la disminución de la producción láctea (Groenendaal *et al.*, 2002; Ott *et al.*, 1999). Otros aspectos que influyen en el impacto económico de esta enfermedad son la disminución en la fertilidad (Buergelt and Duncan, 1978; Stabel, 1998; Garcia-Ispierto, 2016) y el incremento en la

susceptibilidad al padecimiento de mamitis (Buergelt y Duncan, 1978; McKenna *et al.*, 2006). Además, hay que señalar que la infección produce un deterioro general del estado sanitario de los animales aumentando la tasa de mortalidad por otras patologías en los rebaños infectados (Arrazuría *et al.*, 2014; Ott *et al.*, 1999) llegando a ascender a un 3 % (Johnson-Ifearulundu *et al.*, 1999).

Algunos autores han estimado pérdidas de entre 903$ y 979$ por vaca afectada en lo que a pérdida de producción láctea se refiere (Chiodini *et al.*, 1986; van Schaik *et al.*, 1996). Sin embargo, otros autores estiman cifras menores, de 100$ a 200$ por vaca lechera (Ott *et al.*, 1999). Un estudio reciente considerando las opiniones de ganaderos y veterinarios estadounidenses, estimó que las pérdidas ocasionadas por la paratuberculosis ascendían a 235$ a 250$ por cada vaca infectada (Bhattarai *et al.*, 2013). En general, se estima que en la industria lechera bovina de EEUU las pérdidas debidas a la infección por *Map* pueden alcanzar el billón y medio de dólares, pudiendo ascender hasta los 15,4 billones de dólares anuales en zonas con elevadas prevalencias (Stabel, 1998).

Existen pocos estudios que estimen las pérdidas económicas ocasionadas por la paratuberculosis en otras especies animales. Sin embargo, un estudio realizado en España estimó que en ovino la disminución de un 10% en la producción de leche ocasionaba unas pérdidas económicas de 25 millones de pesetas anuales (Aduriz *et al.*, 1994). Un estudio posterior estimó que las pérdidas por cada animal en fase clínica de paratuberculosis en ovino de carne y de leche ascendían a 60 y 150 dólares, respectivamente (Juste, 1997). En otro estudio llevado a cabo en ovinos en Australia se calcularon unas pérdidas anuales de 13.715 dólares por granja y año, abarcando dos tercios de las pérdidas totales asociadas a bajas por cualquier enfermedad (Bush *et al.*, 2006).

II.1.3. PATOGENIA

Conocer y entender los mecanismos patogénicos de una enfermedad es esencial para prevenir la infección y tratar adecuadamente a los animales infectados, además de que permite avanzar en el diagnóstico y control de la enfermedad. A pesar de que se ha dedicado mucho esfuerzo al estudio de la patogenia de la paratuberculosis, a día de hoy

sigue habiendo muchos interrogantes. El avance en este área probablemente se ha visto impedido por el largo periodo de incubación de la enfermedad y por la dificultad para reproducir experimentalmente sus características típicas (Coussens, 2004). En la Figura 3 se recogen los principales eventos que ocurren en la infección por *Map* y de los cuales pasaremos a hablar a continuación.

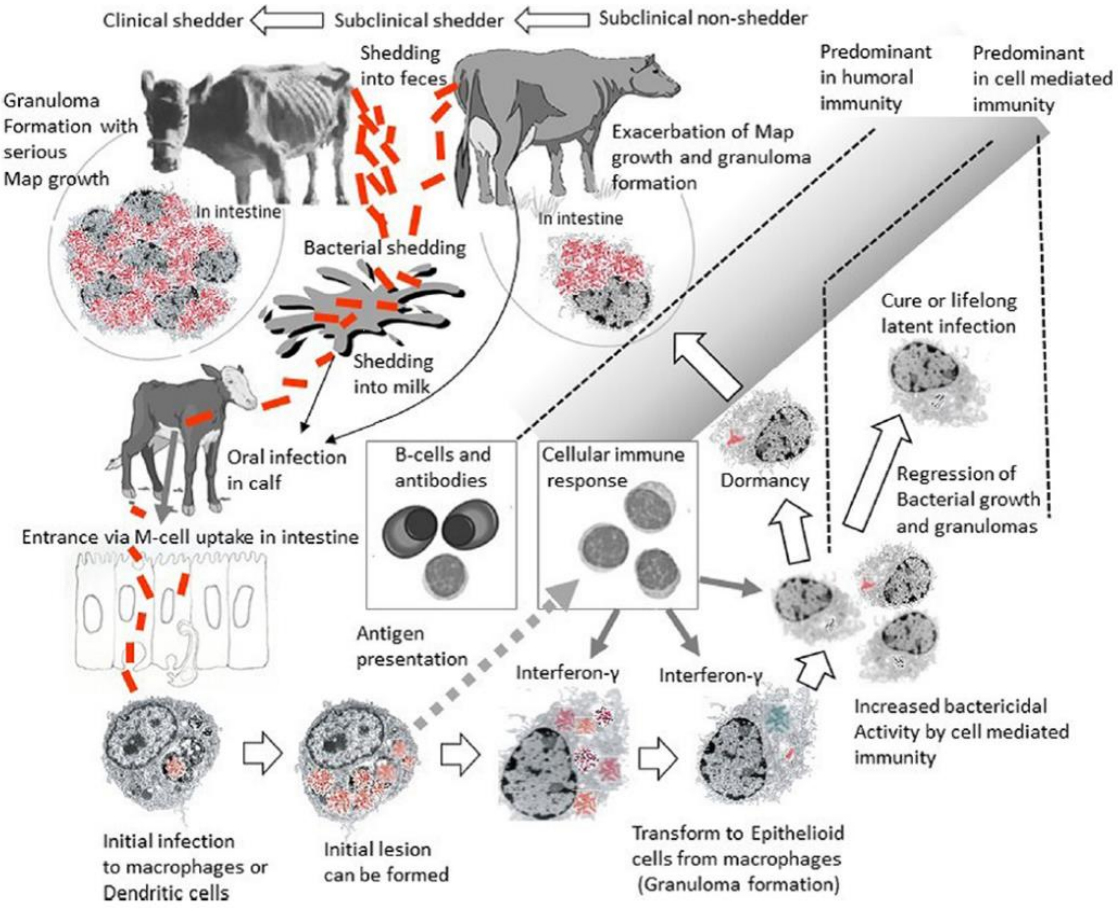

Figura 3. Representación esquemática de la complejidad de la dinámica de la infección de *Map* en el ganado bovino. Fuente: Koets y Gröhn, 2015

II.1.3.1 Entrada y supervivencia de *Map* en el hospedador

Como se ha mencionado anteriormente, el mecanismo natural de entrada de *Map* en el hospedador es por vía oral, penetrando en el organismo a través del tejido linfoide intestinal o placas de Peyer (Momotani *et al.*, 1988; Sigurdardóttir *et al.*, 2001). El hecho de que este tejido este más desarrollado en animales jóvenes hace que estos sean más susceptibles a la infección que los animales de mayor edad, en los que se da una involución o disminución de la extensión del tejido linfoide intestinal.

La entrada de *Map* al organismo ocurre al penetrar el microorganismo a través de un tipo especial de células epiteliales, las células M, las cuales recubren las cúpulas de las placas de Peyer (Momotani *et al.*, 1988; Sigurdardóttir *et al.*, 2001). Antes de penetrar, *Map* debe adherirse a las células, y aunque los mecanismos implicados en este proceso aún no están del todo claros, se ha identificado en la superficie de *Map* una proteína, la llamada proteína de acoplamiento a la fibronectina (*Fibronectin Attachment Proteins*, FAP), que le permite unirse a las integrinas β1 de las células M a través de un puente de unión con la fibronectina (Lee *et al.*, 2009b; Secott *et al.*, 2001, 2004). Las células M, están especializadas en la captación de antígenos luminales y debido a que no poseen lisosomas ni enzimas hidrolíticas, son capaces de transportar la micobacteria intacta en el interior de vacuolas hasta la submucosa (Miller *et al.*, 2007). Es por ello que muchas de las propiedades antigénicas de *Map* permanecerían inalteradas tras atravesar estas células. La captación y transporte del bacilo transcurre de manera muy rápida habiéndose observado que tras la inoculación directa del microorganismo en la luz intestinal, la mayor parte de las micobacterias atraviesan las células M en menos de una hora (Sigurdardóttir *et al.*, 2001). Esta vía de entrada no es exclusiva, ya que se ha demostrado el paso de la micobacteria a través de otras células epiteliales o enterocitos (Bermudez *et al.*, 2010; Ponnusamy *et al.*, 2013; Pott *et al.*, 2009; Sangari *et al.*, 2001; Sigurdardóttir *et al.*, 2005), aunque se estima que esta vía tiene una menor relevancia.

Una vez que la micobacteria ha traspasado la capa epitelial, es fagocitada por los macrófagos subepiteliales (células diana en la infección paratuberculosa) activados por mecanismos innatos que se describen posteriormente.

La supervivencia de *Map* dentro de los macrófagos depende de muchos factores. Algunas de las estrategias que se considera que emplea esta micobacteria para eludir la respuesta defensiva del hospedador son: 1) inhibir la maduración del fagosoma y la fusión fagosoma-lisosoma (Arsenault *et al.*, 2014; Hostetter *et al.*, 2003), 2) inhibir la acidificación del fagosoma-lisosoma: por medio de la reducción de los niveles de reactivos oxigenados (óxido nítrico) y de la síntesis de hidrolasas (Kuehnel *et al.*, 2001; Miller *et al.*, 2004; Weiss *et al.*, 2004; Woo y Czuprynski, 2008), 3) condicionar los fenómenos de apoptosis (Abendaño *et al.*, 2013; Allen *et al.*, 2001; Arsenault *et al.*, 2014; Kabara y Coussens, 2012; Weiss *et al.*, 2004) y 4) desarrollar formas de resistencia (formas de pared defectiva o formas L) (Beran *et al.*, 2006).

II.1.3.2 Respuesta inmune innata

La respuesta inmune innata es la primera línea de defensa inmunitaria que presenta un animal frente a la invasión de microorganismos con potencial patogénico. Durante años este tipo de respuesta se ha considerado secundaria o de menor importancia (Medzhitov y Janeway, 1997). Actualmente, se cree que este tipo de inmunidad es relevante (Kleinnijenhuis *et al.*, 2011) y que puede controlar y dirigir la respuesta inmune adaptativa (Vasselon, 2002).

La activación de la respuesta innata se inicia con el reconocimiento de moléculas altamente conservadas presentes en la mayoría de los microorganismos, denominados patrones moleculares asociados a patógenos (*Pathogen-Associated Molecular Patterns*, PAMP) y que pueden consistir en lipopolisacáridos de la pared celular, lipoproteínas, proteínas o ácidos nucleicos (Akira *et al.*, 2006). Las células inmunitarias reconocen los PAMPs gracias a los receptores de reconocimiento de patógenos (*Pathogen Recognition Receptor,* PRR) lo que permite que las bacterias sean fagocitadas rápidamente (Akira *et al.*, 2006; Kawai y Akira, 2007).

Una vez de que la micobacteria ha traspasado la capa epitelial, es fagocitada por los macrófagos subepiteliales, que están dotados de diversos receptores, incluyendo los receptores del complemento CR1, CR3 y CR4, los receptores a inmunoglobulinas (FcR), receptores de manosa, el receptor CD14 de unión a los liposacáridos y receptores de transferrina, "*scavenger receptors*" (Sohal *et al.*, 2008). Las micobacterias también

pueden interaccionar con las células dendríticas mediante unión al receptor DC-SIGN (*Dendritic Cell-Specific Intercelular adhesion molecule-3-Grabbing Non-integrin*) (Kleinnijenhuis *et al.*, 2011). Aunque existe un gran número de receptores, el receptor CR3 es uno de los más importantes relacionados con fagocitosis de micobacterias, incluyendo *Map* (Bermudez *et al.*, 1991). Es importante destacar que las diferentes rutas de penetración en las células fagocíticas pueden tener consecuencias importantes para la supervivencia intracelular de *Map*, debido a que los diferentes sistemas de receptores inducen la secreción de un único patrón de citoquinas capaz de producir una respuesta inmune diferenciada (Guirado *et al.*, 2013).

La familia de los receptores Toll like (*Toll Like Receptors*, TLR) representan unos de los PRR más importantes en el reconocimiento micobacteriano (Ferwerda *et al.*, 2007; Kugelberg, 2014). Tras la activación de los TLR se produce la activación de una vía de señales intracelulares que induce rápidamente la producción de citoquinas así como de reactivos nitrogenados y oxidativos intermediarios, iniciando así una respuesta inmune innata.

La dinámica de la expresión de los TLRs en respuesta a la infección natural juega un papel importante en el sistema de protección contra *Map*. Sin embargo, la función específica del sistema de los TLR en la defensa contra *Map* es compleja. De esta manera, la activación de ciertos TLRs, como TLR9, parece que inicia respuestas que son críticas en la defensa contra *Map* (Bafica *et al.*, 2005) mientras que la activación de otros, como TLR2, podría inducir respuestas dirigidas a suprimir la defensa inmune contra *Map* (Noss *et al.*, 2001). Los TLR2 reconocen lipoproteínas y lipopolisacáridos de la pared celular micobacteriana (Schwandner *et al.*, 1999) y polimorfismos del TLR2 han sido asociados con una mayor susceptibilidad a la paratuberculosis (Mucha *et al.*, 2009). Recientemente también se ha demostrado que dos SNPs en el receptor TLR4 están significativamente asociados con la susceptibilidad a la infección por *Map* (Sharma *et al.*, 2015). Los TLR9 tienen un interés particular, debido al papel que juegan en la defensa frente a las micobacterias, así como por el potencial uso de agonistas del TLR9 en la inmunoterapia frente a *Map* (Bafica *et al.*, 2005). Los TLR9 reconocen y se unen al ADN microbiano, identificándolo por la presencia de marcadores CpG no metilados. De esta manera, se ha visto que en macrófagos humanos tratados con

oligodesoxinucleótidos CpG se promueve la maduración del fagolisosoma inhibiéndose parcialmente el crecimiento de *M. tuberculosis* (Wang *et al.*, 2005).

Los receptores "NOD-like" (*NOD-Like Receptors*, NLR) también están implicados en el reconocimiento micobacteriano innato (Delbridge y O'Riordan, 2007). Entre ellos, el gen CARD15/NOD2 ha sido ampliamente estudiado. Se han encontrado mutaciones en este gen con mayor frecuencia en los individuos afectados de una enteritis crónica regional que afecta a los humanos conocida como enfermedad de Crohn (Elguezabal *et al.*, 2012; Hugot *et al.*, 2001; Ogura *et al.*, 2001). Además, se ha encontrado una relación significativa entre la presencia de un polimorfismo en el gen CARD15/NOD2 y la sensibilidad a la paratuberculosis en bovino infectado naturalmente (Pinedo *et al.*, 2009; Ruiz-Larrañaga *et al.*, 2010a). Sin embargo, otros estudios no han encontrado asociaciones significativas entre la presencia de mutaciones en este gen y la mayor o menor resistencia a la paratuberculosis (Taylor *et al.*, 2006).

Otro de los genes que participa en la inmunidad innata frente a micobacterias y cuya mutación podría estar relacionada con una mayor sensibilidad a la paratuberculosis es el que codifica para la "proteína macrofágica asociada a la resistencia natural" (*Natural-resistance-associated macrophage protein* 1, Nramp 1). Tras la fagocitosis de *Map*, Nramp 1 es reclutada hacia la membrana del fagosoma en los macrófagos y neutrófilos lo que impide el retraso de la maduración del fagosoma inducido por la micobacteria (Frehel *et al.*, 2002). En un estudio con 267 vacas infectadas se ha relacionado la detección de dos SNPs en este gen con una mayor sensibilidad a la infección (Ruiz-Larrañaga *et al.*, 2010b).

Las células Tγδ parece que también tienen un papel importante en la inmunidad innata (Pollock y Welsh, 2002; Sohal *et al.*, 2008). El subtipo WC1+ de células Tγδ expresan PRRs (Hedges *et al.*, 2005) y además son capaces de reconocer antígenos no asociados al complejo mayor de histocompatibilidad (CMH) (Hayday, 2000; Machugh *et al.*, 1997). Por otro lado, se ha sugerido que las altas concentraciones de células WC1+ presentes en terneros permiten producir citoquinas de tipo Th1 como la IL-12, TNF-α o IFN-γ favoreciendo la respuesta inmune celular (Baldwin *et al.*, 2000; Baquero y Plattner, 2016; Brown *et al.*, 1994). No obstante, otros autores atribuyen a las células Tγδ un papel inmunoregulador y una actividad citotóxica para las células T CD4+ con

la consiguiente inhibición de la respuesta celular (Chiodini y Davis, 1992; Koets *et al.*, 2002, 2006).

También se ha propuesto que los receptores de IFN-γ puedan jugar un papel importante, puesto que su deficiencia genética o su disminución inducida podría explicar los niveles basales elevados de IFN-γ en sangre de animales con paratuberculosis (Geijo, 2007) y en pacientes con enfermedad de Crohn (Juste, 2010).

II.1.3.3 Respuesta inmune adaptativa

Una vez la infección intracelular se ha establecido, comienza la activación de la respuesta inmune adaptativa o adquirida, mediada por los linfocitos T y B, que se caracteriza por la especificidad y la capacidad de memoria (Medzhitov y Janeway, 1997; Stabel, 2006). Este tipo de respuesta inmunitaria comienza con la presentación de antígenos a los linfocitos T para activarlos, lo que induce una cascada de secreción de citoquinas que a su vez estimula la proliferación de un mayor número de linfocitos (Coussens, 2001, 2004; Stabel, 2000b).

Las células T se clasifican en linfocitos T citotóxicos (Tc) y linfocitos T colaboradores (*T helper*, Th). Los linfocitos T citotóxicos presentan el marcador CD8 en su superficie y reconocen antígenos asociados a los CMH de clase I (Kaufmann, 1991). Los linfocitos Th presentan el marcador CD4 en su superficie, reconocen antígenos presentados por la CMH de clase II y su principal función consiste en amplificar la respuesta inmune mediante la secreción de numerosas citoquinas.

Existen dos perfiles de producción de citoquinas por parte de los linfocitos Th, Th1 y Th2. La población linfocitaria Th1 se caracteriza por la secreción de IFN-γ, IL-2 y TNF-α, citoquinas implicadas en la respuesta inmune de tipo celular, frente a microorganismos intracelulares y cuya secreción parece que está regulada por la IL-12 (Lamont y Adorini, 1996). En cambio, los linfocitos Th2 producen de forma notable IL-4, IL-5 e IL-10, induciendo la producción de inmunoglobulinas por parte de las células B implicadas en la respuesta humoral (Coussens, 2001; Kidd, 2003; Stabel, 2000b, 2006).

En el caso de la paratuberculosis, se ha relacionado la respuesta inmune celular con las primeras fases de la infección y la respuesta inmune humoral con la etapa clínica de la paratuberculosis (Begara-McGorum *et al.*, 1998; Burrells *et al.*, 1998; Ostrowski *et al.*, 2003; Pérez *et al.*, 1997, 1999; Stabel, 2000b). Sin embargo, varios estudios recientes señalan que el cambio entre respuesta Th1 y Th2 no sería tan marcado, coexistiendo ambas durante largos periodos de tiempo, sin necesidad de asociar la respuesta humoral a la fase clínica de la enfermedad (Begg *et al.*, 2011; Vazquez *et al.*, 2013).

II.1.3.3.1 Respuesta inmune celular

El IFN-γ es la principal citoquina efectora de tipo Th1, y desempeña un papel muy importante en la diferenciación de las células que intervienen en este tipo de respuesta, así como en la inducción de la activación de los macrófagos (Arsenault *et al.*, 2012; Stabel, 2000b, 2006). Por lo tanto, la IL-12 implicada en la secreción de IFN-γ juega un papel crucial (Thakur *et al.*, 2013). En el caso de la tuberculosis se ha demostrado que la administración exógena de esta interleuquina confiere resistencia a la infección y el bloqueo de su producción favorece el crecimiento micobacteriano (Cooper *et al.*, 1995, 1997).

La elevada secreción de IFN-γ en fases iniciales de la infección paratuberculosa se ha relacionado con el control de la enfermedad (Begg *et al.*, 2009; Storset *et al.*, 2001). Numerosos trabajos han reflejado la mayor secreción de IFN-γ en animales con infección subclínica paratuberculosa, mientras que cuanto la infección progresa a una fase clínica, la producción periférica y local de esta citoquina disminuye (Stabel, 1996, 2000a; Sweeney *et al.*, 1998). Sin embargo, en otros estudios se ha observado que elevadas cantidades de IFN-γ no son suficientes para controlar la infección por *Map* (Billman-Jacobe *et al.*, 1992; Ganusov *et al.*, 2015; Storset *et al.*, 2001) y que los niveles de esta citoquina no muestran una buena correlación con el grado lesional y el aislamiento bacteriano (Vazquez *et al.*, 2013). Además, se ha demostrado que los macrófagos infectados son incapaces de destruir las micobacterias tras ser tratados con IFN-γ (Weiss *et al.*, 2001). Por todo ello, a pesar de que se considera una citoquina esencial para el control de la enfermedad, su verdadero papel no termina de esclarecerse.

Otras citoquinas como el TNF-α, la IL-1, IL-6 tienen importantes propiedades proinflamatorias mediante la estimulación de las células T (Alzuherri *et al.*, 1996; Coussens, 2001; Dinarello, 1996). El TNF-α se considera una de las citoquinas básicas en las fases subclínicas de la paratuberculosis, estando estrechamente implicado en la formación de granulomas (Kaneko *et al.*, 1999; Palmer *et al.*, 2015). La citoquina IL-1 es esencial para la expansión clonal de las células T, mientras que la IL-6 está implicada en la producción de proteínas de fase aguda (Alzuherri *et al.*, 1996; Dinarello, 1996).

Diferentes estudios han demostrado la mayor secreción de estas citoquinas proinflamatorias IFN-γ, IL-1 e IL-6 en animales infectados de paratuberculosis en comparación con animales controles no infectados (Coussens, 2004; Lee *et al.*, 2001). Aunque la secreción de citoquinas proinflamatorias parece necesaria para controlar la infección inicial, su producción excesiva o demasiado prolongada también puede llegar a ser perjudicial a nivel local debido al riesgo de causar excesivo daño tisular (Clarke, 1997; Macdonald *et al.*, 2012; Stabel, 2006). Por ello, las citoquinas de tipo Th2 desempeñan un papel fundamental en contener las respuestas desmesuradas de tipo Th1. Fundamentalmente se ha estudiado el efecto inmunosupresor de la IL-4 (Magombedze *et al.*, 2015) e IL-10 (Ito *et al.*, 1999; Magombedze *et al.*, 2015; Saraiva y O'Garra, 2010) sobre el IFN-γ. Sin embargo, al estar inhibido el IFN-γ por estas citoquinas Th2, los nuevos macrófagos reclutados hacia el lugar de la infección se vuelven incapaces de controlar la invasión de los bacilos, por lo que sería la propia alteración de la respuesta inmune del hospedador la que resultaría perjudicial, permitiendo finalmente la diseminación de las micobacterias en fases avanzadas. Esto se ha demostrado al observarse una mayor expresión de IL-10 en vacas en estado avanzado de enfermedad en comparación con vacas sanas o en fase iniciales de la enfermedad (Khalifeh y Stabel, 2004). Además, también se evidenció una mayor expresión de IL-4, IL-10 en lesiones multibacilares frente a las paucibacilares en tejidos de vacas infectadas por *Map* (Tanaka *et al.*, 2005).

Para contener el daño tisular causado por un exceso de citoquinas proinflamatorias, el hospedador también dispone de una subpoblación de células T, las llamadas células T reguladoras (T_{reg}). Estas células tienen función inmunosupresora y expresan los marcadores de superficie CD4+ y CD25+, además del factor de transcripción "forkhead box P3" (FoxP3) (Hori *et al.*, 2003). Las células CD4+ que tras la exposición a

antígenos se diferencian a T_{reg} son capaces de secretar elevadas cantidades de IL-10 pasando a denominarse Tr1, o TGF-β denominándose en este caso células Th3 (Beissert *et al.*, 2006). Diferentes estudios, sugieren que en fases subclínicas de la infección, la población de T_{reg} aumentaría en respuesta a *Map*, de tal forma que inhibirían la respuesta inmune celular (de Almeida *et al.*, 2008; Roussey y Coussens, 2014; Sohal *et al.*, 2008; Stabel, 2006; Weiss *et al.*, 2006). Por el momento el mecanismo de acción de estas células en la patogenia de la paratuberculosis no está claro.

Actualmente se conoce un tercer subtipo de células T CD4+, independientes de las Th1 y Th2, denominadas Th17. Estas células aunque en menor medida que las Th1 parecen estar implicadas en la inmunidad protectora del hospedador frente a patógenos intracelulares (Curtis y Way, 2009). La producción de la IL-17 por las células Th17 ha sido estudiada en la tuberculosis, destacando su papel en la inducción y mantenimiento de la respuesta protectora frente a la micobacteria (Khader y Cooper, 2008). Además, se ha visto una menor expresión de esta citoquina en pacientes con tuberculosis latente (positivos a la prueba intradérmica de la tuberculina), en comparación con pacientes sanos (Babu *et al.*, 2010). El papel de esta población celular ha sido también estudiado en la enfermedad de Crohn, encontrándose células T reactivas a *Map* y secretoras de IL-17 e IFN-γ en pacientes enfermos (Olsen *et al.*, 2009). Recientemente también se ha observado el aumento en la expresión de IL-17 en vacas paratuberculosas sugiriendo que a medida que avanza la enfermedad prevalece este tipo de respuesta inmune (Dudemaine *et al.*, 2014).

II.1.3.3.2 Respuesta inmune humoral

La respuesta humoral se ha considerado tradicionalmente asociada a las fases clínicas de la paratuberculosis, en las que se da la excreción de bacilos a través de las heces (Clarke, 1997; Pérez *et al.*, 1997; Storset *et al.*, 2001). En la fase clínica, se produce un cambio de respuesta inmune Th1 a Th2 con la consiguiente inhibición de la secreción de citoquinas de tipo 1 (Stabel, 2000a, 2006). Por ello, la presencia de anticuerpos frente a *Map* no parece resultar en la protección de los animales infectados (Sohal *et al.*, 2008; Stabel, 2000b), aunque estudios más recientes señalan que la respuesta humoral sí podría jugar un papel en el control de las micobacteriosis (Achkar y Casadevall, 2013; Balu *et al.*, 2011) ya que se ha demostrado que algunos anticuerpos pueden potenciar la

respuesta inmune innata y celular frente a las micobacterias (de Vallière *et al.*, 2005). Una vez activadas, las células Th2 estimulan la diferenciación de los linfocitos B para que produzcan anticuerpos IgM y fundamentalmente IgG1, específicos frente a los antígenos de *Map* (Sohal *et al.*, 2008). A su vez, las células B estarían implicadas en la activación de las células CD4+ (Stabel y Khalifeh, 2008).

En infecciones naturales y experimentales se ha observado un aumento de linfocitos B (Lee *et al.*, 2001; Waters *et al.*, 1999) y de anticuerpos específicos frente a *Map* a medida que avanza la infección clínica (Begg *et al.*, 2009; Burrells *et al.*, 1998; Pérez *et al.*, 1997; Storset *et al.*, 2001). A su vez, se ha descrito una mayor capacidad proliferativa de los linfocitos B en respuesta a varios antígenos de *Map* en fases subclínicas en comparación con la fase clínica de la infección (Waters *et al.*, 1999). Así, una de las hipótesis propuestas para explicar la progresión de la infección, implicaría, por un lado, la alteración del fenotipo de los linfocitos B y, por otro, la inhibición de su capacidad proliferativa en respuesta a los antígenos de *Map* en las fases avanzadas de la paratuberculosis (Stabel y Khalifeh, 2008; Waters *et al.*, 1999).

Aunque tradicionalmente se ha señalado que el aumento de la respuesta inmune humoral se da en fases avanzadas de la enfermedad se han observado altos títulos de anticuerpos en infecciones experimentales a los 134 días post-infección en vacas (Waters *et al.*, 2003) y a los 180 días en cabras (Munjal *et al.*, 2005).

Hay que tener en cuenta que los resultados observados en relación a la respuesta inmune humoral dependen mucho de los antígenos de *Map* utilizados para la detección de anticuerpos así como del isotipo de inmunoglobulina a detectar (Bannantine *et al.*, 2008; Koets *et al.*, 2001). La observación de la respuesta humoral concomitante con la celular, ha propiciado que el paradigma del paso de una respuesta Th1 a una Th2 sea cuestionado. Así, recientemente se ha sugerido que la disminución de la respuesta inmune celular que se observa en los animales con lesiones multibacilares podría ser debido al comienzo del deterioro general de la respuesta inmune, viéndose afectada en primer lugar la respuesta celular (Begg *et al.*, 2011).

II.1.4. CUADRO CLÍNICO Y LESIONAL

II.1.4.1 Cuadro clínico

Debido al carácter crónico de la paratuberculosis y a pesar de que los animales se infectan en las primeras semanas de vida, los signos clínicos de la enfermedad no se hacen patentes hasta la edad adulta. Las sintomatología clínica, aparece con mayor frecuencia entre los dos y cinco años y a menudo tras el parto, durante la primera o la segunda lactación (Chiodini *et al.*, 1984; Dennis *et al.*, 2011; Pérez y Corpa, 2000). Cabe destacar que la mayoría de los animales infectados no llegan a desarrollar nunca la fase clínica de la enfermedad, pudiendo permanecer en la fase subclínica (Pérez y Corpa, 2000).

Desde un punto de vista clínico y teniendo en cuenta las herramientas diagnósticas disponibles hoy en día, la infección por *Map* se puede dividir en tres etapas (infección silente, subclínica y clínica). Sin embargo, debido a la sensibilidad y especificidad de las técnicas diagnósticas existentes, la diferenciación de las fases de la enfermedad en condiciones naturales puede resultar complicada.

La infección silente o latente se caracteriza por la ausencia de signos clínicos. Tras la infección, los animales pueden permanecer en esta fase de 2 a 10 años. Recientemente se ha demostrado que es la fase predominante en los animales adultos (Vazquez, 2014). Esta fase es difícil de diagnosticar *in vivo*, requiriéndose un diagnóstico post mortem.

En la infección subclínica se puede observar un aumento en la respuesta celular frente a *Map*. Se puede producir descenso de la producción láctea, alteraciones reproductivas y susceptibilidad a padecer mamitis. En esta fase se da la excreción intermitente de micobacterias en las heces, aunque no se observan síntomas característicos, como diarrea y pérdida de peso.

La enfermedad clínica se considera la fase terminal de una infección crónica subclínica, y el paso de una forma a otra puede estar desencadenado por numerosas situaciones causantes de estrés, tales como la gestación, el parto, un manejo inadecuado, una mayor exigencia productiva, desnutrición, infecciones parasitarias, o el pastoreo en suelos secos y deficientes en minerales (Blood y Radostits, 1989; Chiodini *et al.*, 1984). No

obstante, no se conocen con certeza todos los factores y mecanismos implicados en el establecimiento de la fase clínica.

El signo clínico clásico y más característico de la paratuberculosis en rumiantes es la progresiva pérdida de peso y condición corporal (Allen *et al.*, 1974). Otro de los signos clínicos que se puede encontrar es el edema hipoproteinémico en las partes declives, siendo muy típico el edema submandibular, que suele preceder incluso los demás síntomas (Blood y Radostits, 1989; Chiodini *et al*., 1984).

En el ganado bovino, es muy frecuente que la pérdida de peso se acompañe de una profusa diarrea líquida, no sanguinolenta, en ocasiones maloliente, que no responde a tratamiento con antibióticos. Estos episodios de diarrea pueden ser crónicos, pero también es frecuente que se presenten de forma intermitente, con períodos de remisión de semanas o incluso meses, para volver a reaparecer de forma más grave, generalmente tras un periodo de estrés como el parto (Blood y Radostits, 1989; Chiodini *et al.*, 1984; Corpa *et al.*, 1998; Pérez y Corpa, 2000). En cambio, la diarrea no es un signo característico constante de la paratuberculosis en las especies ovina y caprina, en las que a menudo el único signo clínico observado es la pérdida de peso progresiva, que suele manifestarse generalmente después del parto (García-Marín y Pérez, 1994; Valentin-Weigand y Goethe, 1999). Además, mientras el curso clínico de la enfermedad puede durar de 3 a 6 meses o incluso prolongarse durante más tiempo en el ganado vacuno, éste suele ser mucho más corto en los pequeños rumiantes (Chiodini *et al.*, 1984) produciéndose muertes por goteo en el rebaño.

Es importante destacar que como en el caso de otras enfermedades crónicas y subclínicas, en la paratuberculosis se produce el efecto iceberg. Así, cuando se observa un animal en la fase clínica de la enfermedad se estima que hay un mayor número de animales infectados en el rebaño (Fecteau y Whitlock, 2010). La estimación del número de animales infectados en las diferentes fases de la enfermedad se recoge en la Tabla II.

Tabla II. El "efecto iceberg" de la paratuberculosis. Fuente: Fecteau y Whitlock, 2010.

Estado de la infección	% de animales
Enfermedad clínica	1-3
Enfermedad subclínica	6-8
Infección latente	15-25

II.1.4.2 Cuadro lesional

Las lesiones características de la paratuberculosis se observan principalmente en los tramos distales del intestino delgado (íleon y válvula ileocecal) y linfonodos mesentéricos asociados. Sin embargo, en ocasiones los segmentos más distales del yeyuno, el ciego o incluso los segmentos más craneales del colon también pueden verse afectados (Carrigan y Seaman, 1990).

El hallazgo macroscópico más característico de la paratuberculosis en el ganado bovino es un marcado engrosamiento de la pared intestinal, adquiriendo el aspecto de un "tubo de goma" (Pérez y Corpa, 2000) (Figura 4A). Al corte, se puede observar la mucosa tumefacta, edematosa, muy engrosada y formando pliegues muy característicos que se asemejan a las circunvoluciones cerebrales, que persisten tras la tracción del intestino (Figura 4B).

Otro hallazgo característico, especialmente en ovinos y caprinos, es la presencia de linfangitis y linfangiectasia en los tramos afectados (Brown *et al.*, 2007; Pérez y Corpa, 2000), observándose los vasos linfáticos como cordones blanquecinos o transparentes sobre la serosa intestinal, y en casos extremos a través del mesenterio (Brown *et al.*, 2007; Fecteau y Whitlock, 2010; Kurade *et al.*, 2004) (Figura 4C). Hay que destacar que en ocasiones, en pequeños rumiantes, este puede ser el único cambio macroscópico apreciable (Brown *et al.*, 2007). Asimismo, los linfonodos mesentéricos yeyunales e ileocecales aparecen tumefactos y edematosos (Figura 4D). Además, en ovinos, y especialmente en el ganado caprino, se ha señalado la aparición de focos de necrosis

caseosa y calcificación en los nódulos linfáticos mesentéricos (Clarke, 1997; Tafti y Rashidi, 2000; Valheim *et al.*, 2002).

En animales con infecciones subclínicas, no se suelen apreciar cambios evidentes en el intestino o linfonodos asociados; no obstante, algunos individuos pueden mostrar un leve engrosamiento de la pared intestinal o tumefacción de los linfonodos mesentéricos, muy difícil de detectar en la necropsia si se carece de experiencia previa (Pérez y Corpa, 2000). Cabe mencionar que el grado de lesión macroscópica intestinal no siempre se corresponde con la gravedad de la sintomatología clínica observada, hecho que se ha descrito principalmente en el ganado ovino y caprino (Blood y Radostits, 1989; Brady *et al.*, 2008; Clarke y Little, 1996; Clarke, 1997).

En el conejo, especie animal en la que se centra la presente tesis doctoral, se han observado lesiones macroscópicas en los linfonodos mesentéricos, apéndice cecal, *sacculus rotundus* y ciego. Estas lesiones consisten en el aumento de tamaño de tamaño y tumefacción de linfonodos mesentéricos, engrosamiento parietal del *sacculus rotundus*, apéndice vermiforme y ciego; así como la presencia de múltiples focos lesiónales granulomatosos de pequeño tamaño (19 mm de diámetro como máximo) con aspecto que varía de sólido a purulento o caseoso, localizados en linfonodos mesentéricos, apéndice cecal y *sacculus rotundus* (Maio *et al.*, 2011). Sin embargo, hay estudios en los que no se han observado lesiones macroscópicas a pesar de haberse confirmado la infección (Beard *et al.*, 2001b; Greig *et al.*, 1999).

Las lesiones microscópicas propias de paratuberculosis se corresponden con una enteritis y/o linfadenitis de tipo granulomatoso, caracterizadas por un infiltrado focal o difuso de naturaleza mayoritariamente histiocítica, formado por macrófagos de citoplasma espumoso y acompañados en menor medida de linfocitos, células epiteliodes, células gigantes multinucleadas de Langhans y eosinófilos (Brown *et al.*, 2007; Delgado *et al.*, 2013; Pérez y Corpa, 2000; Vázquez, 2014). Además, se puede observar una alteración de la estructura normal intestinal, apreciándose una dilatación de las vellosidades (debido a la infiltración inflamatoria), así como una obliteración de las criptas o glándulas de Lieberkuhn (debido al acúmulo de detritus celular).

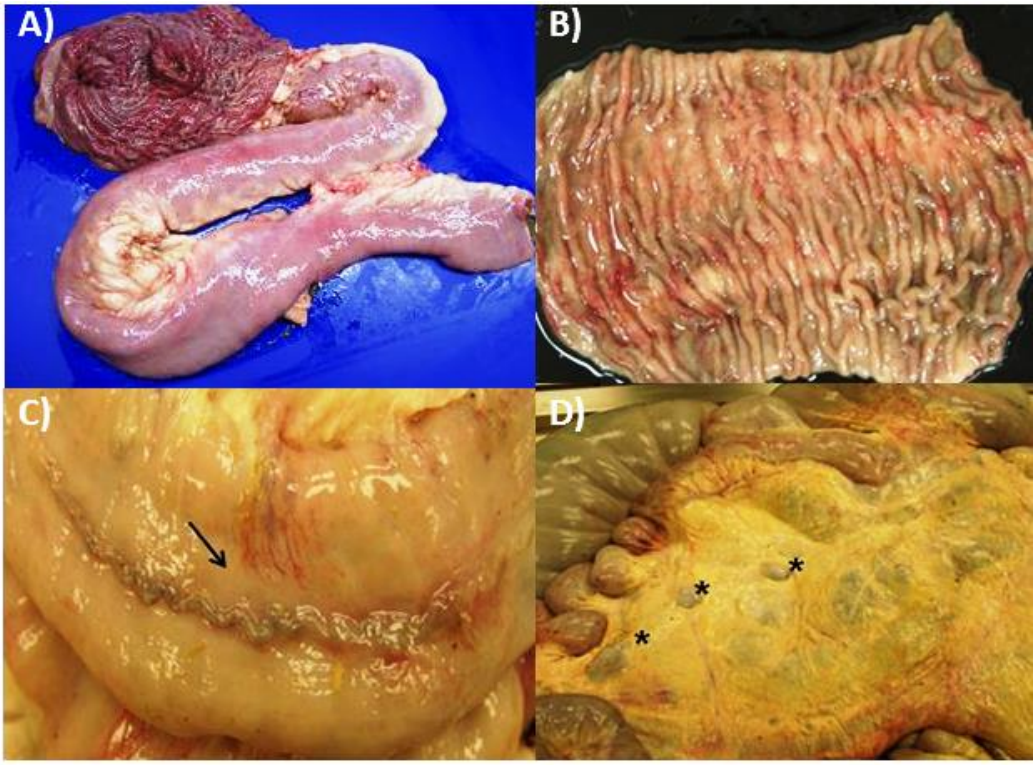

Figura 4. Lesiones macroscópicas observables en la paratuberculosis. A) Aspecto de tubo de goma del íleon distal y engrosamiento de la mucosa del área de la válvula ileocecal. B) Engrosamiento en un tramo de íleon. C) Detalle de vaso linfático con marcada linfangiectasia (→). D) Aumento de tamaño de los linfonodos mesentéricos (*). Fuente: Vázquez, 2014.

En los nódulos linfáticos mesentéricos, también se puede observar la presencia de lesiones granulomatosas que se extienden hacia la zona medular, llegando a alterar irreversiblemente su arquitectura (Buergelt y Duncan, 1978; Corpa *et al.*, 2000a; Delgado *et al.*, 2013; González *et al.*, 2005; Pérez y Corpa, 2000; Pérez *et al.*, 1996; Vázquez, 2014).

Actualmente, no existe una clasificación lesional unificada para las diversas especies susceptibles a la infección por *Map*. En ovino, la caracterización lesional de la paratuberculosis que ha tenido y sigue teniendo especial relevancia es la propuesta inicialmente por García-Marín (1991) y ampliada posteriormente por Pérez *et al.* (1996). Este trabajo sentó las bases para caracterizar las lesiones paratuberculosas no sólo en la especie caprina (Corpa *et al.*, 2000a) y bovina (González *et al.*, 2005), sino

también en gamos (Balseiro *et al.*, 2008) y conejos (Maio *et al.*, 2011), única especie monogástrica en la que se ha empleado esta misma metodología de clasificación.

En el ganado bovino, la clasificación lesional más extendida clasifica las lesiones en *focales*, *multifocales* y *difusas*, y a su vez subdivide estas últimas en: *multibacilares*, *linfocíticas* (o *paubacilares*) e *intermedias* dependiendo de la composición celular del infiltrado inflamatorio y de la cantidad de BAAR (González *et al.*, 2005). Sin embargo, recientemente se ha propuesto una nueva clasificación en base a las formas epidemio-patogénicas en la que se recogen las formas latentes, patentes y aparentemente libres (Vazquez, 2014). Las formas latentes se caracterizan por la presencia de lesiones focales, se asocian con una respuesta inmune predominante de tipo celular y con una limitada detección micobacteriana, siendo las formas más prevalentes en los animales de edad adulta. Las formas patentes se caracterizan por lesiones de tipo multifocal y difuso, elevada seropositividad y elevada presencia de *Map* en tejidos, siendo más frecuentes que las formas latentes en los animales jóvenes. Finalmente, las formas aparentemente libres se caracterizan por la ausencia de lesiones histopatológicas compatibles con la infección por *Map*, pudiendo existir respuesta celular y escasa presencia de *Map* en tejidos en un bajo número de animales.

Asimismo, esta es la única clasificación que se ha relacionado con polimorfismos genéticos, permitiendo observar diferencias en la progresión de la enfermedad y por lo tanto en la longevidad de los animales (Juste *et al.*, 2016).

En cuanto a la clasificación lesional en conejos salvajes infectados naturalmente se ha tenido en cuenta la localización, la magnitud y el tipo de células inflamatorias, así como el número de micobacterias observadas mediante la tinción ZN (Maio *et al.*, 2011). Debido a que la presente Tesis Doctoral se centra en el conejo como modelo animal de infección paratuberculosa en la Tabla III y la Figura 5 se detallan los diferentes tipos de lesiones histopatológicas descritas en esta especie animal.

Tabla III. Principales características lesionales descritas en conejos silvestres infectados naturalmente. Fuente: Maio *et al.*, 2011.

Lesión	Principales características
Lesiones focales	Lesiones consistentes en granulomas pequeños, bien delimitados, situados únicamente en el tejido linfoide intestinal y en las zonas interfoliculares de los linfonodos mesentéricos. Granulomas formados por macrófagos grandes, con citoplasma pálido y ligeramente espumoso y núcleos con cromatina dispersa. Con frecuencia se observan linfocitos y células gigantes de Langhans en los granulomas. Ausencia de micobacterias mediante la tinción ZN.
Lesiones multifocales	Lesiones consistentes en granulomas bien delimitados en el tejido linfoide (Figura 5A) y la lámina propia intestinal. No se observa pérdida significativa de la arquitectura normal del intestino. Presencia de algunos granulomas en la zona interfolicular de los linfonodos mesentéricos. En ocasiones se pueden observar micobacterias mediante la tinción ZN (Figura 5B).
Lesiones difusas	Lesiones características de una enteritis granulomatosa severa y linfadenitis. De acuerdo a la naturaleza de las células presentes en el infiltrado y la cantidad de BAAR se dividen en dos subtipos: **Difusas multibacilares:** Engrosamiento de la pared intestinal con un infiltrado inflamatorio consistente en células epitelioides, macrófagos, linfocitos y numerosas células gigantes multinucleadas de Langhans. Frecuentemente, se observa la fusión de las vellosidades intestinales debido al infiltrado inflamatorio. La submucosa también se ve afectada y las placas de Peyer muestran un infiltrado compuesto principalmente macrófagos y células gigantes de Langhans que invaden los folículos linfoides (Figura 5C). La serosa esta menos afectada aunque se pueden observar infiltrados granulomatosos multifocales. Los linfonodos mesentéricos muestran una linfadenitis granulomatosa severa y difusa con macrófagos y numerosas células gigantes de Langhans, con una alteración significativa de la arquitectura normal. Mediante la tinción ZN se pueden observar un gran número de micobacterias en la mucosa y la submucosa en el intestino (Figura 5 D y E) y linfonodos asociados. Además, también pueden existir lesiones hepáticas consistentes en granulomas formados principalmente por linfocitos y macrófagos conteniendo BAAR. **Difusas intermedias:** Engrosamiento de la pared intestinal con un infiltrado consistente en células epitelioides, macrófagos, linfocitos y numerosas células gigantes de Langhans. Estas últimas, en menor proporción que en las lesiones multibacilares difusas. Infiltrado inflamatorio de la submucosa consistente en células plasmáticas, macrófagos y linfocitos. La serosa no se ve afectada. En linfonodos se observa una linfadenitis granulomatosa. Presencia de BAAR aunque en menor número que en las lesiones del tipo difusas multibacilares.

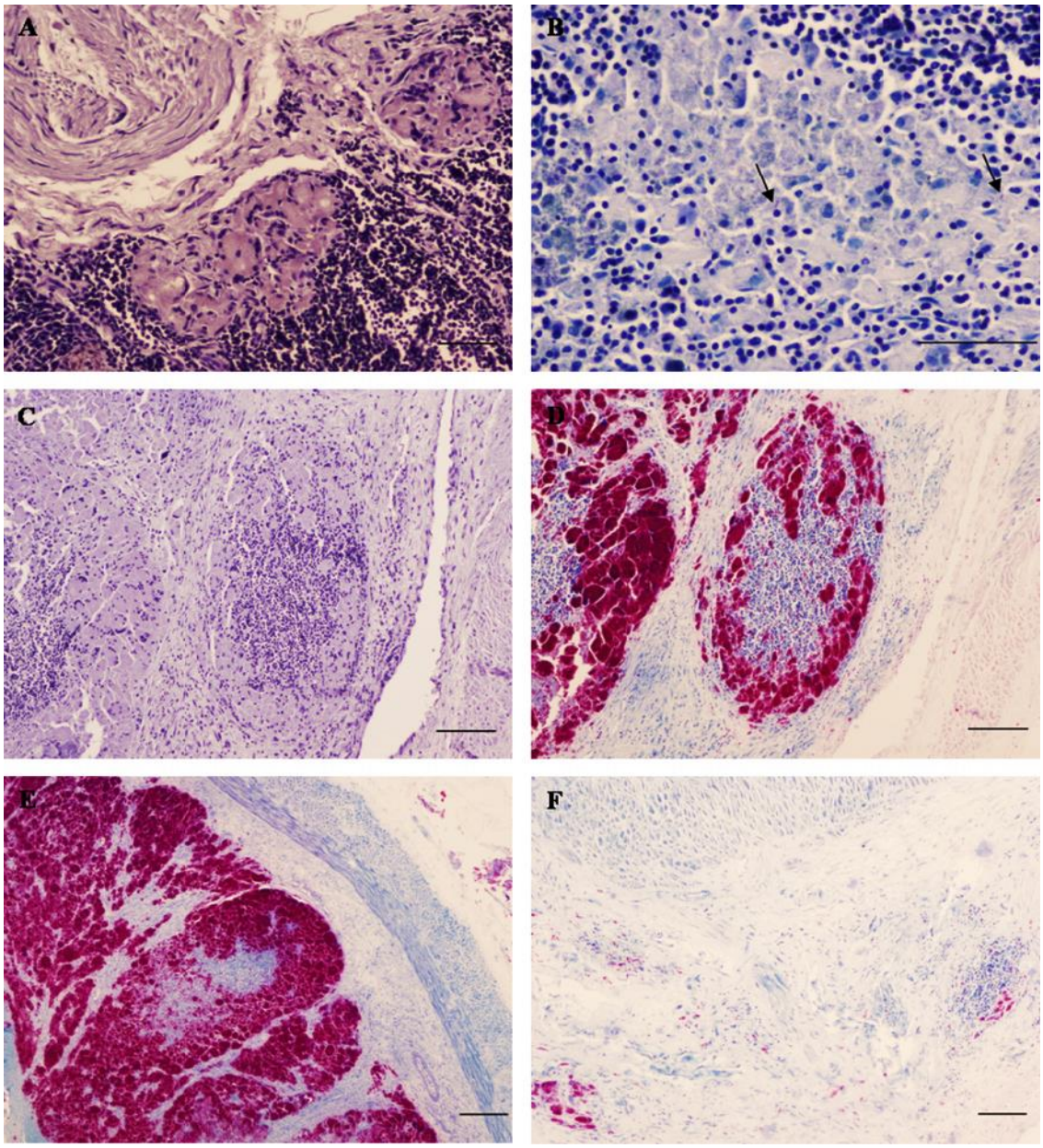

Figura 5. Secciones histológicas intestinales de conejos mostrando lesiones multifocales y difusas multibacilares. **Lesiones multifocales**: A) Granulomas bien demarcados localizados en el *sacculus rotundus* (tejido linfoide) presentando principalmente células gigantes de Langhans y macrófagos. B) Escasa presencia de BAAR en granulomas (flechas). **Lesiones difusas multibacilares:** C) Infiltrado inflamatorio difuso compuesto por macrófagos, linfocitos y células de Langhans en la pared del *sacculus rotundus* y de la placa de Peyer. D) Gran número de micobacterias en mucosa y submucosa. E) Íleon con gran cantidad de células gigantes de Langhans lleno de numerosos BAAR. F) Serosa con infiltrado multifocal granulomatoso y numerosos BAAR. Tinción ZN: Imágenes B, D, E y F. Tinción HE: Imágenes A y C. Tamaño de la línea de referencia: 50 µm (A y B); 100 µm (C y D) y 200 µm (E y F). Fuente: Maio *et al.*, 2011.

II.1.5. DIAGNÓSTICO

El diagnóstico de la infección en animales que presentan sintomatología clínica puede estar orientado por el diagnóstico clínico epidemiológico. No obstante se requiere una posterior confirmación laboratorial.

II.1.5.1 Diagnóstico anatomopatológico

En la necropsia de animales infectados se pueden observar lesiones macroscópicas consistentes en el engrosamiento de la pared intestinal, en el que la mucosa adquiere un aspecto cerebriforme (Figura 4B), y se produce linfagiectasia y aumento del tamaño de los linfonodos asociados al tracto gastrointestinal (Buergelt y Duncan, 1978). En todo caso, debido a que estas lesiones macroscópicas observadas pueden no ser patognomónicas, el diagnóstico post mortem debe de ir acompañado por otras técnicas diagnósticas que permitan confirmar la infección.

Para realizar un examen histopatológico completo se deben de examinar el íleon y linfonodos asociados sobre preparaciones sometidas a la tinción de eosina-hematoxilina y ZN, lo que permite observar cambios histológicos asociados a la inflamación y poner de manifiesto la presencia de BAAR (Corpa *et al.*, 2000a; González *et al.*, 2005; Pérez *et al.*, 1996; Vázquez, 2014).

El estudio histopatológico puede ser completado con otras técnicas como la inmunohistoquímica (IHQ) (Brees *et al.*, 2000; Coetsier *et al.*, 1998; Delgado, 2010; Lee *et al.*, 2009a; Martinson *et al.*, 2008) o la hibridación in situ (HIS) (Delgado *et al.*, 2009; Sechi *et al.*, 2001). A pesar de que el examen histopatológico generalmente se realiza post mortem, también es posible realizarlo en animales vivos mediante la obtención de biopsias quirúrgicas (Collins, 1996). Sin embargo, debido al coste del procedimiento de obtención de la muestra no está indicado como método diagnóstico rutinario (Buergelt y Duncan, 1978).

Hay que tener en cuenta que el estudio histopatológico puede resultar poco sensible para la detección de la infección subclínica que frecuentemente cursa con lesiones focales (Gilardoni *et al.*, 2012). Además, la falta de sensibilidad en la identificación de enteritis de grado leve se acentúa aún más en los pequeños rumiantes (Pérez *et al.*, 1996).

Generalmente, en animales asintomáticos se observan formas delimitadas de infección o tuberculosas, asociadas a la detección de un bajo número de BAAR, mientras que en animales en fase clínica de la enfermedad se observan formas difusas o lepromatosas, con un gran número de BAAR asociados y un aumento en el nivel de anticuerpos (Bastida y Juste, 2011).

II.1.5.2 Diagnóstico microbiológico

La baciloscopia o visualización de *Map* mediante microscopía óptica, en frotis de heces, leche, calostro o tejidos (mucosa intestinal o linfonodos) teñidos mediante ZN, es un método sencillo y barato que permite la rápida detección de BAAR (Figura 6). Sin embargo, esta técnica puede presentar problemas de especificidad con otras micobacterias saprófitas, especialmente en las muestras de heces bovinas (Coelho *et al.*, 2010; Gilardoni *et al.*, 2012; Zimmer *et al.*, 1999). Así mismo, la sensibilidad de la técnica disminuye notablemente en las infecciones paucibacilares o subclínicas (Zimmer *et al.*, 1999) siendo una herramienta diagnóstica útil siempre que los niveles de excreción asociados a los animales con sintomatología clínica no sean inferiores 10^5 UFC/ gramo de heces (Vázquez y Garrido, 2012).

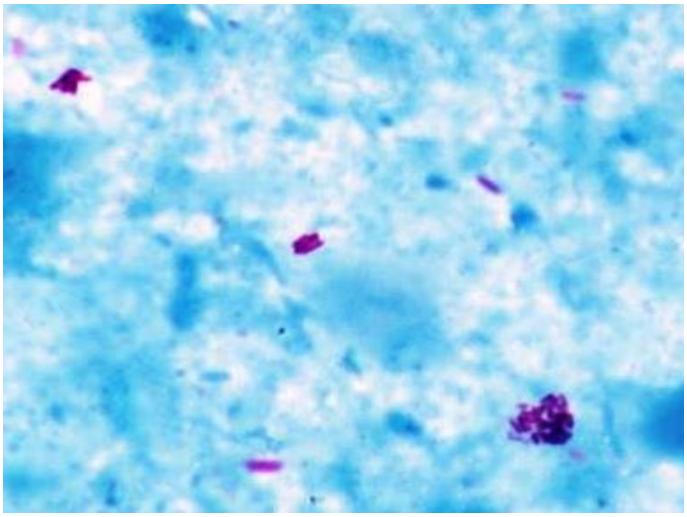

Figura 6. Imagen de frotis fecal teñido con ZN. 100X. Se observan los agregados de BAAR, teñidos por la fucsina, adoptando la disposición en grumos característica. Fuente: Vázquez, 2014.

Otra técnica orientada al diagnóstico microbiológico directo es el aislamiento de *Map* a partir de muestras de heces y/o tejidos (Collins *et al.*, 2006; Timms *et al.*, 2011; Whittington, 2010). Al igual que con las otras técnicas diagnósticas, la sensibilidad de la técnica varía dependiendo de la fase de infección y del tipo de tejido, asumiéndose una sensibilidad media del aislamiento de *Map* en bovinos del 60% en muestras de tejidos (Vansnick *et al.*, 2007), del 16-37% en muestras de leche de tanque (Gao *et al.*, 2005) y en torno al 30-40% en heces (Whitlock *et al.*, 2000) la cual en fases clínicas de la enfermedad puede llegar a alcanzar el 100% (Alinovi *et al.*, 2009a).

El cultivo bacteriológico, a pesar de estar considerada desde hace tiempo como la técnica "gold standard", no resulta una herramienta diagnóstica ideal debido a que requiere largos tiempos de incubación (18-20 semanas para excluir resultados negativos) y presenta un elevado coste económico. El cultivo automatizado basado en sistemas radiométricos (Cousins *et al.*, 1995; Damato y Collins, 1990; Whittington, 2010) y no radiométricos (Grant *et al.*, 2003; Stich *et al.*, 2004) ha permitido aumentar la sensibilidad de la técnica y obtener el aislamiento bacteriano en un periodo de tiempo más razonable (de 4 a 7 semanas) (Whittington, 2010). Cabe destacar que el aislamiento bacteriológico requiere de la confirmación molecular (Whittington *et al.*, 2013).

Para el cultivo bacteriológico se requiere de la utilización de medios de cultivo específicos. Generalmente, se utilizan medios a base de huevo como el HEYM (*Herrold egg yolk medium*) (Kim *et al.*, 1989; Merkal *et al.*, 1964) o el medio de Löwestein-Jensen (Juste *et al.*, 1991b; Merkal *et al.*, 1964; Whittington *et al.*, 2000a). También se pueden emplear medios a base de suero como el Dubos (Gunnarsson y Fodstad, 1979) o medios sintéticos como el Middlebrook 7H9 (Adúriz *et al.*, 1995), 7H10 (Damato y Collins, 1990) o 7H11 (Whittington *et al.*, 1999). Los medios de cultivo deben de suplementarse con una combinación de sustancias antimicrobianas (como anfotericina, cloranfenicol, penicilina, polimixina, trimetropin, ácido nalixilico, etc.), para evitar el crecimiento de otros microorganismos no deseados. Para el óptimo crecimiento de *Map*, generalmente los medios de cultivo suelen ser suplementados con micobactina J, OADC (Oleico-Albúmina-Dextrosa- Catalasa) o en algunos casos piruvato sódico.

Previamente a la realización del cultivo bacteriológico se debe de realizar la descontaminación de la muestra con el fin de eliminar otros microorganismos

contaminantes. Aunque *Map* presenta una gran resistencia a ciertas sustancias bactericidas, en determinadas condiciones este paso puede afectar a la viabilidad de la bacteria (Garrido *et al.*, 2000; Reddacliff *et al.*, 2003). A pesar de que se han probado muchas sustancias, y algunas de ellas con resultados prometedores como la enzima lítica C(18)- carboxipropilbetaina (Thornton *et al.*, 2002), las sustancias más empleadas en la actualidad son el cloruro de piridinio (*hexadecylpyridinium chloride*, HCP) al 0,75% (Dundee *et al.*, 2001; Juste *et al.*, 1991b; Whipple *et al.*, 1991) y el hidróxido sódico (Bradner *et al.*, 2014; Kalis *et al.*, 1999).

Gracias al desarrollo de la biología molecular, se comenzó a utilizar la técnica de la PCR para el diagnóstico molecular de la paratuberculosis. La PCR puede ir dirigida a diferentes dianas genéticas y una de las más usadas tradicionalmente ha sido la secuencia de inserción IS 900 (Collins *et al.*, 1989; Green *et al.*, 1989) debido a que se encuentra en 15-20 repeticiones en el genoma de *Map (Li et al., 2005)*. Sin embargo, debido al descubrimiento de secuencias similares en otras micobacterias, denominadas IS900-like (Cousins *et al.*, 1999; Englund *et al.*, 2002), se comenzaron a utilizar otras secuencias más específicas de *Map* como son el gen HspX (Ellingson *et al.*, 2000, 2005) las secuencias de inserción ISMav2 (Strommenger *et al.*, 2001) e ISMap02 (Sevilla *et al.*, 2014; Stabel y Bannantine, 2005) o el gen F57 (Bosshard *et al.*, 2006; Schönenbrücher *et al.*, 2008; Tasara y Stephan, 2005). Cabe destacar que la utilización de genes que se encuentran en una sola copia en el genoma de *Map*, como es el caso del gen F57 (Vansnick *et al.*, 2004) ha permitido desarrollar la PCR cuantitativa (*quantitative PCR*, qPCR) (Schönenbrücher *et al.*, 2008) que ha servido de base para desarrollar kits que permiten determinar el grado de infección (ParaTBKuanti-VK).

II.1.5.3 Diagnóstico inmunológico

Las técnicas diagnósticas orientadas a la detección de la respuesta inmune adaptativa (celular y/o humoral) producida por la infección, se utilizan ampliamente. Estas técnicas permiten realizar un diagnóstico rápido y económico tanto a nivel individual como a nivel de rebaño.

El test de liberación de interferón-gamma (IFN-γ) es la prueba inmunológica *in vitro* más empleada para medir la respuesta inmunitaria de tipo celular. Esta técnica consiste

en la cuantificación de los niveles del IFN-γ liberados por los linfocitos T tras su estimulación con un derivado proteínico purificado (*Purified Protein Derivative*, PPD) micobacteriano; generalmente de *Map* (PPD johnina) o *M. avium* (PPD aviar) (Billman-Jacobe *et al.*, 1992). En vacuno, esta prueba debe ser empleada en animales mayores de quince meses (Jungersen *et al.*, 2002), ya que en terneros carece de especificidad a consecuencia de la liberación inespecífica de esta citoquina por parte de las células NK (Nielsen, 2010; Olsen *et al.*, 2005). Esta técnica permite detectar animales en fase de infección temprana (Jungersen *et al.*, 2002), estimándose que la sensibilidad y la especificidad son del 41% y 98% respectivamente en la fase subclínica de la infección (Nielsen y Toft, 2008).

Otra alternativa para evaluar la inmunidad celular *in vivo* frente a *Map* es la prueba de la intradermorreacción (IDR) simple o comparada, que permite cuantificar la respuesta de hipersensibilidad retardada de tipo IV que desarrolla el ganado tras la inoculación intradérmica de la PPD johnina o de la PPD aviar (Benedictus y Kalis, 2003; Garrido *et al.*, 2002). Aunque la IDR es una herramienta clave para la erradicación de la tuberculosis bovina, la adaptación de la técnica al diagnóstico de la paratuberculosis bovina presenta una baja sensibilidad y especificidad (Olsen *et al.*, 2002), por lo que su uso es cada vez más restringido (Kalis *et al.*, 2003).

Existen otras técnicas más complejas que miden la inmunidad celular como el test de transformación linfocitaria, la inhibición de la migración linfocitaria (Bendixen, 1978; de Lisle y Duncan, 1981) o el test de detección del receptor de IL-2 (Whist *et al.*, 2000), que son únicamente empleadas con fines científicos.

La respuesta humoral frente a *Map* se evalúa generalmente mediante el test de ELISA. Este test permite medir el nivel de anticuerpos circulantes frente a *Map* en suero o plasma sanguíneo, aunque también se ha desarrollado para la detección de anticuerpos anti-*Map* en muestras de leche de tanque o a nivel individual (Nielsen y Toft, 2008). Aunque se han ensayado diversos antígenos para la detección de la respuesta inmune humoral, los más ampliamente utilizados son el polisacárido Lipoarabinomanano (LAM) (Sweeney *et al.*, 1994), y el antígeno protoplasmático 3 (PPA-3) (Yokomizo *et al.*, 1983).

Esta técnica diagnóstica es rápida, barata y permite la automatización por lo que su uso está muy extendido. Su utilización está enfocada a la detección de la infección en los animales excretores o en aquellos que presentan clínica o enteritis de tipo severo (Garrido *et al.*, 2000; Harris y Barletta, 2001; Vázquez y Garrido, 2012). Sin embargo, la sensibilidad de esta técnica ronda el 45%, con valores extremos de un 15% y 87% para animales con enfermedad subclínica y enfermos clínicos respectivamente. Aunque la especificidad diagnostica se considera mayor, esta puede fluctuar del 40 al 100% en suero, y del 83 al 100% en leche (Nielsen y Toft, 2008). Con el fin de reducir el número de falsos positivos debidos a similitudes entre *Map* y otras micobacterias ambientales se añade una fase previa de preadsorción de los sueros problema con una micobacteria ambiental (*Mycobacterium phlei)*, eliminando gran parte de las reacciones cruzadas incrementando así la especificidad de la técnica (Olsen *et al.*, 2002; Yokomizo *et al.*, 1985).

La implantación de esta técnica ha reemplazado el uso de otros métodos para la detección de la respuesta humoral como la fijación de complemento (Juste y Aduriz, 1990; Kalis *et al.*, 2002) y la inmunodifusión en gel de agar (IDGA) que llegaron a ser bastante populares e incluso oficiales.

II.1.5.4 Técnicas diagnósticas emergentes

El avance científico ha conllevado el desarrollo de nuevas técnicas diagnósticas, las cuales podrían aplicarse al estudio de la paratuberculosis ya que han mostrado ser útiles en el diagnóstico de otras enfermedades complejas como la tuberculosis (Lamont *et al.*, 2014) o la enfermedad de Crohn (Zahm *et al.*, 2011).

Recientemente se ha revisado la aplicación de varias técnicas diagnósticas emergentes en el diagnóstico de la paratuberculosis (Britton *et al.*, 2016). Algunas de las técnicas que se podrían utilizar en un futuro en el diagnóstico de la paratuberculosis podrían ser: el análisis de viabilidad mediante bacteriófagos (Botsaris *et al.*, 2013; Swift y Rees, 2013); el uso de nuevos antígenos recombinantes específicos de *Map* en el test de ELISA (Biet *et al.*, 2008) y en el de IFN-γ (Hughes *et al.*, 2013; Mikkelsen *et al.*, 2011); la utilización de marcadores proteicos del hospedador (You *et al.*, 2012); los análisis transcriptómicos (David *et al.*, 2014a, 2014b); el análisis de micro RNA (miRNA) (De

Guire *et al.*, 2013) y por último el análisis de la microbiota (Derakhshani *et al.*, 2014). Actualmente, algunas de estas técnicas solo son aplicables en investigación debido a su alto coste económico y complejidad analítica. Sin embargo, en un futuro podrían dar solución a los inconvenientes que presentan las técnicas clásicas de diagnóstico.

De entre estas técnicas, cabría destacar el atractivo del análisis de la microbiota, puesto que la infección paratuberculosa afecta a un aparato, el digestivo, en el que la flora bacteriana juega un papel indiscutible. En este sentido, la interacción de dicho aparato con la dieta, se viene postulando cada vez más ampliamente que tiene lugar en gran medida a través la modificación de la microbiota. Esto se debe a que el estudio y caracterización de ésta ha experimentado un avance espectacular en los últimos años, debido a la irrupción en el mercado de los secuenciadores masivos. El empleo de esta tecnología tiene como ventaja el poder estudiar la comunidad microbiana no cultivable, superando las limitaciones de los métodos microbiológicos convencionales. En los últimos años se han abaratado los costes sustancialmente y se ha mejorado el aparataje dotándolo de una mayor capacidad analítica. A pesar de ello sigue siendo una técnica costosa, tanto desde el punto de vista económico como analítico.

Los estudios metagenómicos de la microbiota digestiva pueden estar enfocados a la caracterización de la comunidad bacteriana presente en una muestra, mediante la secuenciación del ARNr 16S o a la secuenciación de la totalidad del ADN presente en la muestra. Este último tipo de estudios permite identificar todos los microorganismos presentes en la muestra (procariotas y eucariotas), además de permitir realizar un estudio completo de su composición genética, permitiendo caracterizar la funcionalidad de la comunidad microbiana.

Se ha sugerido que la secuenciación del ARNr 16S podría utilizarse en el diagnóstico indirecto de la paratuberculosis (Derakhshani *et al.*, 2014), aunque podría tener un mayor potencial en el estudio de la progresión de la infección. Actualmente se sabe que la microbiota intestinal juega un papel importante en la exclusión competitiva de patógenos y en el desarrollo y la maduración de la inmunidad de la mucosa intestinal (Kau *et al.*, 2011; Stecher y Hardt, 2011). Así mismo, son varios los estudios que han descrito diferencias en la composición de las comunidades microbianas entre hospedadores enfermos y sanos (Clemente *et al.*, 2012; Karlsson *et al.*, 2013; Knights *et*

al., 2013). Por lo tanto, la alteración del microbiota no es solo un marcador de enfermedad, sino que puede contribuir activamente en la patogenia de la enfermedad (Chassaing *et al.*, 2012).

El análisis de la microbiota digestiva ha adquirido notable relevancia en el estudio de otras enfermedades gastrointestinales en los últimos años. Así, se ha observado que los pacientes de Crohn presentan unos índices de diversidad microbiana digestiva significativamente menores que los individuos sanos (Dicksved *et al.*, 2008; Manichanh *et al.*, 2006). Estos avances, han conducido al estudio de terapias orientadas al restablecimiento de la microbiota digestiva para el control de la enfermedad. En este sentido, los trasplantes fecales, han mostrado unos resultados prometedores en el tratamiento de enfermedades intestinales crónicas, como la colonización por *Clostridium difficile* (Aroniadis *et al.*, 2015) o la mencionada enfermedad Crohn (Suskind *et al.*, 2015).

Por otro lado, la composición de la microbiota digestiva está estrechamente ligada a la dieta ingerida. La dieta, tiene la capacidad de modificar la comunidad microbiana del aparato digestivo (Graf *et al.*, 2015; Xu y Knight, 2014), pero además también ejerce un efecto directo sobre el sistema inmunitario. En un estudio reciente en ratones, se ha observado que la dieta ejerce un efecto en la microbiota digestiva modulando las enfermedades inmunomediadas. En ese estudio la dieta no ejerció ningún efecto en los ratones libres de gérmenes (Ooi *et al.*, 2014).

Una revisión reciente ha concluido que la ingesta diaria de altos niveles de grasas totales, ácidos grasos poliinsaturados, ácidos grasos omega-6 y carne se asociaron con un mayor riesgo de padecer la enfermedad de Crohn y colitis ulcerosa. La ingesta de una dieta con un alto contenido en fibra y frutas se asoció con una disminución del riesgo de padecer la enfermedad de Crohn, y un consumo elevado de hortalizas se asoció con una disminución del riesgo de padecer colitis ulcerosa (Hou *et al.*, 2011). Si estos hallazgos se llegasen a confirmar en un futuro, pondrían de manifiesto que la composición de la dieta ejerce un papel importante en el establecimiento de las enfermedades inflamatorias intestinales.

II.1.6. TRATAMIENTO Y CONTROL

II.1.6.1 Quimioprofilaxis

Actualmente no existe un tratamiento eficaz para la paratuberculosis. Sin embargo, existen diversos agentes farmacológicos que pueden disminuir la sintomatología clínica. Los principales fármacos con acción bactericida o bacteriostáticos se podrían emplear de forma combinada (St Jean, 1996), habiéndose descrito que algunos de los más eficaces son la isoniacida, rifampicina, clofazimina, antibióticos aminoglucósidos (streptomicina, kanamicina, gentamicina y amikacina) y dapsona (DDS), siendo los tres primeros los más relevantes (Fecteau y Whitlock, 2011).

En los últimos años se han realizado ensayos *in vitro* para evaluar la eficacia de sustancias antimicrobianas empleadas en el tratamiento de la enfermedad de Crohn como el metotrexato (Greenstein *et al.*, 2007a), 6 mercaptopurina (6-MP) (Greenstein *et al.*, 2007a; Krishnan *et al.*, 2009b), ciclosporina A, rapamicina, tacrolimus (Greenstein *et al.*, 2008) o ácido 5 aminosalicílico (5-ASA) (Greenstein *et al.*, 2007b). Otra de las sustancias que ha mostrado una efectividad parcial en el control de la infección paratuberculosa es el antibiótico iónoforo Monensina, el cual introducido como aditivo en la dieta disminuye la carga bacteriana fecal y hace que la susceptibilidad de las terneras a la infección disminuya (Fecteau y Whitlock, 2011; Greenstein *et al.*, 2009; Hendrick *et al.*, 2006a, 2006b). Sin embargo, esta sustancia, se encuentra prohibida como aditivo en las dietas del ganado bovino en la Unión Europea desde el año 2006 (Reglamento (CE) Nº 1831/2003, artículo 11).

Actualmente la terapia antimicrobiana no se considera una opción viable para el tratamiento de la infección por *Map* porque resulta un procedimiento caro, es preciso administrar los fármacos durante un largo periodo de tiempo y cuando cesa el tratamiento la enfermedad recurre (Stabel, 1998). Además se añade la dificultad de que no existe un único fármaco totalmente eficaz (Aduriz *et al.*, 2000; St-Jean y Jernigan, 1991), por lo que tradicionalmente su uso sólo se ha visto justificado únicamente en animales de alto valor genético reproductivo o productivo (Fecteau y Whitlock, 2011; Harris y Barletta, 2001). Además, se debe de considerar siempre que los animales tratados pueden ser una fuente de contagio para el resto de la explotación y su aplicación en animales destinados a consumo humano resulta cuestionable, dada la

creciente preocupación por el desarrollo de resistencias antimicrobianas de diversos patógenos.

Debido a que a día de hoy no existe un tratamiento profiláctico efectivo y económicamente viable, recientemente se han realizado algunos ensayos de tratamiento con sustancias no estrictamente farmacológicas. Así, se han ensayado probióticos del tipo de *Dietzia* subsp C79793-74, observándose la disminución de la intensidad de la respuesta humoral anti-*Map*, la extensión de la vida productiva y la remisión de la sintomatología clínica (Click y Van Kampen, 2009, 2010; Click, 2011a, 2011b, 2012). Además, se ha descrito que diversas sustancias naturales como el carvacol o el trans-cinamaldehído, presentes en altas concentraciones en el aceite de orégano y canela (Wong *et al.*, 2008) o en el aceite de naranja bajo determinado tipo de tratamiento (Crandall *et al.*, 2012) producen la inhibición del crecimiento *in vitro* de *Map*, sugiriendo que estas sustancias podrían utilizarse como medida terapéutica.

II.1.6.2 Medidas higiénico-sanitarias

Las medidas higiénico-sanitarias constituyen un punto crítico en los programas de control y de su correcta aplicación dependerá la posibilidad de frenar la paratuberculosis en un rebaño (Groenendaal *et al.*, 2002). Por ello se recomienda su aplicación junto a la eliminación de animales excretores o la vacunación (Kalis *et al.*, 2001). Estas medidas van encaminadas a cortar el ciclo epidemiológico de Map (Kennedy y Benedictus, 2001), disminuyendo el contacto directo entre animales susceptibles y animales excretores.

Dentro de las medidas más importantes a instaurar en un rebaño con paratuberculosis, se encuentran: evitar el contacto de los purines con los animales, alimentos y bebida (Goodger *et al.*, 1996); separar la zona de partos del resto de la explotación (Kalis *et al.*, 2001); separar los recién nacidos de las madres lo antes posible y mantenerlos en instalaciones y pastos separados al menos durante los 6 primeros meses de vida (Collins *et al.*, 1994; Goodger *et al.*, 1996), o incluso hasta los 12 meses de edad (Windsor y Whittington, 2010); el aislamiento de los animales con sintomatología clínica (Chiodini *et al.*, 1984; Goodger *et al.*, 1996; Muskens *et al.*, 2003); e introducir en la explotación

únicamente animales no infectados y a ser posible de explotaciones libres de la enfermedad (Aduriz *et al.*, 2000; Carpenter *et al.*, 2004; Chiodini *et al.*, 1984).

Debido a que las medidas higiénico-sanitarias no se implementen por igual en todas las explotaciones, recientemente se ha propuesto su inclusión en análisis de peligros y puntos de control críticos (APPCC) de las explotaciones (McAloon *et al.*, 2015). Por lo tanto se estima crucial la concienciación e implicación de los ganaderos y veterinarios (Benedictus y Kalis, 2003). No obstante, cabe destacar que esta estrategia no es efectiva como único método de control (Ritter *et al.*, 2016).

II.1.6.3 Control mediante eliminación de animales infectados

El diagnóstico y sacrificio de los animales infectados (saneamiento o también llamado "*test and cull*" en inglés) es uno de los métodos más utilizados en los programas de control de la paratuberculosis. El objetivo consiste en llegar a la erradicación de la infección en el rebaño (Kalis *et al.*, 2001), pero recientemente se ha señalado que con esta estrategia es muy poco probable lograr la erradicación (Smith *et al.*, 2015). Esta estrategia de control ha sido empleada con resultados poco definidos en diferentes países de Europa como el Reino Unido (Wilesmith, 1982), Dinamarca (Jørgensen *et al.*, 1984), Holanda (van Schaik *et al.*, 1996), Francia (Guilbert *et al.*, 1999), Suecia (Sternberg y Viske, 2003) así como en EEUU (Whitlock *et al.*, 1994), pero los resultados muestran que se está lejos de la erradicación de la enfermedad.

Con este tipo de programas se pretende eliminar de la explotación aquellos animales que estén infectados, especialmente los que excretan grandes cantidades de micobacterias, ya que son los que suponen una mayor fuente de contagio (Dorshorst *et al.*, 2006). Sin embargo, debe tenerse en consideración también que la eliminación de animales que excretan pequeñas cantidades de micobacterias podría ser más costosa que las nuevas infecciones que éstos podrían causar (Dorshorst *et al.*, 2006).

Para que este procedimiento de control sea efectivo, es necesario que las pruebas de diagnóstico utilizadas sean sensibles y especificas (Whitlock *et al.*, 2000) y no se espacien mucho en el tiempo (Lu *et al.*, 2008). El cultivo fecal ha sido la base de varios programas de control durante muchos años, pero, debido al largo tiempo de incubación

que requiere para la obtención de resultados, ha sido sustituido por otras técnicas como el ELISA o la PCR (Geraghty *et al.*, 2014; Sevilla *et al.*, 2014). Se ha sugerido, que la estrategia más efectiva para reducir la prevalencia a nivel de rebaño sería el desvieje tras el primer ELISA positivo (Kudahl *et al.*, 2011). Sin embargo, algunos problemas añadidos a la utilización del ELISA son las diferentes especificidades y sensibilidades de los diferentes kits comerciales (Diéguez *et al.*, 2009; Garrido *et al.*, 2002) y la influencia que el manejo de las muestras tiene sobre los resultados de esta técnica (Alinovi *et al.*, 2009b). La utilización de la técnica de la PCR a partir de materia fecal permite obtener resultados de forma rápida, pero debe de tenerse en cuenta que con esta técnica se podría detectar ADN de *Map* en heces de animales que sin estar infectados han ingerido micobacterias en un medio altamente contaminado (Sweeney *et al.*, 1992a).

Dependiendo del objetivo de control, de los recursos económicos y de personal, y del tiempo para conseguir dichos propósitos se pueden establecer diferentes niveles de actuación en las explotaciones (Alonso-Hearn *et al.*, 2012; Kudahl *et al.*, 2011). Por ello, algunos autores proponen un programa de control básico frente a *Map,* que podría utilizarse como un referente internacional mínimo y que constaría de tres pasos: 1) Diagnóstico de todos los casos de diarrea y desvieje de los animales con clínica, 2) Instauración de medidas higiénico-sanitarias (Intensidad variable dependiendo de los recursos), 3) Seguimiento periódico del estatus sanitario del rebaño, con especial atención a los animales eliminadores (Khol y Baumgartner, 2012).

II.1.6.4 Vacunación

La primera vacuna frente a paratuberculosis fue desarrollada por Vallée y Rinjard en 1926. Esta vacuna, que consistía en una cepa atenuada de *Map* junto con tres sustancias adyuvantes (parafina líquida, aceite de oliva y polvo de piedra pómez) fue probada en la especie bovina. Fue en este primer ensayo donde se observó que la vacunación disminuía la aparición de la sintomatología clínica y el número de animales excretores (Vallée y Rinjard, 1926).

Actualmente son numerosos los estudios en los que se describe una reducción en la sintomatología asociada a la paratuberculosis en rebaños donde se procede a la

vacunación (Alonso-Hearn *et al.*, 2012; Bastida y Juste, 2011; Fridriksdottir, 2000; Griffin *et al.*, 2009; Gwozdz *et al.*, 2000; Harris y Barletta, 2001; Santema *et al.*, 2013; van Schaik *et al.*, 1996; Singh *et al.*, 2013a; Wentink *et al.*, 1994). Además, se ha observado una disminución en la excreción de *Map* a través de las heces (Alonso-Hearn *et al.*, 2012; Dhand *et al.*, 2013; Dimareli-Malli *et al.*, 1997; Eppleston *et al.*, 2011; Griffin *et al.*, 2009; Harris y Barletta, 2001; Hüttner *et al.*; Juste *et al.*, 2009a; Knust *et al.*, 2013; Körmendy, 1994; Kreeger, 1991; Reddacliff *et al.*, 2006; Singh *et al.*, 2007, 2013a; Tewari *et al.*, 2014; Windsor *et al.*, 2014), una disminución en la colonización de tejidos intestinales tras su exposición a *Map* (Alonso-Hearn *et al.*, 2012; Brotherston *et al.*, 1961; Hines *et al.*, 2007; Hore *et al.*, 1971; Juste *et al.*, 1994; Kathaperumal *et al.*, 2008, 2009; Sweeney *et al.*, 2009; Uzonna *et al.*, 2003) y una disminución de la frecuencia de presentación de lesiones histopatológicas graves (Corpa *et al.*, 2000b; Reddacliff *et al.*, 2006).

Las vacunas basadas en células enteras han demostrado que incrementan tanto la inmunidad humoral como la celular en bovino (Kohler *et al.*, 2001; María Muñoz de Frutos, 2014; Muskens *et al.*, 2002; Stabel *et al.*, 2011), ovino (Begg y Griffin, 2005; Corpa *et al.*, 2000b) y caprino (Hines *et al.*, 2014). En consecuencia, la vacunación se ha asociado con una reducción de la mortalidad debida a esta enfermedad (Corpa *et al.*, 2000c; Eppleston *et al.*, 2004; Reddacliff *et al.*, 2006) y se ha utilizado como herramienta en el control de la enfermedad a nivel de rebaño.

Aunque es ampliamente aceptado que la vacunación no proporciona una protección completa frente a la infección (Reddacliff *et al.*, 2006; Windsor y Eppleston, 2006), se acepta generalmente que tiene un claro efecto de aumento de la resistencia a la progresión a la enfermedad clínica. Los efectos positivos de la vacunación son notables en infecciones clínicas y aunque en menor medida también en infecciones subclínicas (Reddacliff *et al.*, 2006), resultando en balances económicos rentables (Cho *et al.*, 2012; Juste, 2012) incluso teniendo en cuenta los costes indirectos producidos por la posibles reacciones cruzadas con el diagnóstico de la tuberculosis bovina en áreas donde la prevalencia de la tuberculosis es baja (Groenendaal *et al.*, 2015).

La posible interferencia de la vacunación con el diagnóstico oficial de la tuberculosis bovina, debida a una reactividad cruzada entre antígenos micobacterianos (Muskens *et*

al., 2002), ha hecho que su uso en el ganado vacuno haya sido limitado. Actualmente se están ensayando nuevos antígenos más específicos para la detección de animales infectados de tuberculosis entre animales vacunados de paratuberculosis (Casal *et al.*, 2012; Pérez de Val *et al.*, 2012). Además, hay estudios que sugieren que mediante el empleo de la intradermorreacción comparada (IDRc) se pueden diferenciar los animales vacunados de paratuberculosis de los infectados de tuberculosis (García Marín *et al.*, 1999; Garrido *et al.*, 2013; Nedrow *et al.*, 2007). La IDRc es un test oficial de acuerdo a la OIE y a la legislación de la UE y consiste en la estimulación simultánea con dos inyecciones intradérmicas en diferentes sitios con PPD bovina y PPD aviar. Si se obtiene una mayor reactividad a la PPD aviar que a la PPD bovina indicaría una infección o vacunación con una micobacteria aviar y descartaría una infección tuberculosa (Bastida y Juste, 2011).

En pequeños rumiantes, se viene empleando la vacunación desde hace muchos años, llegando incluso a utilizarse en los programas de control oficiales de algunos países, como Islandia (Fridriksdottir, 2000), Noruega (Saxegaard y Fodstad, 1985), o Australia (Kennedy y Citer, 2010). Concretamente, en Islandia la obligatoriedad de vacunar a todas las corderas de reposición en las áreas infectadas, ha contribuido significativamente a suprimir el impacto económico de la enfermedad (Fridriksdottir, 2000). En España, en los últimos años se ha observado un incremento en el empleo de la vacunación frente a *Map* como medida de control en la cabaña caprina (Lozano De Arcenegui *et al.*, 2012).

Generalmente la vacunación se aconseja en animales jóvenes, basándose en que de esta manera los animales desarrollarían una respuesta inmune protectora en el momento de entrar en contacto por primera vez con la micobacteria (Bastida and Juste, 2011; Larsen *et al.*, 1964; Saxegaard and Fodstad, 1985). Sin embargo, en diversos estudios se ha visto que los efectos beneficiosos pueden observarse también vacunando a animales adultos (Alonso-Hearn *et al.*, 2012; Corpa *et al.*, 2000c; Santema *et al.*, 2013; Singh *et al.*, 2010, 2013a).

De hecho, diferentes estudios (Gwozdz *et al.*, 2000; Windsor, 2006), sugieren que la vacunación con células enteras podría tener un efecto terapéutico, reduciendo la carga bacteriana en tejidos. Además, otros trabajos han reportado que la vacunación en

animales adultos induce una mayor respuesta inmune (Corpa *et al.*, 2000b), disminuye la enfermedad clínica (Singh *et al.*, 2010, 2013), mejora la producción láctea (Juste *et al.*, 2009; Tamayo *et al.*, 2010) y la longevidad de los animales (Alonso-Hearn *et al.*, 2012; Santema *et al.*, 2013).

Debido a las características de las infecciones micobacterianas, disponer de una vacuna con efecto terapéutico sería de gran ayuda para el control de la diseminación de la infección. Sin embargo, la infección previa de los animales puede producir una respuesta inmune exagerada, produciendo lesiones severas e incluso la muerte, hecho que se observó al inicio de la utilización inmunoterapéutica de la tuberculinización de manera indiscriminada en humanos (Vilaplana y Cardona, 2010). Poco después se descubrió que la dosis del inmunógeno juega un papel determinante (Vilaplana y Cardona, 2010).

Actualmente, según recoge una revisión sistemática, algunas vacunas frente a la tuberculosis que se encuentran en fases avanzadas de estudio muestran un efecto terapéutico incluso en infecciones latentes (Gröschel *et al.*, 2014).
Aunque inicialmente frente a la paratuberculosis se utilizaban sobre todo vacunas vivas, actualmente todas las vacunas que se comercializan son vacunas inactivadas. Estas son: Gudair® (CZV, Porriño, Spain); Mycopar® (Boehringer Ingelheim Vetmedica) y Silirum® (CZV, Porriño, Spain). Todas ellas llevan como adyuvantes emulsiones de aceites minerales, capaces de inducir una respuesta inmune mayor y más persistente. Este tipo de adyuvante induce la formación en el punto de inoculación de un nódulo fibroso con cavidades que contienen material necrótico, que en ocasiones puede salir al exterior por medio de una fístula (García Marín *et al.*, 1999; Hines *et al.*, 2007). Un estudio sobre el impacto económico de este tipo de lesiones a nivel de matadero concluyó que estas lesiones no tiene un efecto significativo en la devaluación de la canal y por lo tanto no tiene una repercusión económica (Eppleston y Windsor, 2007).

Las vacunas que actualmente se comercializan se administran vía subcutánea, y a pesar de que se han evaluado diferentes rutas de administración como la vía oral (Hines *et al.*, 2014), la vía conjuntival (Marly *et al.*, 1988) o la intramuscular (Körmendy, 1994), la vía subcutánea es la que mayor nivel de protección ha mostrado. No obstante, recientemente se ha estudiado en ovino una nueva vacuna formulada en lípido

metabolizable de administración intraperitoneal que permite eliminar las reacciones en el sitio de inoculación, además de inducir una buena respuesta celular (Griffin *et al.*, 2009).

Un problema asociado a la vacunación que se debe de tener en cuenta es la auto-inyección de la persona que realiza la inoculación, ya que puede ocasionar una inflamación granulomatosa prolongada en el lugar del incidente, requiriéndose la escisión quirúrgica e incluso drenaje del material inoculado para evitar una extensa necrosis (Richardson *et al.*, 2005; Windsor *et al.*, 2005). A pesar de todos los inconvenientes anteriormente señalados, la vacunación es una de las mejores estrategias de control de la paratuberculosis disponibles en la actualidad (Bastida y Juste, 2011; Juste, 2012).

En los últimos años, debido a que el impacto económico de la paratuberculosis sigue en aumento, se han realizado numerosos esfuerzos en el desarrollo de vacunas más efectivas y que no produzcan reacciones cruzadas en el diagnóstico de las infecciones micobacterianas. Así, algunos grupos científicos han mostrado su interés por las vacunas vivas atenuadas (Ghosh *et al.*, 2015). En los últimos años, muchos estudios han utilizado la mutagénesis para atenuar la virulencia bacteriana. Esta es llevada a cabo mediante la utilización de fagos, transposones o a través del intercambio alélico (Park *et al.*, 2016).Las vacunas vivas atenuadas tiene la ventaja de que estimulan tanto la respuesta innata como la adaptativa (Park, 2016). Su producción es menos laboriosa y con un menor coste-beneficio que otras vacunas como las de subunidades (Ghosh *et al.*, 2015). Sin embargo, generalmente las vacunas atenuadas interfieren con el diagnostico rutinario de la paratuberculosis y tuberculosis y suponen un riesgo potencial de reversión a la patogenicidad.

Las vacunas de subunidades, están formadas por proteínas recombinantes de *Map* o ADN que codifica para antígenos inmunogénicos. Este tipo de vacunas pretenden solventar el problema de las reacciones cruzadas en el diagnóstico además de estimular la respuesta inmunitaria del hospedados (Rosseels y Huygen, 2008). A pesar de que se han identificado varias proteínas para su uso como vacunas, una de la más extensamente estudiada es la proteína Hsp70. La vacunación con esta proteína ha inducido una reducción en la carga bacteriana en infecciones experimentales (Koets *et al.*, 2006).

Además, se ha reportado que con la adición de un paso previo de adsorción con la proteína Hsp70 en las técnicas serológicas, se consigue disminuir la reactividad cruzada frente a *Map*, y que la intradermorreacción para el diagnóstico de la tuberculosis no muestra reacciones cruzadas (Santema *et al.*, 2009).

Las vacunas de ADN han mostrado resultados prometedores y tienen la ventaja de que son muy estables, lo que facilita el almacenamiento y distribución. Sin embargo, han sido únicamente evaluadas en el modelo de ratón (Park, 2016).
Generalmente la evaluación de las vacunas se lleva a cabo en rumiantes, sin embargo, debido al coste de producción de algunas de las vacunas ensayadas en los últimos años, se ha comenzado a utilizar otros modelos animales más económicos. Así, el uso del modelo murino para el estudio de productos vacunales se ha ido extendiendo (Chen *et al.*, 2008; Ghosh *et al.*, 2015), a pesar de que este modelo animal no reproduce las caracteristicas tipicas de la infeccion. Por lo tanto los resultados obtenidos podrían estar sesgados y podrían no observarse los mismos efectos en rumiantes en condiciones de campo.

II.1.7. MODELOS ANIMALES DE INFECCIÓN EXPERIMENTAL

La utilización de modelos animales para el estudio de la paratuberculosis tiene la ventaja de que permite estudiar los diferentes factores que pueden influir en la infección de una forma controlada. El éxito del modelo animal en el caso de la paratuberculosis se encuentra influenciado por la susceptibilidad a la infección inherente a los propios animales (Singh *et al.*, 2013b) y el largo periodo de tiempo necesario para desarrollar lesiones evidentes o signos clínicos (Chiodini *et al.*, 1984; Clarke, 1997), siendo frecuente que haya animales que no muestren signos clínicos de infección (Gilmour *et al.*, 1977).

Por otro lado, el poder reproducir la paratuberculosis en animales en condiciones experimentales depende de factores como el tipo del inóculo, la dosis, la vía de administración, el número de infecciones e intervalo de tiempo que transcurre entre ellas, así como de la selección de los animales (Begg y Whittington, 2008). Debido a que la paratuberculosis es una enfermedad que afecta principalmente a los rumiantes se ha reproducido experimentalmente en bovinos, ovinos, caprinos y cérvidos. En la Tabla

IV se recogen las principales características de algunos de los estudios realizados en estas especies.

La infección experimental con *Map* en rumiantes domésticos ha contribuido enormemente a los avances en el conocimiento de la enfermedad. Entre otros aspectos, ha permitido estudiar la respuesta génica del hospedador a la infección (Khare *et al.*, 2012), la eficacia de prototipos vacunales (Hines *et al.*, 2014) e incluso la identificación de biomarcadores bacterianos (Facciuolo *et al.*, 2013) y del hospedador (David *et al.*, 2014a). Sin embargo, los rumiantes requieren de amplias instalaciones para la realización de los ensayos experimentales, lo que unido al coste económico de los propios animales y de su mantenimiento (considerando que se requieren varios años para poder observar sintomatología clínica) hace que los costes sean difíciles de asumir y que el avance en el conocimiento de la enfermedad se vea ralentizado.

Tabla IV. Principales características de infecciones experimentales llevadas a cabo en rumiantes.

Animal	Cepa	Edad	Ruta	Duración	Sintomatología	Infeccion[a]	Referencias
Bovino	Bovina	1 m	iv	4 años	66,6 %	100%	(Deans Rankin, 1958)
Bovino	Ovino	6-8 s	Oral	32-34 m	60 %	0 %	(Gilmour y Angus, 1976)
Bovino	Bovino	21 d	Oral/iv/sc	153 d	-	100 %	(Johnson *et al.*, 1977)
Bovino	-	3-4 años	Intrauterina	1 d- 4 s	0 %	38 %	(Merkal *et al.*, 1982)
Bovino	-	2 s	Oral	28-33 m	30 %	70 %	(Lepper *et al.*, 1989)
Bovino	Conejo	1-5d	Oral	6 meses	0 %	87,5 %	(Beard *et al.*, 2001c)
Bovino	Bovino	2 s	Intratonsilar	320 d	0 %	100 %	(Waters *et al.*, 2003)
Bovino	Bovino	2 d	Oral	21-49 d	0 %	100 %	(Uzonna *et al.*, 2003)
Bovino	Bovibo/Bison	2 s p.d	Sondaje gástrico	6 m	0 %	83-100 %	(Stabel *et al.*, 2003)
Bovino	Bovino	4-5 s	Sc	150 d	0 %	-	(Simutis *et al.*, 2005)
Bovino	Bovino	21 d	Oral	21-23 d	0 %	100 %	(Sweeney *et al.*, 2006)

Animal	Cepa	Edad	Ruta	Duración	Sintomatología	Infeccion[a]	Referencias
Ovino	Ovino	5 m	Oral	5-27 m	3 %	63 %	(Gilmour *et al.*, 1977)
Ovino	Bovino	4 m	Oral	15-220 d	-	100 %	(Juste, 1990)
Ovino	Ovino	4-6 s	Sondaje gástrico	8-12 m	50 %	64 %	(Gwozdz *et al.*, 2000)
Ovino	Ovino	3-4 m	Oral	7-14 s	0 %	33 %	(Reddacliff y Whittington, 2003)
Ovino	Ovino	8-12 s	Oral	10-330 d	15 %	35 %	(Kurade *et al.*, 2004)
Ovino	Ovino	3 m	Intratonsilar	7-16 m	0 %	66 %	(Begg *et al.*, 2005)
Caprino	Caprino	5-8 s	Oral	106-117 s	14 %	71 %	(Storset *et al.*, 2001)
Caprino	Caprino	7-10 s	Oral	15-270 d	40 %	10 %	(Munjal *et al.*, 2005)
Caprino	Bovino	5 m	Oral	54 m	40 %	100 %	(Stewart *et al.*, 2006)
Cérvido	Bovino	4 m	Oral	44 s	-	50-100 %	(O'Brien *et al.*, 2006)
Cérvido	Bovina	3-15 m	Oral	53-65 s	0-37 %	94-100 %	(Mackintosh *et al.*, 2010)

[a]: Determinado mediante cultivo de tejidos o histopatología; -: Dato no reportado; pd: postdestete; d: día, s: semana; m: mes; iv: intravenoso, sc: subcutáneo.

Para lograr un avance más rápido y con idea de reducir los costes asociados a los modelos experimentales de rumiantes se han realizado ensayos de infección con *Map* en diferentes animales de laboratorio que además presentan una mayor facilidad de manejo como hámsteres (Francis, 1943; Larsen y Miller, 1978), cobayas (Francis, 1943; Merkal *et al.*, 1982), jerbos (Larsen *et al.*, 1976), ratas (Chandler, 1961; Koets, 2000), ratones (Collins *et al.*, 1983; Hamilton *et al.*, 1991; Thomsen *et al.*, 2001) y conejos (Francis, 1943; Mokresh y Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005).

Actualmente no existe un modelo animal de laboratorio establecido, aunque sin duda han sido los ratones y los conejos los que se han investigado en mayor profundidad. A pesar de que el modelo experimental murino no mimetiza fielmente la infección que se da en los rumiantes, este modelo se ha empleado para la evaluación de vacunas (Bannantine *et al.*, 2014; Chen *et al.*, 2008; Roupie *et al.*, 2012; Settles *et al.*, 2014), para testar la virulencia de mutantes de *Map* (Bannantine *et al.*, 2014; Shin *et al.*, 2006) y para el estudio de la interacción *Map*-hospedador durante la infección (Scandurra *et al.*, 2010; Shin *et al.*, 2015). Además, se ha utilizado para analizar la implicación de determinados genes del sistema inmune del hospedador en la mayor o menor susceptibilidad a la infección con *Map* (Koc *et al.*, 2014; Tanaka *et al.*, 2000) y junto con el modelo de conejo para el estudio de la antigenicidad de determinadas proteínas de *Map* que permitirían mejorar las técnicas diagnóstica (Paustian *et al.*, 2004).

La infección en ratones produce lesiones granulomatosas en el hígado a las 3-6 semanas post infección (Shin *et al.*, 2006). Asimismo, se han descrito lesiones intestinales leves consistentes en la infiltración inflamatoria de la lámina propia con presencia de BAAR en ratones inmunodeprimidos (*Severe Combined Immunodeficiency*; SCID) tras la infección intraperitoneal (Mutwiri *et al.*, 2001). A pesar de las ventajas del modelo murino, existen grandes diferencias anatómicas, fisiológicas e inmunológicas entre los rumiantes y los ratones, que hacen que este modelo animal no muestre los signos clínicos que se observan en el ganado, ni las lesiones intestinales características de la enfermedad. Por ello, el conejo supone una alternativa. En la Tabla V se recogen las principales características de ambas especies como modelos de infección de la paratuberculosis permitiendo su comparación.

Tabla V. Comparación de las principales características del ratón y conejo como modelo animal de la paratuberculosis. Adaptado de Talaat, 2010.

Características	Ratón	Conejo
Raza	C57/BL6, BALB/c, SCID	Neozelandés blanco
Vía de infección	Intraperitoneal, oral	Oral
Dosis de infección	10^7-10^9 UFC/animal	10^5-10^9 UFC/animal
Toma de muestras	Hígado, bazo, intestino, linfonodos, suero.	Hígado, bazo, intestino, linfonodos, tejido linfoide digestivo, suero.
Diarrea	Nunca	Frecuente
Excreción fecal	Raramente	Ocasionalmente
Criterios de valoración	UFC, histopatología, estudio inmunológico	UFC, histopatología, estudio inmunológico, signos clínicos
Desventajas	Ausencia de sintomatología de paratuberculosis.	Posible falta de reactivos.

Los conejos son susceptibles a la infección por *Map* en condiciones naturales (Angus, 1990; Beard *et al.*, 2001b; Greig *et al.*, 1997, 1999; Maio *et al.*, 2011), lo que hace de esta especie animal un modelo atractivo para el estudio de la paratuberculosis. Cabe destacar que los conejos son animales herbívoros no rumiantes. Su sistema digestivo está adaptado para la utilización de la fibra de manera eficiente, mediante la fermentación cecal y la cecotrofia. Por lo tanto, la fisiología digestiva de este animal es más similar a la de los rumiantes que la de otros animales monogástricos como los ratones. Además, este animal se ha utilizado y se utiliza actualmente como modelo animal de otras micobacteriosis, hecho que se ha revisado en profundidad el apartado "II.2 MICOBACTERIOSIS IN RABBITS" de la presente revisión.

A pesar de que se han realizado diversos experimentos en conejos (Mokresh y Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005), este modelo animal no se ha llegado a utilizar ampliamente por la comunidad científica. Debido al carácter crónico de la

infección para observar sintomatología clínica evidente se requieren de 5 a 20 meses (Mokresh y Butler, 1990; Mokresh *et al.*, 1989; Vaughan *et al.*, 2005). Se ha sugerido que factores estresantes como la inmunosupresión o los cambios de dieta podrían acelerar la aparición de la sintomatología clínica (Vaughan, 2005). Además, se ha comprobado en ratones que pequeños cambios en la dieta pueden disminuir las enfermedades inmunomediadas (Ooi *et al.*, 2014) poniendo de manifiesto la importancia de la dieta en la inmunidad.

Aparte de los animales de laboratorio, también se han investigado otras especies monogástricas como modelos experimentales de la paratuberculosis. Una de estas especies es el caballo, que al igual que el conejo es un animal herbívoro monogástrico. La infección experimental en potros por vía endovenosa y oral ha demostrado que ambas vías son adecuadas para conseguir la infección (Larsen *et al.*, 1972). Algunos animales infectados por vía endovenosa presentaron diarrea y adelgazamiento a los 35 y 84 días post infección, y todos los animales del experimento desarrollaron lesiones granulomatosas, acompañadas en ocasiones por una enteritis y linfadenitis granulomatosa difusa y engrosamiento de la porción distal del intestino delgado (Larsen *et al.*, 1972).

En las aves se han llevado a cabo infecciones experimentales tanto con cepas de *Map* aisladas de vacuno (Larsen y Moon, 1972; Valente *et al.*, 1997) como de pacientes con la enfermedad de Crohn (Van Kruiningen *et al.*, 1991), encontrándose lesiones principalmente en bolsa de Fabricio, hígado (Van Kruiningen *et al.*, 1991; Valente *et al.*, 1997), pero también en pulmón, ciego (Van Kruiningen *et al.*, 1991) e intestino (Valente *et al.*, 1997).

Finalmente, el cerdo también se ha utilizado como modelo de infección utilizando tanto cepas aisladas de ganado porcino (Jorgensen, 1969) como de ganado vacuno (Jorgensen, 1969; Larsen *et al.*, 1971). Aunque no se han observado signos clínicos de paratuberculosis en esta especie, si se han hallado lesiones en intestino y linfonodos asociados tras la infección oral (Jorgensen, 1969), e intravenosa, observándose en algunos casos, excreción bacteriana en heces (Larsen *et al.*, 1971).

II.1.8 IMPLICACIONES ZOONÓTICAS DE *Map*

El potencial zoonótico de *Map* ha sido tema de debate desde hace más de un siglo. La hipótesis de que *Map* podría ser el agente causal de la enfermedad inflamatoria intestinal humana fue sugerida por Dalziel en 1913, siendo revisada posteriormente por Chiodini en 1989.

El término de "Enfermedades inflamatoria intestinal" (EEI) engloba tres enfermedades humanas que tienen la característica común de presentarse en forma de inflamación crónica y local del intestino y son: 1) Enfermedad de Crohn (EC), 2) Colitis ulcerosa, 3) Colitis crónica indeterminada o sin clasificar. De estas tres enfermedades, la EC es la que más se ha relacionado con la infección por *Map*.

La sugerencia de que *Map* podría ser el agente causal de la EC fue inicialmente debida a las similitudes clínico-patológicas de esta enfermedad con la paratuberculosis. Las principales características clínico-patológicas de ambas enfermedades se resumen en la Tabla VI.

La EC presenta una mayor prevalencia en individuos de edades comprendidas entre los 15 y 30 años, si bien se ha señalado que existe una proporción cada vez mayor de personas afectadas con más de 60 años (Quezada *et al.*, 2013). Hay que destacar que la incidencia y prevalencia de esta enfermedad parece ir en aumento, debido a que se describen un número creciente de casos a nivel mundial (Molodecky *et al.*, 2012).

Actualmente la causa de la EC no ha sido esclarecida. Una de las hipótesis más aceptadas va enfocada hacia una etiología multifactorial, donde una respuesta inmune inadecuada del huésped, posiblemente desencadenada por algún agente infeccioso, tendría un papel importante en el establecimiento de la inflamación local (Atreya *et al.*, 2014; Davis, 2015; Naser *et al.*, 2014; Sechi y Dow, 2015). Se cree que otra serie de factores como la dieta (Knights *et al.*, 2013) y la predisposición genética (Quirke, 2001) juegan un papel determinante.

Tabla VI. Características clínico-patológicas de la enfermedad de Crohn y la paratuberculosis. Adaptado de Rideout *et al.*, 2003.

	Enfermedad de Crohn	**Paratuberculosis**
Síntomas clínicos		
Diarrea crónica	Sí (más de tres semanas)	Sí (no en ovino)
Dolor abdominal	Sí	Desconocido
Sangre en heces	Infrecuente	No
Obstrucción	Sí	No
Remisión y recidiva	Sí	Sí
Lesiones macroscópicas		
Localización lesional	Esófago, cavidad oral, íleon, colon, linfonodos asociado, recto y ano.	Íleon, yeyuno, linfonodos asociados y recto en casos avanzados.
Edema parietal	Sí	Sí
Perforación	Sí	En casos muy avanzados
Aspecto de la mucosa intestinal	Apariencia en forma de "adoquin".	Ondulada, aspecto "cerebriforme".
Pseudopólipos	Sí	No
Lesiones microscópicas		
Fibrosis	Sí	No
Lesiones granulomatosas	Sí	Sí
Agregados linfoides	Sí	Sí
Caseificación	No	Normalmente no (varía según especies).
Presencia de bacilos acido-alcohol resistentes	Infrecuente	Sí (infrecuente o ausente en la forma paucibacilar).

Durante muchos años se ha intentado demostrar la etiología micobacteriana en la EC, sin embargo no siempre se han obtenido resultados satisfactorios (Kallinowski *et al.*, 1998; Parrish *et al.*, 2009). Uno de los principales problemas para demostrar la relación de *Map* con esta enfermedad radica en que no se conocen en profundidad los mecanismos patogénicos de la paratuberculosis (Davis, 2015). Además, hay que tener en cuenta que *Map* no produce una infección epidémica en la cual todos los animales infectados desarrollan la enfermedad clínica (Davis, 2015). Por lo tanto no es sencillo demostrar la implicación de un agente infeccioso en una enfermedad crónica aplicando los clásicos postulados de Robert Koch (Juste Jordán, 2012). Por otra parte, las técnicas diagnósticas no han ayudado a resolver la controversia, debido a que *Map* suele encontrarse en forma de pared defectiva (esferoplastos) en los pacientes de Crohn, no siendo posible la detección mediante la tinción ZN y haciendo más complicado aún el aislamiento bacteriológico (Chiodini *et al.*, 1986; Grant, 2005) .

Aunque la controversia se ha mantenido durante más de un siglo, actualmente gracias al avance en las técnicas diagnósticas y en el conocimiento de la enfermedad, la posible asociación de *Map* con la EC cuenta con argumentos más sólidos.

Diversos metaanálisis de estudios epidemiológicos concluyen que *Map* se detecta con mayor frecuencia en el tejido intestinal de los pacientes con la EC que de voluntarios sanos (Abubakar *et al.*, 2008; Feller *et al.*, 2007; Naser *et al.*, 2014). Además, se ha demostrado que estos pacientes responden favorablemente a fármacos con actividad anti-*Map* (Borody *et al.*, 2002, 2007; Greenstein *et al.*, 2007a, 2007b, 2008, 2014; Hermon-Taylor, 2002).

Aunque el aislamiento de *Map* en cultivo bacteriológico es complicado, se ha observado una mayor frecuencia de aislamiento en biopsias de tejidos (Bull *et al.*, 2003; Mendoza *et al.*, 2010), sangre (Naser *et al.*, 2004) y heces (Sohal *et al.*, 2008) de pacientes con la EC en comparación con individuos sanos. Asimismo, en los últimos años son numerosos los estudios que han demostrado la presencia de ADN de *Map* en estos pacientes mediante el uso de técnicas moleculares. Concretamente, mediante hibridación *in situ* (HIS), se ha observado una mayor prevalencia de *Map* en las secciones histológicas de pacientes con EC que en sanos (Hulten *et al.*, 2001; Sechi *et al.*, 2001, 2004). Mediante la PCR también se ha identificado *Map* en sangre (Bentley *et*

al., 2008; Elguezabal *et al.*, 2012; Juste *et al.*, 2008, 2009b), tejidos (Bull *et al.*, 2003; Collins *et al.*, 2000; Gan *et al.*, 1997) e incluso biopsias orales (Molicotti *et al.*, 2013) de estos pacientes.

Por otra parte también hay algunos estudios en los que se detecta una mayor respuesta serológica frente a antígenos de *Map* en pacientes con EC que en personas sanas (Collins *et al.*, 2000; Olsen *et al.*, 2001; Juste *et al.*, 2007).

Sin embargo existen también otros estudios en los que no se logró identificar la presencia de *Map* en pacientes con la EC (Baksh *et al.*, 2004; Chiba *et al.*, 1998; Ellingson *et al.*, 2003; Kanazawa *et al.*, 1999) o en los que se ha identificado pero en una menor proporción que en pacientes sanos (Bernstein *et al.*, 2003; Elguezabal *et al.*, 2012; Juste *et al.*, 2008, 2009b; Parrish *et al.*, 2009; Tzen *et al.*, 2006). Además, existen estudios en los que tampoco se han detectado diferencias significativas en la respuesta inmune inducida por antígenos de *Map* entre pacientes con o sin la EC (Bernstein *et al.*, 2004; Juste *et al.*, 2009b). No obstante, debemos tener en cuenta que el tratamiento frente a la EC se asocia con una reducción en la frecuencia de detección de ADN en sangre (Elguezabal *et al.*, 2012; Juste *et al.*, 2009b).

Otro de los argumentos en contra de la asociación de *Map* con la EC es que no se ha observado mayor incidencia de casos entre ganaderos, veterinarios o cualquier personal directamente expuesto a casos clínicos de paratuberculosis (Jones *et al.*, 2006). Sin embargo recientemente en la India se ha descrito una alta prevalencia de *Map* entre cuidadores de ganado con alteraciones gastrointestinales (Singh *et al.*, 2011).

Finalmente, la EC se ha relacionado con otros agentes infecciosos como diversos virus, levaduras y bacterias entre las que se encuentran *Escherichia coli, Listeria monocytogenes, Chlamydia trachomatis, Pseudomonas maltophilia, Bacteroides fragilis* y *Mycobacterium kansasii* (Naser *et al.*, 2014). Esto pone de manifiesto que a pesar de haber cada vez mayores indicios de la asociación de *Map* con la EC, ésta actualmente no está del todo aceptada entre la comunidad científica.

Además de con la enfermedad de Crohn, *Map* se ha asociado a otras patologías inflamatorias autoinmunes como por ejemplo, la sarcoidosis (el-Zaatari *et al.*, 1996; Gupta *et al.*, 2007), la tiroiditis de Hashimoto (Masala *et al.*, 2014; Sisto *et al.*, 2010), el

síndrome Blau (Dow y Ellingson, 2011), la diabetes tipo 1 (Naser *et al.*, 2013; Sechi *et al.*, 2008) o la esclerosis múltiple (Cossu *et al.*, 2011; Frau *et al.*, 2013) aunque a día de hoy no está claro el papel que juega esta micobacteria en todas estas enfermedades. No obstante se cree que el papel de *Map* en estas enfermedades es debido a la expresión bacteriana de proteínas antigénicas similares a las del huésped, lo que produciría un desequilibrio en la respuesta inmune del hospedador (Sechi y Dow, 2015).

La confirmación del papel de *Map* en la EC, tendría una gran repercusión en la salud pública debido a la presencia de la micobacteria en la cadena alimentaria. Diversos estudios ya han confirmado la presencia de esta micobacteria en la canal de ganado vacuno (Alonso-Hearn *et al.*, 2009; Meadus *et al.*, 2008), en formulados de leche en polvo para lactantes (Botsaris *et al.*, 2016; Hruska *et al.*, 2011), el agua (Aboagye y Rowe, 2011; Pierce, 2009; Pistone *et al.*, 2012) así como en vegetales y frutas (Mihajlovic *et al.*, 2011) u otros alimentos derivados de las producciones ganaderas, fundamentalmente leche y derivados lácteos (Ellingson *et al.*, 2005; Faria *et al.*, 2014; Ikonomopoulos *et al.*, 2005; O'Reilly *et al.*, 2004), que se integran en la cadena alimenticia humana. Debido a ello, la ingestión de estos productos supondría la principal vía de exposición para el hombre. Además, hay que destacar que aunque algunos tratamientos físico-químicos que se emplean en la industria alimentaria tienen como finalidad garantizar la calidad higiénico-sanitaria de los productos, diversos estudios han evidenciado que pueden resultar insuficientes para inactivar a *Map* (Grant *et al.*, 2005; Hammer *et al.*; Pearce *et al.*, 2001; Pedley, 2013).

II.2 MICOBACTERIOSIS IN RABBITS

Transboundary and Emerging Diseases

REVIEW ARTICLE

Mycobacterial Infections in Rabbits: From the Wild to the Laboratory

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Keywords:
rabbits; animal model; mycobacteriosis

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Received for publication September 18, 2015

doi:10.1111/tbed.12474

Summary

Tuberculous mycobacterial diseases such as leprosy and tuberculosis are ancient diseases that currently continue threatening human health in some countries. Non-tuberculous mycobacterial (NTM) infections cause a series of well-defined pathological entities, as well as some opportunistic diseases that have also increased worldwide, being more common among immunocompromised patients but rising also in immunocompetent individuals. Reports on natural infections by mycobacteria in rabbits are scarce and mainly involve NTM such as *Mycobacterium avium* subsp. *avium* in pigmy rabbits in the United States and *Mycobacterium avium* subsp. *paratuberculosis* in wild rabbits in Europe. Rabbits have been used as laboratory animals through the years, both to generate immunological reagents and as infection models. Mycobacterial infection models have been developed in this animal species showing different susceptibility patterns to mycobacteria in laboratory conditions. The latent tuberculosis model and the cavitary tuberculosis model have been widely used to elucidate pathogenic mechanisms and to evaluate chemotherapy and vaccination strategies. Rabbits have also been used as bovine paratuberculosis infection models. This review aimed to gather both wildlife and experimental infection data on mycobacteriosis in rabbits to assess their role in the spread of these infections as well as their potential use in the experimental study of mycobacterial pathogenesis and treatment.

Introduction

Taxonomy

Mycobacterium is a genus of the phylum Actinobacteria that is included in the Mycobacteriaceae family of the order Actinomycetales and that is phenotypically related to the genera *Nocardia*, *Rhodococcus* and *Corynebacterium*. The genus *Mycobacterium* includes more than 150 species made up of strict and opportunistic pathogens that infect both humans and animals (http://www.bacterio.net/mycobacterium.html). Mycobacteria are Gram-positive, non-spore-forming, aerobic, immobile, intracellular and pleomorphic bacilli, that are extremely resistant to environmental conditions. The high lipid content cell wall representing 40% of the dry weight of the bacteria is responsible for the property of acid resistance in some staining techniques. Given the wide range of microbiological and clinical characteristics of mycobacteria, numerous classifications have been used to systematize their differences. Runyon (Runyon, 1959) classified non-tuberculous mycobacteria based on growth rate, yellow pigment production and light requirements for its production. Growth speed is still a useful criterion and defines two large groups: fast and slow growers. Fast growers require <7 days to produce colonies on solid agar, whereas slow growers may require from weeks to months. All pathogenic mycobacteria are slow growers.

According to clinical features in humans, pathogenic mycobacteria have been divided into three groups: (i) *M. leprae*, which causes Hansen's disease or leprosy; (ii) *M. tuberculosis* complex (MTC), that cause tuberculosis and including a variety of members isolated from a wide range of hosts such as *M. tuberculosis*, *M bovis*, *M. africanum, M. microti, M. canetti, M. caprae, M. pinnipedii*, *M. orygis*, *M. mungi*, (Rodriguez-Campos et al.,

2014) *M. suricattae* (Parsons et al., 2013) and for some authors, *M. marinum and M. ulcerans* (Stragier et al., 2005); and (iii) non-tuberculous mycobacteria (NTM), comprising *M. avium, M. intracellulare, M. marinum, M. ulcerans* and a large number of so-called atypical mycobacteria (Rastogi et al., 2001; Reavill and Schmidt, 2012). *Mycobacterium avium* complex (MAC) is a group of closely related NTM species and subspecies which includes both veterinary and opportunistic human pathogens. The most relevant are *M. avium* subsp. *avium* (Maa), *M. avium* subsp. *paratuberculosis* (Map), *M. avium* subsp. *silvaticum, M. avium* subsp. *hominisuis* and *M. intracellulare* (Brosch et al., 2001).

Mycobacteriosis in perspective

Mycobacterial diseases in humans and animals and their zoonotic potential have great relevance. Leprosy, the first discovered bacterial disease in humans, is a chronic granulomatous disease of the peripheral nerves and mucosa of the upper respiratory tract and eyes. Skin lesions are the primary external sign. Although it is an ancient disease and its global prevalence has declined from 5.2 million in the 1980s to 200 000 today (Smith and Aerts, 2014), it currently continues being an important threat to human health. Transmission of leprosy occurs between humans, but the exact mechanisms remain unknown. Recent observation of the frequent occurrence of nasal carriers (Klatser et al., 1993) suggests a respiratory route that is gaining acceptance (Job et al., 2008).

Tuberculosis (TB) is a potentially fatal contagious disease. TB is generally used to name a variety of affections caused by MTC members that can affect humans and other organisms on almost any part of the body but that tends to show up more frequently as a respiratory disease. The route of dispersion of the infection depends greatly on the host species type although in the majority of cases it is transmitted by respiratory secretions and aerosols, by oronasal contact with infected materials and foods and in some cases entrance of the microorganism is achieved via skin traumas. TB, both human, caused by MTC members, and bovine, caused by *M. bovis*, are diseases that have a high impact in human health and in the economy worldwide. According to the World Health Organization, TB affects over 8 million new people every year and ranks among the leading infectious diseases with an annual clinical incidence rate of 1% (World Health Organization, 2013). Although cattle are considered to be the true host of *M. bovis*, it is often also reported in humans and causes the most widespread mycobacterial disease as it has been observed in many other domestic and wildlife species (Francis, 1958; Pavlik et al., 2002). This carries a broad economic, biological and social impact as it limits livestock production, affects some endangered species, and exposes humans in underdeveloped countries or suffering immunocompromising conditions. These reasons have led governments to establish eradication and control programmes in animals.

Historically, human TB reached its peak in the late XVIII and early XIX centuries and when the advances in prevention diagnostics and effective anti-biotherapy led to think that it was nearly controlled. Current data show that this has not been so and that, actually, even the prevalence decrease in wealthy countries has stopped because of TB persistence in some specific social environments where multiresistant variants have arisen that challenge the efficacy of standard treatment protocols.

NTM follow a similar course with a worldwide increase during the last three decades, mainly as post-traumatic and post-surgical infections, or recently as localized and disseminated infections (Garcia Martos and García-Agudo, 2012). A variety of mycobacterial species, including *M. avium, M. simiae, M. kansasii* and *M. haemophilum* that had been considered opportunistic, have become more common among immunocompromised patients (Rastogi et al., 2001) and have recently emerged among immunocompetent individuals too (Griffith et al., 2007; Varghese et al., 2013). The study of NTM in animals has also increased in recent years because of animal health concerns and their zoonotic potential as reservoirs (Bercovier and Vincent, 2001; Durnez et al., 2008).

Map causes paratuberculosis, also named Johne's disease in domestic and wild ruminants, although non-ruminant species both domestic and wild have also shown to be susceptible (Marco et al., 2002; Alvarez et al., 2005; Reyes-Garcia et al., 2008). Paratuberculosis is primarily transmitted by the faecal–oral route, but it can also be transmitted vertically (Seitz et al., 1989; Sweeney, 1996) or by aerosolization (Eisenberg et al., 2011). Paratuberculosis is found worldwide and causes significant losses to the livestock industry through reduced production and compromised animal welfare (Barkema et al., 2010). In addition, Map has been related to Crohn's disease, type I diabetes (Sechi et al., 2008; Rosu et al., 2009) and multiple sclerosis (Cossu et al., 2011) in humans, suggesting that Map might have a possible role in public health.

Rabbits and mycobacteria: summary

The European rabbit (*Oryctolagus cuniculus*) is a species of rabbit native to south-western Europe, regions which include the Iberian peninsula (Spain and Portugal), small areas of France and north-west Africa (Morocco and Algeria). It is an invasive species, which has been introduced in all continents with the exception of Antarctica and sub-Saharan Africa (Wilson and Reeder, 1993). The domestic and laboratory rabbits were derived from the European

rabbit. New Zealand White Rabbit is the most used breed in biomedical sciences.

Rabbits are true non-ruminant herbivores. Their digestive system increases the efficiency of utilization of fibrous diets by caecum fermentation and caecotrophy. The latter is the consumption of soft faeces called caecotrophs and produced only in the early morning hours. This maximizes fibre digestion and secures a supply of vitamins of bacterial origin (Davies and Davies, 2003). Occasionally, when no other food is available rabbits might also reingest hard faeces (Hirakawa, 2001). Another rabbit physiological particularity is that they have two unique lymphoid organs associated with the gastrointestinal system that account for more than 50% of the total lymphoid tissue in these animals: the sacculus rotundus and the vermiform appendix.

Rabbits have been used as animal models in almost all areas of biomedical research, ranking just behind mice and rats in numbers. These are large enough to sample adequate quantities of tissue without the need of pooling groups, and blood sampling is easily performed; thanks to its accessible ear veins. It represents an excellent compromise between the relatively large and slow-breeding of larger domestic animals on the one hand and the relatively small and rapidly breeding rodents on the other (McMillian, 1986).

Rabbit was the species that provided first evidence of transmissibility of TB (Villemin, 1867). However, it has not been a widely used experimental species for mycobacteriosis until recently when it has been seen that rabbits can act as a carrier of mycobacteria in natural conditions and therefore has prompted the development of specific pathological models. Studies of rabbit mycobacteriosis, both natural and experimental, may help understand the mechanisms of disease resistance and latency, as well as the cofactors that shift the defensive responses from resistant or carrier states to clinical disease in slow infections where the microbial agent is necessary but not sufficient to cause disease. Rabbit models of mycobacteriosis may also aid new drug and vaccine testing and fine-tuning.

This narrative review was conducted from May 2014 to May 2015 performing a computerized search of the databases PubMed, Google Scholar, Science Direct and Web of Science using keywords: Mycobacterium, rabbit, infection and infection model. The review will focus on all the aspects mentioned before, trying to give a quick view of all the work carried out and reported in the literature.

Infection by *Mycobacterium leprae*

Natural infection

To our knowledge, there are no published cases of natural leprosy in domestic and/or wildlife rabbits. However, in a case–control study carried out with 28 leprosy patients and 59 controls, a statistical analysis showed an association between cleaning rabbits' carcasses and leprosy in humans. Subjects were evaluated for having hunted, cleaned, eaten, or having direct or indirect contact in some way with armadillo, rabbits, deer, birds and squirrels. Only those having been in contact with armadillo and rabbits showed a statistically significant association with leprosy (Clark et al., 2008).

The lack of knowledge about the transmission of leprosy requires more epidemiological surveys taking into account wild, domestic and pet rabbits.

Experimental infection

When Hansen studied leprosy in 1874 (Hansen, 1874), he tried to inoculate this unculturable pathogen into rabbits in order to demonstrate that this disease was caused by an infectious agent and that it was not a hereditary disease. In this case, he was not looking for an animal model but for a proof of concept of transmissibility. Unfortunately, Hansen failed to produce disease in rabbits. And therefore only when Neisser achieved *M. leprae* staining in the laboratory (Neisser, 1880), the scientific community accepted that the disease was produced by a microorganism. Afterwards, numerous experimental infections with *M. leprae* were carried out in rabbits with variable success (Damsch, 1883; Bayon, 1912).

The anterior eye chamber showed the best site of inoculation to develop disease although subcutaneous and intraperitoneal inoculation was also attempted (Bayon, 1912). Rabbits infected by human leprous nodules into the eye died after 139–219 days (Damsch, 1883), showing deposits of acid-fast bacilli in the spleen, liver, caecum, pleura, and pericardium where caseation and giant cells were present, thus demonstrating that a proliferation of the original micoorganisms had occurred (Bayon, 1912). These results, although proving transmissibility, resulted rather impractical and did not lead to much experimentation thereafter.

This animal model was replaced by others in which *M. leprae* replicates easily. Actually, in 1971, armadillos were found to be natural hosts susceptible to this disease and have been used to grow *M. leprae* thereafter (Storrs, 1971), causing the abandonment of the rabbit model of leprosy. From then on, rabbits have been used to produce polyclonal antibodies for diagnosis (Harboe et al., 1977; Oskam et al., 1995) and as an immunological tool for research (Araoz et al., 2006; Spencer and Brennan, 2011) in leprosy, but not as an infection model.

Infection by *Mycobacterium tuberculosis* complex

Natural Infection by *Mycobacterium tuberculosis* and *Mycobacterium bovis*

Not much work has been carried out related to the implication of wild rabbits in TB caused by *M. tuberculosis* or

M. bovis; however, it has been enough to show that it is not a common event meaning that other wildlife mammals may play a more important role in the epidemiology of this disease (Delahay et al., 2002; Gortazar et al., 2008).

Reports concerning detection of natural TB infection in rabbits include *M. bovis* isolation in a rabbit fur farm in the UK (Griffith, 1939), isolation of mycobacteria similar to *M. bovis* not confirmed by molecular typing in Ireland (Delahay et al., 2002) and the finding of one free-living wild rabbit exhibiting advanced and generalized tuberculous disease with involvement of lungs, liver, kidneys and prescapular lymph node in New Zealand (Gill and Jackson, 1993).

On the contrary, MTC was not isolated from 146 wild rabbit carcasses examined in Southern England and Wales by the Ministry of Agriculture, Fisheries and Food in the United Kingdom (MAFF, 1976–97), nor was *M. bovis* detected in over 1200 rabbits from an area in New Zealand where infection was endemic in other mammals (Allen, 1991).

The lack of evidence of TB infection in wild rabbits even in areas of high TB prevalence in ungulates has led the scientific community to think that they may be resistant or that their grazing behaviour limits the exposure to *M. bovis* (Allen, 1991).

Experimental infection models

Experimental infection of rabbits with *M. tuberculosis* or *M. bovis* produces a spectrum of disease that reproduces or resembles different stages of human disease (Manabe et al., 2003). Actually, in the early era of TB research, the rabbit model was used to differentiate between these two mycobacterial species (Soltys et al., 1952). A lot of work has been carried out from then on, and many types of models have been developed for pathogenesis elucidation and for therapy evaluation. A brief overview of these studies and the principal characteristics of the models are summarized in Table 1.

Resistance to M. tuberculosis infection: Lurie's experiments

The observation of a possible natural resistance of rabbits to *M. tuberculosis* promoted experimental studies to prove it. The most informative studies on natural resistance to

Table 1. Experimental tuberculosis infections in rabbits

Study objective	Route	Sensitization	Infection strain	Rabbit strain	Model	References
TB genetic resistance study	Aerosol	No	Mtb H37Rv	Inbred NZW	PuTB and LTB	(Lurie et al., 1952)
TB strain virulence study	Aerosol	No	Mtb Erdman, Mtb H37Rv, Mtb CDC1551	Outbred NZW	LTB	(Manabe et al., 2003)
TB latency and reactivation study	Aerosol	No	Mtb H37Rv	Outbred NZW	LTB	(Manabe et al., 2008)
Gene expression in TB persistence and reactivation	Aerosol	No	Mtb H37Rv	Outbred NZW	LTB	(Kesavan et al., 2009)
Study of spontaneous LTB	Aerosol	No	Mtb CDC1551	NZW not specified	LTB	(Subbian et al., 2012)
TB and host-response interaction	Aerosol	No	Mtb CDC1551, Mtb HN878	NZW not specified	LTB	(Subbian et al., 2013)
Molecular immunology in LTB	Aerosol	No	Mtb CDC1551	NZW not specified	LTB	(Subbian et al., 2013)
M. vaccae-based vaccine evaluation	Aerosol	No	Mbv Ravenel S	Inbred NZW Non-inbred NZW	PuTB	(Converse et al., 1998)
CTB description and analysis	Aerosol	No	Mbv Ravenel S	Outbred NZW Inbred III VO/JU	CTB	(Converse et al., 1996)
Short-course CTB rabbit model description	Bronchoscopic	Yes[a]	BCG, Mbv AF122, Mbv Ravenel, Mtb CDC1551, Mtb H37Rv	Outbred NZW	CTB	(Nedeltchev et al., 2009)
Effect of sensitization in gross pathology	Bronchoscopic	Yes[a]	Mbv AF122, Mbv Ravenel	Outbred NZW	CTB	(Jassal et al., 2011)
Liquefaction study and mycobacteria virulence evaluation	Skin	No	Mtb CDC1551, H37Ra, *M. smegmatis*	NZW not specified	Liquefaction	(Zhang et al., 2010)
Vaccine evaluation	Intrathecal	No	Mtb H37Rv	Outbred	TB Meningitis	(Tsenova et al., 2006)

Mtb, *Mycobacterium tuberculosis*; Mbv, *Mycobacterium bovis*; TB, tuberculosis; LTB, latent tuberculosis; PuTB, pulmonary tuberculosis; CTB, cavitary tuberculosis; NZW, New Zealand White Rabbit.

[a]Sensitization with heat-killed Mbv subcutaneously.

M. tuberculosis were performed by Max B. Lurie, who studied inbred rabbit families for resistance and susceptibility to TB (Lurie, 1941; Lurie et al., 1952). Lurie was successful in his breeding progeny and observed that resistant families developed cavitary TB and susceptible families developed disseminated TB, also showing that *M. bovis* could be spread by aerogenous route in a laboratory rabbit colony, most frequently resulting in involvement of the lungs, pleura, intestines and kidney (Lurie, 1944). Lurie′s studies have been revised by other scientists that have focused on the role of innate resistance concluding that because up to 20–40% of the exposed rabbits from all family groups did not develop disease at the end of the experiment, these animals were probably resistant to the infection in a manner that would be genetically different from acquired resistance (Werneck-Barroso, 1999). Lurie′s results were corroborated in a study where *M. bovis* and *M. tuberculosis* infectivity was compared in rabbits and where results showed that extrapulmonary dissemination almost exclusively occurred among rabbits infected with *M. bovis* (Ravenel and AF2122 strains) but not with *M. tuberculosis* (Nedeltchev et al., 2009).

Lurie's genetically susceptible rabbit strain has disappeared, susceptibility to TB infection has been compared in inbred and outbred New Zealand White rabbits, showing that inbred animals have an impaired ability to contain the disease, including more grossly visible and larger tubercles, a higher amount of mycobacteria per tubercle, more caseous necrosis and more visible bacilli than those observed in outbred rabbits contributing to the genetic resistance hypothesis (Dorman et al., 2004). Regarding strain origin, some studies suggest that certain clinical strains of *M. tuberculosis* that seem to be of higher virulence can establish a progressive infection in rabbits (Tsenova et al., 2006).

Latent TB model

Rabbit's resistance to *M. tuberculosis* has allowed the study of the immunological mechanisms driving latent TB. Latent TB is characterized by the absence of clinical manifestation while the *M. tuberculosis* cells are in a dormant or non-replicating stage. In this stage, individuals are not currently infectious, but may develop active disease if their immune status weakens. One-third of the world's human population is estimated to be latently infected with *M. tuberculosis*. Studies show that 5–20% of those infected people will develop active TB at some point in their lifetime, with the majority developing TB disease within 2–5 years of the initial infection (World Health Organization, 2013). Latently infected people represent an enormous reservoir of potential TB reactivation, which can spread to other people.

The evidence that *M. tuberculosis* (CDC1551)-infected rabbits achieve latent TB is based on the ability of the bacilli to be reactivated upon host immune suppression. Rabbits showed early activation of T cells, macrophages and an early peak in the *TNFα* transcription levels, which decreased in association with clearance of bacilli from the lungs (Subbian et al., 2012). Recent studies suggest that stimulation of the innate immune response is absolutely necessary for induction of adaptive immune responses that promote establishment of latent TB infection in *M. tuberculosis* CDC1551-infected rabbits. In this case, upregulation of genes involved in dendritic cell and natural killer cell recruitment but not significant activation of inflammation was reported (Subbian et al., 2012, 2013).

Other studies with the latent TB rabbit model showed that aerosol infection with *M. tuberculosis* (H37Rv) and reactivation with corticosteroid induced immunosuppression, after, paucibacillary infection could be established in 72% of animals (Manabe et al., 2008). In this study, they also developed an immune reconstitution inflammatory syndrome, resembling many clinical aspects observed in severely immunosuppressed HIV-infected patients (Manabe et al., 2008). Using this same model, Kesavan and co-workers studied gene expression and observed that genes involved in hypoxia response (fdxA), resuscitation-promoting factors (rpfB) and DNA repair pathways (Rv2191) had a role in bacillary persistence (Kesavan et al., 2009).

Skin liquefaction model

Liquefaction is the key deteriorating process in TB that may evolve into cavitation, often making the caseum an excellent reservoir of tubercle bacilli for subsequent spreading to new hosts. The mechanism of liquefaction is unclear. This process can be reproduced in monkeys and rabbits, but not in mice, which develop strong cell-mediated immunity and granuloma lesion that leads to fibrosis. Rabbits provide a rapid model to study the mechanisms of liquefaction.

Rabbit skin liquefaction model has been used to evaluate virulence of different mycobacteria and to study the mechanism itself (Zhang et al., 2010). In that work, live bacilli of all tested strains, BCG, H37Ra and *M. smegmatis,* were able to induce liquefaction if sufficient amount was inoculated, and BCG demonstrated to be most virulent producing ulcers in less time causing higher liquefaction levels compared to the rest of the strains. When the bacteria inoculated were previously heat-killed, these did not produce liquefaction and granulomas were smaller. This model may be useful to assess mycobacteria pathogenicity or as proposed by Dannenberg (Dannenberg, 2009) as a screening method for drugs. Effective drugs in the skin could then be evaluated in the lungs of rabbits for their ability to prevent cavities or impede their progress.

Cavitary TB model

Human cavitary TB is a pulmonary type of TB, which involves the upper lobes of the lung. Cavitation drives a progressive lung destruction that leads to the formation of cavities or enlarged air spaces where *M. tuberculosis* replicates best. This form is highly contagious in humans, and the cavities are the hallmark of post-primary TB meaning that cavitation is a consequence of reactivation.

Mainly because *M. tuberculosis* produces established pulmonary infections that may form cavities, but that eventually may regress and heal (Dannenberg, 2006), *M. bovis*, which rabbits are more susceptible to, has been used as surrogate for *M. tuberculosis* in rabbit models. Also, the rabbit *M. bovis* infection model of TB has been studied in depth because it closely resembles human TB (Converse et al., 1996, 1998), with granulomas of similar organization, caseous necrosis and cavity formation. Actually, rabbits are the only small laboratory animal species that easily develop pulmonary cavitation, resulting in bronchial spread of the pathogen to the environment. Studies of the mechanisms of cavity formation in the lungs are relevant to transmissibility in humans because cavities harbour high levels of bacilli that can gain access to the bronchial tree and spread by the aerogenous route. This is corroborated by the close correlation between sputum culture positivity and contagiousness in humans (Kaplan et al., 2003).

Previous sensitization has been shown to enhance the development of these lesions (Wells and Lurie, 1941; Ratfliffe and Wells, 1948; Yamamura et al., 1954; Yamamura, 1958). Such sensitization involves administering heat-killed *M. bovis* (Wells and Lurie, 1941; Yamamura et al., 1954; Yamamura, 1958) or live *M. bovis* in a low dose through the aerogenous route (Ratfliffe and Wells, 1948). Afterwards, challenges may be carried out by intrathoracic infection of *M. bovis* at low dose (Wells and Lurie, 1941) or high dose (Ratfliffe and Wells, 1948). Recently, sensitization with heat-killed *M. bovis* has shown to be necessary to generate lung cavities by bronchoscopic injection (Jassal et al., 2011).

Rabbits develop an even wider variety of lesion types being appropriate models to test drugs specifically designed to treat cavitary lesions (Basaraba, 2008) or vaccines (Converse et al., 1998).

Infection by Non-Tuberculous Mycobacteria

Natural infection

Mycobacterium genavense

Although spontaneous mycobacteriosis in rabbits has been considered rare, there are reports of rabbits naturally infected by NTM.

A confirmed clinical case in a juvenile dwarf rabbit which presented dyspnoea and suspected ascites was reported. At necropsy serosanguineous pleural effusion, whitish foci in the lungs and multifocal scars in the renal cortex were observed. Histopathologically, granulomatous pneumonia with severe intra-alveolar infiltration by inflammatory cells (foamy macrophages and multinucleated giant cells of Langhans-type), and fibromuscular thickening of the alveolar septa, alveolar lining cell hyperplasia with mild interstitial lymphocellular and plasmacellular infiltration was observed. PCR and 16S rRNA gene sequences confirmed the presence of *Mycobacterium genavense*. As the lungs were found to be affected, the authors proposed inhalation as the infection route (Ludwig et al., 2009).

Mycobacterium avium

There are also reported cases of *Mycobacterium avium* infections in domestic rabbits. In one case report, an affected rabbit presented chronic disease during 4 years consisting in respiratory disorders at nasal level and from where MAC bacteria was isolated by tissue culture (Kelleher, 2008). In Germany, Maa was isolated from rabbits that had been housed with chickens, ducks and doves. Rabbits were in a poor body condition and suffered from lameness due to swollen joints. At necropsy, nodular and partially fibrino-necrotic inflammatory lesions were seen in the joints, intestines, regional lymph nodes, kidneys and lungs. Areas of granulomatous to necrotizing inflammation with intralesional acid-fast bacteria were found histologically. Maa was identified using bacterial culture and molecular techniques. The authors suggested that rabbits had been infected by faeces from birds, which had a similar symptomatology (Lehmbecker et al., 2010).

In another study, eight domestic rabbits living in a breeding facility showed lesions compatible with tuberculosis that led to further microbiological investigation. Two nodular lesions of 3 cm in diameter and pyogranulomatous inflammation, characterized by the presence of neutrophils and macrophages surrounding a central necrotic area of the pyogranuloma with lymphocytic infiltrates and hyperplasia of the bile ducts in the periportal spaces of the parenchyma were found in liver of one adult male rabbit; however, direct *Ziehl–Neelsen* and culture were negative. Using triplex qPCR, Maa was identified in a liver sample of one juvenile rabbit, without gross or microscopic lesions. This finding suggests that Maa infection can occur early in juvenile animals (Kriz et al., 2011).

More recently in a survey carried out on slaughtered rabbits in northern Spain, Maa was detected by PCR in gut associated lymphoid tissue of 16.6% of the analysed animals; however, no isolation was achieved from any of the slaughtered rabbits (Arrazuria et al., 2015b).

Disseminated mycobacteriosis produced by MAC was the most common cause of death (ranging 29–37% depending on the season) in adult captive pygmy rabbits from a zoo and a park in Oregon and Washington (Harrenstien et al., 2006, 2011). The pygmy rabbit (*Brachylagus idahoensis*) is the smallest of any North American rabbit species, and it digs its own burrow meaning that it is highly exposed to environmental mycobacterial species (Keinath and Mcgee, 2004). In this case, the clinical course was long, and non-specific symptoms included pale mucous membranes due to anaemia and dyspnoea associated with granulomatous pneumonia, as well as lactation in non-pregnant does (11% of cases). Haematologic and biochemical findings were also non-specific. Neutrophilia was the most common finding, and decreased albumin–globulin ratio was found in approximately 50% of serum biochemistry panels (Harrenstien et al., 2006).

The susceptibility of the pygmy rabbit to lethal disseminating mycobacteriosis seems a result of underlying cell-mediated immune function deficiency, which explains the high morbidity and mortality (McClure, 2012). Cell-mediated immunity, assessed by cytokine and lymphocyte stimulation assays, showed a Th1/Th2 imbalance as pygmy rabbits produce lower amounts of gamma-interferon, which would result in decreased macrophage activation and have elevated levels of Th2 cytokines, which inhibit Th1 responses, necessary for an effective immune defence against mycobacteria (Harrenstien et al., 2006).

Mycobacterium avium subsp. paratuberculosis

The first record of rabbit infection with Map was made in Spain in 1988 when Fuentes and Cebrian (Fuentes and Cebrian, 1988) reported an isolation of Map from wild rabbits collected by hunters in grazing grounds used by small ruminant paratuberculosis infected flocks. Since then, numerous studies from Scotland have shown natural paratuberculosis infection in rabbits from farms with a history of paratuberculosis in domestic ruminants (Angus, 1990; Greig et al., 1997). More recently, in the Iberian peninsula Map infection has been described in hunter-harvested wild rabbits (Maio et al., 2011). Although these reports prove that wild rabbits are susceptible to infection by Map in natural conditions, difficulty in finding macroscopic lesions (Greig et al., 1997; Beard et al., 2001a) and assessing clinical manifestations in fallen animals have fuelled controversy about rabbits suffering the disease or being non-symptomatic carriers. In any case, transmission between rabbits may occur vertically, pseudovertically and horizontally as Map has been isolated from the testes, uterus, placenta, foetuses, milk and faeces of affected animals (Judge et al., 2006). Molecular genetic typing techniques could not discriminate between selected rabbit and cattle isolates, suggesting that the same strain may infect both species and that inter-species transmission may occur (Greig et al., 1999). Moreover, evidence of Map infection was detected in 22% of the examined rabbits, whereas the organism was cultured from 17 of 110 rabbits, histopathological lesions consistent with Map infection were noted in 18 of 98 rabbits, but no macroscopical lesions suggestive of infection were observed in any of the animals (Beard et al., 2001a).

In the study mentioned above in southern Spain where 237 wild rabbits were analysed, 2.5% were seropositive to Map in a paratuberculosis ELISA (Maio et al., 2011). In two of 80 necropsied rabbits, gross lesions compatible with paratuberculosis were observed (one of which was confirmed by PCR). Histopathology revealed Map compatible lesions in 8 of 10 examined rabbits.

A recent study concluded that the high prevalence of paratuberculosis in local populations of wild rabbits could be associated with cattle farms that have difficulties controlling paratuberculosis (Shaughnessy et al., 2013). Although the authors' conclusions suggest that Map-infected rabbits may contribute to the persistence of paratuberculosis in domestic livestock and undermine control strategies that focus on livestock alone, the opposite can also be true, that is, that rabbits become infected if cattle infection is not efficiently controlled. After all, cattle bacterial burden and shedding amounts are vastly larger than what can be expected from rabbits. On the other hand, Map has been found in environments without rabbits, but not in rabbits without infected ruminants. This hypothesis gains support by other studies that indicate that inter-species transmission between rabbits and cattle occurs (Stevenson et al., 2009). Plus the experimental inoculation of young calves with an isolate of Map recovered from a free-living rabbit has been demonstrated (Beard et al., 2001b).

Cattle show no aversion to rabbit contaminated swards and are thus likely to contact fresh rabbit faeces while grazing (Daniels et al., 2001). As the ingestion of a single faecal pellet can contain sufficient bacteria to constitute an infective dose, this suggests that grazing contaminated pasture poses a significant risk of paratuberculosis to cattle and that wildlife reservoirs should therefore be considered when formulating control plans for the disease. However, the amount of bacteria shed by a few infected rabbits is just negligible compared to the amounts of bacteria shed by infected cattle with multibacillary forms.

In North America, the potential role of wild rabbits as sources of Map for Minnesota dairy herds has been evaluated (Raizman et al., 2005). The prevalence of Map in rabbit faeces was low (2%) but the diagnostic technique used was culture and it may be possible that some rabbits were shedding below detection limits of this method. The authors proposed that the primary risk was from cattle to rabbits pointing out that farm practices such as manure

spreading on crop fields or pasture may increase the spread of infection to rabbits. This theory is contrary to what has been suggested in Scotland but much more plausible (Daniels et al., 2001).

Experimental infection: models

Although most experiments have been performed predominantly focusing on TB, rabbits also have been used as a model for other mycobacteriosis.

Mycobacterial keratitis model

Mycobacterial keratitis can occur after trauma or surgery such as penetrating keratoplasty, radial keratotomy, refractive surgery and laser keratomileusis. NTM keratitis represents a challenge in diagnosis and treatment because it is indolent and the susceptibility to conventional antibacterial therapy is poor. Mycobacterial biofilms may play a role in evading host defence mechanisms and promoting resistance to conventional disinfection (O'Brien, 1996).

Rabbits can easily develop mycobacterial keratitis, providing a good laboratory animal model for pathogenesis studies and treatments assays. An experimental study of NTM keratitis in rabbits was performed focusing on clinical manifestations and pathological findings at different time points. In it, it was seen that the clinical feature of rabbit keratitis is the result of multifocal dense superficial stromal infiltrates where CD4 + T lymphocytes may play an important role (Liang et al., 2007). More recently, this rabbit model of NTM keratitis has been used for experimental studies on antibiotic treatment with amikacin, gatifloxacin or azithromycin with promising results (Yin et al., 2013).

In addition, rabbits have been used to develop a model of infection with *Mycobacterium chelonae*. In this case, eleven of 12 experimentally infected rabbits developed corneal disease from which *M. chelonae* could be isolated in nine rabbits, meaning that corneal infection was successfully induced and that it could be used for the analysis of therapeutic responses (Adan et al., 2004).

Mycobacterium avium subsp. paratuberculosis

For many years, rabbits have been used to generate antibodies against Map antigens, as they develop a strong immune response (Stabel et al., 1996). Success of the use of rabbits in immunodiagnostics of Map has led this species to be employed for research of paratuberculosis immunopathogenesis. Added to this, the rabbits are attractive because they are naturally susceptible to Map.

Although rabbits are true non-ruminant herbivores and paratuberculosis clinical symptoms appear mainly in ruminants, rabbits' digestive tract resembles that of ruminants more closely when compared to other laboratory animals because of the similarity of the sacculus rotundus with the ileocaecal valve. Many experimental infections with Map in rabbits have been carried out, but even the use of diverse infection routes, at various ages and different detection techniques, has not resulted in a well-established animal model for paratuberculosis. Key aspects of the most relevant studies involving Map infection are summarized in Table 2.

The first Map inoculation in rabbits was performed intraperitoneally with a cattle strain in 1943 by Francis (Francis, 1943). Later other experiments were carried out by infecting rabbits intraperitoneally and intravenously (Hirch, 1956). Rabbit models of Map pathogenesis more recently reported include oral administration of inoculum, mimicking the route of natural infection (Mokresh et al., 1989; Mokresh and Butler, 1990; Vaughan et al., 2005; Arrazuria et al., 2015a).

Table 2. Experimental paratuberculosis infection studies in rabbits

Source of strain	Age at challenge	Dose	dose number	Route	Animal number	Necropsy w pi	[a]Infected %	References
Cattle	10 days	8.9 mg	2	IP	4	12–27	100	(Francis, 1943)
Rabbit/hamster	4–5 weeks	1.6–5.6 mg	7	Oral	22	4–40	6–50	(Hirch, 1956)
Rabbit/goat	4–6 weeks	0.2 mg	1	IP	10	≤42	<50	(Hirch, 1956)
Rabbit/goat	8–10 weeks	0.5 mg	2	IV	10	1–33	100	(Hirch, 1956)
Cattle	1–2 days	7×10^6 cfu	5/10	Oral	21	32–40	43	(Mokresh et al., 1989)
Cattle	1–2 days	3.6×10^8 cfu	1	Oral	15	2 –36	38	(Mokresh and Butler, 1990)
Cattle	1–2 days	2.6×10^8 cfu	1	Oral	5	2 –36	100	(Mokresh and Butler, 1990)
Cattle	3 months	5×10^8 cfu	3	Oral	4	104–128	50	(Vaughan et al., 2005)
Cattle	2 weeks	1×10^8 cfu	3	Oral	16	8–84	19	(Vaughan et al., 2005)
Cattle	8 weeks	1×10^9 cfu	3	Oral	15	16–20	40–87	(Arrazuria et al., 2015a)

cfu, colony-forming units; wpi, weeks post-infection; IP, intraperitoneal; IV, intravenous.

[a]Percentage infection determined primarily by tissue culture at necropsy, when these data were not available, then other methods of detection such as histology, faecal culture and PCR were taken into account.

The hallmarks of paratuberculosis in ruminants (weight loss, faecal shedding and diarrhoea) have been reproduced to more or less extent in the rabbit oral infection models. In some cases, clinical symptoms such as weight loss in 4.76% of the animals at 12 mpi (Mokresh et al., 1989) and in 25% of the animals at 24 mpi (Vaughan et al., 2005) or diarrhoea in 67% (Mokresh et al., 1989) and 69% (Mokresh and Butler, 1990) of infected rabbits have been observed. Faecal shedding has been confirmed by faecal culture on solid media in 30–50% of rabbits (Mokresh et al., 1989; Mokresh and Butler, 1990), by radiometric faecal culture in BACTEC^{TM} (Vaughan et al., 2005) and more recently by PCR in 46% of animals (Arrazuria et al., 2015a).

As for post-mortem paratuberculosis gross changes, these may comprise thickened and often corrugated walls of small intestine, enlarged and oedematous mesenteric and other regional lymph nodes. Gross lesions consisting of pale-white reactive spots and thickened walls have been observed in some rabbits, mainly in sacculus rotundus and vermiform appendix in experiments performed with end points set at 4 (Arrazuria et al., 2015a) and 12 months (Vaughan et al., 2005). Histopathological analysis at different ages showed that the most affected tissues were the sacculus rotundus, the vermiform appendix and the mesenteric lymph nodes, compounding a picture of granulomatous enteritis with aggregates of macrophages and scattered polymorphonuclear cells and lymphocytes where acid-fast bacilli were rather scarce (Mokresh et al., 1989; Mokresh and Butler, 1990; Vaughan et al., 2005; Arrazuria et al., 2015a).

Map isolation from tissues has been achieved in all oral infection route studies, mainly in sacculus rotundus and vermiform appendix were 19–40% (Mokresh and Butler, 1990; Vaughan et al., 2005), 33% (Mokresh and Butler, 1990) and 40% (Arrazuria et al., 2015a) of samples yielded Map colonies.

Overall, rabbits could provide a practical and close to natural disease animal model to study key aspects of paratuberculosis that cannot be investigated in other laboratory animals because many symptoms such as diarrhoea, faecal shedding and granuloma formation can be reproduced in this model. However, reliable reproduction of clinical, pathological and microbiological typical findings in diseased ruminants has not been achieved and requires further investigation.

Rabbits as Reservoirs of Mycobacteria

Pathogens that are transmitted between the environment, wildlife, livestock and humans represent major challenges for the protection of human and domestic animal health, the economic sustainability of agriculture, and the conservation of wildlife. TB and leprosy are ancient diseases and even though large efforts to control them have been made, they remain real threats to human health. Even though there are very few reports of natural infection of TB in rabbits, these animals may be a useful tool because they provide a broad spectrum of TB lesions in experimental conditions. Additionally, as TB control in some regions is proving to be much more difficult than initially expected, it is necessary to study all possible reservoirs and routes of transmission, including the possible role of rabbits. Regarding leprosy, rabbits naturally infected by *M. leprae* have never been reported. However, given that contact with this species' carcasses has appeared as a statistical risk (Clark et al., 2008) its involvement in leprosy epidemiology requires further exploration especially because transmission mechanisms of this disease are poorly understood.

NTM species are ordinary inhabitants of a wide variety of hosts, and their role in human and animal diseases has been fully recognized (Biet et al., 2005). As mentioned earlier, the role of rabbits in the epidemiology of paratuberculosis has been shown by several studies in Scotland (Greig et al., 1999; Shaughnessy et al., 2013). Although it is not clear whether wild rabbits have clinical paratuberculosis, there is no doubt that they develop typical intestinal lesions and can shed Map in faeces that can contaminate pastures and water. Map and other mycobacteria survive water chlorination (Aronson et al., 1999; Collins et al., 2001; Whan et al., 2001), and selection of resistant mycobacteria strains is likely to occur (Primm et al., 2004); hence, drinking water can be a human exposure route. Rabbits may play a role as a reservoir of Map contributing to the maintenance of the infection in ruminant grazing grounds. Also as noted above, rabbits can be affected by other mycobacteriosis; however, the lack of surveys in epidemiology of these in natural conditions makes it difficult to assess whether rabbits could be reservoirs of infection for other animals and humans.

Conclusions

Spontaneous mycobacteriosis in rabbits are considered to be rare mainly because a limited number of reports have been published. However, rabbits have been used for many years as an animal model for the study of mycobacteriosis and they prove to be suitable to study different pathological and immunological aspects of TB that cannot be studied in other laboratory animals, also showing to be appropriate for chemotherapy testing and vaccine screening. The possible role of rabbits in mycobacterial epidemiology has received little attention, indicating that more studies are needed and that this species should not be ruled out as a reservoir of *Mycobacterium* spp.

Acknowledgements

This work was supported with funds from the Ministerio de Economía y Competitividad from the Spanish Government (AGL 2012-39818-C02-02) and from the Departamento de Medio Ambiente, Planificiación Territorial, Agricultura y Pesca of the Basque Government. RA acknowledges support from the Departamento de Educación y Universidades of the Basque Government (BFI-2012-237). The authors thank Iker A. Sevilla and Joseba M. Garrido for comments on the manuscript.

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III. RESULTADOS / RESULTS

ESTUDIO I / STUDY I

Detection of *Mycobacterium avium* subspecies in the gut associated lymphoid tissue of slaughtered rabbits. R Arrazuria, IA Sevilla, E Molina, V Pérez, JM Garrido, RA Juste, N Elguezabal. BMC Vet Res. 2015, 11:130.

Arrazuria et al. BMC Veterinary Research (2015) 11:130
DOI 10.1186/s12917-015-0445-2

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Detection of *Mycobacterium avium* subspecies in the gut associated lymphoid tissue of slaughtered rabbits

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Abstract

Background: Rabbits are susceptible to infection by different species of the genus *Mycobacterium*. Particularly, development of specific lesions and isolation of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis*, both subspecies of the *M. avium* complex, has been reported in wildlife conditions. Although, rabbit meat production worldwide is 200 million tons per year, microbiological data on this source of meat is lacking and more specifically reports of mycobacterial presence in industrially reared rabbit for human consumption have not been published. To this end, we sought mycobacteria by microbiological and histopathological methods paying special attention to *Mycobacterium avium* subsp. *paratuberculosis* in rabbits from commercial rabbitries from the North East of Spain.

Results: *M. avium* subsp. *paratuberculosis* was not detected either by culture or PCR. However, *Mycobacterium avium* subsp. *avium* was detected in 15.15 % (10/66) and *Mycobacterium avium* subsp. *hominissuis* was detected in 1.51 % (1/66) of gut associated lymphoid tissue of sampled animals by PCR, whereas caecal contents were negative. 9 % (6/66) of the animals presented gross lesions suggestive of lymphoid activation, 6 % (4/66) presented granulomatous lesions and 3 % (2/66) contained acid fast bacilli. Mycobacterial isolation from samples was not achieved, although colonies of *Thermoactinomycetes* sp. were identified by 16s rRNA sequencing in 6 % (4/66) of sampled animals.

Conclusions: Apparently healthy farmed rabbits that go to slaughter may carry *M. avium* subspecies in gut associated lymphoid tissue.

Keywords: Animal pathogens, Epidemiology, *Mycobacterium avium* subsp, *Mycobacterium avium* complex, *Thermoactinomyces* sp, Rabbits, Slaughter

Background

Rabbits have been found to be naturally susceptible to *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection in the wild [1–4], to *Mycobacterium avium* subspecies infection in natural conditions, specifically *Mycobacterium avium* subsp. *avium* (Maa) in pigmy rabbits [5] and moderately susceptible to Map in laboratory conditions [6, 7].

Map and Maa are subspecies of *Mycobacterium avium*, as well as *Mycobacterium avium* subsp. *silvaticum* (Mas) and *Mycobacterium avium* subsp. *hominissuis* (Mah) [8]. From an epidemiologic point of view, Maa is typically virulent for birds and small terrestrial mammals causing a range of lesions that go from characteristic tuberculous lesions in parenchymatous organs, to lymphadenitis and disseminated infection [9–11]. Mah has the human [12] and the pig [13] as its primary hosts and it is frequently found in soil [14]. Map is the causal agent of paratuberculosis in ruminants and it has been controversially associated with human inflammatory bowel disease, more precisely with Crohn's disease [15–17] and also diabetes

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mellitus [18]. Its prevalence in slaughtered cattle in Europe has been estimated to be up to 50 % [19]. Mas has been isolated from wood pigeons [20], roe deer [21] and horses causing tuberculous-like lesions in these animals [22]. However, since there is a controversy about the real existence of Mas as a unique subspecies independent of Maa [23], for the present work we will consider Mas as part of Maa, and jointly refer to them as Maa/Mas.

Mycobacterium avium subspecies members are widely spread in the environment and often enter in contact with animals and humans. Transmission from animals to humans can occur either through the consumption of contaminated foods or *via* direct contact with an infected animal. Apparently healthy animals thereby may represent a reservoir for *Mycobacterium avium* subspecies and these pathogens may enter the food chain during slaughter. Most work focused on the detection of *M. avium* subspecies in animal species for human consumption has been performed on meat products. Map has been detected in beef, pork and chicken [24], whereas Mah has been detected in beef, pork and lamb [24]. Detection of non-tuberculous mycobacteria or *Mycobacterium avium* subspecies at slaughter has been described in lymph nodes of pigs [25–27] and detection of Map has been reported in lymph nodes, muscle and faeces of both dairy and beef cattle [28].

Mycobacterium avium subspecies presence in rabbits in wild conditions led us to hypothesize that these mycobacteria could also be present in commercial rabbits that go to slaughter and thus represent a route of exposure for humans. The aim of the present study was to carry out a small survey on the frequency of mycobacterial microorganisms detected by solid and liquid culture and by a tetraplex real-time PCR for *Mycobacterium* genus, *M. avium* subspecies and *M. tuberculosis* complex in gut associated lymphoid tissue (GALT) and caecal contents of apparently healthy rabbits at slaughter.

Methods

Ethics statement

Animals used in this study did not undergo any manipulation prior to stunning for standard industrial slaughter according to the pertinent legislation. For this reason, no specific ethical approval was required.

Animals and sampling

We contacted the official veterinarian of the nearest rabbit slaughterhouse to set-up a sampling schedule. This study was based on samplings that took place from May to October of 2013 (decontamination procedure set-up and PCR evaluation) and during January and February of 2014 (proper study) in a rabbit slaughterhouse (Basque Country, Spain) processing 1300000–1400000 rabbits annually. Rabbits are typically slaughtered with an average age of two months and average live weight around 1.8-2.2 kg (http://www.magrama.gob.es/es/ganaderia/temas/produccion-y-mercadosganaderos/INDICADORES_ECONÓMICOS_SECTOR_CUNÍCOLA_2013_tcm7-330314.pdf). Breeding rabbits are slaughtered at about 2–2.5 years of age. Production rabbits are often outbred in order to maximize meat production [29]. This slaughterhouse was operated by an agriculture cooperative company and complied with the pertinent Basque (Basque Government Decree 454/1994), Spanish (Spanish Government Law 32/2007 and Royal decree 731/2007) and European (Council Regulation (EC) No 1099/2009) legislation on animal welfare under the supervision of official veterinarians and the samples obtained were authorized by the slaughterhouse managers.

A total of 12 animals (decontamination procedure set-up and PCR evaluation) and 66 animals (proper rabbit slaughterhouse study) from 21 farms scattered in 6 provinces of North-East Spain were sampled. Samples were drawn from two young animals from each batch of sacrifice and from a third additional breeding animal, if the batch included breeding rabbits. Samples were kept in refrigeration for 6-12h before being processed.

Decontamination procedure

In order to determine the optimum NaOH concentration for decontamination initial set-up experiments were performed. Mucosa from sacculus rotundus and vermiform appendix from 12 rabbits were used to test decontamination with 2 %, 4 %, 6 % and 8 % NaOH. Decontamination procedures were run for 10 and 15 min. Contamination was observed in both solid and MGIT culture at NaOH concentrations lower than 6 % for both 10 and 15 min. 6 % NaOH for 15 min was the lowest concentration that resulted in no contaminated samples. For this reason, the final decontamination procedure was performed with these conditions. Also manufacturer's instructions do not recommend running decontamination for more than 15 min or NaOH concentrations higher than 6 % (BACTEC™ MGIT ™960).

10 ml of sterile water were added to 2 g of sample tissue. Homogenization was then performed on a Stomacher blender for 1 min at medium speed. Afterwards, the homogenized solution was transferred to a tube and 10 ml of 6% NaOH were added. After a vortex mix, the suspension was incubated for 15 min at room temperature before neutralization with 15 ml of phosphate buffer. The suspension was mixed well and centrifuged for 20 min at 2,885 × g and the supernatant was discarded. Pellets were suspended in 2 ml of sterile water.

Culture

Solid culture

Four drops (150 μl)/per tube of the decontaminated suspension were seeded on solid media: Herrold's Egg Yolk Medium (HEYM); Middlebrook 7H10 with penicillin, amphotericin B, and chloramphenicol; Lowenstein-Jensen with penicillin, amphotericin B and supplemented with mycobactin J and Tsukamura minimal media with cycloheximide [30]. All seeded tubes were incubated at 37+/−1 °C and checked for growth at 8, 12, 16 and 20 weeks. After seeding on solid culture, the remaining inoculum was used for liquid culture.

Liquid culture

Pellets destined to liquid culture were seeded on BBL Mycobacteria Growth Indicator Tubes (MGIT) supplemented with BACTEC MGIT growth supplement and BBL MGIT PANTA (Becton, Dickinson and Company). Tubes were incubated for 45 days in a BACTEC MGIT 960 System and time to detection (TTD) values were recorded.

PCR evaluation

PCR performance on spiked mucosa was evaluated since this specimen is known to be tricky because of the presence of PCR inhibitors.

Inocula preparation for tissue spiking

Mah Strain 104 was grown in Middlebrook 7H9 (M7H9) broth supplemented (v/v) with 10 % Middlebrook OADC enrichment (Becton, Dickinson and Company, MD, USA), 0.2 % glycerol and 0.05 % Tween 80 (Sigma-Aldrich, Co. Ltd., Haverhill, UK). Mycobacteria were harvested by centrifugation at 2,800 × g. Pellets were washed twice in phosphate buffered saline (PBS). Mycobacteria were resuspended in PBS containing 0.2 % glycerol and 0.05 % Tween 80 (PBS-GT). Bacterial suspensions were adjusted to 1 McFarland unit after turbidity measurements were performed with a Densicheck densitometer (Bio-Mérieux, Marcy L'Etoile, France). Five ten-fold dilutions of the bacterial suspensions were prepared in PBS-GT and the highest, medium and lowest, with a 2 log difference between each of them were used to inoculate negative tissues. To verify the viable number of CFU, the inocula were plated onto appropriately supplemented agar-solidified M7H9 flasks.

Mucosa spiking

Mucosa was scraped from vermiform appendix tissue from 12 apparently healthy rabbits previously confirmed to be negative for the presence of mycobacteria by PCR, culture and histopathology and it was thoroughly mixed and homogenized. The blended tissues were divided in batches of 1 g of mixture per stomacher bag. The stomacher bags were inoculated with 100 μl of one of each of the previously adjusted bacterial suspensions (high, medium and low). 300 mg of the homogenate spiked tissues were extracted as described in the DNA extraction section and MycMavMtc PCR [31]. In cases where PCR inhibition took place ½ and ¼ dilutions of the extracted DNA were performed previous to PCR reaction and this showed to be enough to prevent inhibition.

Slaughterhouse sample study

A total of 66 animals (48 young and 18 breeding animals) were sampled and different steps that are summarized on Fig. 1 were taken to investigate the specimens.

Briefly, sacculus rotundus and vermiform appendix were scraped for mucosa and caecal contents were collected. Part of each sample was used for direct molecular detection and part for decontamination and subsequent culture. Based on set-up results, the final decontamination procedure was performed with 6 % NaOH as described in the decontamination procedure section because it was the lowest concentration found to inhibit contamination. Both solid and liquid cultures were performed followed by PCR confirmation and sequencing of the 16Sr RNA gene when non-mycobacterial colonies were isolated.

Histopathological analysis was performed on samples of sacculus rotundus and vermiform appendix of a total of 22 rabbits (14 young rabbits and 8 breeding rabbits) including animals with and without macroscopic lesions.

DNA extraction

Spiked tissues and fresh rabbit slaughterhouse samples

DNA extraction was performed on spiked mucosa (PCR evaluation), sacculus rotundus, vermiform appendix and caecal content (slaughterhouse study), using

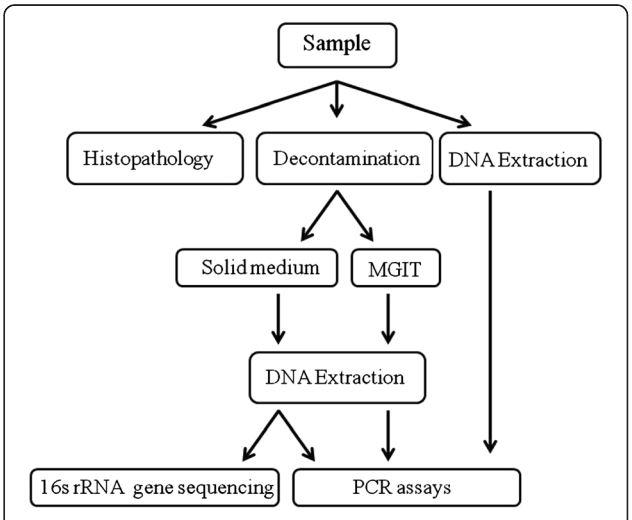

Fig. 1 Experimental scheme showing methods followed at each stage

DNA Extract-VK (Vacunek S.L, Bizkaia, Spain) following manufacturer's instructions with modifications. Briefly, 300 mg of tissue or caecal content were weighed in a microtube. 250 μl of sterile distilled water, plus 250 μl of VK-Lysis buffer and 300 mg of VK-extraction beads were added to the tissue and homogenized at 30Hz during 10 min on TissueLyser II (Qiagen). Following homogenization, samples were centrifuged at 7,000 × g for 5 min and 200 μl of the supernatant transferred to a new vial. 25 μl of Proteinase K were added to the previous solution and tubes were incubated at 56 °C for 15 min. Lysis was performed adding 200 μl of Lysis Buffer VL-LB3 to the previous solution and mixing thoroughly. The solution was incubated at 70 °C for 10 min. Then, 210 μl of ethanol (96-100 %) were added and mixed thoroughly. The final mixtures were loaded on VK-DNA binding columns and centrifuged at 11,000 × g for 1 min. Pass-through liquid was discarded. Washing steps were performed with 500 μl of Wash Buffer VK-WB1 and 600 μl of Wash Buffer VK-WB2 with a 11,000 × g 1 min centrifuging step in between. After washes were done, 100 μl of Elution Buffer were added to the column and the column left for 1 min. DNA was recovered by centrifuging at 11,000 × g for 1 min. Extracted DNA was stored at −20 °C until PCRs were performed. Negative DNA extraction controls (for every 23 samples), no template and positive PCR controls (for every PCR) were included in all downstream PCR assays.

Solid medium isolated colonies

A loopful of cells from each colony growing on solid medium was placed in 100 μl of sterile water. Vials were incubated at 95 °C for 10 min and centrifuged at 13,800 × g for 5 min. The supernatant was used as template for further PCR assays.

Positive MGIT liquid medium

One millilitre of vortexed liquid culture from the MGIT tubes was collected and centrifuged at 13,800 × g for 5 min, the supernatant was discarded and after a washing with 1 ml of sterile distilled water, the pellet was suspended in 250 μl of sterile water. Then 300 mg of zirconium beads were added and homogenized at 30Hz for 10 min on TissueLyser II (Qiagen). Following homogenization, samples were centrifuged at 7500 × g for 15 min and the supernatant was collected.

Molecular detection

Detection of mycobacteria by Tetraplex real-time PCR (MycMavMtc)

For initial screening purposes to test the presence of *Mycobacterium* sp., a fourplex real-time PCR described by Sevilla *et al.* [31] was performed. This PCR is able to detect the genus *Mycobacterium* and then distinguish between the *M. tuberculosis* complex and *M. avium* subspecies. The target genes were the internal transcribed spacer (ITS) between 16S and 23SrRNA genes, insertion sequence IS1311 and the devR genr for *Mycobacterium* genus, *M. avium* subspecies and *M. tuberculosis* complex members respectively.

Reactions were carried out in a final volume of 25 μl containing 5 μl of template DNA, and 1X TaqMan Universal PCR MasterMix without AmpErase uracil-DNA glycosylase (Applied Biosystems), 0.3 μM of each primer, 0.2 μM of each probe. An internal amplification control (IAC) was included in the reaction to rule out false negative results due to inhibitors. ROX (6-carboxyl-X-rhodamine) dye was used as passive reference reporter. Amplification was carried out in a 7500 Real-Time PCR instrument (Applied Biosystems) and consisted of one denaturation and polymerase activation cycle of 10 min at 95°C, and 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60°C for 1 min. Results were analyzed using the 7500 System SDS software v. 1.4 (Applied Biosystems). Threshold cycle (C_T) and baseline were automatically determined by the software and verified by visual examination of the threshold line in amplification plots.

M. avium subsp. paratuberculosis detection by IS900 & ISMap02 real-time PCR

To identify Map positive samples, a real time multiplex PCR described by Sevilla *et al.* [32] was performed. This PCR detects IS*900* and IS*Map02* DNA sequences of *M. avium* subsp. *paratuberculosis*.

Briefly, the reaction mixture contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300–400 nM of each primer, 200 nM of each probe, and 5μl of DNA extract in a final volume of 25 μl. An IAC is also included in the PCR reaction.

Amplification and real time measurement were performed in the same system and conditions as described in the previous PCR protocol.

M. avium subsp. hominisuis and M. avium subsp. avium/M. avium subsp. silvaticum detection by IS1245 & IS901 PCR

In order to identify other *M. avium* subspecies, a previously published real time PCR was carried out, based on the detection of IS*901* and IS*1245* insertion sequences [33]. Maa/Mas organisms are positive for IS*1245* and IS*901*, while Mah is only positive for IS*1245*. The PCR did not include DNA concentration standards for quantification purposes because the objective was just to detect and identify Mah and Maa/Mas.

The final reaction mixture contained the same PCR ingredients at the same concentration indicated above for Map PCR but primers and probes were those previously

described by Slana *et al.* [33]. Amplification and real time measurement were performed in the same system and conditions as described in the previous PCR setting.

16S rRNA gene sequencing of colonies on solid medium

The identification of colonies grown on solid media that were not mycobacteria as assessed by previously described PCR methods was performed by 16S rRNA gene sequencing. Amplification of target DNA was performed by PCR using bacterial universal primers pAF (5′- AGA GTT TGA TCC TGG CTC AG-3′) and 530R (5′-CCG CGG CKG CTG GCAC-3′). Amplification was carried out in a 2700 GeneAmp PCR Instrument (Applied Biosystems) and consisted of one denaturation and polymerase activation cycle of 4 min at 94 °C, and 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 40 s. The PCR product was cleaned with Illustra Exoprostar 1-Step (GE Healthcare Life Sciences) following manufacturer's instructions.

The sequencing reaction was carried out with BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) and resolved with a 3130 ABI *(*Applied Biosystems*)* capillary sequencer. Sequencing conditions consisted of 30 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min. Sequence alignment was performed using BLAST (Basic Local Alignment Search Tool) and identification with 100 % of coverage and 99-100 % of identity was made.

Gross pathology

The whole digestive system of the rabbits was inspected for gross anatomical change. Samples from sacculus rotundus and vermiform appendix were collected for solid and/or liquid culture and histopathological analysis. Samples from sacculus rotundus, vermiform appendix and caecal contents were collected for detection of mycobacteria by PCR.

Histopathology

Histopathological analysis was performed on samples of sacculus rotundus and vermiform appendix and on other samples that presented macroscopic lesions. The tissues were fixed in 10 % neutral buffered formalin for a minimum of 24 h, trimmed, dehydrated through graded alcohols, embedded in paraffin wax and sectioned at 5μm. These sections were mounted on glass slides stained with haematoxylin and eosin (HE) or by the Ziehl-Neelsen (ZN) method for acid fast bacteria (AFB) according to standard procedures. Slides were examined under the microscope for granuloma formation and AFB presence.

Statistical analysis

Association of mycobacteria detection and age was tested with the Chi square or Fisher exact probability (FET) tests for the frequencies of PCR results according to age using the PROC FREQ of the SAS statistical package (SAS Institute Inc., Cary, NC, USA). For all analyses, a p value of <0.05 was considered to be statistically significant.

Results

PCR evaluation

MycMavMtc PCR results of the spiked rabbit mucosa are shown in Table 1. Inhibition was observed at all bacterial concentrations when samples were not diluted and only probe IS*1311* was detected in the highest concentration. After dilution steps were performed, inhibitory effects were mitigated. In the medium concentration bacterial suspension a ½ dilution was enough to detect *M. avium* sp. DNA by the IS*1311* probe. However, in the low concentration inoculum after ½ and ¼ dilutions the IS*1311* probe was not positive and only the internal amplification control gave significant C_T values.

Slaughterhouse sample study

Gross pathology and digestive system parameters

Of the 66 necropsied animals, 6 (9 %) showed visible pathological lesions consistent with mycobacterial infection. They were characterized by the presence of focal thickenings of the intestinal wall in the sacculus rotundus and vermiform appendix together with the presence of pale-whitish spots consistent with lymphoid follicle hyperplasia. Jejunal and ileal wall thickening was observed in one and three animals respectively, affecting some areas of the intestine in these regions. Representative photographs of lesions observed in three different animals are shown in Fig. 2.

Molecular detection

Of the 66 analyzed rabbits, 11 were positive for *M. avium* in GALT (16.66 %). Of these, six animals (54.54 %) were positive in vermiform appendix only, 2 (18.18 %) in sacculus rotundus only, and 3 (27.27 %) in both tissue types. C_Ts for IS*1311* probe were 36.48+/−2.32 for undiluted samples and 37.12+/−1.01 for diluted samples. Attending to tissue type, C_Ts were 36.94+/−0.83 for vermiform appendix and 35.83+/−3.88 for sacculus rotundus. Mycobacteria were not detected by PCR in caecal contents.

The 14 *M. avium* positive samples were further analyzed by IS*900* & Is*Map02* PCR for Map detection and IS*1245* & IS*901* PCR for differentiation between Maa/Mas and Mah. All these samples were negative for the first real-time PCR, meaning that Map was not detected in these rabbits. In contrast 13 of the *M. avium* positive samples were identified as Maa/Mas and 1 was identified as Mah according to the results obtained with IS*1245* & IS*901* PCR (Table 2).

Table 1 *M. avium* subsp *hominissuis* spiked rabbit mucosa C_Ts of MycMavMtc PCR

		Mah spiked rabbit mucosa		
		high	medium	low
Dilution	DNA Probe	7.66×10^6 (CFU/g)	7.66×10^4 (CFU/g)	7.66×10^2 (CFU/g)
1/1	ITSdenak	UD	UD	UD
	IS*1311*	31.77	UD	UD
	IAC	UD	UD	UD
1/2	ITSdenak	43.56	UD	UD
	IS*1311*	28.5	36.66	UD
	IAC	35.1	41.19	36.00
1/4	ITSdenak	39.7	UD	UD
	IS*1311*	28.85	34.50	UD
	IAC	32.62	33.99	33.63

Mah: *M. avium* subps. *hominissuis*, ITSdenak: detects *Mycobacterium* genus, IS*1311*: detects *Mycobacterium avium*, IAC: internal amplification control, UD: undetermined no threshold-crossing fluorescence, devR detecting Mtb complex members was UD for all assayed conditions

Culture

The culture of samples in BACTEC MGIT recorded contamination of 19.69 % of sacculus rotundus and 7.5 % of vermiform appendix samples. The remaining samples gave TTD readouts compatible with mycobacterial growth patterns on an average of 7.36 days for samples of sacculus rotundus and 6.95 days for samples of vermiform appendix but with a negative result in the MycMavMtc PCR. As a consequence, all MGIT cultures were finally recorded as contaminated.

No contamination on solid media was observed. Four samples cultured on Lowenstein-Jensen solid medium yielded colonies that were negative in the MycMavMTC. Sequencing of 16S rRNA gene identified them as *Thermoactinomycetaceae bacterium W8742* in three cases and *Thermoactinomyces sanguinis* in one case (Table 3).

Histopathology

Most significant lesions were found in the sacculus rotundus and vermiform appendix lymphoid tissue. In some cases, they were formed by well demarcated granulomas compatible with mycobacterial infection, composed of between 10–50 macrophages with occasional multinucleated Langhan's type giant cells, located in the interfollicular areas of the lymphoid tissue. In other animals, granulomatous lesions were categorized as unspecific and were formed by small granulomas, also seen in the interfollicular areas of lymphoid tissue, with less than 15 macrophages, harbouring variable amounts of a brown pigment in their cytoplasm. Microscopical examination of tissues revealed that wall thickening observed on ileum and jejunum corresponded to reactive lymphoid follicles in the ileal and jejunal Peyer's patches. Remaining samples were

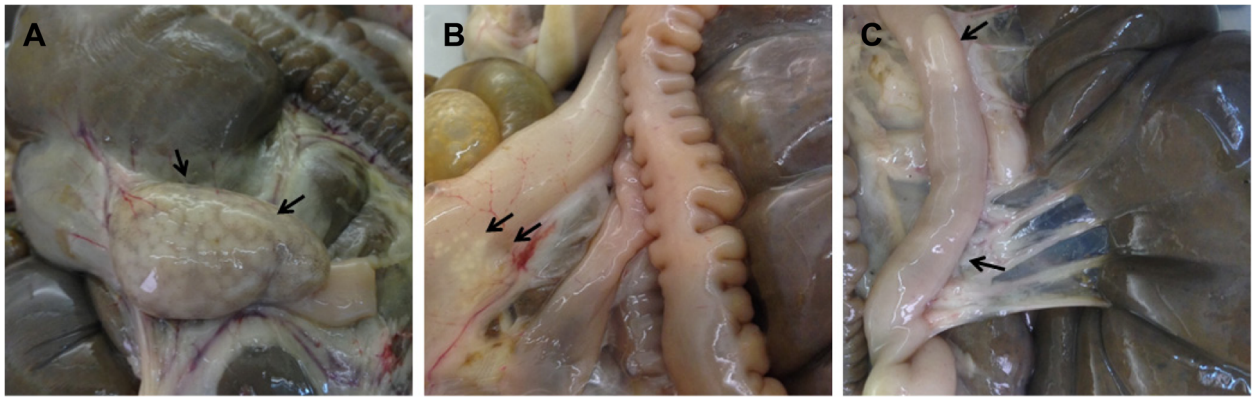

Fig. 2 Macroscopic lesions found in slaughtered rabbits. **a** sacculus rotundus with pale white spots and thickened wall (animal D2.B2), **b** vermiform appendix with white spots and thickened wall (animal E1.Y2), **c** ileum with thickened walls and a reactive Peyer's patch (animal C1.Y2)

Table 2 Gross pathology and molecular detection of sacculus rotundus and vermiform appendix from analyzed slaughtered rabbits

		Gross pathology	Molecular detection	
	Tissues (n)	Animals (n (%))	Animals (n (%))	Identity
Young rabbits	SR (48)	2[a] (11.1)	3 (4.2)	Maa/Mas
			1 (2.0)	Mah
	VA (48)	1 (5.5)	7 (14.6)	Maa/Mas
Breeding rabbits	SR (18)	3[b] (16.6)	2 (11.1)	Maa/Mas
	VA (18)	0 (0)	1 (5.5)	Maa/Mas

n: number of tissues or animals, %: percentage of positive results, SR: sacculus rotundus, VA: vermiform appendix, Maa: *M. avium* subsp. *avium*, Mas: *M. avium* subsp *silvaticum*, Mah: *M. avium* subsp *hominissuis* [a]Lesions spread to the ileum in both animals, [b]Lesions spread to the ileum in one animal

classified as reactive hyperplasic or absence of lesion when tissues presented a normal form. AFB were detected on two samples that were classified as absence of lesion and were also PCR positive. Complete results for all animals are shown in Table 3.

Association analysis

Analysis of age and gross pathology results showed that 6 out of 48 (6.25 %) of the young rabbits and 3 out of 18 (16.7 %) breeding rabbits showed some gross changes. However, these frequencies were not statistically significant (FET $p = 0.333$).

Microbiological results showed that 9 of 48 (18.7 %) young rabbits and 2 of 18 (11.1%) breeding animals were *M. avium* subspecies positive in the PCR tests, but again, these frequencies were not statistically significant (FET $p = 0.713$).

Worth mentioning is that 50 % (2/4) of the samples that presented focal lesions were PCR positive.

The geographical origin of sampled rabbits is shown on Fig. 3a. All geographical areas except for F presented at least one positive farm as shown on Fig. 3b. 8 out of 21 farms were positive (38 %), when positive was considered as having at least one *M. avium* subspecies PCR positive animal. Noticeable, is geographical area E where

Table 3 Molecular analysis, gross pathology and histopathology results of animals with at least one positive result in any of the methods

MycMavMtc PCR			Identity	16s rRNA gene sequencing	Gross pathology	Histopathology
Animal	SR	VA				
E1.Y1	-	-	-	*Thermoactinomyceteae*[a]	-	SR (0)/ VA (0)
E1.Y2	-	+	Maa/Mas	NI	VA	SR (0)/ VA (0)
B1.B1	-	-	-	*Thermoactinomyceteae*[b]	-	NA
B1.B2	-	-	-	NI	SR	SR (0)/ VA (0)
E2.Y2	-	-	-	NI	SR/IL[c]/JE[c]	SR (3)/ VA (0)/ IL (1)/ JE (0)
C1.Y2	+	+	Maa/Mas	NI	SR/IL[c]	SR (0)[d]/ VA (1)/ IL (1)
C2.Y1	-	+	Maa/Mas	NI	-	SR (1)/ VA (3)
C2.Y2	-	+	Maa/Mas	NI	-	SR (0)/ VA (0)[d]
B2.Y2	-	-	-	*Thermoactinomyceteae*[a]	-	SR (0)/ VA (0)
A1.Y2	-	+	Maa/Mas	NI	-	NA
E3.Y1	-	+	Mah	NI	-	NA
E3.Y2	-	+	Maa/Mas	NI	-	NA
B7.Y1	-	-	-	*Thermoactinomyces sanguinis*[b]	-	NA
B5.B1	+	+	Maa/Mas	NI	-	NA
D2.Y2	+	+	Maa/Mas	NI	-	NA
D2.B1	-	-	-	NI	SR/IL[c]	SR (2)/ VA (0)/ IL (1)
D2.B2	+	-	Maa/Mas	NI	SR	SR (2)/ VA (0)
D3.Y1	+	-	Maa/Mas	NI	-	NA

SR: sacculus rotundus, VA: vermiform appendix, IL: ileum, JE: jejunum, NI: no isolation. [a]Identified from a colony isolated from sacculus rotundus, [b]Identified from a colony isolated from vermiform appendix. [c]intestinal wall thickening, NA: Not analyzed. (0): Without lesions, (1): Reactive hyperplasia, (2) Unspecific granulomatous lesions (3): Granulomatous lesions,[d]: Ziehl Neelsen positive

only 2 animals were sampled and one was positive for Maa and the other for Mah.

Discussion

There is not much information in the veterinary literature concerning microbiological findings from rabbits at slaughter. A few studies have assessed *Listeria*, *Salmonella*, and *E.coli* among others [34, 35]. It is not surprising then, that data from mycobacteria and more specifically *M. avium* subspecies is scarce and mainly limited to other animal species such as cattle [28] or pigs [26, 27]. Therefore, to the best of our knowledge, this is the first study reporting *M. avium* subspecies detection in slaughtered rabbits.

In rabbits, apart from mesenteric lymph nodes and Peyer's patches, the GALT which accounts for 50 % of the lymphoid tissue includes two specific structures: the sacculus rotundus, and the vermiform appendix. Both tissue types present M cells [36] which actively uptake and present particular antigens and microorganisms to the immune cells of the lymphoid follicle to induce an effective immune response [37]. Map has been reported to enter the domes of the Peyer's patches through M cells [38] so orally ingested mycobacteria should end up in these sites and/or in caecal content and therefore we chose these specimens for our analysis.

Regarding PCR evaluation, the possibility of inhibition was the main concern. MycMavMtc PCR performed well at 10^4-10^6 CFU/g of tissue. According to our results if

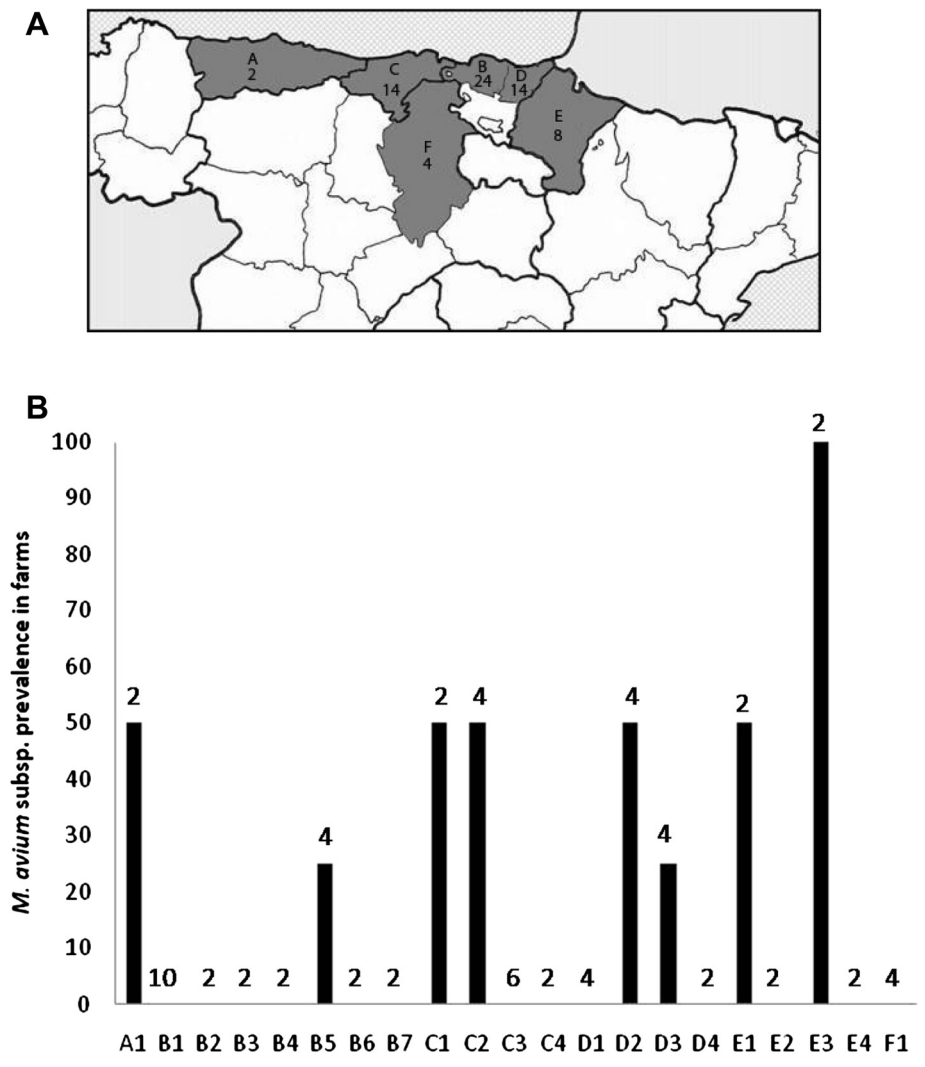

Fig. 3 a Geographical origin of sampled slaughtered rabbits. A: Asturias, B: Bizkaia, C: Cantabria, D: Gipuzkoa, E: Navarra, F: Burgos and number of analyzed animals per area. **b** *M. avium* subspecies positivity of each farm identified as letters (A-F) representing each geographical area and a number (1–7) identifying the farm. Black bars represent prevalence (%), numbers on bars represent total number of analyzed animals from each farm

we had +/−10^2 CFU/g of mycobacteria we would probably not detect them at least in our tissue type because we would necessarily have to perform dilution steps. The detection limit for this PCR for *M. avium hominissuis* in a mixture of bovine tissues has been reported as 1.59×10^2 CFU/g [32] which is in near agreement with our findings. We must add that the DNA extraction methods of both studies are not coincident and that the bovine tissue mixture may present a more favorable chemistry for the DNA extraction and PCR reaction compared to rabbit mucosa. In any case, for the slaughterhouse study we decided to dilute template DNA if inhibition took place, maybe missing positive samples in return.

In the slaughterhouse study, although gross pathology compatible with mycobacterial infection was seen, histological analysis of GALT did not reveal presence of granulomas in all gross pathology positive animals and AFB detected by ZN were only found in two cases. The presence of granulomas in GALT associated to Mah have been reported before in sheep [39]. The unspecific granulomas may not be due to infectious agents and they may be "garbage" granulomas or on the other hand they can be ancient granulomas that accumulate waxy pigments that have been attributed to mycobacteria in previous studies [40].

DNA of *M. avium* was present in 16.6 % of the analyzed GALT samples. If we compare C_T values in sampled tissues with the PCR of artificially spiked tissues we can estimate that the bacterial load is probably below 10^4 CFU/g. In an experimental infection in rabbits with Map, isolation was variable, since some sacculus rotundus and vermiform appendix samples that had bacterial loads of 10^2-10^6 genomic equivalents/g estimated by qPCR gave positive solid culture results, whereas the same tissue site samples from other animals with 10^1-10^5 genomic equivalents/g gave negative culture results (unpublished observations). Of course, isolation variability can be affected by the irregular distribution of AFB in the analyzed tissues among other factors. As for, liquid cultures, they were eventually contaminated and other bacteria could have used up the nutrients necessary for mycobacterial growth. MycMavMtc PCR of MGIT cultures was negative meaning that mycobacterial load was possibly under $1{,}59\times10^2$ CFU/g, since this was the reported detection limit for MGIT culture in a bovine tissue mixture using 2 % NaOH [31]. If more than 2 % NaOH were to be used as in the present study (6 % NaOH), it would probably affect mycobacterial viability and interfere with isolation in both solid and liquid media. In solid media, only *Thermoactinomyces*, bacteria were isolated. *Thermoactinomyces* species have been implicated as causal agents of farmer's lung diseases [41] since the disease appears in farmers that are in direct contact with mouldy hay and cereal grains where these bacterial species are known to be abundant. Also they are found in soil, rivers and dairy products [42]. We are not sure about the meaning of this finding but it could be that these bacteria are present in the grains that the rabbits are fed or in the straw litter that maybe used for nesting before kindling. In any case, it is worth mentioning that these bacteria survive decontamination techniques used for mycobacterial culture and that no macro or microscopic signs of pathology in the digestive system were detected in the animals these bacteria were isolated from.

Association analysis revealed that neither gross pathology nor mycobacterial presence was affected by age. Maa has been detected before in rabbits that had been housed with infected pigeons [43] or with doves, ducks and chickens [44] suggesting in both cases that transmission had probably occurred from infected fowl to rabbits. In our case, although not specifically examined, given the standard production housing characteristics in the studied region, it is most probable that rabbits did not have direct contact with fowl. In any case, young rabbits do present Maa implicating an early contact in their life that might point to water or feeding.

The results from this study show that *M. avium* subspecies are widely spread and in contact with farmed rabbits since only 2–3 animals analyzed from each batch of 21 farms has shown 16.6 % positivity among animals and 38 % positivity among farms. Detection of mycobacterial DNA was achieved in 50 % of the tissues with focal lesions, slightly higher than what has been reported for Map in cattle [40, 45]. At this point, we are not sure about the real significance of detecting *M. avium* subspecies in GALT tissue of slaughtered rabbits either from an animal pathological and epidemiological or Public Health perspective. Although detected granulomas suggest the generation of lesions, the focal nature of these granulomas could be compatible with a latent and controlled infection. Rabbit GALT does not enter the food chain, but if it occurred, properly cooked affected parts should not pose a risk to healthy or immunocompromised individuals. Scarcity of previous reports indicates that the problem probably does not compromise rabbit production to a big extent. Lack of other information on the infected animals such as clinical status or weight at slaughter impedes drawing conclusions. It is noteworthy to state that no Map was detected or isolated, therefore indicating that its presence in wild rabbits might be related to environmental factors that do not occur in more controlled farm conditions or that its prevalence is lower and a higher number of animals should be tested.

Conclusions

This study should be considered as a preliminary survey that might draw attention to a hitherto undetected potential problem. Future studies should consider including other intestinal sites, associated lymph nodes and respiratory tissues as well.

The detection MAC bacteria other than Map indicates that a niche for this group of mycobacteria does exist in farmed rabbits.

Abbreviations

Map: *Mycobacterium avium* subsp. *paratuberculosis*; Maa: *Mycobacterium avium* subsp. *avium*; Mah: *Mycobacterium avium* subsp. *hominissuis*; MAC: *Mycobacterium avium* complex; AFB: Acid fast bacilli; GALT: Gut associated lymphoid tissue; HE: Haematoxylin and eosin; ZN: Ziehl-Neelsen; HEYM: Herrold's Egg Yolk Medium; PBS: Phosphate buffered saline; PBS-GT: PBS 0.2 % glycerol 0.05 % Tween 80; MGIT: Mycobacteria Growth Indicator Tubes; TTD: Time to detection; FET: Fisher exact probability; CFU: Colony forming unit; PCR: Polymerase chain reaction; C_T: Threshold cycle; IAC: Internal amplification control; ROX: 6-carboxyl-X-rhodamine; UD: Undetermined; NI: No isolation; NA: Not analyzed; SR: Sacculus rotundus; VA: Vermiform appendix; IL: Ileum; JE: Jejunum.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

RA collected the samples, participated in all the microbiological and pathological studies and collaborated in the interpretation of the data and writing of the paper, IS participated in the analysis of the data, interpretation of the results and writing of the paper, EM contributed to the sample collection and microbiological studies, VP collaborated in the histopathological interpretation and paper drafting, JG collaborated in the interpretation of the results and the paper drafting, RJ conceived and designed the experiment, performed data analysis and drafting of the paper, NE conceived and designed the experiment, participated in sample collection, pathological studies, interpretation of the results and performed data analysis and writing of the paper. All the authors read and approved the final manuscript.

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Acknowledgements

The authors thank the official veterinarian, Susana Rodríguez and all the employees at the rabbit slaughterhouse for their collaboration in collecting the samples and also Dr. Patricia Vazquez for technical assistance with the histopathological analysis.

Funding

This study was supported by grants (AGL2012-39818-CO2-02) and (INIA, RTA2010-00022) from the Spanish Ministry of Economy and Competitiveness (MINECO) and the Spanish National Institute for Agricultural and Food Research and Technology, respectively. Rakel Arrazuria held a pre doctoral fellowship (BFI-2012-237) from the Basque Government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received: 18 February 2015 Accepted: 20 May 2015
Published online: 11 June 2015

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ESTUDIO II / STUDY II

Effect of various dietary regimens on oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* in a rabbit model. R Arrazuria, E Molina, M Mateo-Abad, I Arostegui, JM Garrido, RA Juste, N Elguezabal. Res Vet Sci. 2015, 101:80-3.

Research in Veterinary Science 101 (2015) 80–83

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/yrvsc

Effect of various dietary regimens on oral challenge with *Mycobacterium avium* subsp. *paratuberculosis* in a rabbit model

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ARTICLE INFO

Article history:
Received 27 January 2015
Received in revised form 10 June 2015
Accepted 14 June 2015

Keywords:
Mycobacterium avium sbsp. *paratuberculosis*
Johne's disease
Infection
Rabbit
Animal model
Diet

ABSTRACT

Rabbits are susceptible to infection by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in both wildlife and experimental conditions. Under the hypotheses that nutrient balance might influence the establishment of infection, we designed an experiment where MAP intestinal colonization was assessed under three dietary regimens: high fiber, high protein, and regular diet in New Zealand white rabbits submitted to oral challenge with MAP.

Lowest weight gain ($F = 5.17$, $p = 0.024$), higher tissue culture positivity rates ($\chi^2 = 7.43$, $p = 0.024$) and especially extended MAP-compatible lesions ($F = 5.78$, $p = 0.017$) were detected in the regular diet.

Taken altogether, results indicate that paratuberculosis infection was achieved affecting mostly regular diet animals and showing that dietary changes may modulate the course of the infection.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is responsible for a chronic granulomatous enteritis named paratuberculosis (PTB), distributed worldwide (Kennedy and Benedictus, 2001; Manning and Collins, 2001). Although PTB has been historically linked to domestic ruminants there is evidence that wild-life species, both ruminant (Chiodini and Van Kruiningen, 1983) and non-ruminant (Beard et al., 2001a; Greig et al., 1997) are susceptible to natural infection. Association of MAP with Crohn's disease makes PTB a human health concern, as well (Hermon-Taylor, 2001).

The pathogenic mechanisms of PTB have not been fully elucidated probably due to the lack of an appropriate laboratory animal model and the slow infection characteristics including the lengthy incubation period of the disease (Juste, 2012).

Rabbits are a convenient laboratory species and natural PTB infections of wild rabbits (Angus, 1990; Beard et al., 2001b; Greig et al., 1997) have been described. Intestinal lesions consistent with PTB (Harding, 1959; Mokresh and Butler, 1990; Mokresh et al., 1989; Vaughan et al., 2005) as well as diarrhea (Mokresh and Butler, 1990; Mokresh et al., 1989) and fecal shedding (Mokresh et al., 1989) have been reported previously in rabbits orally inoculated with MAP in laboratory conditions. Susceptibility to MAP in both experimental (Harding, 1959; Mokresh and Butler, 1990; Mokresh et al., 1989; Vaughan et al., 2005) and natural (Beard et al., 2001b; Greig et al., 1997) infection conditions suggests that rabbits may be a suitable model for PTB.

Dietary changes have been shown to have an effect on infectious diseases caused by bacteria (Fox and Wang, 2014; Zumbrun et al., 2013). We hypothesized that dietary changes could influence MAP infection. Therefore, the aim of this study was to evaluate the effects of diet shifts during MAP challenge to gather information that might aid further investigation regarding the potential use of rabbits as a PTB model. To test the hypothesis, three different diet conditions: regular rabbit (R), high fiber (HF), high protein (HP) were tested simultaneous to oral challenge with MAP strain K10. Infection progression was evaluated and monitored by MAP isolation on solid media and quantitative polymerase chain reaction (qPCR) of feces and tissues, as well as by histopathological examination of tissues.

MAP strain K10 was cultured on Middlebrook 7H9 (7H9) liquid media supplemented with OADC and mycobactin J (MJ) for 4 weeks at 37 +/− 1 °C. Bacterial concentration was adjusted in PBS by turbidometry. Colony forming unit (CFU) counts were confirmed on 7H9 OADC MJ agar plates. The final challenging dose was 4×10^8 CFU of MAP.

New Zealand rabbits were purchased from an accredited animal dealer (Granja San Bernardo, Navarra) arriving at the animal facilities

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http://dx.doi.org/10.1016/j.rvsc.2015.06.006
0034-5288/

at an age of 6 weeks (1.5 kg). After a two week adaptation period fed with weaning pellets, animals were tattooed for identification and started feeding with diet R for 10 days. They were then divided into three diet groups: R (n = 5), HF (n = 5) and HP (n = 5). Diet compositions are detailed on Supplementary Table 1. Four days after commencing these diets, weight was recorded and feces were collected from all animals (S_0, day 0). On three consecutive days (days 1, 2 and 3) animals were orally administered a single challenging dose per day. Feces were collected on day 3 (S_{0A}) to check MAP pass through the digestive tract. Also, from day 3 on, all animals were fed diet R until the end of the experiment (day 114). Monitorization consisted of weight recording along with feces collection twice a month (S_1–S_8).

The study was designed following European, National and Regional Law and Ethics Committee regulations and it underwent ethical review and approval by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAMOD-6278-BFA).

At the endpoint, animals were injected with xylazine (5 mg/kg) and ketamine (35 mg/kg) intramuscularly for sedation. Then pentobarbital was injected intracardiacly.

For microbiological examination, samples from ileum, jejunum, sacculus rotundus (SR), ileocecal junction, vermiform appendix (VA), liver, spleen, muscle, tonsils and cecal contents were collected and stored at −20 °C. For histopathological examination, all previously mentioned samples except for cecal contents were taken and processed as described by Vazquez et al. (2013).

Slides were examined under the microscope and granuloma extension was measured using Image J software (http://imagej.nih.gov/ij/) (Schneider et al., 2012) on two micrographs of each section. The lesion index was calculated as the total area of granulomatous lesion divided by the total area of the micrograph.

Two grams of freshly collected feces was cultured on solid Herrold Egg Yolk Medium (HEYM) as described by Aduriz et al. (1995). For culture on 7H9 OADC MJ penicillin, anfotericin and cloramphenicol, the decontaminated suspension was washed twice with sterile water (2885 ×g during 10 min). Four drops/tube of the final pellet suspended in 2 ml of water were seeded.

For tissue culture, tonsils, spleen, liver, and muscle were spliced into tiny pieces whereas VA, SR, ileum and jejunum were scraped for mucosa. Previously mentioned samples along with cecal content were weighed and identical protocol as for feces was followed.

DNA extraction from feces was done following manufacturer's instructions of DNA Extract-VK (Vacunek S.L.). For tissues, brief modifications described by Arrazuria et al. (2015) were performed. In both cases, extracted DNA was stored at −20 °C for PCR analysis.

MAP detection was performed following a MAP F57 PCR (Schonenbrucher et al., 2008). Samples yielding C_T values equal or below 37 for F57 probe were considered positive. In these cases, MAP genomic equivalents (GE) were estimated by ParaTB Kuanti-VK qPCR (Vacunek, S.L.) as described by Elguezabal et al. (2011).

For weight, fecal PCR MAP tissue PCR and lesion analysis ANOVA approach based on summary measures was used (weight gain: the difference between S_8 weight and S_0 weight, total fecal shedding: total GE/g of MAP detected in feces by each animal during the experiment, total MAP in tissues: the sum of MAP GE/g in all examined tissues, total lesion index (TLI): sum of the lesion extension in all examined tissues). For dichotomous variables such as tissue culture, logistic regression was used taking R as the reference category. For weight and fecal PCR, analysis of repeated measurements was done by mixed-effect regression, including individuals as random-effect. Multiple Correspondence Analysis (MCA) was used as a multivariate exploratory analysis to detect and graphically represent underlying structures in the data (Benzécri, 1969). All the in vivo and post mortem measurements were included in the MCA as categorical versions of the original variables. All statistical analyses were performed using R statistical software (3.1.0) and significance of the differences among groups for all variables was stated at $p < 0.05$.

During the in vivo follow-up no overt clinical signs were observed and weight loss between samplings was minimal and exceptional. This was expected since weight loss has shown to be rare in long term experiments (Mokresh et al., 1989; Vaughan et al., 2005). Diet R animals gained less weight than animals that had been on the other two diets during challenge and significant differences were observed both when weight gain among groups was analyzed ($F = 5.17$, $p = 0.024$). Moreover, considering all the measurements over time, diet R has significantly less weight than HF (Supplementary Table 2).

MAP passed through the digestive tract demonstrated by culture and q-PCR of sampling $S0_A$ feces (Table 1), with no significant differences in bacterial load among diet groups suggesting that challenge was achieved equally in all animals.

No episodes of diarrhea were observed and fecal culture was positive only in sampling $S0_A$ in 66.6% of the animals being negative in all cases thereafter. In previous experimental infections, MAP was either not isolated from feces (Mokresh and Butler, 1990; Vaughan et al., 2005) or isolated from 30.7% of infected animals (Mokresh et al., 1989). Unsuccessful fecal isolation could be due to low detection limit by culture, light shedding or low sampling frequency. qPCR showed higher detection capacity since all animals were positive in $S0_A$ and MAP shedders were detected throughout experiment samplings. Total MAP shedding tended to be higher in diet group R although significant differences were not detected.

Gross lesions consisting in pale-white reactive spots were detected in the SR and VA in diet R (40%) and diet HF (20%) animals contrary to previous studies where macroscopic lesions were not reported (Mokresh and Butler, 1990; Mokresh et al., 1989; Vaughan et al., 2005).

Microscopically, animals presented granulomatous infiltrates in the SR and VA located in the follicular and/or interfollicular regions depending on the diet. Well demarcated granulomas with a huge variability in size were detected (Supplementary Fig. 1 A, B and C). These findings are consistent with PTB infection and could be equivalent to focal and multifocal lesions detected in subclinically infected goats (Corpa et al., 2000) or sheep (Pérez et al., 1996). AFB were detected in SR of only one rabbit from diet group R indicating a low bacterial colonization, a dormancy-related loss of acid-fastness (Zhang, 2004) or too short duration of the experimental trail.

The TLI was higher in diet group R (0.550 +/− 0.359) compared to diet HF (0.100 +/− 0.068) and diet HP (0.196 +/− 0.108) showing significant differences ($F = 5.78$, $p = 0.017$) (Supplemental Fig. 1 D), suggesting that diet R could favor MAP tissue reaction or that diets HF and HP were able to limit lesion extension.

Mucosa from VA, SR, ileum and jejunum was MAP positive by culture (Table 1). Tissue locations are in agreement with previous works showing MAP culture positive results for VA (Mokresh et al., 1989; Vaughan et al., 2005) and SR and ileum (Mokresh et al., 1989). Diet group R showed a higher MAP culture positivity rate (60%) ($\chi^2 = 7.43$, $p = 0.024$). Differences between diet group R and HF were observed in both VA ($p = 0.035$) and SR ($p = 0.008$), and differences between diet R and diet HP in SR ($p = 0.008$). Bacterial load measured by qPCR was variable among specimens and individual animals showing no significant differences among groups.

MCA analysis gave a picture of the infection outcome, by explaining 62% of the variability in the measurements (Fig. 1). The resulting two dimensional map clearly shows that diet R animals are correlated to higher rates of infection since most positive results and high rates were concentrated on the right side of the graph where 80% of the animals with diet R appeared, whereas negative results and low indexes were in the left side, where 80% of the animals from the other diets were located.

In conclusion Diet R performed best at aiding infection in the assayed conditions and the two diet changes could be modifying the course of infection in a way that we cannot explain at the moment. These results suggest that there is a strong interaction between diet and exposure to MAP that should be further investigated.

Table 1
Microbiological findings in feces through in vivo follow up and in post mortem tissues.

Diet	Animal	MAP levels by qPCR (GE/g)									MAP culture		
		Feces						Tissues			Tissues		
		$S0_A$	S1	S4	S5	S6	S8	VA	SR	IM	VA	SR	IM
	NT	$1.45\ 10^5$	0	$1.58\ 10^3$	$3.99\ 10^3$	0	0	$4.52\ 10^2$	$1.49\ 10^4$	0	+	+	−
	0	$1.38\ 10^5$	0	0	$1.35\ 10^3$	0	0	$1.22\ 10^4$	$1.43\ 10^4$	0	+	+	+
R	1	$3.75\ 10^5$	0	0	$4.07\ 10^3$	$5.64\ 10^3$	0	0	$1.35\ 10^5$	0	−	−	−
	2	$3.50\ 10^5$	0	0	0	0	0	0	$4.44\ 10^2$	0	−	+	−
	3	$2.01\ 10^5$	0	0	0	0	0	0	$1.41\ 10^5$	0	−	−	−
	4	$2.00\ 10^5$	0	0	$2.23\ 10^3$	0	101.25	$3.89\ 10^2$	$3.57\ 10^4$	0	−	−	−
	5	$1.98\ 10^5$	0	0	0	0	540	0	$1.31\ 10^2$	0	−	−	−
HF	6	$1.56\ 10^5$	0	0	0	0	0	$5.88\ 10^3$	$3.91\ 10^6$	$3.93\ 10^2$	+	+	−
	7	$7.13\ 10^2$	0	0	0	0	0	54.3	$1.06\ 10^3$	0	−	−	−
	8	$7.27\ 10^5$	0	0	0	0	0	0	$4.10\ 10^2$	0	−	−	−
	9	$2.00\ 10^5$	0	0	0	0	0	0	0	0	−	−	−
	12	$1.80\ 10^5$	0	0	0	0	0	0	0	0	−	−	−
HP	13	$2.65\ 10^5$	$1.40\ 10^3$	0	0	0	0	75	$4.15\ 10^5$	0	−	−	−
	14	$6.46\ 10^5$	0	0	$1.31\ 10^3$	0	0	0	$2.89\ 10^2$	0	−	−	−
	15	$4.97\ 10^5$	0	0	0	0	0	23	$7.38\ 10^2$	0	−	+	−

R: regular diet; HF: high fiber diet; HP: high protein diet, S: sampling, VA: vermiform appendix, SR: sacculus rotundus, IM: ileum mucosa.
S0, S2, S3, and S7 did not yield positive fecal culture results. Tonsils, spleen, liver, cecal content, ileum, jejunum and muscle were negative by culture.
Tonsils and spleen were not determined by qPCR and liver, muscle, cecal content and ileocecal valve were all negative by this technique.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rvsc.2015.06.006.

Competing interests

The authors declare that they have no competing interests.

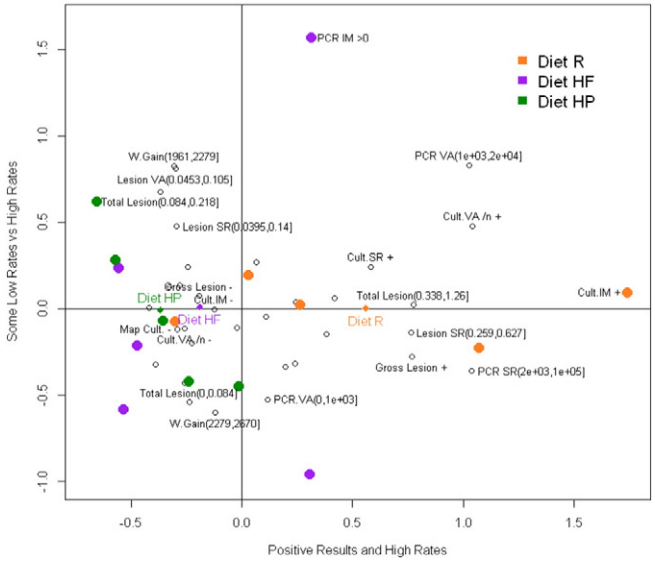

Fig. 1. Map created by the first two components derived from the multiple correspondence analysis. Interpretation of the Multiple Correspondence Analysis (MCA) map: The right side contains close to positive results (positive Map culture) and high rates (high Map load in feces, high Map load in tissues, large lesion extension), whereas left side contains close to negative (negative Map culture) and low rates (low Map load in feces, low Map load in tissues, small lesion extension). Empty circles in the plane represent the categories of the measurements included in the MCA analysis being only the most representative ones were labeled. The relative position of the category points indicates the level of similarity or association between the categories. The closer the points are, the stronger the relationship between categories is. Diet was included in the map with illustrative purposes and all the individuals were also projected into the map. Relative positions of the subjects in this plane are represented by large circles in different colors (Diet R: orange, Diet HF: purple and Diet HP: green) and small rhombus in the same colors represents mean position of each diet group. Most Diet R individuals are on the right side of the Map implying a higher association with parameters that could represent a close to fulfilled infection status whereas Diet HF and Diet HP individuals lay on the left side of the map indicating a mild infection or a not properly achieved infection.

Acknowledgments

The authors thank Felix Blanco from the animal facilities (NEIKER-Tecnalia) for assistance and Esmeralda Minguijón (NEIKER-Tecnalia) and Valentín Pérez (University of León) for technical advice with histopathological techniques.

This study was supported with funds from the Ministerio de Economía y Competitividad from the Spanish Government (AGL 2012-39818-C02-02), from the Departamento de Medio Ambiente, Planificación Territorial, Agricultura y Pesca of the Basque Government and from the Departamento de Educación, Política Científica y Cultura of the Basque Government (IT620-13). RA acknowledges support from the Departamento de Educación y Universidades of the Basque Government (BFI-2012-237).

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ESTUDIO III / STUDY III

***Mycobacterium avium* subspecies *paratuberculosis* infection modifies gut microbiota under different dietary conditions in a rabbit model.** R Arrazuria, N Elguezabal, RA Juste, H Derakhshani, E Khafipour. Front Microbiol. 2016, 7:446.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 March 2016
doi: 10.3389/fmicb.2016.00446

Mycobacterium avium Subspecies *paratuberculosis* Infection Modifies Gut Microbiota under Different Dietary Conditions in a Rabbit Model

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Systems Microbiology, a section of the journal Frontiers in Microbiology

Received: *30 November 2015*
Accepted: *18 March 2016*
Published: *31 March 2016*

Citation:
Arrazuria R, Elguezabal N, Juste RA, Derakhshani H and Khafipour E (2016) Mycobacterium avium Subspecies paratuberculosis Infection Modifies Gut Microbiota under Different Dietary Conditions in a Rabbit Model. Front. Microbiol. 7:446. doi: 10.3389/fmicb.2016.00446

Mycobacterium avium subspecies *paratuberculosis* (MAP) the causative agent of paratuberculosis, produces a chronic granulomatous inflammation of the gastrointestinal tract of ruminants. It has been recently suggested that MAP infection may be associated with dysbiosis of intestinal microbiota in ruminants. Since diet is one of the key factors affecting the balance of microbial populations in the digestive tract, we intended to evaluate the effect of MAP infection in a rabbit model fed a regular or high fiber diet during challenge. The composition of microbiota of the cecal content and the sacculus rotundus was studied in 20 New Zealand white female rabbits. The extracted DNA was subjected to paired-end Illumina sequencing of the V3-V4 hypervariable region of the 16S rRNA gene for microbiota analysis. Microbial richness (Chao1) in the cecal content was significantly increased by MAP infection in regular diet rabbits ($p = 0.0043$) and marginally increased ($p = 0.0503$) in the high fiber group. Analysis of beta-diversity showed that MAP infection produces deeper changes in the microbiota of sacculus rotundus than in the cecal content. A lower abundance of Proteobacteria in the cecal content of infected animals fed the high fiber diet and also lower abundance of Bacteroidetes in the sacculus rotundus of infected animals fed the regular diet were observed. Based on OPLS-DA analysis, we observed that some bacteria repeatedly appear to be positively associated with infection in different samples under different diets (families Dehalobacteriaceae, Coriobacteriaceae, and Mogibacteriaceae; genus *Anaerofustis*). The same phenomenon was observed with some of the bacteria negatively associated with MAP infection (genera *Anaerostipes* and *Coprobacillus*). However, other groups of bacteria (Enterobacteriaceae family and ML615J-28 order) were positively associated with infection in some circumstances and negatively associated with infection in others. Data demonstrate that MAP infection and diet changes do interact and result in shifts in the microbiota of the cecal content and sacculus rotundus of rabbits.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, rabbit, animal model, high fiber diet, gut microbiota, inflammation

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of paratuberculosis (PTB) a chronic granulomatous inflammation of the gastrointestinal (GI) tract of ruminants. The infection may be cleared by the host or develop into subclinical or clinical disease causing significant economic losses in livestock (Clarke, 1997; Kennedy and Benedictus, 2001).

Although PTB has been historically linked to domestic ruminants, wild-life ruminant, and non-ruminant species are also susceptible to infection (Chiodini and Van Kruiningen, 1983; Beard et al., 2001a; Forde et al., 2013). In addition, association of MAP with Crohn's disease (CD; Naser et al., 2004; Juste et al., 2008) and Type 1 diabetes (Masala et al., 2011; Naser et al., 2013) in humans adds further interest to the role of this disease as a potential zoonosis.

It is known that the gut microbiome plays an important role in competitive exclusion of pathogens and in development and maturation of intestinal mucosal immunity (Kau et al., 2011; Stecher and Hardt, 2011). Many studies have documented differences in the composition of host associated microbial communities between healthy and diseased states (Clemente et al., 2012; Karlsson et al., 2013; Knights et al., 2013). It is recognized that an altered microbiome is not just a marker of disease but that it also actively contributes to pathogenesis (Chassaing et al., 2012). Reduced diversity of both fecal and mucosa-associated microbiota has been extensively reported in patients suffering from chronic intestinal inflammatory disease (Manichanh et al., 2006; Dicksved et al., 2008; Walker et al., 2011). Moreover, the composition of gut microbial communities may also modulate Type 1 diabetes (Wen et al., 2008).

Additionally, it is important to note that diet is one of the key factors affecting the balance of microbial populations in the digestive tract (Xu and Knight, 2014; Graf et al., 2015). Dietary nutrients are the principal substrates for the microbial population and also have a direct effect on the immune response (Faria et al., 2013). In laboratory mice, dietary-mediated effects are evident in a GI infection model (Ooi et al., 2014). Although the diet role in MAP infection has been scarcely studied, in previous experiments we observed that short term dietary shifts can modulate PTB infection in a rabbit model, showing differences in histopathological lesion extension and bacterial load on gut associated lymphoid tissue (GALT; Arrazuria et al., 2015a).

Rabbits are naturally susceptible to the development of MAP infections causing severe PTB-associated inflammatory responses in its wild habitats (Beard et al., 2001b; Maio et al., 2011), and milder pathological changes under experimental conditions (Vaughan et al., 2005; Arrazuria et al., 2015a). These characteristics, together with their size and handling easiness make rabbits a convenient experimental species to study chronic intestinal diseases of animals and humans. One specific feature of rabbit GALT is that it is composed of two additional special structures: the sacculus rotundus, which is located at the ileo-cecal junction, and the vermiform appendix, located at the end of the cecum. These two lymphoid organs account for more than 50% of the total lymphoid tissue in the rabbit (Rees Davies et al., 2003). These tissues can induce an effective immune response and it has been demonstrated that a specific microbial composition on the vermiform appendix can induce inflammation (Shanmugam et al., 2005). The sacculus rotundus closely resembles the ileocecal-valve in ruminants, and in previous MAP infection studies with rabbits we detected higher MAP loads in sacculus rotundus compared to vermiform appendix and any other sites in the intestine (Arrazuria et al., 2015a).

Recent advances in next generation sequencing technologies have enabled researchers to investigate the complete microbial composition of the GI tract under different conditions (Schuster, 2008). In the present study, high-throughput sequencing of the 16S rRNA gene was performed to determine MAP infection associated shifts in the microbial composition of cecal content and sacculus rotundus.

Previous results from our group have shown that diet shifts can modulate MAP infection (Arrazuria et al., 2015a). In humans, high fiber diets have been shown to promote and increase the gut microbiota diversity (Steinle et al., 2013) and also to diminish inflammatory responses by a mechanism that includes shaping the intestinal microbiome and indirectly affecting the immune system (Kuo, 2013). On the basis of previously obtained results, in which a high fiber diet during challenge produced MAP infection modulation (Arrazuria et al., 2015a), we again performed this dietary shift to investigate if it produces changes in gut microbiota that could help understanding MAP infection modulation.

We hypothesized that as in other chronic intestinal diseases, MAP infection may produce changes in the gut microbiota. Therefore, our aim was to evaluate changes in gut microbiota in rabbits challenged with MAP and fed either a regular or a high fiber diet.

MATERIALS AND METHODS

Animals and Experimental Designs

New Zealand white female rabbits ($n = 20$) were purchased from authorized experimental animals dealers, Granja San Bernardo, Tulebras, Spain and arrived at NEIKER animal facilities, Derio, Spain, at the age of 6 weeks and 1.5 kg of body weight. After a 2-week adaptation period during which rabbits were fed special weaning pellets, all animals started taking a regular growing diet containing neither antibiotics nor coccidiostatics (**Table 1**). To prevent obesity, feeding was limited to 30–35 g/day of Dry Matter/kg of live weight throughout the experiment. Two

TABLE 1 | The nutrient composition of the diets.

Feed Composition	Regular diet	High fiber diet
Dry Matter (%)	90.26	93.3
Crude Protein (%)	16.32	2.69
Crude Fat (%)	3.04	1.02
Crude Fiber (%)	14.36	35.05
Crude Ash (%)	7.04	8.40

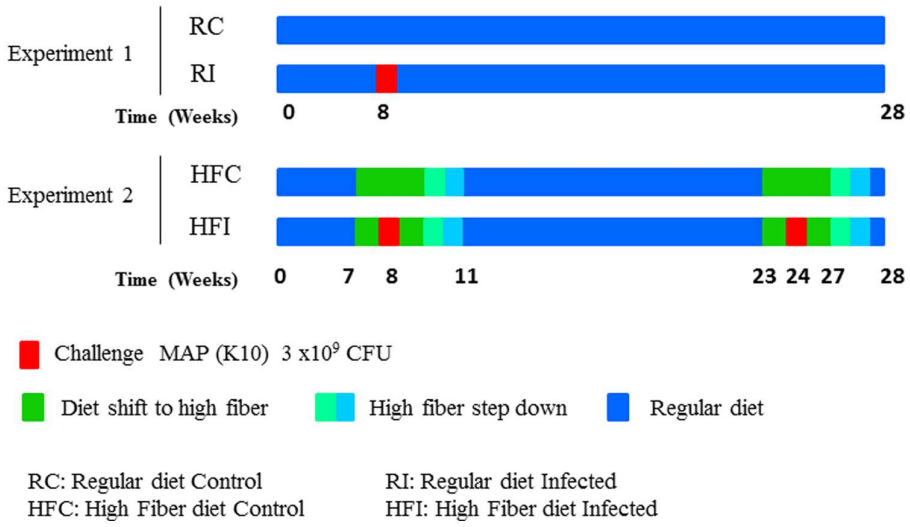

FIGURE 1 | Experimental design diagram.

different experiments were carried out. The experimental scheme is detailed on **Figure 1**.

In experiment 1, in which all animals were fed a regular diet (**Table 1**), rabbits were split in two groups ($n = 5$/treatment); uninfected control (RC: Regular diet Control) and infected with MAP (RI: Regular diet Infected). The RI group was orally challenged 8 weeks after the beginning of the experiment (**Figure 1**) with 2 ml of 2×10^8 cfu/ml of MAP strain K10 for three consecutive days as described previously (Arrazuria et al., 2015a).

In the second experiment, rabbits were also split in two groups ($n = 5$/treatment): (a) uninfected controls taking high fiber diet during the same period as their infected mates (HFC, high fiber control), and (b) twice infected with MAP taking high fiber diet during challenge (HFI, high fiber infected). In this experiment, regular diet was switched to a high fiber diet (**Table 1**) from week 7 to week 11 and from week 23 to week 26 in both groups (**Figure 1**) in order to modulate MAP infection as previously observed (Arrazuria et al., 2015a). The high fiber diet was switched back to the regular diet by replacing 25% of high fiber diet with regular diet weekly. The HFI group was orally challenged at week 8 and 24 with 2 ml of 2×10^8 cfu/ml of MAP strain K10 for three consecutive days as described previously (Arrazuria et al., 2015a).

All four groups were housed in different rooms, with direct contact between animals belonging to the same group, and *ad libitum* water available on accessible containers fixed to the room walls. All animals were euthanized at week 28 of the experiment (36 weeks of age) by intracardiac pentobarbital injection after deep sedation with xylazine (5 mg/kg) and ketamine (35 mg/kg). Animals were exsanguinated and then necropsied.

This study was carried out following European, National, and Regional regulations on animals used in experimentation and other scientific purposes. The procedure was evaluated by the Ethics Committee of the institution (NEIKER-OEBA-2014-0001) and authorized by the Regional Council (BFA-4269).

Sampling, DNA Extraction, and PCR for MAP Detection

The lymphoid tissues: sacculus rotundus, vermiform appendix, and mesenteric lymph node were excised and stored at −20°C until further analyses.

DNA extraction was performed using DNA Extract-VK (Vacunek S.L, Bizkaia, Spain) following manufacturer's instructions as described previously (Arrazuria et al., 2015b). Briefly, DNA extraction was carried out on 300 mg of tissue, with a combination of mechanical disruption of the cell walls using glass microspheres (100 μm) agitation and a chemical lysis with SDS and proteinase K. DNA purification was achieved via silica based spin columns (Vacunek S.L, Bizkaia, Spain).

To identify MAP positive samples, DNA was subjected to a real-time multiplex PCR assay targeting the IS*900* and IS*Map02* DNA sequences of MAP (Sevilla et al., 2014). The reaction mixture contained 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Madrid, Spain), 300–400 nM of each primer, 200 nM of each probe, and 5 μl of DNA extract in a final volume of 25 μl. Negative DNA extraction controls, non-template DNA and a positive PCR control were included in each PCR assay. A competitive internal amplification control (IAC) was also included in the PCR reaction. Amplification was carried out in a 7500 Real-Time PCR instrument (Applied Biosystems, Madrid, Spain) and consisted of one denaturation and polymerase activation cycle of 10 min at 95°C, and 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Results were analyzed using the 7500 System SDS software v. 1.4 (Applied Biosystems). Threshold cycle (C_T) and baseline were automatically determined by the software and verified by visual examination of the threshold line in amplification plots. C_T values equal or below 38 for both IS*900*

and IS*Map02* probes were considered positive, C_T values over 38 for both targets probe and under 38 for IAC probe were considered negative.

Sampling and DNA Extraction for 16S rRNA Gene Sequencing

To obtain the samples of cecal content, a small incision about 5 cm long was made in the middle of the cecum and approximately one gram of cecal content was taken and immediately saved in a sterile microtube. The sacculus rotundus was carefully excised and washed with sterile PBS to remove the digesta residues. A fragment was divided and saved immediately in a sterile microtube. All samples were stored in liquid nitrogen until further processing.

The frozen cecal content was defrosted and total DNA was extracted from 150 mg of each sample using a ZR Fecal DNA MiniPrep (Zymo Research, Freiburg, Germany) according to manufacturer's instructions. This extraction kit includes a bead-beating step for the mechanical lysis of the cells.

The DNA extraction from the sacculus rotundus was carried out with Ultra-Deep Microbiome Prep kit (Molzym, Bremen, Germany) following manufacturer's instructions. This extraction method allows the enrichment of bacterial DNA and removal of animal and "dead" microbial DNA. Briefly, 0.25 cm^2 of tissue sample was treated for host cell lysis under chaotropic conditions. The released DNA was then enzymatically degraded, and then the degrading enzymes inactivated. After bacterial cell wall degradation the DNA was extracted and purified via silica based spin columns. DNA concentration and purity of all samples were determined with ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) by measuring the $A_{260/280}$. DNA quality was also evaluated by gel electrophoresis after standard PCR using universal primers pAF (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 530R (5′-CCG CGG CKG CTG GCAC-3′). DNA extracts were stored at −20°C until they were processed.

Library Construction and MiSeq Illumina Sequencing

The V3–V4 region of 16S rRNA gene was targeted for PCR amplification using a modified F338 and barcoded R806 primers (Caporaso et al., 2012) as described previously (Derakhshani et al., 2016b). Briefly, PCR reaction for each sample was performed in duplicate and contained 1.0 μl of pre-normalized DNA (20 ng/μl), 1.0 μl of each forward and reverse primers (10 μM), 12 μl HPLC grade water (Fisher Scientific, ON, Canada) and 10 μl 5 Prime Hot MasterMix (5 Prime, Inc., Gaithersburg, MD, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 30 amplification cycles at 94°C for 45 s, 62°C for 60 s, and 72°C for 90 s; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit (Zymo Research, Irvine, CA, USA) to remove primers, dNTPs and reaction components. The V3–V4 libraries were then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, New York, NY, USA) and diluted to a final concentration of 5 pM, measured by Qubit 2.0 Fluorometer (Life technologies, Ottawa, ON, Canada). In order to improve the unbalanced and biased base composition of the generated 16S rRNA libraries, 15% of PhiX control library was spiked into each amplicon pool. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGAC TGACT-3′) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V3 (600-cycle; Illumina, San Diego, CA, USA). The 300 paired-end sequencing reactions were performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada. The sequencing data were deposited into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed via accession number SRR2962702.

Bioinformatics and Statistical Analyses

The FLASH assembler (Magoč and Salzberg, 2011) was used to merge overlapping paired-end Illumina fastq files. All the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME version 1.9.0 (Caporaso et al., 2010b). Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with ambiguous calls and those with phred quality scores (Q-scores) below 20 were discarded. Chimeric reads were filtered using UCHIME (Edgar et al., 2011) and sequences were assigned to operational taxonomic units (OTU) using the QIIME implementation of UCLUST (Edgar, 2010) at 97% pairwise identity threshold. Taxonomies assignment of representative OTUs and alignment to

TABLE 2 | *Mycobacterium avium* subspecies *paratuberculosis* (MAP) PCR results in the infected animals.

		PCR results for indicated site		
Group	**Animal ID**	**SR**	**VA**	**MLN**
RI	16	−	−	−
	17	+	−	−
	18	−	−	+
	19	−	−	+
	20	+	−	+
HFI	11	−	−	+
	12	+	+	+
	13	−	−	+
	14	−	+	+
	15	−	−	+

RI, regular diet infected; HFI, high fiber diet infected; SR, sacculus rotundus; VA, vermiform appendix; MLN, mesenteric lymph node.

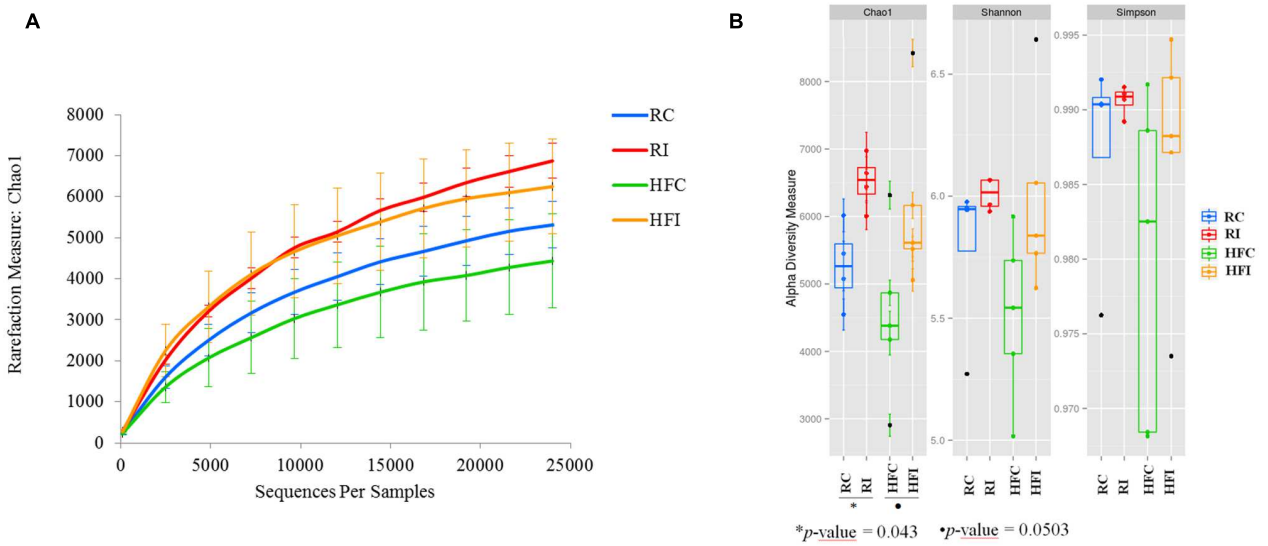

FIGURE 2 | Cecal content diversity analysis. (A) Cecal content microbiota rarefaction curve generated using Chao1 richness estimator. Samples have been rarified at an even depth of 24,500 sequences per sample. Error bars indicate the 95% confidence intervals. **(B)** Cecal content alpha-diversity; richness (Chao1) and diversity (Shannon and Simpson) indices box plot.

Silva reference database (Quast et al., 2013) were performed using PyNAST algorithms (Caporaso et al., 2010a). Phylogenetic trees were built with FastTree 2.1.3 for further comparisons between microbial communities (Price et al., 2010).

Within community diversity (alpha-diversity) was calculated using QIIME scripts. An even depth of 24,500 and 12,400 sequences per sample was used for calculation of species richness (Chao1; Chao, 1984) and diversity indices (Shannon and Simpson) for the cecal content and sacculus rotundus, respectively. Alpha rarefaction curves were generated using Chao1 estimator of species richness (Chao, 1984) with 10 sampling repetitions at each sampling depth. UNIVARIATE procedure of SAS (SAS 9.3, SAS Institute Inc., Cary, NC, USA) was used to test the normality of residuals for alpha-diversity indices and the average of bacterial phyla. A logarithmic transformation was used to normalize the data when necessary. Comparisons between groups were performed using Student's *t*-test.

The diversity between animals and treatments (beta-diversity) was compared using weighted and unweighted UniFrac distances (Lozupone and Knight, 2005) based on phylogenetic differences. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER V6 software (Warwick and Clarke, 2006) and permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005) was used to calculate *p*-values and test for differences between microbial communities.

Microbial community between infected and non-infected animals was analyzed through orthogonal projection to latent structures discriminant analysis (OPLS-DA) using the SIMCA 14 software suite (Umetrics, Malmö, Sweden). The OPLS-DA is an extension of PLS-DA, featuring an integrated orthogonal signal correction (OSC) filter to remove variability not relevant to class separation (Trygg and Wold, 2002). In OPLS-DA a regression model is calculated between the multivariate data and a response variable that only contains class information. The OPLS-DA models were fitted with one predictive and two orthogonal components (1 + 2). The quality of the models was evaluated with R^2Y and Q^2, indicating the percent of variation of the training set explained by the Y-predictive components (infection status) and the cross-validated predicted variation, respectively. An R^2Y-value close to 1 (explained variation) and Q^2 value larger than 0.5 is indicative of a good model with good predictability (Trygg and Wold, 2002).

For all analyses, a *p*-value of <0.05 was considered to be statistically significant and <0.1 was considered as a trend.

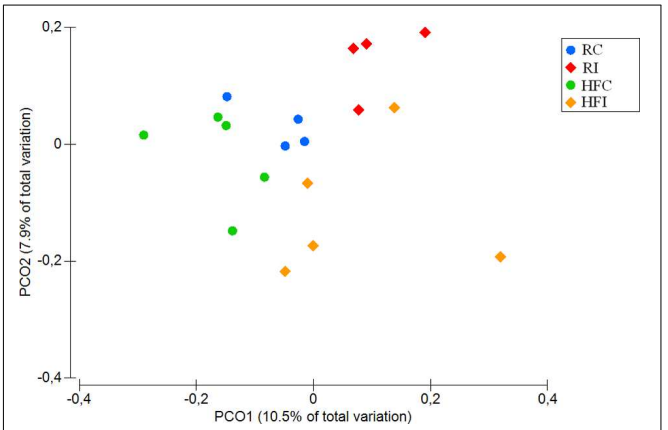

FIGURE 3 | Cecal content principal coordinates analysis (PCoA) of unweighted UniFrac distances. Significant *p*-value = 0.01 for Infected vs. non-infected comparison was obtained from PERMANOVA.

TABLE 3 | Average percentages of bacteria phyla in the cecal content.

Phyla	Mean values, %, under indicated conditions (SEM)				*p*-values	
	Treatments				RC vs. RI	HFC vs. HFI
	RC	RI	HFC	HFI		
Firmicutes	71.01 (3.59)	69.05 (2.16)	64.75 (0.56)	64.99 (0.59)	0.725	0.918
Bacteroidetes	13.73 (4.70)	12.21 (1.70)	16.86 (1.36)	14.16 (1.37)	0.755	0.554
Cyanobacteria	4.23 (1.00)	1.72 (0.82)	4.19 (1.03)	5.18 (1.08)	0.118	0.731
Actinobacteria	0.39 (0.07)	0.56 (0.05)	0.51 (0.01)	0.48 (0.02)	0.100[2]	0.817
Proteobacteria	0.16 (0.10)	0.29 (0.09)	0.07 (0.00)	0.06 (0.00)	0.797	0.019[1]
TM7	0.16 (0.03)	0.26 (0.04)	0.47 (0.02)	0.16 (0.02)	0.853	0.147
Tenericutes	0.43 (0.13)	0.16 (0.05)	0.34 (0.06)	0.78 (0.07)	0.955	0.052[2]
Unassigned	9.89 (0.93)	15.75 (2.61)	12.81 (1.04)	14.18 (1.42)	0.132	0.732

SEM, standard error of the mean. RC, regular diet control; RI, regular diet infected; HFC, high fiber diet control; HFI, high fiber diet Infected. [1]*$P < 0.05$ was considered significant.* [2]*$P < 0.1$ was discussed as trend.*

RESULTS

Determination of MAP Infection

Mycobacterium avium subspecies *paratuberculosis* infection was confirmed following detection of MAP by PCR in at least one of the three lymphoid tissues (**Table 2**). All non-challenged animals yielded MAP negative PCR results, whereas all challenged group animals except one belonging to the RI group, were positive for at least one lymphoid tissue. Samples from the animal that rendered the negative PCR result were removed from all downstream analyses, and as such, only animals that presented at least one tissue positive in MAP PCR were included in analyses in their respective infected groups.

Cecal Content Microbiota

Following quality control and removal of chimeric reads, an average of 40,986 ($SD = 12{,}744$) high quality sequences were obtained for cecal content samples and used for downstream analyses. Analyses of microbial communities revealed differences in richness (Chao1) between infected and control animals (**Figure 2A**). The RI animals presented higher richness values than RC (6873 ± 481 vs. 5313 ± 651; $p = 0.0043$; **Figure 2B**). An increasing trend was also observed in the Chao1 richness estimates of HFI compared to HFC group (6274 ± 1299 vs. 4434 ± 1273; $p = 0.0503$). However, the Shannon and Simpson indices were not significantly different between infected and control animals in any of the diet groups.

The beta-diversity analysis of unweighted UniFrac distances revealed distinct clustering pattern for the cecal content microbiota of MAP infected and control animals ($p = 0.01$; **Figure 3**). However, when the weighted UniFrac distances were analyzed, no significant differences were observed between the microbiota composition of infected and non-infected animals ($p = 0.2$; **Supplementary Figure S1**).

Alignment of OTUs at 97% similarity threshold against Silva database resulted in identification of seven bacterial phyla and 51 bacterial genera in the cecal content (**Table 3**, **Figure 4**). While majority of OTUs were identified at the genus level, some were only classified at the phylum, class, order, or family. The cecal content microbiota was dominated by members of phylum Firmicutes followed by members of phylum Bacteroidetes. Among all phyla, only the abundance of Proteobacteria significantly declined following MAP infection in animals fed high fiber diet ($p = 0.019$).

The OPLS-DA of cecal content bacteria for Y variable (infection) yielded a suitable model for microbial comparison between infected and non-infected animals fed the regular ($R^2Y = 1$; $Q^2 = 0.678$) or the high fiber diets ($R^2Y = 0.988$; $Q^2 = 0.733$; **Figure 4**).

In RI animals most of the bacteria positively associated with MAP infection belonged to the phylum Firmicutes including family Dehalobacteriaceae, Mogibacteriaceae, Lachnospiraceae (including genera *Anaerostipes* and *Moryella*) and Eubacteriaceae (including genus *Anaerofustis*). In addition, family Coriobacteriaceae (phylum Actinobacteria) was more abundant in RI group than in RC (**Figure 4A**, **Supplementary Figure S2A**). On the other hand bacterial phyla showing negative association with MAP infection while positively related with RC group were more diversified including genus *Lachnobacterium* (phylum Firmicutes); family Enterobacteriaceae (phylum Proteobacteria); family S24-7 (phylum Bacteroidetes); orders YS2 and Streptophyta (phylum Cyanobacteria); order ML615J-28 (phylum Tenericutes), and unclassified members of phylum Lentisphaerae (**Figure 4A**, **Supplementary Figure S2A**).

Animals fed the high fiber diet showed a different pattern of bacteria positively associated with MAP infection (**Figure 4B**, **Supplementary Figure S2B**). Within the phylum Firmicutes, several taxa including genera *Ruminococcus*, *Clostridium,* and *Butyrivibrio*, and family Erysipelotrichaceae were positively associated with HFI. In this group, members of phylum Proteobacteria were more abundant than in RI group. Examples included family Enterobacteriaceae (class Gammaproteobacteria); family Comamonadaceae; order Burkholderiales (class Betaproteobacteria); and genus *Bilophila* (class Deltaproteobacteria). Bacterial lineages belonging

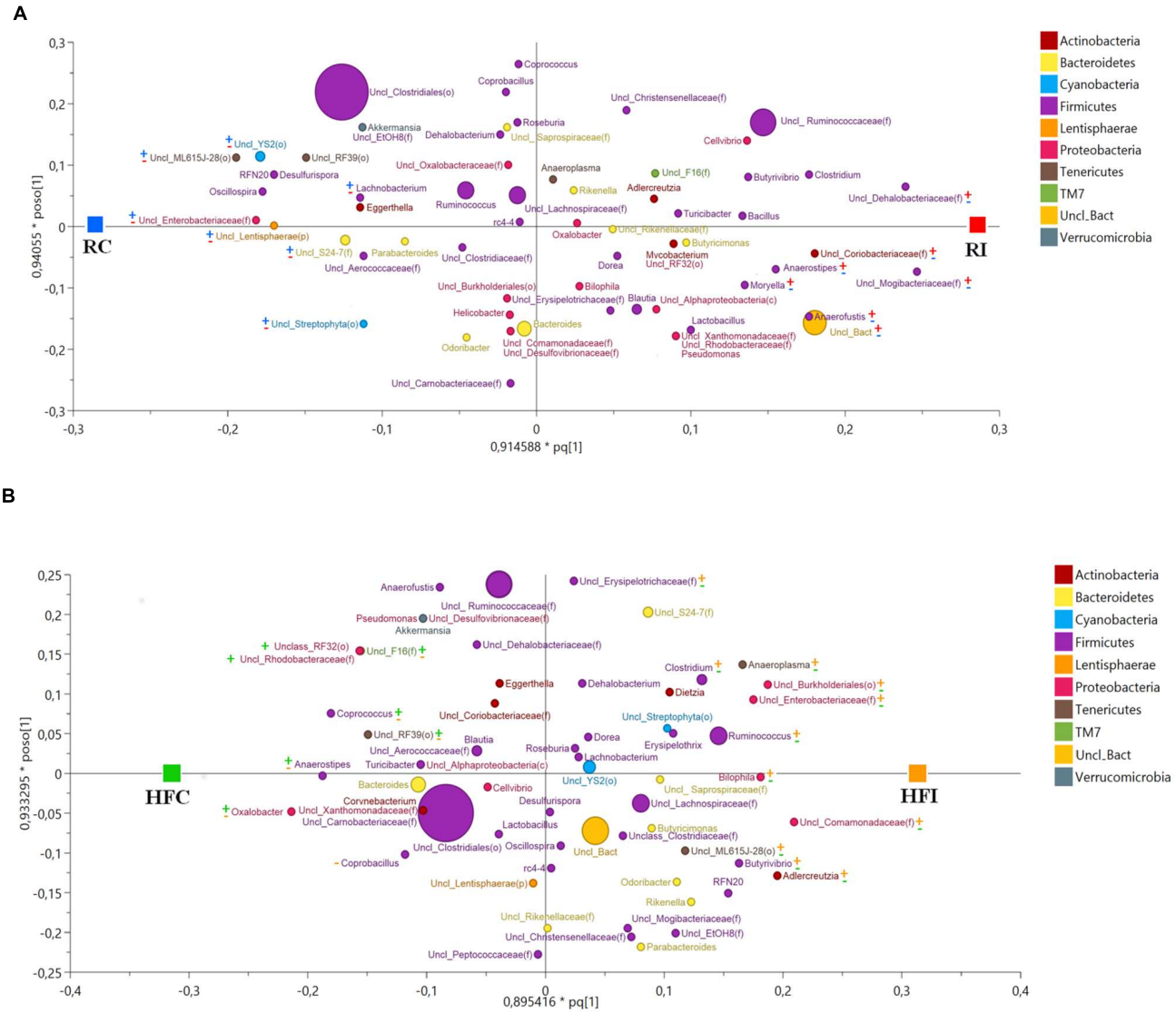

FIGURE 4 | Cecal content microbiota orthogonal projection to latent structures discriminant analysis (OPLS-DA) scatter plot. The size of the spheres represents abundance. **(A)** OPLS-DA Scatter Plot for cecal content samples of animals fed with regular diet ($R^2Y = 1$; $Q^2 = 0.678$). The taxa with significant positive association with MAP infected animals are labeled with (+) and with negative association with MAP infected animal with (–). The taxa with significant positive association with control animals are labeled with (+) and with negative association with control animals with (–). **(B)** OPLS-DA scatter plot for cecal content samples of animals fed with high fiber diet ($R^2Y = 0.988$; $Q^2Y = 0.733$). The taxa with significant positive association with MAP infected animals are labeled with (+) and with negative association with MAP infected animal with (–). The taxa with significant positive association with control animals are labeled with (+) and with negative association with control animals with (–).

to the phylum Actinobacteria (genus *Adlercreutzia*) and phylum Tenericutes (genus *Anaeroplasma* and family ML615J-28) were also more abundant in the HFI group compared to HFC.

The bacterial taxa showing negative association with MAP infection in animals fed high fiber diet and therefore more abundant in the HFC group, were different from those described in the RC group. These included genera *Coprococcus*, *Anaerostipes*, and *Coprobacillus* (phylum Firmicutes); genus *Oxalobacter*, family Rhodobacteraceae, and order RF32 (phylum Proteobacteria); order RF39 (phylum Tenericutes); and family F16 (phylum TM7; **Figure 4B**, **Supplementary Figure S2B**).

Sacculus Rotundus Microbiota

An average of 26,900 (SD = 10,284) of high quality sequences were obtained from sacculus rotundus samples. There were no significant differences in richness (Chao1 index), or other alpha-diversity indices (Shannon and Simpson) when infected and non-infected animals were compared (**Figure 5**). The beta-diversity analysis of UniFrac distances revealed differences between infected and non-infected animals in both weighted

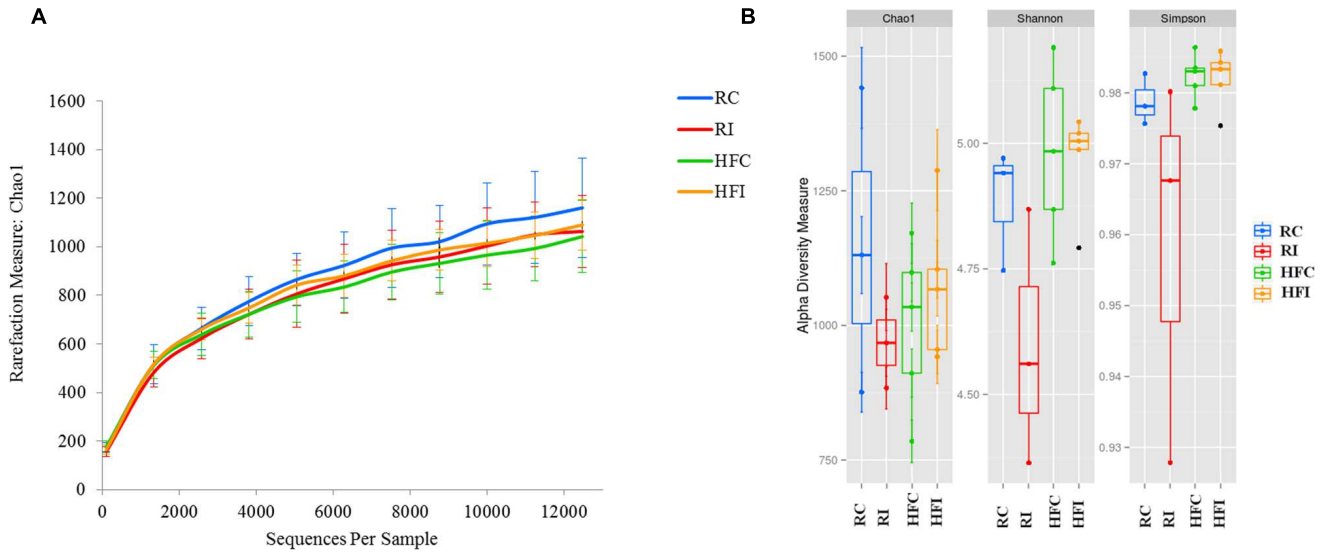

FIGURE 5 | Sacculus rotundus diversity analysis. (A) Sacculus rotundus rarefaction curve generated using Chao1 richness estimator. Samples have been rarified at an even depth of 12,400 sequences per sample. Error bars indicate the 95% confidence intervals. **(B)** Sacculus rotundus alpha-diversity; richness (Chao1) and diversity (Shannon and Simpson) indices box plot.

($p = 0.048$) and weighted ($p = 0.032$) measures (**Figures 6** and **7**). In this lymphoid tissue, 7 bacteria phyla and 63 bacteria genera were identified.

Similar to the cecal content, sacculus rotundus microbiota was mainly composed of phylum Firmicutes (**Table 4**). In contrast, phylum TM7 was the second most abundant phylum in the sacculus rotundus ranging from 2.3 to 5.9% of community, while its relative abundance ranged from 0.16 to 0.47% in the cecal content. The only phylum that significantly shifted with infection was Bacteroidetes, with declining abundance in MAP infected rabbits fed the regular diet. The composition of the OTUs at the genus level in the four groups is presented in **Figure 8**.

As in the cecal content, the OPLS-DA analysis of bacterial community in sacculus rotundus for *Y*-variable (infection) yielded a model with high goodness of fit (R^2Y) and predicted value (Q^2) both for animals fed with regular diet ($R^2Y = 0.995$, $Q^2 = 0.765$) and high fiber diet ($R^2Y = 0.921$, $Q^2 = 0.511$; **Figure 9**).

Several bacterial taxa were positively associated with MAP infection and therefore more abundant in the RI compared to the RC group. This included the families Mogibacteriaceae, Dehalobacteriaceae, and Coriobacteriaceae and the genus *Stenotrophomonas*. A large number of taxa had negative association with MAP infection and therefore were overrepresented in the RC group comparing to RI one (**Figure 9**, **Supplementary Figures S3A,B**). Among these taxa, three also had negative association with infection in the cecal content and belonged to the phylum Tenericutes (order ML615J-28) and phylum Cyanobacteria (orders Streptophyta and YS2; **Figure 9A**, **Supplementary Figure S3A**).

Among animals fed the high fiber diet, genus *Anaerofustis* and family Lachnospiraceae were more abundant in the HFI group compared to the HFC one and therefore they were positively

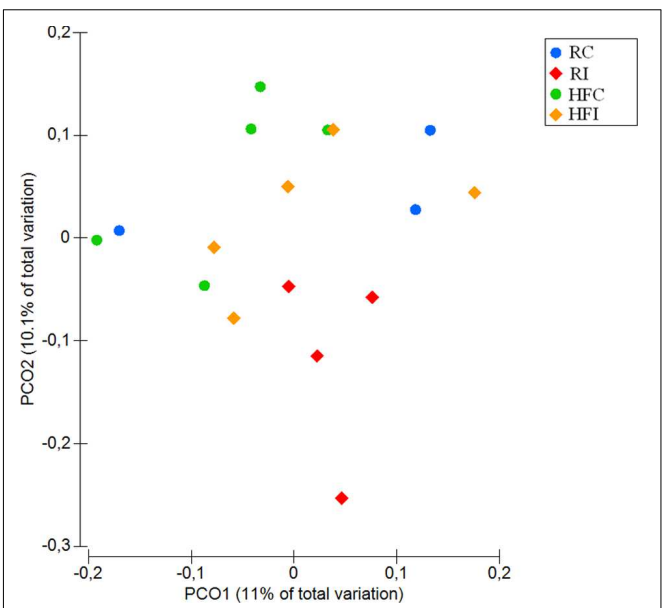

FIGURE 6 | Principal coordinates analysis (PCoA) of unweighted UniFrac distances of samples from sacculus rotundus. Significant *p*-value = 0.032 for Infected vs. non-infected comparison was obtained from PERMANOVA.

associated with infection. The two taxa with negative association with infection and therefore more abundant in the HFC group, belonged to the phylum Firmicutes, including families Erysipelotrichaceae (genus *Coprobacillus*) and Lachnospiraceae (genus *Anaerostipes*; **Figure 9B**, **Supplementary Figure S3B**). These two genera were also more abundant in the cecal content of HFC group and thus negatively associated with MAP infection.

TABLE 4 | Average percentage of bacteria phyla in the sacculus rotundus samples.

Phyla	Mean values, %, under indicated conditions (SEM)				*p*-values	
	Treatments				RC vs. RI	HFC vs. HFI
	RC	RI	HFC	HFI		
Firmicutes	89.28 (5.37)	89.55 (1.61)	81.78 (3.56)	87.86 (1.80)	0.414	0.178
TM7	2.71 (1.74)	4.27 (1.19)	5.97 (1.65)	2.30 (0.69)	0.821	0.065[2]
Bacteroidetes	2.13 (0.11)	1.49 (0.25)	0.93 (0.27)	1.75 (0.75)	0.014[1]	0.261
Actinobacteria	0.95 (0.35)	2.14 (0.64)	1.17 (0.20)	0.99 (0.19)	0.150	0.378
Tenericutes	0.83 (1.11)	0.27 (0.09)	0.95 (0.39)	0.79 (0.20)	0.398	0.504
Cyanobacteria	0.66 (1.11)	0.20 (0.10)	5.14 (1.95)	1.36 (0.54)	0.467	0.212
Proteobacteria	0.49 (0.64)	0.17 (0.03)	0.40 (0.15)	0.17 (0.04)	0.428	0.136
Unassigned	2.97 (0.82)	1.91 (0.08)	3.66 (0.60)	4.76 (1.16)	0.242	0.620

SEM, standard error of the mean. RC, regular diet control; RI, regular diet infected; HFC, high fiber diet control; HFI, high fiber diet infected. [1]*P < 0.05 was considered significant.* [2]*P < 0.1 was discussed as trend.*

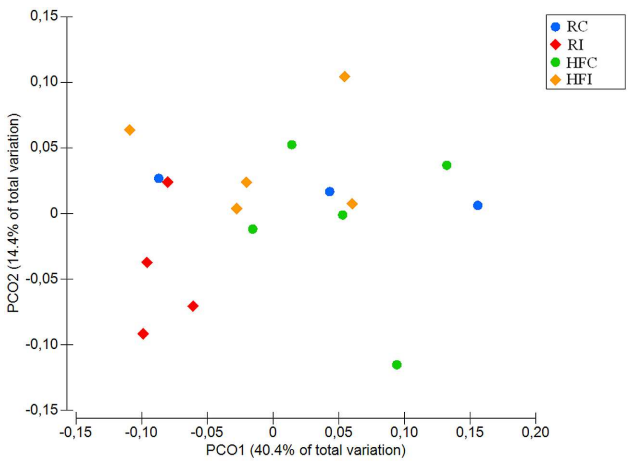

FIGURE 7 | Principal coordinates analysis (PCoA) of weighted UniFrac distances of samples from sacculus rotundus. Significant *p*-value = 0.048 for Infected vs. non-infected comparison was obtained from PERMANOVA.

DISCUSSION

In the present work, we attempt to study the impact of MAP infection on gut microbiota composition under two different dietary scenarios. Variations in the microbial community were observed depending on the dietary group and in response to MAP infection.

Cecal microbial community of rabbit has been assessed in recent studies (Bäuerl et al., 2014; Zhu et al., 2015) but to the best of our knowledge, this is the first work describing the microbiota of rabbit sacculus rotundus. This organ unique to rabbits is characterized by an ampoule-like enlargement of the terminal ileum. Due to its location and lymphoid nature it might play a similar role to the ileocecal-valve in ruminants (Besoluk et al., 2006) and can be assumed to bear microbiota with an important role in immune regulation.

Studies using high-throughput sequencing technology to explore the gut microbiota in rabbits are not abundant. These limited studies indicate that GI tract of rabbits is a highly diverse environment (Bäuerl et al., 2014; Zhu et al., 2015). In the present study, we observed that even with an average sequencing depth of 24,700 reads/sample in the cecal content and 12,400 reads/sample in the sacculus rotundus, rarefaction curves did not reach a plateau phase indicating that the ecosystem is highly diverse. These findings further support previous observations on high microbiota diversity in rabbit cecal content (Bäuerl et al., 2014; Zhu et al., 2015) and feces (Eshar and Weese, 2014; Zeng et al., 2015).

Microbiota richness, evenness and diversity have been reported as characteristic features determining state of health or disease. A decreased in richness and diversity indices has been reported in CD (Manichanh et al., 2006; Dicksved et al., 2008; Walker et al., 2011) and Type 1 diabetes patients (Giongo et al., 2011), whereas bacterial vaginosis (Ling et al., 2010) and helminthic parasitosis (Lee et al., 2014) have been associated with increased richness. In our study, the alpha-diversity indices in sacculus rotundus microbiota, did not differ between infected and non-infected animals. However, in the cecal content, we found higher values of richness (chao1 index) in infected animals whereas the abundance-based diversity measures (Shannon and Simpson indices) did not vary between infected and non-infected animals. It must be taken into account that cecal content DNA extraction did not allow removing DNA from dead bacteria whereas, the extraction method used for sacculus rotundus did, and this could have influenced results.

The diversity analyses between animals (beta-diversity) revealed that the microbial composition between infected and non-infected animals varied in the cecal content and sacculus rotundus in response to MAP infection. In the cecal content, the significant differences were observed only in unweighted UniFrac distances, suggesting that MAP infection has an impact on the presence and/or absence of certain members of the microbial community rather than on the abundance of these. Due to rabbits' practice of cecotrophy, the composition of the microbial community of the cecal content may play a role in the modulation

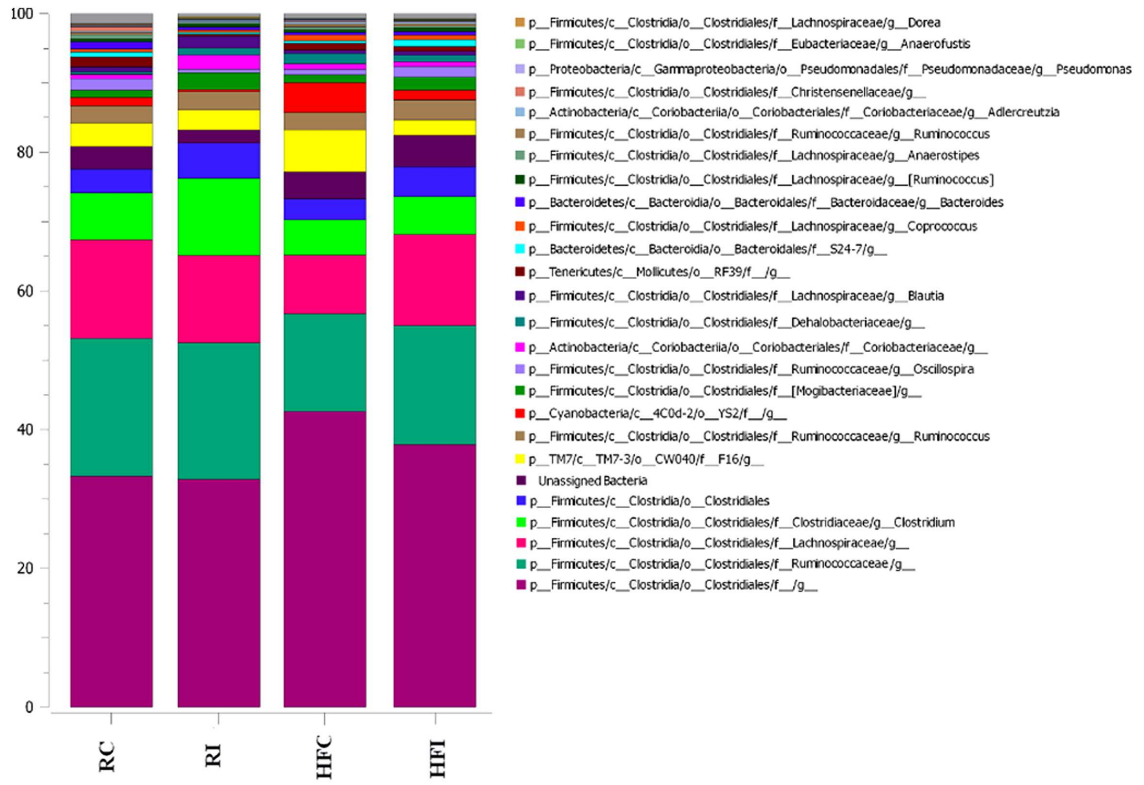

FIGURE 8 | Stack-bar of the most abundant OTUs in sacculus rotundus. Vertical bars demonstrate the proportion (%) of the most abundant bacterial taxa (>0.1% of community). Taxonomic classification of each taxon is identified at phylum (p_), class (c_), order (o_), family (f_), and genus (g_) level.

of nutrient absorption. In contrast, in sacculus rotundus, we observed changes in beta-diversity using both weighted and unweighted UniFrac distances, suggesting that MAP infection both impacted the presence and/or absence of certain taxa as well as their abundance. These observed changes may play a role in inflammation as demonstrated in previous reports where specific microbial compositions in rabbit's lymphoid tissue have been seen to induce inflammation (Shanmugam et al., 2005).

It was not surprising that rabbit cecal content was dominated by the phylum Firmicutes as reported in recent studies (Bäuerl et al., 2014; Zhu et al., 2015). This phylum contains some of the major cellulolytic and fibrolytic organisms (Daly et al., 2001) that can be also found in other hindgut fermenters, such as horses (Costa et al., 2012).

At the phylum level, we only observed significant differences in the phyla Proteobacteria and Bacteroidetes. The phylum Proteobacteria decreased in the cecal content of HFI group compared with HFC. However, in the animals fed with high fiber diet there were more bacteria belonging to this phylum positively associated with MAP infection than in animals fed with regular diet. Proteobacteria are considered to be minor and opportunistic components of the gut ecosystem. The proportion of this phylum increases in both IBD patients (Gophna et al., 2006) and animal models of IBD (Munyaka et al., 2016) suggesting these bacteria may play a role in pathogenesis of IBD (Mukhopadhya et al., 2012).

In our study, the phylum Bacteroidetes was decreased in the sacculus rotundus of RI compared with RC. This phylum is reported to decrease in experimentally MAP-infected calves (Derakhshani et al., 2016a) and in CD patients (Gophna et al., 2006). The phylum Bacteroidetes has a major role in degrading complex polysaccharides (Terrapon and Henrissat, 2014) that are normally present in high fiber diets. Studies have shown that high fiber based diets promote the abundance of Bacteroidetes in rabbits gut (Nicodemus et al., 2005; Zhu et al., 2015). In our previous study, we reported that a shift to a high fiber diet during MAP challenge may decelerate the progression of infection (Arrazuria et al., 2015a). The increase of Bacteroidetes may be in part responsible for this finding.

Genus *Mycobacterium* was only detected in one cecal content sample from an animal belonging to the infected group fed the regular diet (**Figure 4A**, **Supplementary Figure S2A**). Since the classification was at the genus level we cannot confirm that it is MAP. In slaughtered rabbits, detection of other members of *Mycobacterium avium* complex has been reported in lymphoid tissue, although no mycobacteria were found in the cecal content (Arrazuria et al., 2015b). The differences in MAP detection in sacculus rotundus by PCR (Arrazuria et al., 2015b) and sequencing could be due to the standardization of amount of DNA for sequencing, the choice of primers used (universal bacterial primers in case of sequencing vs. MAP

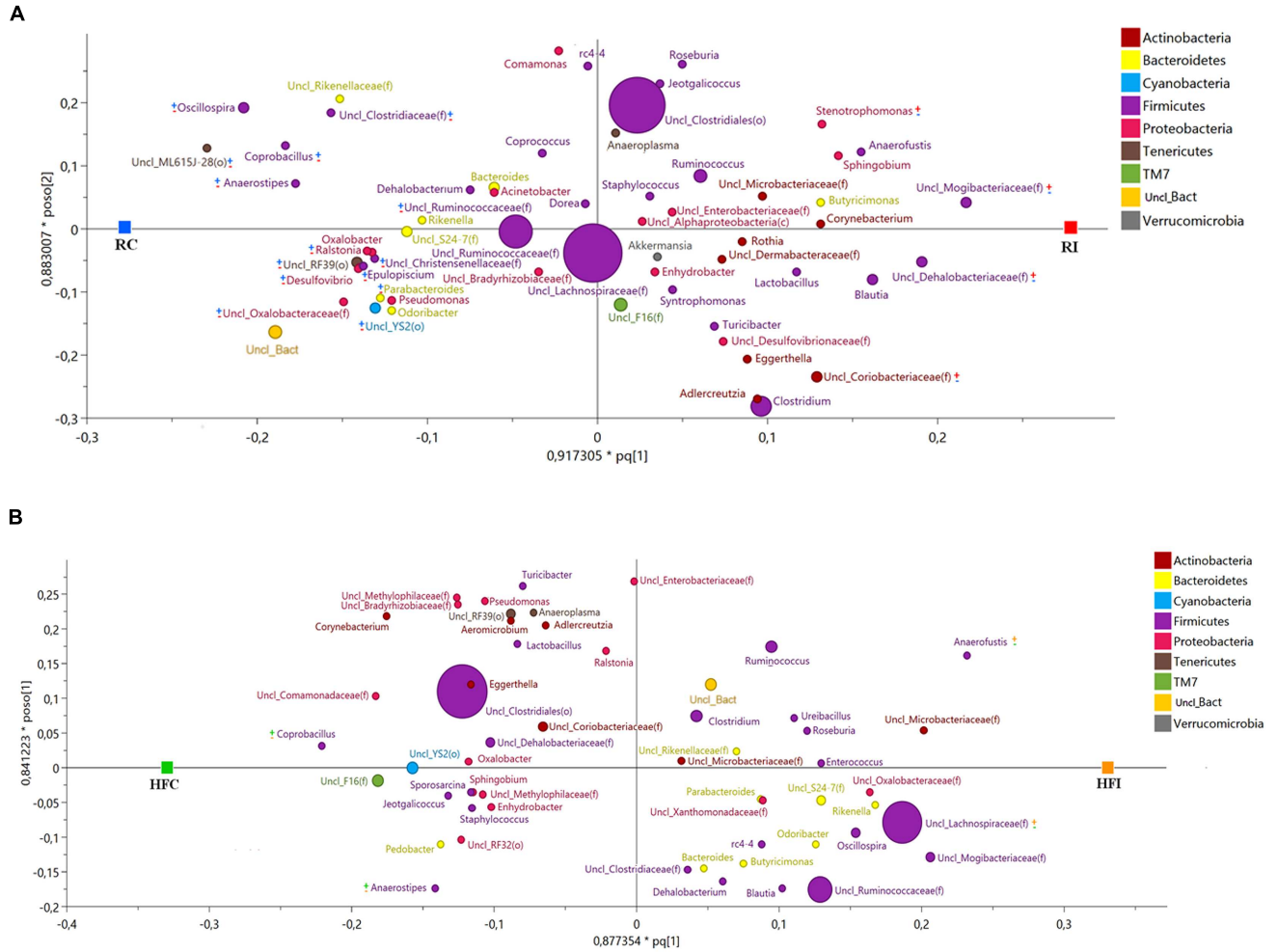

FIGURE 9 | Sacculus rotundus microbiota orthogonal projection to latent structures discriminant analysis (OPLS-DA) scatter Plot. The size of the spheres represents abundance. **(A)** OPLS-DA scatter plot for sacculus rotundus samples of animals fed with regular diet ($R^2Y = 0.995$; $Q^2 = 0.765$). The taxa with significant positive association with MAP infected animals are labeled with (+) and with negative association with MAP infected animal with (–). The taxa with significant positive association with control animals are labeled with (+) and with negative association with control animals with (–). **(B)** OPLS-DA scatter plot for sacculus rotundus samples of animals fed with high fiber diet ($R^2Y = 0.921$; $Q^2 = 0.511$). The taxa with significant positive association with MAP infected animals are labeled with (+) and with negative association with MAP infected animal with (–). The taxa with significant positive association with control animals are labeled with (+) and with negative association with control animals with (–).

specific primers for PCR) or because the relative abundance of MAP in infection is not too high since the disease is of chronic nature.

We used an OPLS-DA model to determine which bacteria were positively or negatively associated with MAP infection. Using this approach, we observed that three families, Coriobacteriaceae, Dehalobacteriaceae, and Mogibacteriaceae were positively associated with MAP infection in both sacculus rotundus and the cecal content of the RI group. Some members of the family Coriobacteriaceae have been found in vaginosis, bacteriemia, and periodontitis, being considered pathobionts (Clavel et al., 2014). In the context of PTB, the increase of this family of pathobionts may have an implication in disease progression. Derakhshani et al. (2016a) reported that the family Mogibacteriaceae was the main overrepresented bacteria in the ileum mucosa of MAP infected calves. Unfortunately there is a lack of knowledge on the role that this family could play in gut inflammation.

The genus *Anaerostipes* was found to be more abundant in the cecal content of the RI group. It was also more abundant in sacculus rotundus of the RC and in cecal content and sacculus rotundus of HFC animals. The genus *Anaerostipes* can utilize lactate to produce butyrate (Sato et al., 2008), which can suppress pro-inflammatory cytokine production by intestinal macrophages (Chang et al., 2014) and it is beneficial to colonic health providing protection against colitis (Segain et al., 2000). Also, this genus was found in significant lower abundance in smoker CD patients compared to non-smokers (Morgan et al., 2012). These findings suggest that this genus may play an important role in GI health.

The genus *Stenotrophomonas* was overrepresented in sacculus rotundus of RI. It has been described in CD, and has been recognized in many clinical specimens and inflammatory conditions like pneumonia, bacteremia, and urinary tract infection (Denton and Kerr, 1998; Knösel et al., 2009). This species also appears to be increased in mice females infected with MAP (Karunasena et al., 2014). These findings are in agreement with our results.

The genus *Coprobacillus* was more abundant in the control groups (sacculus rotundus of RC and in sacculus rotundus and cecal content of HFC). This genus has been found to be beneficial through maintaining intestinal stability and conferring resistance against *Clostridium difficile* colonization (Stein et al., 2013).

In the present study, sacculus rotundus and cecal content of the RC group showed higher levels of two orders that belong to the phylum Cyanobacteria (orders Streptophyta and YS2). Since Cyanobacteria has been detected in the GI tract of human and other mammals (Ley et al., 2008) the efforts to better characterize the function of members of this phylum are increasing (Rienzi et al., 2013; Soo et al., 2014). Streptophyta, which derive from chloroplasts within plant matter has been detected in high proportion in stool from a patient with resistant tuberculosis treated with broad-spectrum antibiotics (Dubourg et al., 2013), suggesting that the critical reduction of bacterial sequences detected had permitted the amplification of DNA from green plant foods absorbed by the patient. A recent genomic study showed that YS2 does not have photosynthetic ability. Metabolic analysis demonstrated that YS2 has many special functions including obligate anaerobic fermentation, syntrophic H2-production, nitrogen fixation, and synthesis of vitamin B and K (Rienzi et al., 2013). The infection may lead to the establishment of an ecosystem that is not favorable for this order and it could support the pathogenesis of the disease.

As shown in **Figures 4** and **9**, we found additional taxa that show positive or negative relation with MAP infection only under specific dietary regimens. Diets containing different amounts of fiber are likely to differentially modulate the composition of the intestinal microbiota (Serino et al., 2011; Zhu et al., 2015). We cannot rule out that some of the detected microbes were taken up with the diet. DNA from food adsorbed by the host has been reported as mentioned previously (Dubourg et al., 2013). Microbes taken up with food may succeed to survive, especially in the high fiber diet groups where the diet shift itself could have favored their survival. Finally, we cannot, speculate if the associated taxa with MAP-infection, only observed in animals fed the high fiber diet, are consequence of the short diet change, or on the contrary, are the result of MAP reinfection.

CONCLUSION

The MAP infection and dietary changes shift microbiota of the cecal content and the sacculus rotundus of rabbits. While some taxa seem to be positively associated with MAP infection in different sites or conditions, there are others that show opposite patterns. Knowing the composition of the microbial community alone does not lead to an understanding of its function. Therefore, it would be helpful to confirm and complete the results presented in the present study with a shotgun metagenomic or metatranscriptomics approach that could help to better understand the role of microbiota composition and function. Further research is required to understand the pathogenesis of MAP associated to chronic intestinal inflammation and the role that intestinal microbiota plays during this process.

AUTHOR CONTRIBUTIONS

RJ and NE conceived and designed the experiment. RA and NE conducted the animal experiment. RA and HD performed lab analyses. RA, HD, and EK developed the bioinformatics and statistical models. RA and HD analyzed the data. All authors drafted the manuscript. All authors carefully read and approved the final version of the manuscript.

FUNDING

The research was funded by grant (AGL2012-39818-C02-02) from the Spanish Ministry of Economy and Competitiveness (MINECO) and University of Manitoba Start-up fund. RA held a predoctoral fellowship (BFI-2012-237) and a visiting fellowship (EP_2015_1_53) from the Department of Education, Universities and Research of the Basque Government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00446

FIGURE S1 | Cecal content principal coordinates analysis (PCoA) of weighted UniFrac distances. PERMANOVA analysis showed no significant differences between Infected vs. non-infected animals p-value = 0.2.

FIGURE S2 | Cecal content OPLS-DA coefficient plot; bars with negative values indicating bacteria that are significantly lower in MAP infected animals and bars with positive value indicating bacteria which are significantly higher in MAP infected animals than in control animals. Only bacteria with a confidence interval that did not cross the zero line were significantly changed $*p < 0.05$. **(A)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed with regular diet ($R^2Y = 1$; $Q^2 = 0.678$). **(B)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed high fiber diet ($R^2Y = 0.988$; $Q^2Y = 0.733$).

FIGURE S3 | Sacculus rotundus OPLS-DA coefficient plot; bars with negative values indicating bacteria that are significantly lower in MAP infected animals and bars with positive value indicating bacteria which are significantly higher in MAP infected animals than in control animals. Only bacteria with a confidence interval that did not cross the zero line were significantly changed $*p < 0.05$. **(A)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed with regular diet ($R^2Y = 0.995$; $Q^2 = 0.765$). **(B)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed high fiber diet ($R^2Y = 0.921$; $Q^2 = 0.511$).

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

ESTUDIO IV / STUDY IV

Vaccination sequence effects on immunological response and tissue bacterial burden in paratuberculosis infection in a rabbit model. R Arrazuria, E Molina, JM Garrido, V Pérez, RA Juste, N Elguezabal. Vet Res. 2016, 47:77.

Arrazuria *et al. Vet Res (2016) 47:77*
DOI 10.1186/s13567-016-0360-y

VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Vaccination sequence effects on immunological response and tissue bacterial burden in paratuberculosis infection in a rabbit model

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Abstract

Paratuberculosis (PTB), a chronic granulomatous enteritis produced by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is considered as one of the diseases with the highest economic impact in the ruminant industry. Vaccination against MAP is recommended during the first months after birth on the basis that protection would be conferred before the first contact with mycobacteria. However, little is known about the therapeutic effect of MAP vaccination in controlled experimental conditions. The current study was designed to evaluate the efficacy of vaccination before and after challenge with MAP in a rabbit infection model. The rabbits were divided into four groups: non-infected control (NIC, $n = 4$), infected control challenged with MAP (IC, $n = 5$), vaccinated and challenged 1 month after with MAP (VSI, $n = 5$) and challenged with MAP and vaccinated 2 months later (IVS, $n = 5$). The results from this study show a quick increase in IFN-γ release upon stimulation with bovine, avian and johnin PPD in animals vaccinated before MAP challenge. All vaccinated animals show an increased humoral response as seen by western blot and ELISA. The final bacteriology index (considering tissue culture and qPCR) shows that the IC group was the most affected. Vaccination after infection (IVS) produced the lowest bacteriology index showing significant differences with the IC group ($p = 0.034$). In conclusion, vaccination against MAP shows positive effects in a rabbit model. However, vaccination after infection shows a slightly stronger protective effect compared to vaccination before infection, suggesting a therapeutic effect. This feature could be applied to previously infected adult animals under field conditions.

Introduction

Paratuberculosis (PTB) also called Johne's disease is a chronic granulomatous enteritis produced by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) that mostly affects ruminants. PTB considered as one of the diseases with the highest economic impact in the ruminant industry can affect many ruminant and non-ruminant wildlife species, as well [1, 2]. In addition numerous studies have suggested that MAP could play a role in human Crohn's Disease [3–5].

PTB is transmitted predominantly through the fecal-oral route. Infected animals in the subclinical stage shed MAP in the environment intermittently, making its control difficult. Current control programs in livestock include test and cull strategies whose implementation alone is not sufficient to control the disease making vaccination the best intervention strategy [6, 7]. Vaccination against MAP reduces the incidence of PTB within herds and the severity of disease in individual animals. In general, it reduces production loss, mortality and histopathological lesions [8, 9] and it diminishes the number of clinical cases in cattle, reducing bacterial shedding in feces contributing to longer productive life and improvement in milk production [10–12]. In small ruminants,

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vaccination has been successfully used as a part of the control programs in sheep in Iceland [13] and Australia [14] and in goats in Norway [15] and Spain [16] allowing a sharper decline in PTB prevalence. However, the fact that vaccination does not provide complete protection from infection is also widely accepted [17, 18].

Vaccination against MAP is recommended during the first months after birth on the basis that protection would be conferred before the first contact with the mycobacteria [6, 15, 19]. Nevertheless, due to the chronic nature of MAP infection when clinical signs are detected vaccination of the whole flock independent of age, including adult animals can be an option [12]. In fact, studies involving vaccination after MAP infection have shown reduction of MAP burden in sheep vaccinated with Gudair® [20] or with a live attenuated vaccine [21]. Although the mechanism of this therapeutic effect is unknown it can be assumed that whole herd vaccination might contribute to a reduction in the level of environmental contamination with MAP allowing faster progress in PTB control.

The therapeutic effect of vaccines could be very useful controlling the spread of infectious diseases in which the number of individuals in a latent or subclinical stage of infection is high. A recently published systematic review has reported that some vaccines against tuberculosis currently under study have a therapeutic effect even in latent infection [22]. When it comes to PTB, vaccination effects after MAP exposure have been understudied in experimental conditions, probably because of the lengthy incubation period of PTB in ruminants. In this sense, rabbits which are naturally susceptible to infection with MAP [23–25] and have shown to be useful short term models in experimental conditions as well [26], may be suitable to study the therapeutic effect of PTB vaccination.

The aim of this study, therefore, was to evaluate the effect of vaccination with a heat inactivated vaccine previous to and after challenge with MAP in a rabbit infection model resembling the subclinical stage of PTB, focusing on changes in bacterial load and both cellular and humoral responses.

Materials and methods

Experimental design

New Zealand white female rabbits ($n = 19$) were purchased from official dealers and arrived at the NEIKER animal facilities at the age of 6 weeks 1.5 kg weight to follow a 2 week adaptation period. The most important aspects of the experimental design are represented in Figure 1. The rabbits were divided into four groups: non-infected control (NIC; $n = 4$), infected control (IC; $n = 5$), infected and then vaccinated with Silirum® (IVS; $n = 5$) and vaccinated with Silirum® prior to infection (VSI; $n = 5$). Silirum® is a heat-inactivated vaccine containing 2.5 mg/mL of MAP strain 316F culture combined with an immunological adjuvant consisting of highly refined mineral oil.

At day 1 of experimentation, animals were sedated with azepromazine maleate intramuscularly at 2 mg/kg of weight and tattooed in the inner part of one of the ears for easier identification during the experiment. Also, on this day, the VSI group rabbits were vaccinated subcutaneously with 1 mL of Silirum®. On three consecutive days (days 28, 29 and 30) all animals except the ones belonging to the NIC group were challenged orally with 10^9 CFU of MAP strain K10 obtained as described previously [26]. Rabbits of the IVS group were vaccinated with Silirum® 71 days after the beginning of the experiment that is, 41 days after challenge.

Animals were monitored by recording weight and blood prior to vaccination (baseline S0 samples) and after challenge twice a month (S1–S10). The fecal consistency was observed in all samplings. Feces were collected after challenge (S0A) and freshly processed. Blood samples were divided for IFN-γ release quantification and for serological testing. In this case, blood was centrifuged at 1500 *g* for 10 min to obtain plasma which was stored at −20 °C until ELISA or Western blot (WB) analyses were performed.

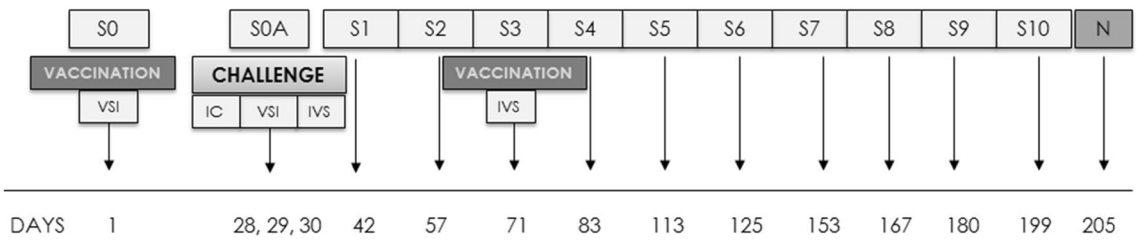

Figure 1 Experimental design diagram. S: sampling, N: necropsy, IC: infected control, VSI: vaccination before infection, IVS: vaccination after infection.

The endpoint of the experiment was set at day 205 and all animals were euthanized administering pentobarbital intracardially after deep sedation with xilazine and ketamine. Then a complete necropsy focusing on a gross pathology in the digestive tract was performed and tissue samples were collected and stored at −20 °C. Handling procedures, sampling frequency and euthanasia procedure were designed to produce minimum stress on subjects following European, National and Regional Law and Ethics Committee regulations. The experiment was reviewed and approved by NEIKER's Animal Care and Use Committee and by the Agriculture Department (PARAMOD-6278-BFA).

Tissue MAP culture

For culture, 2 g of collected tissues were processed. Tonsils, spleen, liver, muscle and mesenteric lymph node were spliced in tiny pieces and weighed, whereas, vermiform appendix, sacculus rotundus, ileum and jejunum were scraped for mucosa and weighed and samples were processed for HEYM [27] as described in Arrazuria et al. [26]. For culture on 7H9 OADC [28] supplemented with MJ penicillin, anfotericin and cloramphenicol, the same decontaminated suspension used for HEYM culture was centrifuged at 2885 *g* during 10 min. Supernatant was discarded and a wash with sterile water was performed. After a new centrifugation step in the same conditions the pellet was suspended in 2 mL of water and four drops/tube were seeded. All seeded tubes were incubated at 37 ± 1 °C and checked for MAP growth at 8, 12, 16 and 20 weeks.

Fecal and tissue DNA extraction

Feces and tissue DNA was extracted with DNA Extract-VK (Vacunek S.L, Derio, Spain) according to the manufacturer's instructions with slight modifications that included an initial hydrating step where 1 g of feces was hydrated with 7 mL of sterile water overnight. The following day, the mixture was vortexed to achieve a homogenous solution and let still for 10–15 min. Then 500 µL of supernatant were pipetted into a new vial and centrifuged at 11 000 *g* for 5 min. The supernatant was discarded and the pellet was suspended in 250 µL of sterile water. From this step on, the manufacturer's instructions were followed.

Collected tissues (tonsils, spleen, liver, muscle, mesenteric lymph node, vermiform appendix, sacculus rotundus, ileum and jejunum) and cecal content stored at −20 °C were thawed overnight at 4 °C and brief modifications were performed as previously described [26].

In both cases extracted DNA was stored at −20 °C until PCR were performed.

MAP PCR. Detection and quantification

For initial screening purposes a real time multiplex PCR detecting IS900 and ISMap02 DNA sequences of MAP was performed with 350 ng/µL as described by Sevilla et al. [29].

PCR results were analyzed using the 7500 System SDS software v. 1.4 (Applied Biosystems, Spain). Threshold cycle (C_T) and baseline were automatically determined by the software and verified by visual examination of the threshold line in amplification plots. C_T values equal or below 38 for both IS900 and ISMap02 probes were considered positive, C_T values over 38 for both targets probe and under 38 for IAC probe were considered negative.

For a quantitative assessment of MAP levels in tissues, this is genomic equivalents (GE), qPCR was done employing ParaTB-Kuanti-VK (Vacunek S.L, Derio, Spain), and the manufacturer's instructions were followed. Briefly, 10 µL of DNA were added to 15 µL of master mix in 96 well plates. Triplicates of dilutions of positive control standards were run in parallel to obtain data points for standard curves. Amplification and real time measurement were performed in a 7500 Real Time PCR System (Applied Biosystems, Spain). The results were analysed with the ABI Prism software version 7500 SDS software v 1.4.

Interferon gamma release assay (IGRA)

To explore the cellular immune response, interferon gamma release quantification was carried out after stimulation of whole blood with bovine, avian and johnin purified protein derivative (PPD) (CZ Veterinaria, Porriño, Spain). Heparinized blood samples collected at S0, S1, S3 and S10 were assayed. Stimulation of whole blood was performed within 8 h of collection. A 25 000 UI/mL concentrations of bovine and avian PPD and 26 446 UI/mL of johnin PPD were used.

Briefly, 35 µL of different PPD (10 µg/mL) or sterile PBS 0.01 M pH 7.2 used as a negative control were added to 500 µL of whole blood from each animal. All samples were mixed slowly and incubated at 37 °C and 5–7% CO_2 for 16–24 h. Plasma was separated by centrifugation at 500 *g* for 10 min and frozen at −20 °C until testing. The IFN-γ production was measured using a commercial kit (Cusabio-Biotech®, China) according to the manufacturer's instructions.

The optical density was determined at 450 and 570 nm to correct for optical imperfections in the plate, using an automated ELISA plate reader (Multiskan EX®, Thermo Lab Systems, Finland). The standard curve was performed to calculate the IFN-γ concentration of each sample. The final IFN-γ concentration was obtained by subtracting the values of the negative control (sterile

PBS) from each tested PPD in order to reduce the background of non-specific IFN-γ release.

PPA-3 enzyme-linked immunosorbent assay (ELISA)

Homemade indirect ELISA was performed using PTB protoplasmatic antigen 3 (PPA-3) (Allied Lab, Fayette, Missouri, USA). Plasma samples were adsorbed with *Mycobacterium phlei* in saline solution (5 g/L) at equal concentrations to reduce cross-reactivity. Checkerboard titration was performed to determine the optimal concentrations of plasma (1:200) and conjugate (0.15 μg/mL) (unpublished data).

Microtiter plates were coated with 0.04 mg/mL of PPA-3 diluted in 0.5% sodium carbonate buffer (pH 9.6). A total of 100 μL of antigen dilution was added to each well and it was incubated at 4 °C overnight. After coating, the wells were washed once with 0.05% Tween 80 in 0.85% sodium chloride (Wash solution).

The plasma samples were diluted 1:200 in 0.05% Tween 80 in PBS (PBS-T) and were assayed (100 μL/well). After 2 h of incubation at RT in a humid chamber, plates were washed three times with 300 μL/well of wash solution. A volume of 100 μL of recombinant protein G peroxidase (0.025 μg/mL, Sigma-Aldrich, Spain) in PBS-T was added to each well and the plates were incubated at RT for 2 h. The plates were washed again three times with 300 μL/well of wash solution and then 100 μL/well of peroxidase substrate (0.01% ABTS, Sigma Aldrich, Spain) were added. The plates were incubated at RT for 30 min in darkness. The reaction was stopped by the addition of 100 μL/well of 2% hydrofluoric acid. The absorbance was measured at 405 and 450 nm using an automated ELISA plate reader (Multiskan EX®, Thermo Lab Systems, Finland).

The reading obtained at 450 nm was subtracted from the reading of 405 nm to reduce optical imperfections in the plate. The results are expressed as a relative absorbance index calculated by dividing the mean absorbance of the sample by the mean absorbance of a negative sample. Optical density (OD) values were normalized across plates using the following calculation: Absorbance index = (Mean sample OD) × (Mean OD of all positive control plates/Mean OD of the positive control plate).

Sds-page

K10 strain proteins were obtained and separated by SDS-PAGE 12%. For protein extraction 50 mL (approximately 1.5 mL of cell pellet) of MAP K10 strain culture at log phase was centrifuged at 3000 *g* 10 min. The supernatant was discarded and the pellet was washed three times with PBS 0.01 M. The pellet was resuspended in 300 μL of complete extraction buffer containing 15 μL of DTT (dithiothreitol), 12 μL of IP25 (Protease inhibitor 25x) and 273 μL of extraction buffer. The extraction buffer was composed of Tris 0.04 M, Urea 8 M and Chaps 0.01 M in Ready Prep proteomics Grade Water (Bio-Rad Laboratories, Spain). This suspension was added to tubes with 300 mg of glass beads and homogenized at 30 Hz during 10 min on TissueLyser II (Qiagen, Spain). Following homogenization, centrifugation at 15 000 *g* during 15 min was performed and the supernatant was transferred to a new vial. The absorbance was measured at 280 nm in a NanoDrop® ND-1000 Spectrophotometer (Thermo scientific, Spain).

For protein separation, 200 μg of K10 strain proteins were loaded in 12% acrylamide gels, and separation by electrophoresis was performed at 150 V. Precision Plus Protein™ Dual Color Standard (Bio-Rad Laboratories, Spain) was loaded in one lane to monitor electrophoretic separation, transfer efficiency as well as molecular weight size.

Protein transfer

The membrane protein transfer was performed according to the guidelines outlined by Towbin [30]. Briefly, proteins were transferred to polyvinylidene fluoride (PVDF) microporous membrane, Immobilon® (Millipore Ibérica, Madrid, Spain) by Trans-Blot® Semi-Dry electrophoretic transfer cell (Bio-Rad Laboratories, Spain) at 15 V.

Western blot (WB)

The membrane was cut in 0.5 cm wide strips that were blocked with 0.1 M Tris-Buffered Saline (TBS) supplemented with 5% nonfat dry milk (TBS-M) overnight at 4 °C.

Plasmas were diluted 1:50 in TBS-M prior to incubation with the membrane for 1 h at 37 °C with slow agitation. After four five minute washes with TBS, membranes were incubated with recombinant protein G peroxidase (Sigma-Aldrich, Spain) 1:8000 in TBS-M for 1 h at 37 °C. The membrane was washed again four times with TBS and enhanced chemiluminescence (ECL) was added. The immunoreactive bands were visualized by autoradiography. After scanning the autoradiography, reactivities were measured with Image J software.

Statistical analysis

Significance of the differences among groups for all variables weight, fecal PCR, MAP tissue PCR, IFN-γ release increase index, ELISA index, western blot reactivity, bacteriology index were assessed using analysis of variance (ANOVA). Some variables were based on summary measures obtained as follows; weight gain: the difference

between S10 and S0 weight, total MAP in tissues: the sum of MAP GE/g in all examined tissues, ELISA index: the difference between S10 and S0 index, Western blot reactivity: the sum of the total reactivity per area for each protein measured with Image J. Non- normally distributed data were subjected to natural log(Ln) transformation prior to statistical analyses. When ANOVA showed significant differences, Tukey's post hoc testing was used to make paired comparisons. Pearson's correlation test was applied to assess the association between ELISA and total western blot reactivity.

IFN-γ response was expressed for each sampling time 1, 3 and 10 (S1, S3, S10) after subtracting the start point sampling (S0). In order to estimate the contribution of vaccination to IFN-γ release, an index was calculated, where mean values from non-vaccinated infected animals were subtracted from the value of each vaccinated and infected animal and divided by the value of each vaccinated and infected animal. Afterwards, ANOVA was performed. The percentage of these indexes may be assumed to be equivalent to the response conferred by vaccination.

In order to take into account both bacteriological outcomes, the results of both post-mortem bacteriological techniques (qPCR of tissues and tissue culture) were used to generate what was termed as the bacteriology index. For this purpose, the number of positive qPCR tissues and culture were placed on the same scale by dividing each category by the maximum value within the category. The sum of the two resulting values for each technique was the final bacteriology index for each animal.

All statistical analyses were performed using R statistical software (3.1.0) and differences among groups for all variables were stated at $p < 0.05$.

Results

In vivo follow up

All animals maintained a healthy status until the experiment endpoint except for one animal belonging to the VSI group. This animal was euthanized prematurely at 153 days after the beginning of the experiment due to an unrelated pathology.

Challenge was achieved in all animals as shown by equal shedding levels in all animals from the third day challenge fecal sampling ($p = 0.373$). No episodes of diarrhea were observed throughout the experiment. However, fecal consistency did vary from hard to soft pellets occasionally in some animals.

Animals maintained normal food intake and only minimal weight loss was observed in some occasions in individuals that eventually recovered from the losses. Significant differences in weight gain among groups were not observed ($p = 0.302$).

Cellular immune response: interferon gamma release assay

As expected, animals from the NIC group did not show an increase of IFN-γ levels with any of the studied PPD and in the IC group, slightly higher IFN-γ levels were observed in the first sampling after challenge with all tested PPD (data not shown). In order, to measure the effect of vaccination on the cellular immune response the percentage of IFN-γ levels due to vaccination were calculated and represented on Table 1. These percentages were superior for the VSI group compared to the IVS group upon stimulation with all PPD at all time points showing significant differences at S10 upon stimulation with johnin ($p = 0.0019$).

Humoral immune response

The antibody production against MAP was assessed by PPA3 ELISA and WB. The results of antibody production expressed as the means of the ELISA Index values of all study groups during the experiment are represented in Figure 2. Vaccination with Silirum® produced an increase in humoral immune response prior to challenge (VSI vs IC; $p = 0.005$) and also after challenge (IVS vs IC; $p = 0.001$). However, infection alone did not raise humoral response, at least during the experimental period (NIC vs CI; $p = 0.999$). Vaccination prior to challenge produced slightly higher levels of antibodies compared to vaccination after challenge; however, at the end of the experiment the level of antibodies was similar in both vaccination schemes (Figure 2).

WB results are shown in Figure 3. We did not find any differences in humoral response against MAP proteins between infected and non-infected animals in both assays. The reactivity per area which was measured

Table 1 Percentage of IFN-γ release index upon stimulation with PPD due to vaccination

	PPD	VSI[a]	IVS[b]	*p* value
S1	AV	54.56	0.00	0.172
	BO	60.97	0.00	0.139
	JO	64.66	0.00	0.296
S3	AV	94.21	62.68	0.969
	BO	0.00	1.27	0.111
	JO	62.09	54.59	0.925
S10	AV	100.00	100.00	0.404
	BO	100.00	100.00	0.431
	JO	33.21	0.00	0.0019*

AV: Avian PPD, BO: bovine PPD, JO: johnin PPD, VSI: vaccinated before infection, IVS: vaccinated after infection, S1: IFN-γ release index first sampling. S3: IFN-γ release index third sampling, S10: IFN-γ release index tenth sampling.

* $p < 0.05$.

[a] Index percentage in relation to the infected control group (VSI-IC)/VSI.

[b] Index percentage in relation to the infected control group (IVS-IC)/IVS.

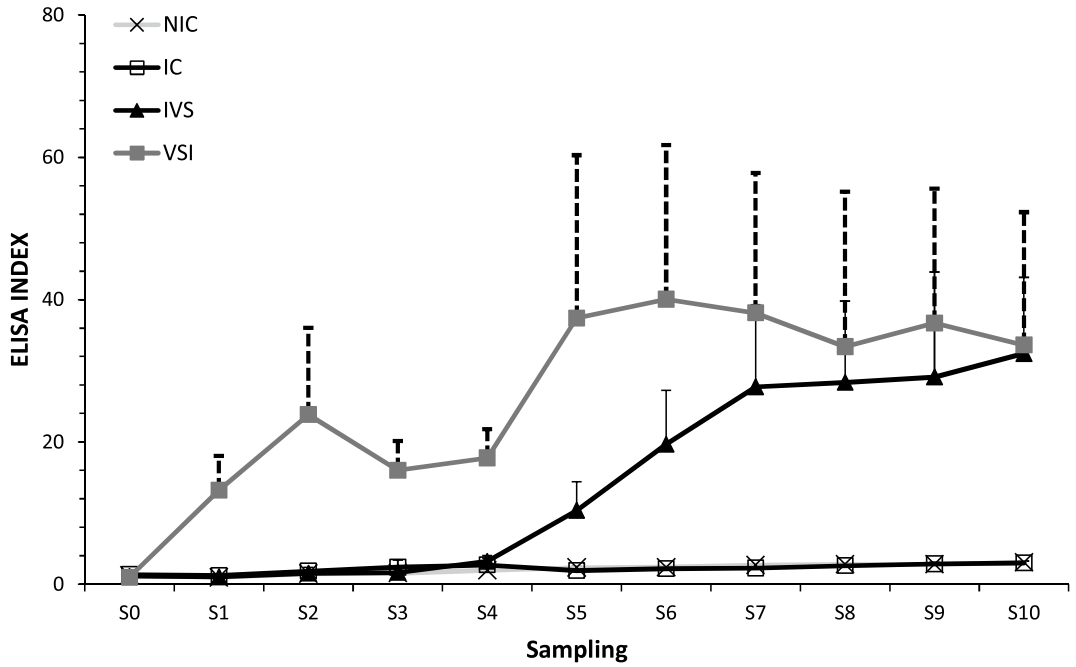

Figure 2 Evolution of mean ELISA index value per group. NIC: non infected control, IC: infected control, IVS: vaccination after infection, VSI: vaccination before infection. Error bars represent standard error of the mean.

attending to the protein weight did show differences when vaccinated animals were compared with infected ones, as well as between both vaccinated groups. Humoral response against ~22 kDa protein was significantly higher in both vaccinated groups, vaccinated after challenge (IVS vs IC, $p = 0.007$) and vaccinated before challenge (VSI vs IC, $p < 0.0001$). The VSI group also had higher antibody levels against ~22 kDa than the IVS group ($p < 0.0001$). Animals vaccinated before challenge (VSI) had higher levels of antibodies against ~35 kDa proteins than those of the infected group (VSI vs IC, $p < 0.0001$) or those vaccinated after challenge (VSI vs IVS, $p < 0.0001$). Vaccinated animals after infection (IVS) showed higher reactivity against ~37 kDa protein than IC animals ($p = 0.008$). Regarding humoral response against ~75 kDa proteins the animals vaccinated before infection (VSI) had higher values than IC animals ($p = 0.030$) and the IVS group animals ($p = 0.014$).

The total reactivity per group in WB had a strong positive correlation with the ELISA assay ($r = 0.993$; $p = 0.006$).

Post mortem findings

A summary of postmortem findings is shown in Table 2. At necropsy, some infected animals presented gross pathology consisting of increased vascularity and pale-white reactive spots in the sacculus rotundus and vermiform appendix as well as wall thickening in the ileum and jejunum compatible with PTB.

MAP was isolated from the sacculus rotundus, vermiform appendix, ileum and/or mesenteric lymph node in 6 of the 14 infected animals. In the IC group, MAP was isolated from at least one tissue in 80% of the animals. Vaccination decreased the bacterial burden in tissues. MAP was not isolated from any tissue of animals belonging to the IVS group whereas two animals of the VSI group presented one MAP positive tissue culture each.

If we attend to bacterial load measured by qPCR, we detected MAP DNA in the sacculus rotundus, vermiform appendix and mucosa. In the IC group, all animals had at least one tissue positive for qPCR with a mean of 150 ± 87 GE/g of MAP. Vaccination decreased the number of positive tissues for MAP qPCR. MAP DNA was detected in at least one tissue in 40% of the animals from the IVS group while 50% of the animals of the VSI group presented positive results to qPCR. However, bacterial load in the IVS group was higher than in the VSI group (953 ± 375 GE/g of MAP, 94 ± 71 GE/g of MAP respectively) although significant differences were not observed among the vaccinated groups.

The bacteriology index considering the number of positive tissues for qPCR and the number of tissues in which MAP has been isolated is represented in Figure 4. Animals of the IC group had the highest values followed by

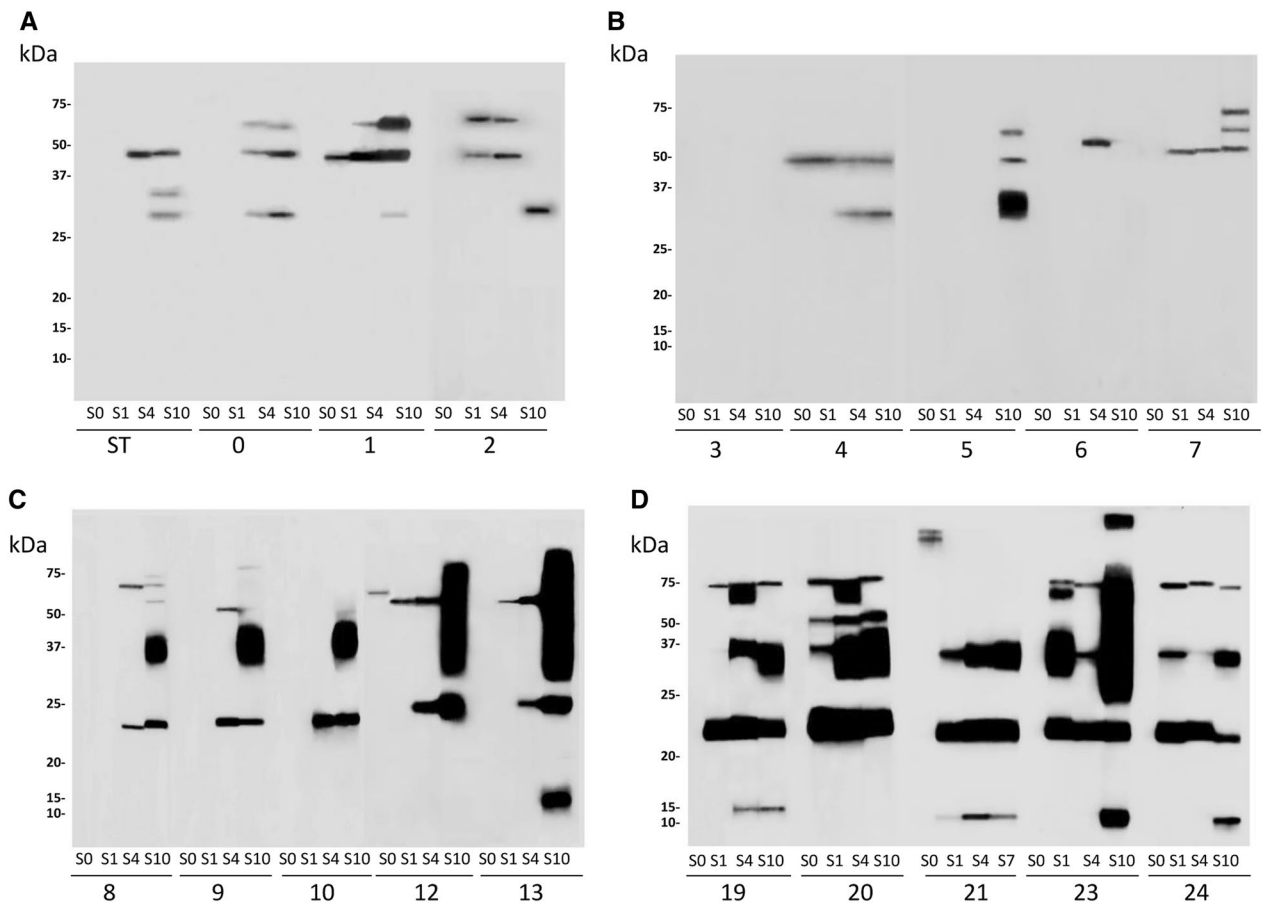

Figure 3 Western Blot of serum of individual animals showing reactivity to MAP K10 extracted proteins. A Non-infected control (NIC), **B** Infected control (IC), **C** Vaccinated after infection (IVS), and **D** Vaccinated before infection (VSI). Strips were incubated with serum dilution 1:50. Recombinant protein G peroxidase was used at dilution 1:8000. Blots were developed by chemiluminescent detection and visualized by autoradiography. Samplings (S0, S1, S4 and S10) of each animal are represented.

animals vaccinated before infection (VSI). Vaccination after infection (IVS) produced the lowest bacteriology index showing significant differences with the IC group ($p = 0.034$).

Discussion

The present study attempts to characterize the effect of vaccination before and after MAP challenge in a rabbit infection model. To our knowledge this is the first time that vaccination against PTB has been evaluated in a rabbit model, showing an increased immune response and reducing bacterial burden in tissues.

All challenged animals had equal chances of being infected due to the absence of significant differences in shedding levels in the fecal sampling immediately after challenge. As in previous studies carried out by our group with this short term model, clinical signs were not observed representing the subclinical stage of infection [26]. In order to observe clinical symptoms the experiment should have gone on for a longer period of time: 5–8 months for diarrhea [31, 32] and up to 2 years for emaciation [33].

PTB whole cell vaccine studies have demonstrated the induction of both cellular and humoral immune response in calves [34–37], sheep [38, 39] and goats [40]. For this reason we have attempted to evaluate both: cellular by IFN-γ release and humoral by ELISA and Western blot. Animals vaccinated before infection (VSI) had a temporary increase in IFN-γ response (at S1 or 42 days after the beginning of the experiment) although significant differences were not observed, probably due to the high variability between animals (non-reactors, low-reactors and high reactors) and in turn by the small sample number. These results are supported by previous studies in which vaccination with a whole cell vaccine increased IFN-γ and IL-10 release, in a murine model [41]. In addition,

Table 2 Summary of post mortem findings

Group	Animal	Gross pathology[a]			Tissue qPCR GE/g			Tissue culture			
		SR	VA	MUC	SR	VA	MUC	SR	VA	ICV.IL	MLN
	ST	–	–	–	–	–	–	–	–	–	–
NIC	0	–	–	–	–	–	–	–	–	–	–
	1	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–
	3	++	++	+	246	42.9	86.7	+	+	+	+
IC	4	+	++	+	–	–	5.13	+	+	–	–
	5	+	+	–	620	–	–	–	–	–	–
	6	+	+	+	–	37.8	–	+	+	–	–
	7	+	+	–	200	105	6.27	+	–	–	–
	8	–	–	–	–	–	–	–	–	–	–
IVS	9	–	++	–	–	–	–	–	–	–	–
	10	–	–	+	–	–	1130	–	–	–	–
	12	–	–	–	–	–	–	–	–	–	–
	13	–	–	–	1690	38.4	–	–	–	–	–
	19	–	–	–	–	258	2.45	+	–	–	–
VSI	20	–	–	–	NA	–	7.16	–	–	–	–
	23	–	–	+	–	–	–	–	+	–	–
	24	–	–	–	–	–	–	–	–	–	–

NIC: non infected control, IC: infected control, IVS: infected before vaccination, VSI: vaccinated before infection, SR: sacculus rotundus, VA: vermiform appendix, MUC: ileum and jejunum mucosa, ICV.IL: ileocecal valve and Ileum, MLN: mesenteric lymph node, NA: not analyzed.

[a] Gross pathology consisting of the following: pale white reactive spots and severe wall thickening (++) and increased vascularity and mild wall thickening (+).

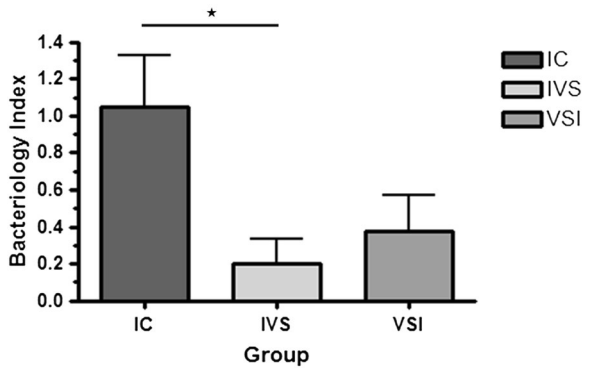

Figure 4 Bacteriology index per group. Bacteriology index was calculated based on the number of MAP qPCR positive tissues and the number of MAP culture positive tissues. IC: infected control, IVS: vaccinated after infection, VSI: vaccinated before infection. Error bars represent standard error of the mean. Significant differences were observed between the IC and IVS group ($p = 0.034$).

in this group (VSI), significant differences between IFN-γ release levels upon stimulation with bovine and avian PPD were not observed as documented in previous studies [34, 42, 43].

The effect of vaccination after challenge in the cellular immune response is not clear, at least on the short term basis. The IVS group was vaccinated at S3 (71 days of the start point of the experiment) and IFN-γ response was measured at S10 (128 days after vaccination). In this period of time IFN-γ response could have increased temporarily and decreased shortly afterwards, and we would not have detected these oscillations at S10. Also, the effect of vaccination itself prior to challenge was missed since a proper sampling was not performed between vaccination and challenge in the VSI group. For this reason, calculating the index in relation to infection was the only way to estimate the contribution of vaccination on IFN-γ release at each sampling time point. The VSI group presented higher percentages of IFN-γ index in comparison to the IVS group meaning that on a long term basis vaccination prior to infection may be more effective in maintaining a cellular immune response. In any case, differences between both groups were only significant for johnin PPD at the final sampling point, so we cannot state if there was any difference in the specificity of cellular immune response related with the sequence of vaccination.

Since MAP is an intracellular organism, a Th1 response and in particular IFN-γ secretion has been thought to be suggestive of protection in PTB, similar to other mycobacterial infections [44]. However, studies reporting high IFN-γ levels in vaccinated animals with high bacterial burden

and severe lesions [34–37] point out that IFN-γ may be a response to MAP antigens in circulating cells more than a correlate of protection. Recently humoral immune responses have shown to play key roles in protection in PTB [45] and against *M. tuberculosis* infection [46, 47]. Therefore, the increased level of antibodies in both vaccinated animal groups observed in our study could be in part responsible for the lower bacterial burden detected in the analyzed tissues. On the contrary, we did not observe an increased humoral response against MAP in the IC group probably because of the subclinical stage of the disease. In these animals the low antibody titers that they could be harboring may be forming immune complexes not detectable by ELISA or WB. In agreement, previous studies have shown low antibody titers during the first 2 years of infection, in naturally MAP infected cows [48].

In the present study, WB analysis was performed to better characterize host immune responses to vaccination and to identify potential markers that might be useful in differentiating MAP vaccinated and MAP infected animals. Unfortunately the absence of humoral immunity in the IC group during the study period impeded prediction of these hypothetical protein markers.

Although ELISA measures antibody levels against the PPA3 antigen and WB shows reactivity against all MAP extracted proteins, both techniques showed a strong correlation when total reactivity in WB was compared to ELISA index values. At the individual protein level, WB analysis showed some differences in humoral response among studied groups. For example, animals vaccinated before challenge had reactivity against ~12 kDa proteins which was absent in animals vaccinated after challenge. Animals vaccinated before challenge had higher reactivity against 22, 35 and 75 kDa proteins compared to all animal groups. These changes were probably related to the intensity of humoral response, and to the fact that vaccination in the VSI group has had more time to develop. A previously characterized MAP lipoprotein of approximately 22 kDa (P22) has demonstrated the ability to induce humoral and cellular responses being detected in vaccinated sheep and in clinically and subclinically infected cows [49]. In our study, both vaccinated groups had reactivity against a ~22 kDa protein absent in infected animals which may suggest that in our subclinical experimental model the ability to stimulate the humoral response by infection may be more limited than in cows in field conditions.

Vaccination has been reported to be the best currently available tool to control PTB in field conditions in recent reviews [6, 50]. We have noted that both groups of vaccinated animals harbor fewer bacteria in tissues than non-vaccinated animals. However, other vaccination studies performed in a murine model have shown poor protection using the whole cell vaccine Silirum® [51], suggesting that the rabbit model could better emulate the effect of vaccination previously observed in ruminants.

Attending to the bacteriology index, we observed that vaccination after infection was more effective than vaccination before infection. Vaccination in older animals has been reported to induce better immune response [39], ameliorate the clinical disease [52, 53] improve milk production [10] and longevity [11, 54]. Although the classic recommendation to vaccinate animals before being infected has proven to be an effective approach in acute diseases, it might not be an effective strategy in chronic diseases. The goal of vaccination in acute diseases is to mimic natural infection and increase the specific immune response in order to enhance immunity and avoid the disease. However, the acquired immune response might be not sufficient to clear the infection in chronic diseases [55]. Furthermore, an increased immunity produced by vaccination in the clinical phase of the disease could have a beneficial effect and it could be used as a short term individual strategy to control the disease in high valued animals when vaccination of the whole flock is restricted.

In the present study we used a rabbit model, showing positive effects of whole-cell vaccination against MAP, increasing immune response and reducing bacterial load in tissues, and although some of the results presented here are in agreement with previous reports, these should be confirmed in a ruminant species. Mainly, because the differences observed in infection between both vaccination strategies could be in part influenced by the age of the animals and the period that the animals were under vaccination effects in each vaccination strategy (animals from the IVS group were vaccinated 2 months after animals of the VSI group meaning that in those 2 months the protective effect of vaccination could have decreased).

On the contrary to what may be traditionally believed, vaccination after infection has shown a slightly stronger protective effect than before infection. In any case, the protective mechanisms that operate behind MAP vaccination are unknown and further research is needed to understand why MAP infection progression and/or vaccination fail in some animals. In this case, rabbits can be a useful and non-expensive laboratory infection model to advance in the knowledge of MAP pathogenesis and vaccination.

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

Authors' contributions
RAJ, NE and JMG conceived and designed the experiment. RA, EM and NE conducted the animal experiment. RA, EM, NE and VP performed lab analyses. RA and NE analyzed the data. All authors discussed results. All authors drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors thank Felix Blanco from the animal facilities (NEIKER-Tecnalia). This study was supported with funds from the Ministerio de Economía y Competitividad from the Spanish Government (AGL 2012-39818-C02-02). RA acknowledges support from the Departamento de Educación y Universidades of the Basque Government (BFI-2012-237).

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Received: 24 February 2016 Accepted: 6 June 2016
Published online: 05 August 2016

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IV. DISCUSIÓN GENERAL

Este trabajo aborda el problema de las infecciones micobacterianas en conejos, tanto en condiciones naturales como experimentales, con el objetivo de revisar un problema de sanidad animal que ha sido poco estudiado y que presenta algunos aspectos contradictorios. Para ello, se han desarrollado varios estudios observacionales y experimentales en los que se analiza la susceptibilidad del conejo a la infección por *Map* así como el efecto de la vacunación y la dieta en la modulación de la infección. Además, para profundizar en los conocimientos científicos de las infecciones micobacterianas naturales y experimentales en los conejos, se ha realizado una revisión de la información disponible en la literatura.

Debido a la susceptibilidad de los conejos a las infecciones micobacterianas y especialmente a *Map*, el primer estudio, de tipo observacional, se centró en la detección de micobacterias en conejos de matadero. El objetivo era, no solo determinar la prevalencia de infecciones micobacterianas en conejos domésticos de tipo industrial, sino hacer una estimación preliminar de la posible exposición de la población humana a micobacterias debido a los productos cárnicos de origen leporino. En el segundo estudio, se pretendía desarrollar un modelo de infección experimental por *Map* en el conejo con el fin de reproducir la enfermedad clínica mediante la exposición a factores naturales como son los cambios de dieta durante el desafío. Este modelo, fue empleado en un experimento posterior diseñado para estudiar la posible interacción de la microbiota intestinal con la infección y la dieta. Finalmente, se llevó a cabo un ensayo en el que se trataba de estudiar el potencial terapéutico de la vacunación frente a la infección paratuberculosa.

Los conejos son susceptibles a la infección por *Map* en condiciones naturales (Fuentes y Cebrian, 1988; Angus, 1990; Beard *et al*., 2001; Greig *et al*., 1997, 1999; Maio *et al*., 2011) y experimentales (Mokresh y Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005), pero no existen en la literatura estudios de prevalencia en explotaciones cunículas.

El aislamiento de micobacterias es una tarea tediosa y delicada, debido a múltiples factores entre los que se encuentra el proceso de descontaminación inicial al que se debe someter la muestra para su cultivo. Este proceso puede afectar la viabilidad

micobacteriana, y por ello se busca el equilibrio entre la eliminación de la flora bacteriana y la preservación de las células micobacterianas. En el Estudio I se utilizó la menor concentración de descontaminante que permitía evitar la contaminación en los medios de cultivo solidos (NaOH al 6%). Sin embargo, la tasa de aislamiento de MAC sugiere que incluso esta concentración puede ser excesiva para el crecimiento de las bacterias de este complejo. Asimismo, la distribución irregular de las micobacterias en los tejidos y la baja carga bacteriana, detectada mediante PCR, también podrían haber influido en los resultados obtenidos.

En el examen histopatológico, se observaron lesiones granulomatosas en el tejido linfoide digestivo del 27,2% de los animales analizados. La presencia de lesiones granulomatosas en esta misma localización debido a la infección por *Maa* se ha documentado con anterioridad en ovino (Benavides *et al*., 2013). Sin embargo, en nuestro estudio no se observaron lesiones histopatológicas compatibles en todos los animales en los que se identificó MAC por PCR y, mediante la tinción ZN, únicamente se detectaron BAAR en dos animales. Estos hallazgos unidos a la naturaleza focal de las lesiones histopatológicas observadas en los tejidos analizados sugieren que la presencia de MAC en conejos podría ser compatible con infecciones latentes o controladas similares a las descritas por Vazquez *et al*. en bovinos sacrificados en el matadero (Vazquez *et al*., 2013).

La ausencia de notificaciones previas de micobacteriosis en las explotaciones sugiere que estas afecciones no comprometen la producción cunícola en gran medida. Cabría pensar que la sintomatología clínica asociada a infecciones crónicas podría no llegar a manifestarse debido al corto ciclo productivo de los conejos destinados a consumo humano (9-11 semanas). No obstante, esta observación estaría en contradicción con las tasas similares de infección en gazapos y en reproductoras observadas, aunque se debe de tener en cuenta el limitado número de reproductoras analizadas. Por lo tanto, la ausencia de formas epidemio-patogénicas patentes indica que su desarrollo depende de la interacción de la infección por MAC con factores que no se han incluido en este estudio en términos cualitativos o cuantitativos.

Las subespecies del MAC, son las micobacterias más comúnmente aisladas en las linfadenitis cervicales pediátricas (van Ingen *et al*., 2009), habiéndose identificado también en pacientes con VIH o enfermedad pulmonar crónica obstructiva (Smole *et al*., 2002). Se ha sugerido que la presencia de MAC en tejidos de bovino (Alonso-Hearn *et al*., 2009; Klanicova *et al*., 2011), corderos (Klanicova *et al*., 2011), pollos (Klanicova *et al*., 2011) y porcino (van Ingen *et al*., 2010) puede suponer una vía de transmisión a los humanos. Sin embargo, debido a que el sistema digestivo de los conejos no entra en la cadena alimentaria la comercialización de estos animales portadores de MAC no debería de suponer un riesgo para la salud pública. En cualquier caso, el Estudio I pone de manifiesto que las explotaciones cunículas son un nicho para las especies del MAC que debe de seguir explorándose.

En nuestro estudio no se confirmó la presencia de *Map* a pesar de haberse detectado otras micobacterias del MAC como son *Maa/Mas y Mah* en el 16,6% de los conejos muestreados. Estas subespecies, así como *Map* pueden estar presente en el agua de bebida (Beumer *et al*., 2010) o en los pastos fertilizados con purín de explotaciones infectadas (Salgado *et al*., 2011) por lo que los resultados aquí presentados podrían interpretarse como el efecto de la entrada de componentes de la ración contaminados en la base alimentaria de las explotaciones cunícolas. Si esto fuese así, una posible explicación sería que la infección previa de los animales con otras bacterias del MAC pudiera tener un efecto protector frente a *Map* la cual ya habría desaparecido en el momento del sacrificio en la mayoría de los animales. Alternativamente, los animales recién nacidos podrían haberse expuesto a bacterias del MAC en los primeros días de vida como mecanismo de cebado microbiológico e inmunológico natural de la especie.

La imposibilidad de detectar *Map* en los animales muestreados en las condiciones de nuestro estudio sugiere que su presencia en conejos silvestres está relacionada con otras causas que no se dan en las situaciones controladas de las explotaciones cunícolas como pueden ser los factores ambientales, genéticos, edad, etc. Por otro lado, se debe de tener en cuenta que en las explotaciones cunículas podría darse la infección por *Map* con una baja prevalencia, lo cual requeriría del muestreo de un mayor número de animales para su detección.

En la búsqueda de modelos animales de laboratorio para estudiar la paratuberculosis, varios grupos han evaluado la utilidad del conejo (Mokresh y Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005). Las diferencias en la edad de los animales, las cepas de *Map* y las técnicas diagnósticas utilizadas han producido resultados inconsistentes y probablemente esta sea una de las razones por la cual el uso de este modelo animal no se ha generalizado.

En los Estudios II, III y IV se ha observado que la infección experimental en conejos de laboratorio se establece a nivel intestinal y del tejido linfoide asociado. Así, se ha detectado *Map* mediante cultivo y/o qPCR en *sacculus rotundus*, apéndice vermiforme, íleon y linfonodos mesentéricos. Estos resultados son coincidentes con los observados en experimentos previos en los que se ha obtenido el aislamiento de *Map* del apéndice vermiforme (Mokresh *et al*., 1989; Vaughan *et al*., 2005), *sacculus rotundus*, íleon (Mokresh y Butler, 1990; Mokresh *et al*., 1989) y linfonodos mesentéricos (Mokresh *et al*., 1989; Vaughan *et al*., 2005).

En el Estudio II, se ha demostrado la eliminación de *Map* a través de las heces mediante la técnica de qPCR en un 46,6% de los animales infectados. La detección de *Map* en heces mediante cultivo tan solo se había señalado en uno de los estudios anteriores, siendo la tasa de aislamiento del 30,7% de los conejos desafiados (Mokresh *et al*., 1989). De esta manera, la qPCR empleada en nuestro estudio puede ser una técnica de gran utilidad a la hora de monitorizar infecciones experimentales ya que ha permitido aumentar la tasa de detección de *Map*, hecho que se había constatado en estudios anteriores con cultivos puros (Elguezabal *et al*., 2011), en vacuno a nivel de matadero (Vazquez *et al*., 2013) y en conejos infectados naturalmente (Maio *et al*., 2011).

En oposición a lo observado en estudios anteriores (Mokresh y Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005), en los Estudios II y IV detectamos la presencia de lesiones macroscópicas, consistentes en zonas reactivas de color blanquecino, linfangiectasia, aumento marcado de la vascularización y engrosamiento de la pared de *sacculus rotundus* y apéndice vermiforme en un 20-53 % de los animales infectados (Anexo VII.3). Estos hallazgos son similares a los observados en conejos infectados naturalmente, en los cuales se han descrito lesiones que consisten en el engrosamiento

parietal del íleon terminal (Angus, 1990), *sacculus rotundus*, apéndice vermiforme y ciego (Maio *et al*., 2011), aumento de tamaño y tumefacción de linfonodos mesentéricos, y presencia de múltiples focos lesiónales granulomatosos en linfonodos mesentéricos, apéndice vermiforme y *sacculus rotundus* (Maio *et al*., 2011).

Según la literatura, los animales infectados naturalmente suelen presentar una mayor diseminación de las lesiones macroscópicas en comparación con los animales infectados de forma experimental, aunque en ambos casos el *sacculus rotundus* y el apéndice vermiforme son los tejidos más afectados. En el conejo estas dos estructuras suponen más del 50% del tejido linfoide asociado al tracto gastrointestinal (Davies y Davies, 2003) y es el lugar donde se asienta inicialmente la infección. Hay que destacar que *Map* podría afectar al *sacculus rotundus* del conejo de la misma manera que afecta la válvula-ileocecal en rumiantes, ya que parecen ser estructuras tisulares linfoides muy similares localizadas justamente la misma región anatómica (Davies y Davies, 2003). Asimismo, el apéndice vermiforme se podría ver afectado debido a que tiene funciones similares a las placas de Peyer ileales en rumiantes (Weinstein *et al*., 1994).

Las lesiones microscópicas observadas consisten principalmente en la presencia de granulomas bien definidos, representando los macrófagos la principal población celular. Se trata de lesiones compatibles con la infección por *Map* que han sido descritas de forma similar en infecciones subclínicas en vacas (Vazquez, 2014), cabras (Corpa *et al*., 2000a) y ovejas (Pérez *et al*., 1996). Mediante la tinción ZN solo se detectaron BAAR en uno de los animales, sugiriendo la presencia de una baja carga bacteriana (formas paucibacilares) o la perdida de la acido alcohol resistencia relacionada con un estado de dormancia o latencia (Seiler *et al*., 2003).

En las infecciones experimentales que se han llevado a cabo en este trabajo, tanto la limitada detección microscópica (Estudio II) como la dificultad en el aislamiento de *Map* (Estudio II y IV) recuerdan a lo reportado en la enfermedad de Crohn en humanos (Ikonomopoulos *et al*., 2000; Jeyanathan *et al*., 2007) en términos microbiológicos. Asimismo, Mokresh y Butler (1990) en infecciones experimentales en conejos aislaron colonias atípicas, caracterizadas por la ausencia de la triple capa exterior de las bacterias que las formaban, que podrían ser equivalentes a las formas deficientes de pared celular postuladas en los pacientes de Crohn (Chiodini *et al*., 1986; Mendoza *et al*., 2010).

Estos hallazgos sugieren que merecería la pena evaluar al conejo como modelo animal de la enfermedad de Crohn sobre todo si se demuestra definitivamente que *Map* es el agente causal de esta enfermedad humana y que este animal reproduce los fenómenos inflamatorios que caracterizan a dicha enfermedad.

En los Estudios II y III se ha observado que cambios en la dieta durante el desafío pueden modular la infección por *Map* en conejos bajo condiciones experimentales. La dieta regular estaba compuesta por una dieta comercial formulada para el crecimiento de conejos en producción, mientras que las dietas hiperproteica y alta en fibra se prepararon de forma exploratoria orientadas al objetivo de causar un estrés nutricional. Estas dietas se diseñaron teniendo en cuenta tanto la hipótesis hiperproteica de la paratuberculosis y la enfermedad de Crohn (Jantchou *et al*., 2010), como las condiciones naturales de los conejos de Castilla La Mancha (Maio *et al*., 2011) y Escocia (Greig *et al*., 1997). Los conejos silvestres podían pasar por periodos de estrés nutricional en los que la única alimentación disponible sería la de tipo fibroso. La ausencia de información preliminar sobre el efecto de duración y simultaneidad de ambos factores (nutrición e infección) determinó que tuviesen que elegirse niveles aleatorios de factores e interacciones, los cuales también se encontraban restringidos por la necesidad de que no fuesen tan extremos que pudiesen causar enfermedades digestivas agudas distintas del modelo que se buscaba.

De las tres dietas ensayadas, regular, hiperproteica y alta en fibra, la dieta regular fue la que estuvo asociada con mayores índices de infección. Los animales que se alimentaron con la dieta regular presentaron mayor eliminación fecal, mayor presencia de lesiones macroscópicas y un mayor índice de lesiones histopatológicas. Unido a lo anterior, la diferencia de la extensión de las lesiones fue estadísticamente significativa en el *sacculus rotundus*, sugiriendo que el cambio de dieta durante la infección podría tener un impacto mayor en este tejido que, como se ha mencionado anteriormente, podría considerarse como un equivalente a la válvula-ileocecal de los rumiantes (Davies y Davies, 2003).

Cabe destacar que la dieta regular formulada para maximizar el crecimiento y la producción en conejos no ha mostrado ser la más efectiva en la prevención de la

infección. Esto puede ser debido a que estas dietas están balanceadas para aumentar la productividad en animales sanos, sin conocerse su efecto en la inmunidad de los animales afectados por determinadas infecciones (Klasing *et al*., 2000). Es decir, la mayor eficiencia metabólica que se espera de una dieta diseñada para mejorar el crecimiento y, por ende, la producción cárnica, podría detraer recursos de otras funciones biológicas como las de defensa frente a infecciones. En consecuencia, los resultados de este estudio sugieren que, bien la ralentización de la función nutritiva mediante el incremento de la proporción de fibra, bien el incremento de los nutrientes proteicos necesarios para construir los elementos celulares responsables de la defensa inmune, podrían ser estrategias simples y baratas para mejorar la resistencia a infecciones lentas como la paratuberculosis.

La dieta tiene un efecto directo sobre el sistema inmunitario, pero además tiene la capacidad de modificar la comunidad microbiana del aparato digestivo (Graf *et al*., 2015; Xu y Knight, 2014), la cual juega un papel importante en la exclusión competitiva de patógenos y en el desarrollo y la maduración de la inmunidad de la mucosa intestinal (Kau *et al*., 2011; Stecher y Hardt, 2011). En el Estudio III hemos utilizado los métodos más actuales y de más amplio espectro y profundidad para el estudio de la flora digestiva de las regiones intestinales de interés. Estos métodos dan una visión amplia de los grupos bacterianos presentes, permitiendo incluso identificar los microorganismos no cultivables. Sin embargo, estas técnicas presentan la limitación de ignorar las poblaciones que se encuentran en bajas concentraciones, así como la imposibilidad de llegar a clasificar la microbiota a nivel de especie.

Mediante esta tecnología se han observado diferencias en la microbiota del *sacculus rotundus* y contenido cecal en animales infectados con *Map*, alimentados tanto con dieta regular como con dieta alta en fibra durante el desafío. Los índices de diversidad microbiana a menudo se utilizan como indicadores de salud de un ecosistema. Así, la disminución de diversidad microbiana intestinal propia de cada sujeto (diversidad alfa) se ha relacionado con la enfermedad de Crohn (Dicksved *et al*., 2008; Manichanh *et al*., 2006) y la diabetes tipo I (Giongo *et al*., 2011), enfermedades en las que se sospecha que *Map* podría jugar cierto papel (Sechi y Dow, 2015). Cabe destacar, que el aumento del número de especies bacterianas observado en el contenido cecal de los animales infectados podría ser reflejo del cambio de composición microbiana, debido a que las

técnicas de extracción que no permiten eliminar el ADN de las bacterias muertas podrían influir en los resultados obtenidos. Sin embargo, también podría ser el resultado de la interacción de *Map* con otros grupos bacterianos o ser consecuencia de los cambios en el ecosistema producidos por la infección.

En lo referente a la diversidad microbiana entre animales (diversidad beta), en el contenido cecal se han observado diferencias cualitativas entre la microbiota de animales infectados y no infectados, mientras que en el *sacculus rotundus* se han detectado diferencias tanto cualitativas como cuantitativas. Estos cambios observados en el tejido linfoide sugieren que la composición microbiana puede jugar un papel importante en la inflamación producida por la infección. Esta hipótesis se ve reforzada por el hecho de que la población microbiana en el tejido linfoide de conejo es capaz de inducir la inflamación tisular (Shanmugam *et al*., 2005).

En relación a la comunidad bacteriana positivamente asociada con la infección por *Map* destaca la familia Mogibacteriaceae la cual recientemente se ha identificado a su vez en mayor proporción en la mucosa ileal de vacuno infectado experimentalmente con *Map* (Derakhshani *et al*., 2016). A un nivel taxonómico más profundo destaca el género *Stenotrophomonas*, positivamente asociado a la infección en animales alimentados con la dieta regular. Este género se ha visto aumentado en los pacientes con la enfermedad de Crohn (Knösel *et al*., 2009) y en ratones infectados con *Map* (Karunasena *et al*., 2014). Además, este género ha sido relacionado con diversos estados inflamatorios como neumonía, bacteriemia e infecciones del tracto urinario (Denton y Kerr, 1998). Lamentablemente, en la actualidad hay un gran desconocimiento de la función biológica de estas familias bacterianas por lo que no se puede aventurar qué papel podrían desempeñar en la inflamación intestinal.

Dentro de los géneros que se han detectado en menor proporción en los animales infectados y que por lo tanto podrían ser indicadores indirectos de la pérdida de sus posibles efectos beneficiosos en condiciones normales, destaca el género *Anaerostipes.* Las bacterias de este género utilizan el lactato para producir butirato (Sato *et al*., 2008), el cual puede disminuir la producción de citoquinas proinflamatorias producidas por los macrófagos intestinales (Chang *et al*., 2014) ejerciendo un efecto beneficioso en casos

de colitis (Segain *et al*., 2000). Además, este género se encuentra en una menor proporción en pacientes con la enfermedad de Crohn (Morgan *et al*., 2012), sugiriendo que podría jugar un papel importante en la salud gastrointestinal. El género *Coprobacillus* también destaca por encontrarse negativamente asociado a la infección por *Map* tanto en animales alimentados con dieta regular como alta en fibra. Interesantemente, se ha sugerido que este género podría tener un papel beneficioso en el mantenimiento de la estabilidad intestinal, confiriendo a su vez resistencia a la colonización por *Clostridium difficile* (Stein *et al*., 2013), agente microbiano relacionado con la colitis pseudomembranosa (Larson *et al*., 1978).

Finalmente, destaca el hecho de que mediante la secuenciación del ARNr 16S, el género *Mycobacterium* únicamente se ha detectado en el contenido cecal de un animal infectado, mientras que por PCR se ha detectado en el *sacculus rotundus* del 30% de los animales infectados. Estas diferencias observadas en la detección de *Map* podrían ser debidas a la estandarización de la concentración del ADN para la secuenciación, a los cebadores utilizados (universales *vs* específicos de *Map*) o a la baja concentración relativa de *Map* en tejido de los animales infectados, por lo que se requeriría de una elevada profundidad de secuenciación para su detección.

Recientemente se ha sugerido que el perfil bacteriano asociado a la infección se podría usar como herramienta diagnóstica de infecciones paratuberculosas subclínicas (Derakhshani *et al*., 2016). Sin embargo, en nuestro estudio se ha observado que algunos grupos taxonómicos muestran una asociación opuesta con la infección, dependiendo del tejido o la dieta ingerida. Por lo tanto, establecer un patrón bacteriano asociado a la infección por *Map* requeriría de la realización de más estudios, con el fin de definir los grupos bacterianos asociados a la infección bajo diferentes condiciones. Asimismo, debido a que una gran parte de la comunidad microbiana observada no es cultivable y hay un gran desconocimiento de su función biológica, sería de gran ayuda la realización de estudios metatranscriptómicos que pudieran ayudar a comprender el papel que juega la microbiota intestinal en el establecimiento y la progresión de la infección por *Map.*

Los hallazgos del presente trabajo podrían ser aplicados para el desarrollo de prebióticos o probióticos compuestos por especies de la flora bacteriana negativamente asociada a

la infección, con el fin de compensar las pérdidas causadas por la infección y ayudar al restablecimiento del equilibrio normal. Dichos prebióticos o probióticos se podrían administrar tanto a animales jóvenes como adultos en combinación con la dieta u otras estrategias de control de la enfermedad más clásicas como es el caso de la vacunación.

La vacunación ha demostrado ser la herramienta de control de la paratuberculosis más efectiva disponible en la actualidad (Bastida y Juste, 2011; Juste, 2012). Así, se ha utilizado satisfactoriamente en los programas de control de paratuberculosis en ovino en Islandia (Fridriksdottir, 2000) y Australia (Kennedy y Citer, 2010) y en caprino en Noruega (Saxegaard y Fodstad, 1985) y España (Corpa *et al*., 2000c). Sin embargo, es bien conocido que la vacunación no proporciona una protección completa contra la infección (Reddacliff *et al*., 2006; Windsor y Eppleston, 2006). Por ello, es importante estudiar otras estrategias que puedan ser utilizadas de forma complementaria, además de ahondar en el conocimiento de los mecanismos patogénicos para intentar formular vacunas más efectivas.

Clásicamente se ha recomendado la vacunación de los animales en los primeros meses de vida, bajo la premisa de que se debe estimular la respuesta inmune antes del primer contacto con *Map* (Bastida y Juste, 2011; Larsen *et al*., 1964; Saxegaard y Fodstad, 1985). La cuestión es que la vacunación previa a la exposición al agente infeccioso supone un abordaje efectivo frente a infecciones agudas, pero podría no ser la estrategia óptima en el caso de las enfermedades crónicas. Por ello, en el último estudio se ha evaluado el efecto de la vacunación frente a *Map* previo y posterior a la infección (efecto terapéutico), en la respuesta inmune y en la carga bacteriana en el modelo de conejo (Estudio IV).

Numerosos estudios han demostrado que la vacunación frente a *Map* estimula la respuesta inmune celular y humoral en vacuno (Kohler *et al*., 2001; María Muñoz de Frutos, 2014; Muskens *et al*., 2002; Stabel *et al*., 2011), ovino (Begg y Griffin, 2005; Corpa *et al*., 2000b), y caprino (Hines *et al*., 2014).

El aumento de la respuesta Th1, y en particular la secreción de IFN-γ se ha considerado que ejerce un efecto protector frente a las micobacteriosis (Flynn, 1993). A pesar de que

clásicamente se haya utilizado esta citoquina como marcador de protección en los estudios vacunales frente a tuberculosis, recientemente se ha sugerido que esta podría no ser una estrategia útil para evaluar la protección inducida por la vacunación, debido a que no se correlaciona con el índice de infección (Nunes-Alves *et al*., 2014). Además, hay que tener en cuenta que esta citoquina proinflamatoria, tiene la capacidad de promover el desarrollo de lesiones progresivamente destructivas (Orme *et al*., 2014). Unido a esto, los elevados niveles de producción de IFN-γ de los animales vacunados, con elevada carga bacteriana de *Map* y lesiones severas (Kohler *et al*., 2001; María Muñoz de Frutos, 2014; Muskens *et al*., 2002; Stabel *et al*., 2011) pone de manifiesto que el aumento de esta citoquina podría no estar relacionada con la protección sino que podría ser únicamente el reflejo de la estimulación causada por los antígenos de *Map* en las células circulantes.

En el modelo de conejo hemos observado que la vacunación estimula la inmunidad celular, tal y como se había reportado previamente en el modelo murino (Ghosh *et al*., 2015). Además, se ha detectado un mayor aumento de la respuesta inmune celular en los animales vacunados previo al desafío con *Map* que en los vacunados posteriormente, pudiendo estar relacionada con los niveles de carga bacteriana en tejidos.

Por otro lado, aunque históricamente se ha considerado que la respuesta humoral no confiere protección frente a las micobacteriosis, recientemente se ha sugerido que ejerce un papel crucial en la protección frente a la tuberculosis (Achkar y Casadevall, 2013; Niki *et al*., 2015) y paratuberculosis (Begg *et al*., 2011) o, al menos, que aparece asociada a la protección en una proporción de la población. Por lo tanto, el aumento del nivel de anticuerpos observado en los conejos vacunados antes y después del desafío podría en parte ser responsable de la menor carga bacteriana detectada en los tejidos de los animales vacunados.

Mediante western blot se ha observado una leve respuesta humoral a partir del primer muestreo en los animales no infectados. Esta respuesta podría ser debida a una leve exposición a *Map* de los animales mediante aerosoles, que también se habría producido en los demás grupos, lo que demostraría que las dosis y vías de exposición a antígenos micobacterianos podrían ser relevantes en el control de la enfermedad. No obstante, esta respuesta inmune podría ser debido a que con la maduración los animales desarrollan

una respuesta humoral inespecífica frente a las proteínas de alto peso molecular de *Map*, lo que explicaría la resistencia de los animales adultos a la infección.

En el modelo de conejo hemos observado que ambas estrategias de vacunación disminuyen el índice bacteriológico (cultivo y qPCR de tejidos). Resultados que están en concordancia con lo observado en rumiantes (Bastida y Juste, 2011). No obstante, la vacunación posterior a la infección ha sido ligeramente más efectiva que la vacunación previa a la infección. De hecho, diferentes estudios (Gwozdz *et al*., 2000; Windsor, 2006), sugieren que la vacunación con células enteras podría tener un efecto terapéutico, reduciendo la carga bacteriana en tejidos. Además, otros trabajos han reportado que la vacunación en animales adultos induce una mayor respuesta inmune (Corpa *et al*., 2000b), disminuye la enfermedad clínica (Singh *et al*., 2010, 2013) y mejora la producción láctea (Juste *et al*., 2009; Tamayo *et al*., 2010) y la longevidad de los animales (Alonso-Hearn *et al*., 2012; Santema *et al*., 2013). Por lo tanto, la vacunación en la fase clínica o subclínica de la enfermedad podría tener un efecto beneficioso y podría ser utilizada como una estrategia de control a corto plazo en animales de alto valor económico y/o en los casos en los que no es posible vacunar a todo el rebaño. Adicionalmente, supone un indicio de que las terapias de inmunización activa podrían utilizarse en medicina humana frente a enfermedades inflamatorias lentas asociadas a infecciones de tipo paratuberculoso.

En la presente Tesis doctoral se ha avanzado en el estudio de la susceptibilidad del conejo a la infección por MAC, así como en la utilización de este animal como modelo animal de laboratorio, lo que ha permitido progresar en el conocimiento de la patogenia y la vacunación frente a *Map*. Así, se ha demostrado que la utilización de diferentes estrategias alimentarias a través de la modificación de la microbiota intestinal puede jugar un papel fundamental en la modulación de la infección por *Map*. Estos hallazgos, sugieren que estas estrategias de modulación de la infección se deberían ensayar con un enfoque holístico, teniendo en cuenta las posibles interacciones y/o efectos aditivos.

Los hallazgos del presente trabajo justifican la necesidad de llevar a cabo estudios que permitan elucidar si el consumo de conejo podría suponer una fuente de exposición a MAC para los humanos y que efecto podría tener en la salud. Además, sería interesante

estudiar el posible impacto de estas infecciones a nivel productivo así como aislar y caracterizar las especies de MAC para determinar el origen de la infección.

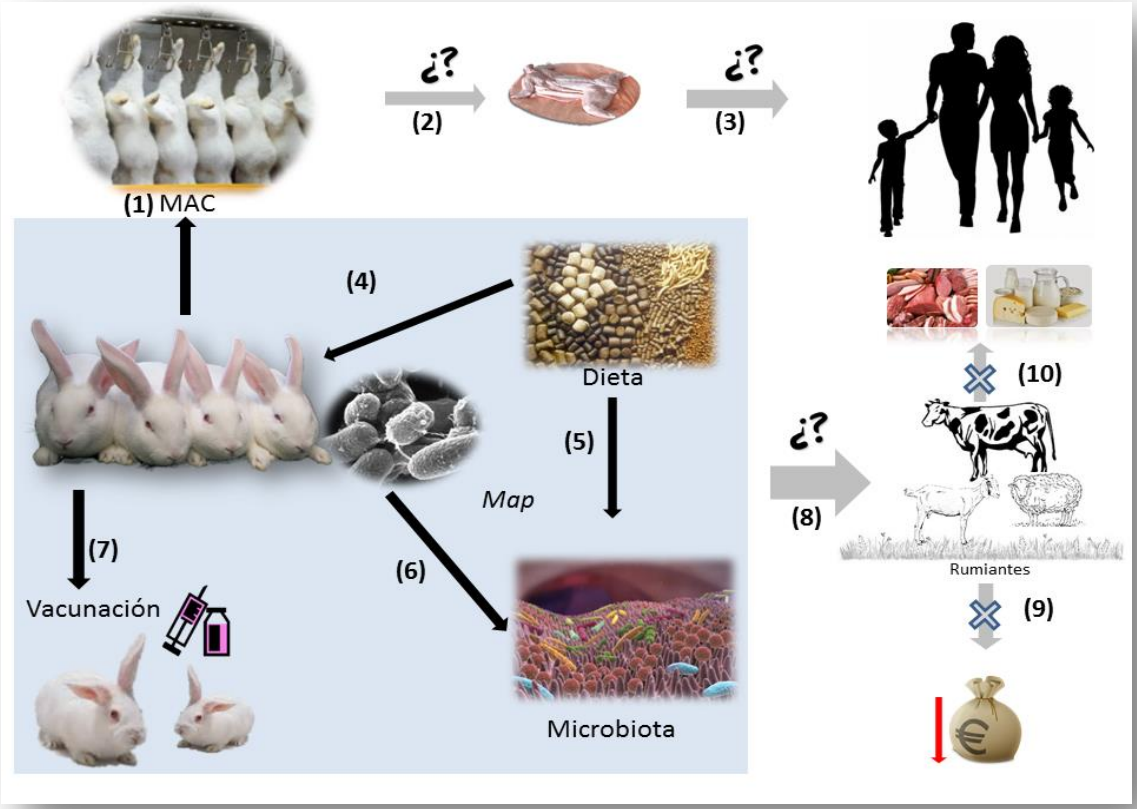
Por otro lado, al observar que la dieta puede influir en la infección por *Map*, se abre un nuevo paradigma de estrategias de intervención frente a la enfermedad. Este hallazgo unido a la descripción de la microbiota digestiva negativamente asociada a la infección abre el camino a nuevos abordajes para el control de la enfermedad, como pueden ser el uso de diferentes dietas en cortos periodos de tiempo que no comprometan la producción o la utilización de dietas suplementadas con determinados prebióticos o probióticos. Adicionalmente se justifica el ensayo de otras estrategias que han obtenido resultados prometedores en humanos, como son los trasplantes fecales. Este tratamiento ha mostrado ser eficaz en otras enfermedades intestinales crónicas, como la colonización por *Clostridium difficile* (Aroniadis *et al*., 2015) o la enfermedad de Crohn (Suskind *et al*., 2015).

Este tipo de intervenciones unidas a la observación de cierto grado de efecto terapéutico de la vacunación sugieren que se deberían ensayar de forma combinada en rumiantes en condiciones de campo. Esto permitiría estudiar los mecanismos inmunológicos implicados en la protección y/o la modulación de la infección con el fin de lograr una estrategia efectiva en el control de la enfermedad.

Finalmente en el presente trabajo hemos demostrado que el conejo es un modelo animal de infección de *Map* útil, que permite avanzar en el estudio de la infección subclínica y por ende podría ser de utilidad en el estudio de otras enfermedades inflamatorias intestinales en humanos. Además, el empleo de este modelo animal permitiría avanzar en el cribado inicial de nuevos productos y pautas vacunales de una manera rápida y económica.

A continuación se resumen los principales hallazgos de este trabajo en la Figura 7 y su posible implicación en la sanidad animal y la salud pública.

Figura 7. Representación esquemática de los principales hallazgos derivados del presente trabajo y su posible implicación en la sanidad animal y en la salud pública.



(1) Los conejos destinados a consumo humano presentan MAC en el tejido linfoide asociado. (2) De esta manera, la canal podría encontrarse infectada, (3) de forma que el consumo de carne de conejo inadecuadamente cocinado podría suponer una exposición de la población humana cuyos efectos son inciertos. (4) La dieta tiene un efecto modulador de la infección por *Map*, y a su vez (5) tiene la capacidad de producir cambios en la microbiota digestiva, la cual (6) se ve alterada significativamente a causa de la infección por *Map*. Tanto la dieta como la microbiota tienen la capacidad de influir en la respuesta inmunitaria del hospedador. (7) La vacunación presenta un efecto protector, produciendo un aumento en la inmunidad humoral y celular. (8) Modificaciones en la dieta y/o microbiota se podrían combinar con la vacunación en los rumiantes domésticos, con el fin de (9) disminuir las pérdidas económicas producidas por la enfermedad y (10) limitar la transmisión del agente infeccioso a la población humana mediante el consumo de alimentos de origen animal, aspecto relevante en el caso de confirmarse la relación de *Map* con la enfermedad de Crohn.

IV. GENERAL DISCUSSION

The present work addresses the problem of mycobacterial infections in rabbits, under both natural and experimental conditions, aiming to review an animal health problem that has been under studied and presents some contradictory aspects. For this purpose, several observational and experimental studies were carried out in which susceptibility of rabbit to *Map* infection and the effect of vaccination and diet in the infection is analyzed. In addition, to go in depth into the scientific knowledge of natural and experimental mycobacterial infections in rabbits, a review of the information available in the literature has been conducted.

Due to the susceptibility of rabbits to mycobacterial infections, especially to *Map*, the first observational study was focused on the detection of mycobacteria in rabbits at slaughterhouse. The aim of this study was not only to determine the prevalence of mycobacterial infections in industrial rabbit's farms, but rather to perform a preliminary estimation of the possible exposure of human population to mycobacteria through consumption of leporine origin meat products. In the second study, the aim was to develop a rabbit model of *Map* experimental infection with the purpose of producing the clinical disease through exposure to natural factors such as diet changes during the challenge. This model was used in a subsequent experiment designed to study the interaction of the intestinal microbiota with infection and diet. Finally, an assay was conducted to study the therapeutic potential of the vaccination against paratuberculosis infection.

Rabbits are susceptible to *Map* infection in both natural (Fuentes y Cebrian, 1988; Angus, 1990; Beard *et al*., 2001; Greig *et al*., 1997, 1999; Maio *et al*., 2011) and experimental conditions (Mokresh y Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005). However, in the literature there are no previous studies of *Map* prevalence on rabbit farms.

The isolation of mycobacteria is a tedious and delicate procedure due to multiple factors, including the initial decontamination process to which the sample must be submitted for culture. This process can affect mycobacterial viability and therefore the balance between bacterial flora removal and preservation of mycobacterial cells is sought. In Study I the lowest concentration of decontaminant (6% NaOH) which was capable of preventing solid culture media contamination was used. However, this

concentration of NaOH could be responsible of not achieving the isolation of MAC. Moreover, the irregular distribution of mycobacteria in tissues and the low bacterial load detected by PCR may have also influenced the results.

In the histopathological examination, granulomatous lesions were observed in the digestive lymphoid tissue of 27.2% of the tested animals. The presence of granulomatous lesions in this same location due to infection of *Maa* has been previously documented in sheep (Benavides *et al*., 2013). However, in our study histopathological lesions were not observed in all the animals in which MAC was identified by PCR and by ZN staining. Acid-alcohol resistant bacilli were only detected in two animals. These findings linked to the focal nature of histopathological lesions observed in analyzed tissues suggest that the presence of MAC in rabbits could be compatible with controlled or latent infections, similar to those described by Vazquez *et al*. in slaughtered cattle (Vazquez *et al*., 2013).

The absence of previous notifications of mycobacteriosis on rabbit farms suggests that these infections do not compromise rabbit meat production to a great extent. It could be that the clinical symptoms associated with chronic infections may not be noticeable because of the short production life cycle of rabbits for human consumption (9-11 weeks). However, this observation could be inconsistent with the similar infection rates observed both in young and breeding rabbits, although it must be taken into account the limited number of breeding animals analyzed. Therefore, the absence of patent epidemio-pathogenic forms indicates that their development depends on the interaction of MAC infection with factors not included in this study in qualitative or quantitative terms.

MAC subspecies are the most commonly isolated mycobacteria in pediatric cervical lymphadenitis (van Ingen *et al*., 2009), having also been identified in patients with HIV or chronic obstructive pulmonary disease (Smole *et al*., 2002). It has been suggested that the presence of MAC in bovine tissues (Alonso-Hearn *et al*., 2009; Klanicova *et al*., 2011), sheep (Klanicova *et al*., 2011), chickens (Klanicova *et al*., 2011) and pigs (van Ingen *et al*., 2010) could be a route of transmission to humans. However, because the digestive system of rabbits does not enter the food chain, MAC-bearing animals should

not be a risk to public health. In any case, Study I highlights that the rabbit farms are a niche of MAC that should be further explored.

In our study, the presence of *Map* was not confirmed is spite of detecting other mycobacteria of MAC such as Maa/Mas and Mah in 16.6% of the sampled rabbits. These subspecies and *Map* may be present in drinking water (Beumer *et al*., 2010) or on fertilized pasture with manure from infected farms (Salgado *et al*., 2011). Therefore, the results presented here could be interpreted as the effect of using contaminated components in rabbit farm food. If this were the case, a possible explanation would be that a previous infection of animals with other bacteria of MAC could have a protective effect against *Map* infection which would already have disappeared at the slaughter time in the majority of the animals. Alternatively, newborn animals could have been exposed to MAC bacteria in the first days of life as a mechanism for natural microbiological immune priming of the species.

The failure to detect *Map* in sampled animals under our study conditions suggests that its presence in wild rabbits is related to other factors that do not occur under rabbit farms controlled circumstances such as environmental conditions, genetic, age, etc. Furthermore, it should be noted that in rabbit farms, *Map* infection could be present with a low prevalence, requiring sampling of a larger number of animals to acheive its detection.

Looking for a laboratory animal model to study paratuberculosis infection, several groups have evaluated the usefulness of the rabbit model (Mokresh y Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005). The differences in the age of the animals, *Map* strains and diagnostic techniques used have produced contradictory results and probably this is one of the reasons for which this animal model has not been established.

In Studies II, III, and IV, it has been shown that experimental infection in laboratory rabbits is established at intestinal and associated lymphoid tissue level. Subsequently, *Map* has been detected by culture and/or qPCR in sacculus rotundus, vermiform appendix, ileum and mesenteric lymph nodes. These results are consistent with those observed in previous experiments in which *Map* isolation has been achieved from vermiform appendix (Mokresh *et al*., 1989; Vaughan *et al*., 2005), sacculus rotundus,

ileum (Mokresh and Butler, 1990; Mokresh *et al*., 1989) and mesenteric lymph nodes (Mokresh *et al*., 1989; Vaughan *et al*., 2005).

In Study II, *Map* shedding in feces has been proved by qPCR in 46.6% of infected animals. *Map* detection in feces by culture has been reported only in one of the previous studies, achieving an isolation rate of 30.7% of challenged rabbits in that case (Mokresh *et al*., 1989). Therefore, the qPCR employed in our study may be a useful technique to follow up experimental infections, allowing the increase of *Map* detection limit, a fact that has been reported in previous studies with pure cultures (Elguezabal *et al*., 2011), in cattle at the slaughterhouse (Vazquez *et al*., 2013) and in naturally infected rabbits (Maio *et al*., 2011).Contrary to the observations made in previous studies (Mokresh and Butler, 1990; Mokresh *et al*., 1989; Vaughan *et al*., 2005), in Studies II and IV, gross pathology consisting of pale white reactive spots, linfangiectasia, increased vascularity and wall thickening of sacculus rotundus and vermiform appendix (Annex VII.3) was detected in 20-53% of infected animals. These findings are in agreement with the descriptions from natural infection in wild rabbits where wall thickening of terminal ileum (Angus, 1990), sacculus rotundus, vermiform appendix and cecum (Maio *et al*., 2011), enlargement and swelling of mesenteric lymph nodes, and the presence of multiple granulomatous foci in mesenteric lymph nodes, sacculus rotundus and vermiform appendix (Maio *et al*., 2011) has been reported.

According to the literature, gross pathology tends to be more thoroughly spread in naturally infected animals compared to experimentally infected ones, although in both cases the sacculus rotundus and vermiform appendix are the most affected tissues. In rabbits, these two structures represent more than 50% of gut associated lymphoid tissue (GALT) (Davies and Davies, 2003) being the region where the infection is initially established. It should be noted that *Map* could affect rabbit sacculus rotundus in the same way that it affects ileocecal valve in ruminants, as they seem to be similar lymphoid tissue structures located in the same anatomical region (Davies and Davies, 2003). Likewise, vermiform appendix could be affected because its function is similar to that of ileal Peyer's patches in ruminants (Weinstein *et al*., 1994).

The microscopic lesions mainly consisted of the presence of well defined granulomas, being the macrophages the main cell population. These kind of lesions are compatible

with *Map* infection as they are similar to those found in subclinical infection in cows (Vazquez, 2014), goats (Corpa *et al*., 2000a) and sheep (Perez *et al*., 1996). By ZN staining, acid-fast bacilli were detected only in one animal, suggesting a low bacterial load (paucibacillary forms) or the loss of acid alcohol resistance related to a dormancy or latency state (Seiler *et al*., 2003).

In the experimental infections carried out in this work, the limited microscopic detection (Study II) and the difficulty in isolating *Map* (Study II and IV) resembles the microbiological outcomes reported in human Crohn's disease (Ikonomopoulos *et al*., 2000; Jeyanathan *et al*., 2007). Furthermore, Mokresh and Butler (1990) have isolated atypical colonies in experimental infection in rabbits, characterized by the absence of the external triple layer of the bacteria, resembling cell wall deficient forms postulated in Crohn's disease patients (Chiodini *et al*., 1986; Mendoza *et al*., 2010). These findings suggest that it would be worthwhile to evaluate rabbit as an animal model of Crohn's disease, especially if it is definitely demonstrated that *Map* is the causative agent of this human disease and that this animal reproduces its characteristic inflammatory features.

In Studies II and III it has been observed that dietary changes during challenge modulate *Map* infection in rabbits under experimental conditions. Regular diet consisted of a commercial diet formulated for growth of rabbits in production, while hiperproteic and high fiber diets were designed in an exploratory manner with the objective of causing nutritional stress. These diets were designed taking into account the hiperproteic hypothesis of paratuberculosis and Crohn's disease (Jantchou *et al*., 2010) as well as the natural conditions of infected rabbits in Castilla La Mancha (Maio *et al*., 2011) and Scotland (Greig *et al*., 1997).

Wild rabbits could have periods of nutritional stress where the only available feeding source would be fibrous. The absence of preliminary information on the effect of both factors (nutrition and infection) determined the choice of random levels of factors and interactions. The diet formulation was also limited by the absence of extreme ranges of nutrients that could themselves be the cause of digestive diseases different from the model that was sought.

Our results showed that of the three diets tested (regular, high protein and high fiber), the regular diet was the one that was associated with higher rates of infection. Animals fed regular diet had higher fecal shedding increased gross pathology and higher rate of histopathological lesions. Additionally, the differences in the lesions extension were statistically significant in the sacculus rotundus, suggesting that diet changes could have a higher impact on this tissue, which has similarities with the ileocecal valve in ruminants (Davies and Davies, 2003) as mentioned above.

It should be noted that regular diet, formulated to maximize rabbit's growth and production, was not the most effective in preventing infection. This could be because the diet is formulated to increase productivity in healthy animals, without knowing its effect on the immunity of animals affected by different infections (Klasing *et al*., 2000). Correspondingly, the higher metabolic efficiency expected of a diet designed to improve growth and, therefore, meat production, could divert resources from other biological functions such as defense against infections. Consequently, the results of this study suggest that both slowdown of the nutritional function by increasing the proportion of fiber and the increase of protein nutrients necessary to conform cellular elements responsible for the immune defense, could be simple and cheap strategies to improve resistance to slow infections such as paratuberculosis.

Diet has a direct effect on the immune system but also has the ability to modify the microbial community of the digestive system (Graf *et al*., 2015; Xu and Knight, 2014), which plays an important role in the competitive exclusion of pathogens and in the development and maturation of the intestinal mucosal immunity (Kau *et al*., 2011; Stecher and Hardt, 2011). In Study III we have used the most current methodology to study the digestive microbial community of the intestinal regions of interest. These methods provide a comprehensive view of bacterial groups present in one ecosystem, allowing the identification of non-culturable microorganisms. However, these techniques present the limitation of ignoring the taxonomic groups that are at low concentrations, as well as the inability to reach the species level in microbial classification. Using this technology we observed differences in the microbiota of sacculus rotundus and cecal content of *Map* infected animals fed with regular and high fiber diet during challenge.

Microbial diversity indexes are often used as heath markers of a ecosystem. Moreover, a decrease of intestinal microbiota diversity (alpha diversity) has been associated with Crohn's disease (Dicksved *et al*., 2008; Manichanh *et al*., 2006) and type I diabetes (Giongo *et al*., 2011), diseases in which it is suspected that *Map* could play a role (Sechi and Dow, 2015). It is noteworthy that the increase in the number of bacterial species observed in the cecal content of infected rabbits could be a consequence of changes in the microbial composition. The DNA extraction methodology used does not remove DNA from dead bacteria and it could therefore influence the results obtained. However, these results could also be due to *Map* interaction with other bacteria and consequence of changes in the ecosystem by infection.

Regarding microbial diversity between animals (beta diversity), in the cecal content qualitative differences in the microbiota of infected and non-infected animal were observed, whereas in the sacculus rotundus both qualitative and quantitative differences were detected. The changes observed in the lymphoid tissue suggest that microbial composition could play an important role in the inflammation caused by *Map* infection. This hypothesis is strengthened by the fact that the microbial composition in rabbit lymphoid tissue is capable of inducing tissue inflammation (Shanmugam *et al*., 2005).

Concerning the bacterial community positively related to *Map* infection, we found the family Mogibacteriaceae which has recently been identified in higher levels in the ileal mucosa of *Map* experimentally infected calves (Derakhshani *et al*., 2016). At a deeper taxonomic level, the genus *Stenotrophomonas* was positively related to infection in animals fed with regular diet. This genus has been found overrepresented in patients with Crohn's disease (Knösel *et al*., 2009) and in mice infected with *Map* (Karunasena *et al*., 2014). In addition, this genus has been linked to many inflammatory conditions such as pneumonia, bacteremia and urinary tract infection (Denton and Kerr, 1998). Unfortunately, at the moment there is a lack of knowledge of the biological function of these bacterial families and consequently it is not possible to venture what role they could play in intestinal inflammation.

The genus *Anaerostipes* was found to be more abundant in infected animals and it could be an indirect marker of the loss of their possible beneficial effects under normal conditions. The bacteria of this genus can utilize lactate to produce butyrate (Sato *et al*.,

2008), which can suppress pro-inflammatory cytokine production by intestinal macrophages (Chang *et al*., 2014) and it is beneficial to colonic health providing protection against colitis (Segain *et al*., 2000). In addition, this genus was found in significant lower abundance in Crohn's disease patients (Morgan *et al*., 2012). These findings suggest that this genus may play an important role in gastrointestinal health.

The genus *Coprobacillus* was negatively associated with *Map* infection in both animals fed regular and high fiber diet. Interestingly, it has been suggested that this genus may be beneficial through maintaining intestinal stability and conferring resistance against *Clostridium difficile* colonization (Stein *et al*., 2013), the bacteria related to pseudomembranous colitis (Larson *et al*., 1978).

Finally, it should be noted that the genus *Mycobacterium* was only detected in the cecal content of one infected animal, while by PCR it was detected in the sacculus rotundus of 30% of infected animals. The differences in *Map* detection could be due to the standardization of the DNA concentration for sequencing, the primers used (bacterial universal primers vs *Map* specific) or the low relative concentration of *Map* in tissue of infected animals, which would require a higher depth of sequencing for its detection.

Recently, it has been suggested that the bacterial pattern associated with infection could be used as a diagnostic tool for subclinical paratuberculosis infection (Derakhshani *et al*., 2016). However, in our study we observed that some taxonomic groups showed an opposite association with the infection, depending on the tissue or ingested diet. Therefore, determining a bacterial pattern associated with *Map* infection would require further studies, in order to define bacterial taxonomic groups associated with infection under different conditions. Moreover, because a high number of the identified bacteria are not cultivable and there is a lack of knowledge of their biological function, the implementation of metatranscriptomic studies would be helpful to help to understand the role of intestinal microbiota in the establishment and progression of *Map* infection.

The findings of this study could be applied to the development of prebiotics or probiotics composed of bacterial community negatively associated with the infection. It could compensate the losses caused by the infection and help to restore the normal balance. These prebiotics or probiotics may be given both to young and adults animals

in combination with diet or other classic control strategies of the disease such as vaccination.

Vaccination has been reported to be the best currently available tool to control paratuberculosis in field conditions (Bastida and Juste, 2011; Juste, 2012). Vaccination has been successfully used as a part of the control programs in sheep in Iceland (Fridriksdottir, 2000) and Australia (Kennedy y Citer, 2010) and in goats in Norway (Saxegaard y Fodstad, 1985) and Spain (Corpa *et al*., 2000c). However, the fact that vaccination does not provide complete protection against infection is also widely accepted (Reddacliff *et al*., 2006; Windsor y Eppleston, 2006). Therefore, it is important to study other strategies that could be used in a complementary manner or go further in our understanding of the pathogenic mechanisms to develop more effective vaccines.

Classically, vaccination has been recommended during the first months after birth on the basis that protection would be conferred before the first contact with *Map* (Bastida and Juste, 2011; Larsen *et al*., 1964; Saxegaard and Fodstad, 1985). The point is that the vaccination prior to exposure to the infectious agent represents an effective approach against acute infections, but may not be the optimal strategy in the case of chronic diseases. Therefore, in the last study the effect on the immune response and bacterial load of vaccination before and after *Map* infection (therapeutic effect) has been studied in the rabbit model (Study IV).

Several studies have shown that vaccination against *Map* induces both cellular and humoral immune response in cattle (Kohler *et al*., 2001; María Muñoz de Frutos, 2014; Muskens *et al*., 2002; Stabel *et al*., 2011), sheep (Begg y Griffin, 2005; Corpa *et al*., 2000b) and goats (Hines *et al*., 2014).

The increase of Th1 response and particularly IFN-γ secretion has been considered to provide a protective effect against mycobacterial infection (Flynn, 1993). This cytokine has been used as a marker of protection in vaccination studies against tuberculosis. However it has recently been suggested that this might not be a useful strategy for assessing the protection induced by vaccination, because there is no correlation between IFN-γ and the rate of infection (Nunes-Alves *et al*., 2014). In addition, this is a proinflammatory cytokine, which has the ability to promote the development of

destructive lesions (Orme *et al*., 2014). Moreover, the high levels of IFN-γ of vaccinated animals, with high *Map* bacterial load and severe lesions (Kohler *et al*., 2001; María Muñoz de Frutos, 2014; Muskens *et al*., 2002; Stabel *et al*., 2011) suggested that the increase of this cytokine could not be related to protection and it could be the consequence of *Map* antigens stimulation in circulating cells.

In the rabbit model we observed that vaccination stimulates cellular immunity, as previously reported in the mouse model (Ghosh *et al*., 2015). In addition, higher increase of cellular immune response in animals vaccinated before than after *Map* challenge has been detected. Subsequently it may be related to bacterial load levels in tissues.

Although it has been historically considered that the humoral response does not provide protection against mycobacteria, it has recently been suggested that it plays a key role in protection against tuberculosis (Achkar and Casadevall, 2013; Niki *et al*., 2015) and paratuberculosis (Begg *et al*., 2011) or, at least, it appears associated with protection in a proportion of population. Therefore, the increased antibody levels observed in vaccinated rabbits before and after challenge could be in part responsible for the lower bacterial levels detected in the tissues.

Using western blot, a slight humoral response was observed in the uninfected animals at first sampling. This response could be due to a light exposure to *Map* by aerosols, which could also have occurred in the other groups of animals. This fact would demonstrate that the doses and routes of exposure to mycobacterial antigens could be relevant in the outcome. However, it could also be related to the maturation of the immune response with age. Animals could develop a nonspecific humoral response against high molecular weight *Map* proteins, which would explain the resistance of adult animals to infection.

In the rabbit model we observed that both vaccination strategies reduced the bacteriological index (tissue culture and qPCR). These results are in agreement with the observed in ruminants (Bastida and Juste, 2011). Moreover, vaccination after infection was slightly more effective than vaccination before infection. Indeed, several studies (Gwozdz *et al*., 2000; Windsor, 2006) have suggested that vaccination with whole cells could have a therapeutic effect, reducing bacterial load in tissues. Other studies have

reported that vaccination in adult animals boosts immune response (Corpa *et al*., 2000b), reduces clinical symptoms (Singh *et al*., 2010, 2013), improves milk production (Juste *et al*., 2009; Tamayo *et al*., 2010) and longevity of animals (Alonso-Hearn *et al*., 2012; Santema *et al*., 2013). Therefore, vaccination in clinical or subclinical disease could have a beneficial effect and could be used as a short term control strategy in high economic value animals and/or when it is not possible vaccinate the whole herd. Additionally, these findings suggest that the active immunization therapies could be used in human medicine against slow inflammatory diseases associated with paratuberculous type infections.

In the present Doctoral Thesis advance has been made in the study of rabbits susceptibility to MAC infection, as well as in the utility of this laboratory animal model, allowing progress in understanding of *Map* pathogenesis and vaccination. Moreover, it has been shown that different feeding strategies could play a role in *Map* infection, modifying the intestinal microbiota. These findings linked to the observed degree of therapeutic effect of vaccination suggest that those strategies should be tested with a holistic approach, taking into account possible interactions and/or additive effects. Moreover, these findings justify the need to carry out more studies to elucidate if eating rabbit products could be a source of MAC exposure for humans and the effects it would have on human health. It would also be interesting to study the possible impact of these infections in rabbit meat production as well as to isolate and characterize the MAC species to determine the source of infection.

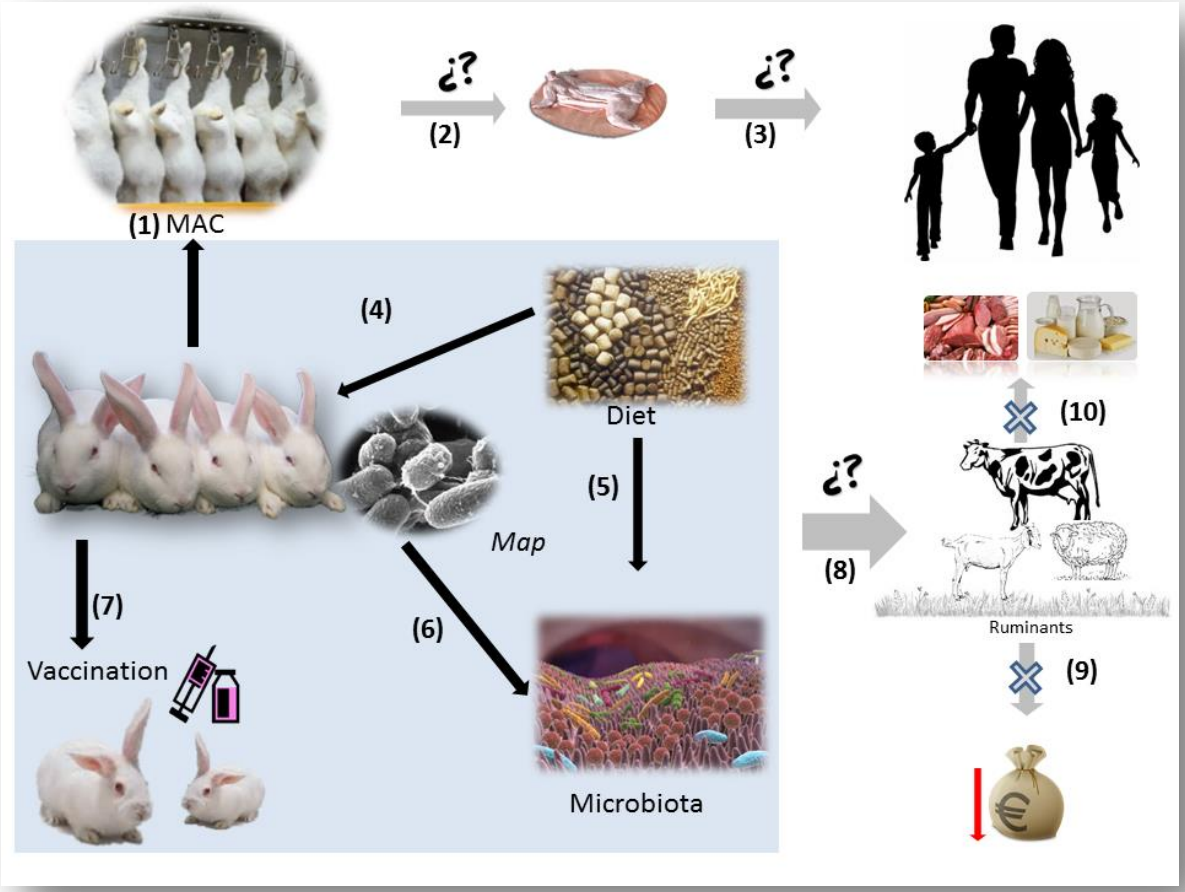
On the other hand, the observation that diet modulates *Map* infection opens a new paradigm of intervention strategies against the disease. This finding together with the description of the digestive microbiota negatively associated with infection opens the way to new approaches for disease control, such as the use of different diets in short periods of time to avoid compromising the animal production system or the use of diets supplemented with prebiotics or probiotics. Additionally, the presented results support the use of other strategies that have shown promising results in humans, such as fecal transplants. This treatment has proven effective in other chronic intestinal diseases, such as *Clostridium difficile* colonization (Aroniadis *et al*., 2015) or Crohn's disease (Suskind *et al*., 2015). This kind of interventions linked to the observation of some degree of therapeutic effect of vaccination suggests that they should be tested in combination

under field conditions in ruminants. This would allow studying the immunological mechanisms involved in the protection and/or modulation of infection in order to achieve an effective strategy to control the disease.

Finally, in this work we have shown that the rabbit is a useful animal model of *Map* infection allowing to advance in the study of subclinical infection. It may also be useful in the analysis of other inflammatory bowel diseases in humans. Additionally, this animal model could allow to progress quickly and economically in new products and vaccine guidelines initial screening.

The main findings of this study and their possible role in animal and public health are summarized in Figure 7.

Figure 7. Schematic representation of the main findings derived from the present work and their possible role in animal and public health.



(1) Rabbits for human consumption presented MAC in the digestive associated lymphoid tissue. (2) The carcass of animals could be infected (3) and the consumption of under cooked rabbit meat by humans could suppose a transmission risk to the human population with uncertain effects. (4) Diet modulates *Map* infection and (5) has a direct effect in the digestive microbiota. (6) *Map* infection produces changes in the digestive microbiota. Both diet and microbiota have an effect on the host immune response. (7) Vaccination shows a protective effect, increasing the humoral and cellular immunity. (8) Diet and/or microbiota modification could be used in a complementary manner with vaccination in domestic ruminants with the objective (9) of reducing the economic losses produced by the infection and (10) of limiting the transmission of the infectious agent to human population through the consumption of food of animal origin, an important aspect if the relation of *Map* with Crohn's disease is confirmed.

V. CONCLUSIONES

1. Los conejos sacrificados para consumo humano presentan una alta tasa de infección por MAC en el tejido linfoide intestinal, cuyo significado precisa seguir siendo explorado. Asimismo, la imposibilidad de detectar *Map* en los conejos en producción podría estar relacionada con la exposición previa a otras bacterias del MAC dando lugar a una sensibilización.

2. La especie cunícola muestra características de la infección por *Map* similares a las observadas en las formas latentes de los rumiantes, como son la eliminación fecal intermitente y la presencia de lesiones macroscópicas y microscópicas en el sistema digestivo y tejido linfoide asociado, siendo un modelo animal útil en el estudio de la paratuberculosis subclínica.

3. Cambios en la dieta pueden modular la infección por *Map*. La dieta convencional formulada para la producción de conejos ha demostrado favorecer el desarrollo de la infección paratuberculosa en comparación con los otros regímenes alimenticios ensayados. Por lo tanto, sería conveniente explorar el impacto de la dieta en la infección por *Map* en condiciones de campo.

4. La infección por *Map* produce alteraciones en la microbiota digestiva, que son más profundas a nivel del tejido linfoide. La existencia de patrones bacterianos positivamente y negativamente asociados a la infección sugiere que la microbiota podría tener un efecto en la modulación de la infección. No obstante, algunos grupos taxonómicos se han relacionado tanto positivamente como negativamente con la infección dependiendo del ecosistema.

5. La vacunación es capaz de inducir tanto la respuesta inmune humoral como la celular. Además, produce reducciones en la carga bacteriana que son marginalmente mayores cuando se aplica después de la infección, lo que podría considerarse como un efecto terapéutico.

V. CONCLUSIONS

1. Slaughtered rabbits for human consumption have a high rate of intestinal lymphoid infection by MAC which significance would require further studies. Likewise, the impossibility to detect *Map* in rabbits in production may be related to previous exposure to other bacteria of MAC leading to a sensitization.

2. Rabbits show *Map* infection features similar to those observed in latent forms in ruminants, such as intermittent fecal shedding and presence of macroscopic and microscopic lesions in the digestive system and associated lymphoid tissue. Rabbit, therefore is a useful animal model to study subclinical paratuberculosis.

3. Dietary changes modulate *Map* infection. Regular diet formulated for growing rabbits has been shown to promote the development of paratuberculosis infection compared to the other tested dietary regimens. Therefore, the impact of diet on *Map* infection should be further explored in field conditions.

4. *Map* infection produces changes in the digestive microbiota that are deeper in lymphoid tissue. The existence of bacterial patterns positively and negatively associated with infection suggests that the microbiota might have an effect on the infection modulation. However, some taxonomic groups have been related both positively and negatively to infection depending on the ecosystem.

5. Vaccination induces both humoral and cellular immune response. It also produces a reduction in tissue bacterial load, slightly higher when vaccination is applied after infection, which may be considered as a therapeutic effect.

VI. RESUMEN

La paratuberculosis es una enteritis granulomatosa crónica de distribución mundial que ocasiona cuantiosas pérdidas económicas. Aunque la infección por *Map* (*Mycobacterium avium* subsp. *paratuberculosis*) afecta principalmente a los rumiantes domésticos y silvestres, se ha descrito en un amplio rango de animales no rumiantes, entre los que se incluyen los conejos. El avance en el estudio de la paratuberculosis se ha visto limitado debido a la falta de un modelo de laboratorio adecuado.

Esta Tesis Doctoral está formada por un compendio de cuatro estudios en los que se analiza la susceptibilidad del conejo a la infección por *Map* así como el efecto de la vacunación y la dieta en la modulación de la infección, utilizando el conejo como modelo animal de laboratorio. Además, para profundizar en los conocimientos científicos de las infecciones micobacterianas naturales y experimentales en los conejos, se ha realizado una revisión de la información disponible en la literatura.

La posible asociación de *Map* con la enfermedad de Crohn en humanos ha hecho que la presencia de este agente infeccioso en las canales de vacuno y en productos de origen animal cobre cierta relevancia, por ser una posible vía de exposición en humanos. De esta manera, el primer estudio de este trabajo, se centró en la detección de micobacterias en el tracto digestivo de conejos de matadero. A pesar de que la presencia de *Map* no se confirmó en las muestras analizadas, mediante PCR se detectaron otras micobacterias del MAC (*Mycobacterium avium* complex), concretamente *Mycobacterium avium avium*/*silvaticum* y *Mycobacerium avium hominisuis* en el 16,6% de los conejos muestreados. No obstante, debido a que el sistema digestivo de los conejos no entra en la cadena alimentaria, la comercialización de estos animales portadores de MAC no debería de suponer un riesgo para la salud pública. De cualquier manera, el primer estudio pone de manifiesto que las explotaciones cunículas son un nicho de MAC que debe de seguir explorándose.

En el segundo estudio se desarrolló un modelo de infección experimental por *Map* en el conejo con el fin de reproducir la enfermedad mediante la exposición a factores naturales como son los cambios de dieta durante el desafío. Gracias a este estudio se pudo comprobar que los cambios en la dieta son capaces de modular la infección por *Map*. De las tres dietas ensayadas (regular, hiperproteica y alta en fibra), la dieta regular fue la que estuvo asociada a mayores índices de infección. En los animales infectados experimentalmente, se observó la presencia de lesiones macroscópicas e

histopatológicas a nivel digestivo, siendo los tejidos más afectados el *sacculus rotundus* y el apéndice vermiforme. Mediante qPCR se detectaron niveles variables de *Map* en tejidos y se demostró la eliminación fecal intermitente, hallazgos que están en concordancia con lo observado en rumiantes infectados naturalmente.

Tras la observación de que la dieta posee un papel modulador de la infección por *Map* y teniendo en cuenta que la dieta tiene un efecto directo sobre la microbiota digestiva, se realizó un experimento posterior en conejos con el fin de ahondar en los posibles mecanismos implicados en el efecto modulador de la dieta. Así, el tercer estudio fue diseñado para estudiar la interacción de la microbiota intestinal con la infección y la dieta. En dicho estudio a través de la secuenciación del RNAr 16S de la microbiota digestiva, se detectaron diferencias significativas en uno de los índices de diversidad microbiana calculados. Además, se identificaron cambios en la microbiota digestiva debidos a la infección por *Map* tanto en el contenido cecal como en el *sacculus rotundus*. Como fruto de este trabajo se han podido caracterizar los grupos taxonómicos asociados tanto positivamente como negativamente a la infección. El efecto modulador de la infección de la dieta y otras estrategias más clásicas de control como la vacunación podrían aplicarse de manera combinada en rumiantes en condiciones de campo.

La vacunación, aunque no proporciona una protección completa contra la infección ha demostrado ser la herramienta de control de la paratuberculosis más efectiva disponible en la actualidad. Con la finalidad de ahondar en el conocimiento de los efectos de la inmunización, en el último estudio se ha evaluado el efecto de la vacunación frente a *Map* previo y posterior a la infección (efecto terapéutico), utilizando el modelo de conejo. En este estudio se observó que ambas estrategias de vacunación estimulan la respuesta inmune celular y humoral y además disminuyen el índice bacteriológico (cultivo y qPCR de tejidos). No obstante, la vacunación posterior a la infección fue ligeramente más efectiva que la vacunación previa a la infección indicando que la vacunación de los animales en la fase clínica o subclínica de la enfermedad podría tener un efecto beneficioso y podría ser utilizada como una estrategia de control a corto plazo en animales de alto valor económico y/o en los casos en los que no es posible vacunar a todo el rebaño.

Por último, los avances en el estudio de la paratuberculosis logrados en la presente Tesis Doctoral mediante la utilización del conejo como modelo animal de infección por *Map*,

ponen de manifiesto la aplicabilidad de este modelo, que podría ser empleado en el estudio de otras enfermedades inflamatorias intestinales en humanos.

VI. SUMMARY

Paratuberculosis is a chronic granulomatous enteritis distributed worldwide that causes significant economic losses. Although *Map* (*Mycobacterium avium* subsp. *paratuberculosis*) infection mainly affects domestic and wild ruminants it has been described in a wide range of non-ruminant animals, including rabbits. The advancement in the knowledge of paratuberculosis has been limited due to the lack of a suitable laboratory animal model.

The present Doctoral Thesis is composed of four studies in which susceptibility of rabbit to *Map* infection and the effect of vaccination and diet in the infection is analyzed using the rabbit as a laboratory animal model. In addition, to go in depth into the scientific knowledge of natural and experimental mycobacterial infections in rabbits, a review of the information available in the literature has been conducted.

The possible relation of *Map* with human Crohn´s disease has increased the importance of the presence of the infectious agent in beef carcasses and animal origin products, because it could be the route of infection to humans. Therefore, the first study of this work focused on the detection of mycobacteria in digestive tract of rabbits produced for human consumption. Although *Map* presence was not confirmed in the analyzed digestive system samples, the presence of other bacteria of MAC (*Mycobacterium avium* complex) such us *Mycobacterium avium avium*/*silvaticum* and *Mycobacterium avium hominisuis* was detected in 16.6% of sampled rabbits. However, because the digestive system of rabbits does not enter to the food chain, MAC-bearing animals should not be a risk to public health. In any case, Study I highlights that the rabbit farms are a niche of MAC that should be further explored.

In the second study, the aim was to develop a rabbit model of *Map* experimental infection with the purpose of reproducing the disease through exposure to natural factors such as diet changes during challenge. Thanks to this study, it was proven that dietary changes are able to modulate *Map* infection. Of the three diets tested (regular, high protein and high fiber), regular diet was the one associated with higher rates of infection. Moreover, in experimentally infected animals the presence of macroscopic and histopathological lesions was observed at digestive level, being sacculus rotundus and vermiform appendix the most affected tissues. Diverse levels of *Map* were detected in tissues and intermittent fecal shedding was proved using qPCR. These findings are in agreement with the observed in natural infected ruminants.

Following the observation of dietary *Map* infection modulation and because of diet has a direct effect on the digestive microbiota, a subsequent experiment was performed in rabbits in order to delve into the possible involved mechanisms. Accordingly, the third study was designed to study the interaction of the intestinal microbiota with infection and diet. In this study through 16S rRNA sequencing of digestive microbiota, significant differences in one of the measured microbial diversity indexes was detected. Moreover, changes in the digestive microbiota due to *Map* infection were detected in cecal content and sacculus rotundus. Therefore, thanks to this work the taxonomic groups associated both positively and negatively to infection have been characterized. The modulatory effect of diet may be applied, in combination with other traditional control strategies such as vaccination, under field conditions in ruminants.

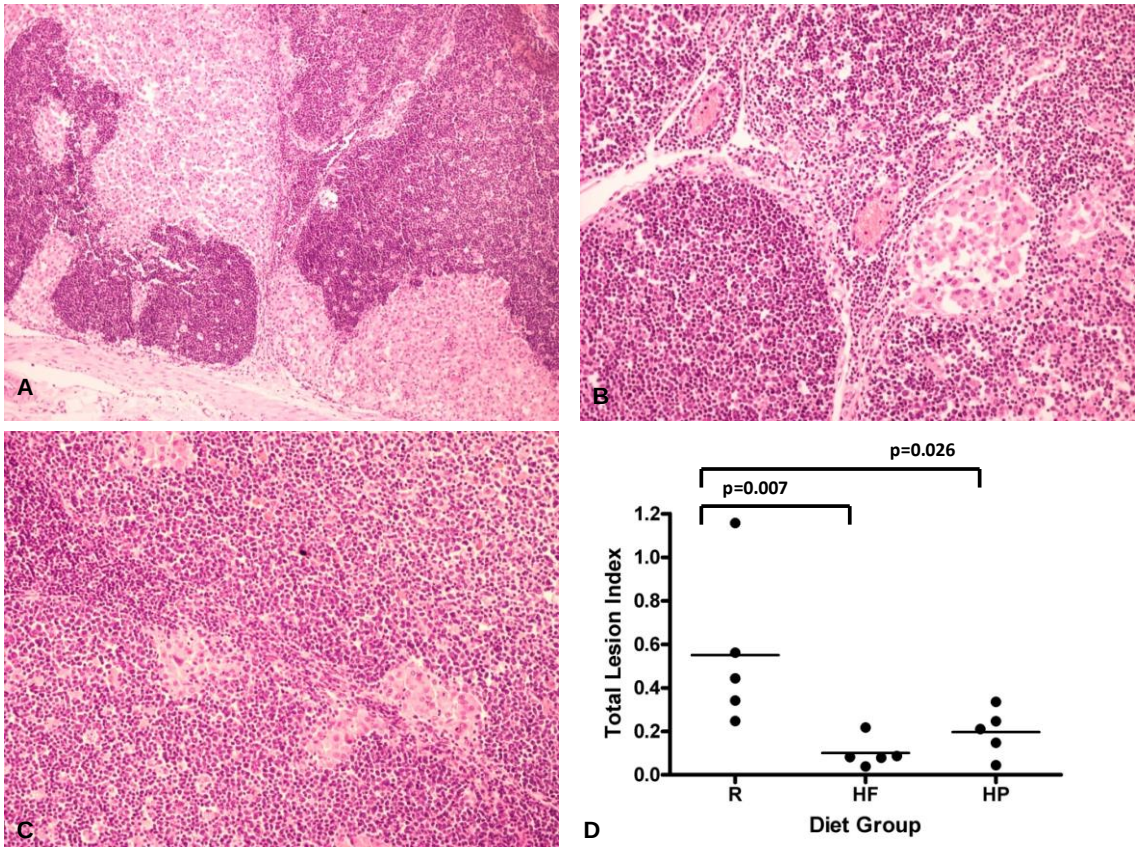
Vaccination has proven to be the most effective control tool currently available, although it is widely accepted that it does not provide complete protection against *Map* infection. In the last study, to further understand the effects of immunization, the effect of vaccination before and after *Map* challenge (therapeutic effect) was assessed using rabbit model. In this study, both vaccination strategies were able to stimulate cellular and humoral immune response, decreasing bacteriological index (tissue culture and qPCR). However, vaccination after infection was slightly more effective than vaccination before infection, suggesting that vaccination in clinical or subclinical disease could have a beneficial effect and could be used as a short term control strategy in high economic value animals and/or when it is not possible vaccinate the whole herd.

Finally, the progress in the study of paratuberculosis achieved in this Doctoral Thesis using the rabbit as an animal model of *Map* infection, demonstrates the applicability of this model, which could be used to study other inflammatory bowel diseases in humans.

VII. ANEXOS / ANNEXES

VII.1 SUPPLEMENTARY MATERIAL STUDY II

Supplemental Fig. 1.



Histopathological findings. Hematoxylin and eosin-stained section micrographs of the sacculus rotundus of a rabbit from: (A) diet group R showing well-demarcated large granulomas containing macrophages as the main cellular population in the follicles and interfollicular region (100 ×), (B) diet group HF showing a few small granulomas (200 ×), and (C) diet group HP showing medium size granulomas in the interfollicular region (200 ×). (D) Total Lesion Index calculated as the sum of the area of lesion divided by the area of the micrograph of all examined specimen sections. The solid lines show the mean values.

Table S1. Diet composition.

Diet	Protein %	Fat %	Fiber %	Ashes%
A	15-18	2.6-9.5	13-17	5-10
R	17.5	3.5	16.8	7.9
HF	15.5	2.3	20.3	12
HP	24	8.5	14.0	10.6

A: acceptable levels, R: regular; HF: high fiber; HP: high protein

Table S2. In vivo follow up and post mortem histopahological analysis.

	Weight gain		Total Fecal qPCR		Histopathology					
					Total		Sacculus Rotundus		Vermiform Appendix	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
R	$1621.6^{HF,HP}$	231.6	3326	4237.3	$0.550^{HF,HP}$	0.359	$0.342^{HF,HP}$	0.135	0.209	0.247
HF	2192.2^{R}	346.4	574.25	1009.6	0.101^{R}	0.068	0.050^{R}	0.052	0.050	0.023
HP	2128.1^{R}	330.9	542	742.8	0.197^{R}	0.109	0.134^{R}	0.112	0.062	0.039
ANOVA Sign.	0.024		0.183		0.017		0.003		0.199	
Mixed model* Sign.	0.042		0.120							

Weight gain was calculated as weight registered at S8 minus the weight registered at S0. Total fecal qPCR was calculated as the sum of genomic equivalents detected in all samplings. Superscripts indicate categories with statistically significant differences
*All the measurements over time were included in the analyses.

VII.2 SUPPLEMENTARY MATERIAL STUDY III

FIGURE S1. Cecal content principal coordinates analysis (PCoA) of weighted UniFrac distances. PERMANOVA analysis showed no significant differences between Infected vs. non-infected animals p-value = 0.2.

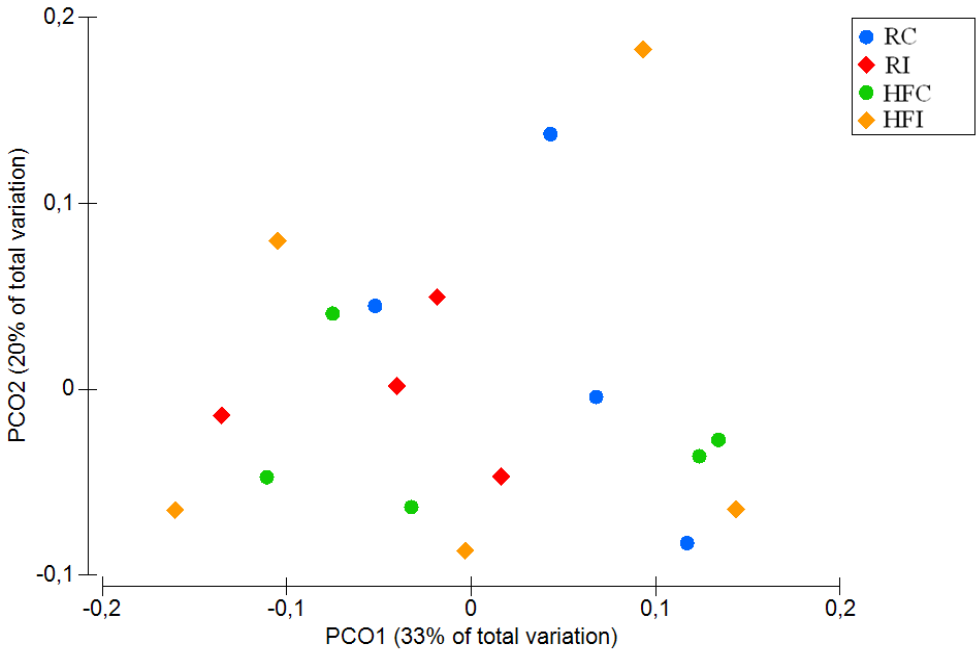


FIGURE S2. Cecal content OPLS-DA coefficient plot; bars with negative values indicating bacteria that are significantly lower in MAP infected animals and bars with positive value indicating bacteria which are significantly higher in MAP infected animals than in control animals. Only bacteria with a confidence interval that did not cross the zero line were significantly changed $^{*}p < 0.05$. **(A)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed with regular diet ($R^2Y = 1$; $Q^2 = 0.678$). **(B)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed high fiber diet ($R^2Y = 0.988$; $Q^2Y = 0.733$).

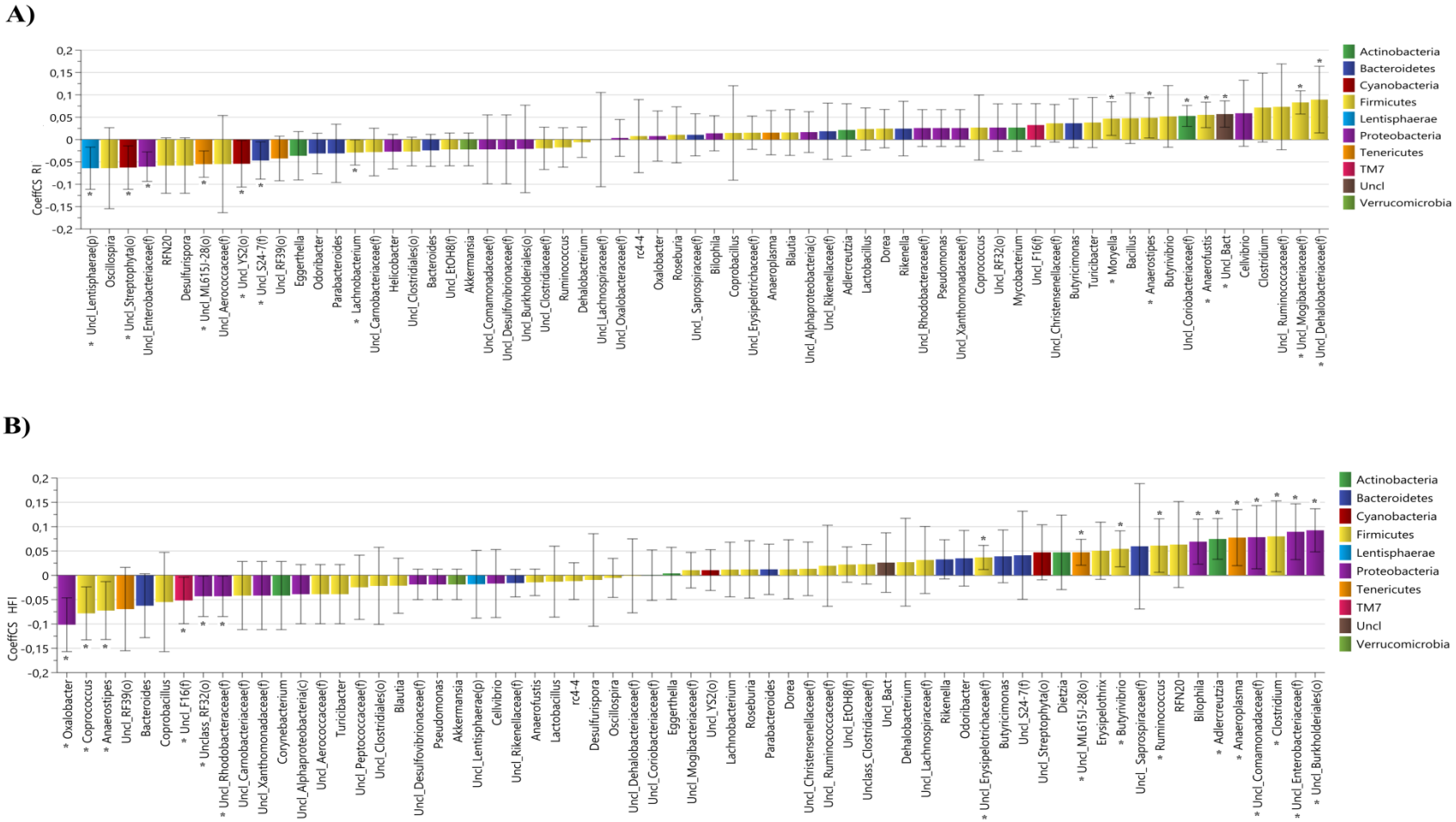
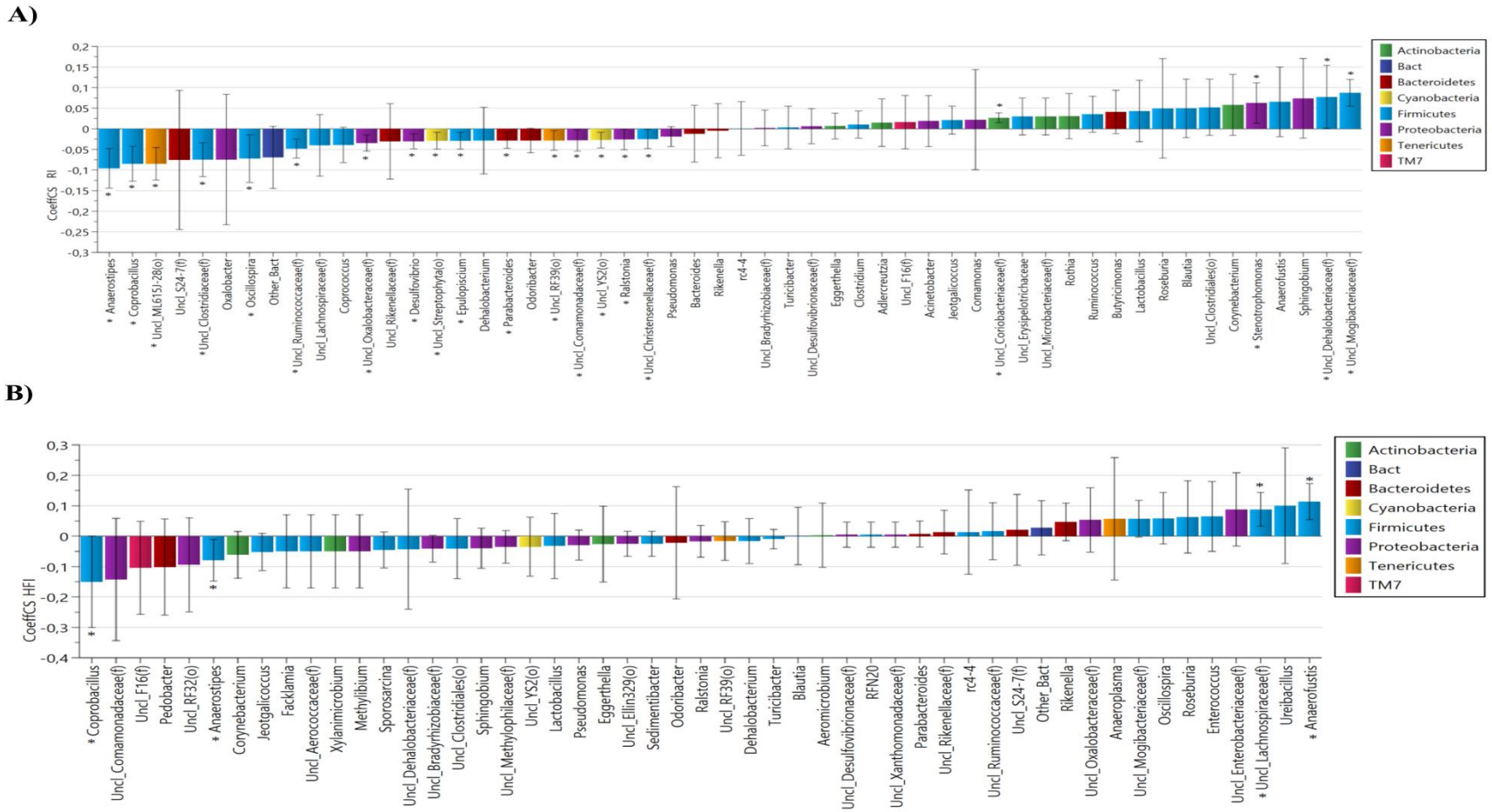
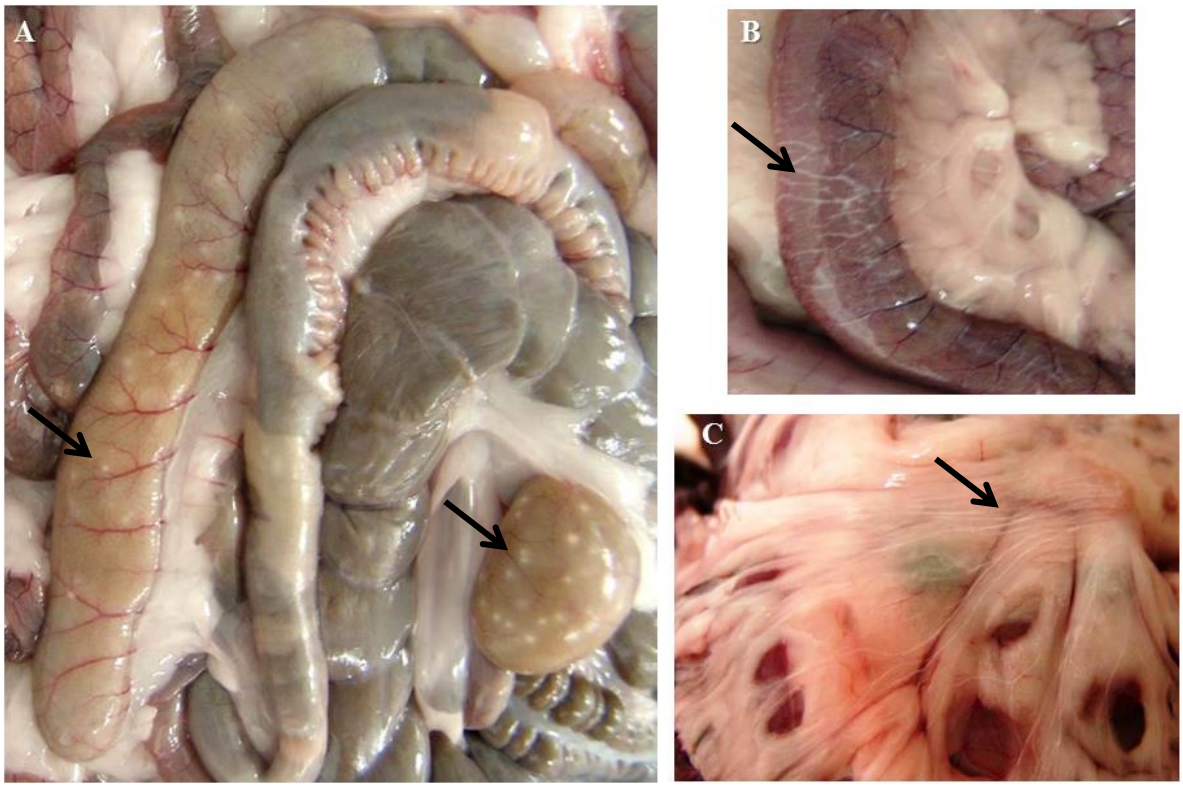


FIGURE S3. Sacculus rotundus OPLS-DA coefficient plot; bars with negative values indicating bacteria that are significantly lower in MAP infected animals and bars with positive value indicating bacteria which are significantly higher in MAP infected animals than in control animals. Only bacteria with a confidence interval that did not cross the zero line were significantly changed $^{*}p < 0.05$. **(A)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed with regular diet ($R^2Y = 0.995$; $Q^2 = 0.765$). **(B)** OPLS-DA coefficient plot for samples of sacculus rotundus from animals infected and fed high fiber diet ($R^2Y = 0.921$; $Q^2 = 0.511$).



VII.3 DIGESTIVE GROSS PATHOLOGY FINDINGS

Figure 1. Digestive gross pathology observed in some *Map* experimentally infected rabbits. A) Pale white reactive spots in sacculus rotundus and vermiform appendix. B) Intestinal lymphangiectasia. C) Dilatation of lymphatic vessels in the mesentery.



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