

Relative abundance of *Mycobacterium* in ovine Johne's disease



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No study has determined what proportion of the total microbiota comprises the genus *Mycobacterium* in ovine Johne's disease (OJD) tissues. We aimed to assess the relative abundance of *Mycobacterium* in the ileocaecal lymph node, involved and uninvolved ileal mucosa from sheep with and without OJD, using three extraction methods. Eight sheep, four with and four without OJD, were recruited. Pyrosequencing of the 16S rRNA gene amplicons for all samples revealed that *Mycobacterium* represented between 0-92% (average

38%) of the total microbiota of samples from sheep with OJD, and 0-85% (average 13%) of sheep without OJD. Only sheep with OJD had samples that were positive for the IS900 (*MAP*) element. *Mycobacterium* strains other than *MAP* may provide competitive exclusion of *MAP* and should be further investigated.

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is the etiologic agent of ovine Johne's disease (OJD), a chronic,

contagious granulomatous enteritis of ruminants. The disease causes significant morbidity and mortality worldwide, resulting in significant economic losses¹.

To our knowledge, no study has determined what fraction of the microbial community of non-OJD and OJD affected tissue comprises *Mycobacterium*. Cheung *et al.* (2013)² found that *Mycobacterium tuberculosis* represented less than 1% of the total microbial community of sputum samples of infected patients. Does *Mycobacterium* comprise such a small fraction of the microbial community in all infectious states and specimens? *MAP* is a putative trigger of Crohn's disease in humans, and is detected in some studies but not others, including a recent study of ours³. Is this because it is not being detected?

While other studies have quantified *Mycobacterium* or *MAP* in clinical and subclinical specimens^{4,5}, no study has determined what fraction of the microbial community comprises *Mycobacterium* in mucosa from sheep with and without OJD. The primary aim is to assess the relative abundance of *Mycobacterium* in the microbial communities of macroscopically normal bowel mucosa obtained >50 cm downstream the ileocaecal valve (sheep without OJD) or involved mucosa (sheep with OJD), involved (or normal for sheep without OJD) ileal mucosa adjacent the ileocaecal lymph node, and the ileocaecal lymph node of sheep with and without OJD.

Samples of involved and uninvolved ileal mucosa, and ileocaecal lymph nodes were collected from eight sheep, four with and four without OJD, from farms around Rutherglen, Australia. Sterile gloves and implements were used to extract the ileocaecal node before the bowel was opened, so that it did not come into contact with the skin or bowel microbiota. Total DNA was extracted from each sample using three different extraction methods. The first protocol followed the Qiagen DNeasy kit protocol and included an enzymatic lysis step and bead-beating step. The second and third extractions were the same as the first, except the second protocol included a freeze-thaw step, and the third protocol a boiling step. Technical replicates were included for the first protocol. All samples were amplified using barcoded primers targeting the universal bacterial 16S rRNA gene, and pyrosequenced using a 454 Genome Sequencer FLX-Titanium platform, as previously described³. Sequences were analysed using Mothur⁶. IS900 PCR was used to determine whether or not *Mycobacterium* detected in a given sample was *MAP*, or not.

Four sheep (1, 2, 4 and 5; for example, see Figure 1) were diagnosed with OJD by qualified veterinarians and pathologists, using methods previously described^{7,8}. Briefly, sheep were initially assessed as



Figure 1. Ovine Johne's disease affected sheep #2, showing physical signs of wasting.

having OJD, or not, based on physical signs, including condition, scouring, ill-thrift, and lethargy during mobility. Diagnosis was confirmed by a NATA-accredited pathology laboratory (AgriBio, Victoria), and included *MAP* culture and DNA analysis, as well as microscopic visualization of acid-fast bacteria using ZN staining. Sheep 1 was initially suspected to have OJD, however negative culture and staining results excluded it from a diagnosis of OJD, and it was found to have metastatic cancer.

Pyrosequencing showed that *Mycobacterium* represented between 0–92% (average 38%) of the total microbiota of samples from sheep with OJD, and 0–85% (average 13%) of sheep without OJD (Figure 2). Mucosal samples from sheep 7 were excluded due to the inability to amplify products from them. Sheep 4 and 5 each had a positive lymph node sample for one extraction, comprising 6% and 68% *Mycobacterium* reads, respectively. These levels were similar to that observed in the mucosal samples from the same animal.

The lymph node and mucosa samples of sheep 1 were negative when tested with the *MAP*-specific primers. Sheep 1 may have had a paucibacillary form of OJD. Samples from all other sheep with OJD were positive, except the normal mucosa sample of sheep 4 (Table 1). All samples from sheep without OJD were negative for the IS900 element.

No significant effect of extraction method was detected for either Simpson or Shannon diversity estimates (Simpson Diversity, AOV: $P > F = 0.384$; Shannon Diversity, AOV: $P > F = 0.443$), nor technical replicates (Simpson Diversity, AOV: $P > F = 0.398$; Shannon Diversity, AOV: $P > F = 0.566$).

Each sample was covered by an average of 2,644 quality sequences. Six phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Synergistetes*, and *TM7*) represented ~98% of all sequences

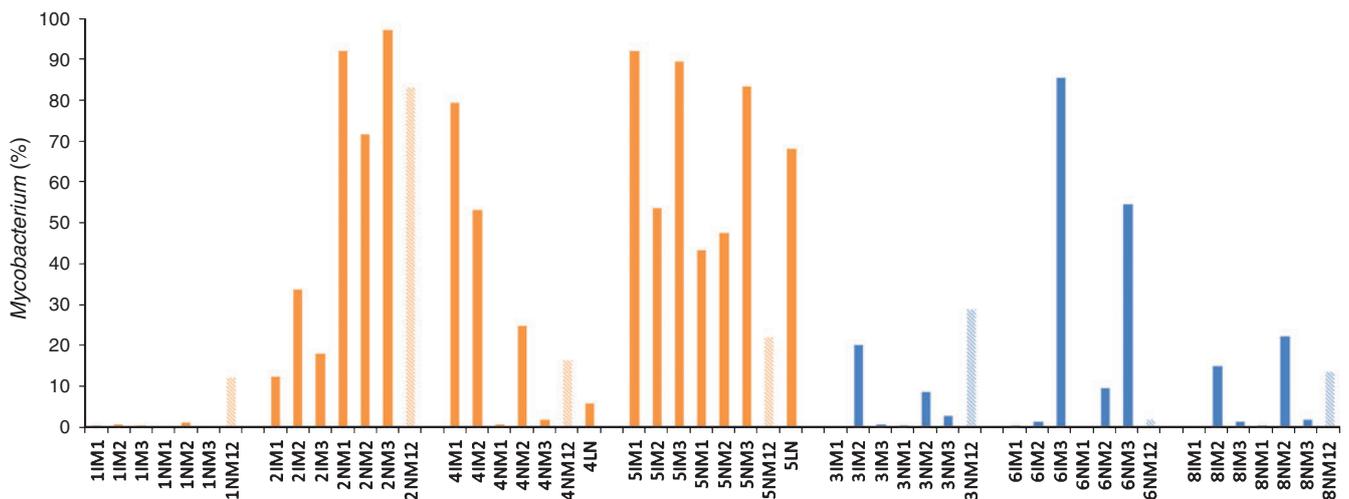


Figure 2. Proportion of involved and normal mucosa and lymph node tissues (sheep 4 and 5) containing *Mycobacterium*. Sheep with Johne’s disease are coloured orange, without blue. Technical replicates (normal mucosa only) have diagonal texturing. The first numeral in the label indicates the animal number; NM, normal mucosa; IM, involved mucosa; LN, lymph node; the second numeral indicates the DNA extraction: ‘1’, ‘2’ and ‘3’ are replicates, ‘12’ is the technical replicate for normal mucosa (DNA extraction 1) only. Sheep 1, 2, 4 and 5 had OJD, sheep 3, 6 and 8 did not.

Table 1. Sheep characteristics, pathology and *MAP*-specific PCR results

Sheep	1	2	3	4	5	6	7	8
Diagnosis	OJD and MIC	OJD	Non-OJD	OJD	OJD	Non-OJD	Non-OJD	Non-OJD
Sex	F	F	F	F	F	F	F	F
Age	7	4	7	7	4	6	3	6
Vaccination	N	Y	N	N	Y	N	Y	N
Breed	Merino	Merino	Merino X Leicester	Merino X Leicester	Merino	Merino	Merino	Merino
Intestinal lesions	Severe	Moderate	None	Moderate	Mild	None	None	None
<i>MAP</i> culture	+	+	-	+	+	-	-	-
AFB in Involved Mucosa	Absent	Occasional	Absent	Numerous	Numerous	Absent	Absent	Absent
AFB in Lymph node	Absent	Absent	Absent	Moderate	Numerous	Absent	Absent	Absent
<i>MAP</i> -specific IS900 PCR								
NM	-	+	-	-	+	-	-	-
IM	-	+	-	+	+	-	-	-
LN	-	+	-	+	+	-	-	-

OJD, ovine Johne’s disease; MIC, metastatic intestinal cancer; *MAP*, *Mycobacterium avium* subspecies *paratuberculosis*; Y, yes; N, no; AFB, acid-fast bacteria; NM, normal mucosa; IM, involved mucosa. All samples were positive for bacterial DNA using the 16S rRNA gene primers, except sheep 7 samples.

(Figure 3). *Actinobacteria*, of which *Mycobacterium* is a member, was overrepresented in sheep with OJD, except sheep 1. Microbial community diversity declined as *Mycobacterium* increased ($R^2=0.705$, $p=0.001$) (Figure 4).

To our knowledge, this is the first study to describe the full microbial community of mucosa from sheep with and without OJD. Although studies have enumerated *MAP* in the feces of sheep using RT-PCR^{5,9}, no study has determined what fraction of the

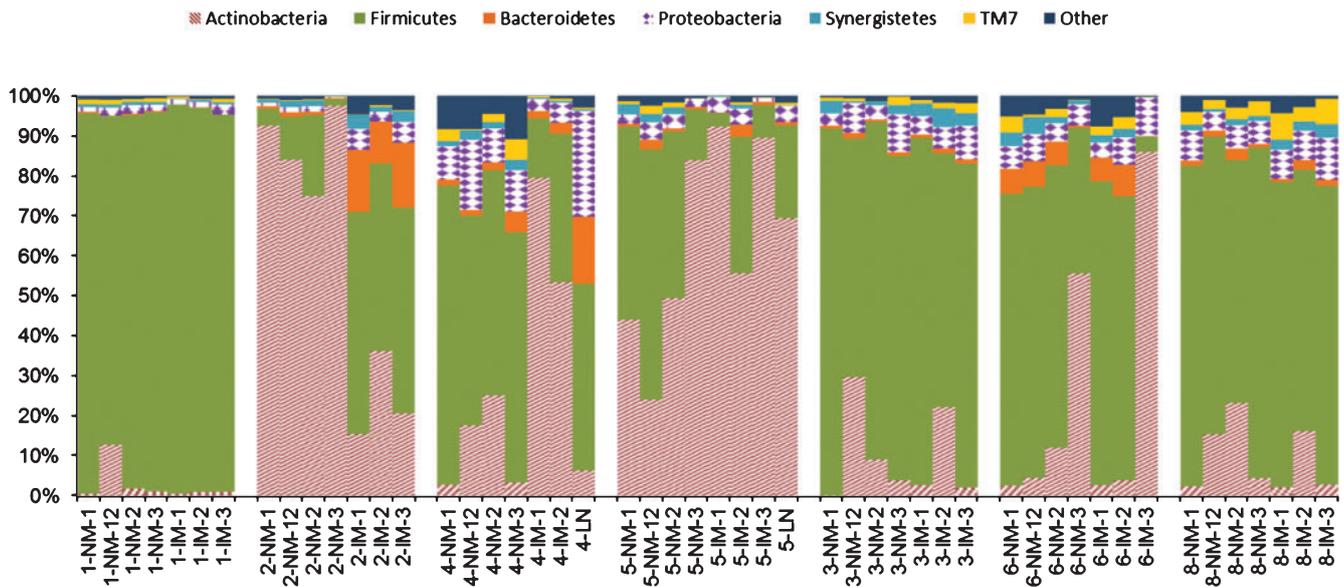


Figure 3. Proportion of the six dominant bacterial phyla in tissues from sheep with and without ovine Johne's disease (OJD), showing a predominance of *Actinobacteria*, of which *Mycobacterium* is a member, in sheep with OJD. The first numeral in the label indicates the animal number; NM, normal mucosa; IM, involved mucosa; LN, lymph node; the second numeral indicates the DNA extraction: '1', '2' and '3' are replicates, '12' is the technical replicate for normal mucosa only. Sheep 1, 2, 4 and 5 had OJD, sheep 3, 6 and 8 did not.

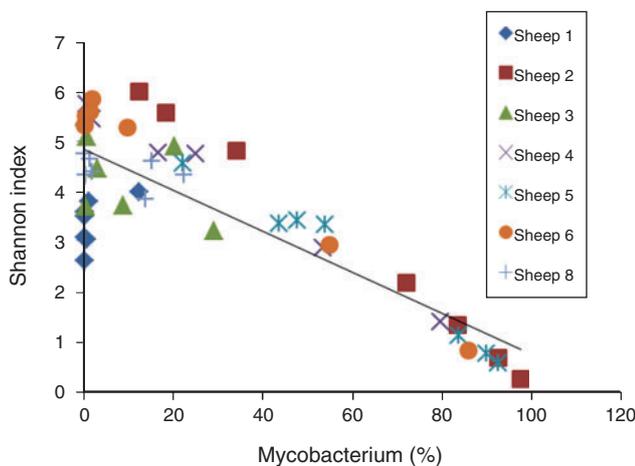


Figure 4. Figure showing a negative correlation between the Shannon diversity index and the proportion of *Mycobacterium* in the mucosal microbiota of sheep. Sheep 1, 2, 4 and 5 were diagnosed with OJD.

microbial community *Mycobacterium* comprises, and whether or not these proportions differ in sheep with and without OJD, as well as various tissue types.

Bacterial translocation to the ileocaecal lymph node was observed in 50% (2/4) of sheep diagnosed with OJD. The microbial community of these nodes was similar to that of the involved and normal mucosa from the same sheep. Bacteria in the node are likely to be secondary invaders, present in the node due to a breakdown in the mucosal barrier. Using the same methods described here, we found that bacterial translocation in Crohn's disease of humans is also non-specific. We also did not detect *Mycobacterium* in this study using 16S rRNA gene sequencing³. This suggests that *Mycobacterium* is unlikely to be a cause of Crohn's disease.

Samples from sheep without OJD were not positive for *MAP* culture or the IS900 element, but other strains of *Mycobacterium* were present, representing on average 13% of the total microbiota. Strains of *Mycobacterium* other than *MAP* could occupy a niche in the sheep gastrointestinal tract that *MAP* would otherwise occupy, thereby protecting the animal from colonization of *MAP*. Future studies should assess the diversity of *Mycobacterium* strains associated with the mucosa of sheep with and without OJD.

We found the abundance of *Mycobacterium*, relative to other bacterial taxa in the mucosal samples, was significantly higher in sheep with OJD. Increases in *Mycobacterium* result in large decreases in *Firmicutes*. Members of the *Firmicutes* are important producers of short-chain fatty acids, which are shown to improve intestinal barrier function and repress inflammation¹⁰. Dysbiosis and reduced diversity would likely exacerbate symptoms of OJD, as beneficial bacteria are decreased and their roles in homeostasis unfulfilled.

Mycobacterium makes up a large fraction of the microbial communities of mucosa from sheep with OJD. Future studies should determine if *MAP* is the sole contributor to these levels of *Mycobacterium* in sheep with OJD, and assess the diversity of *Mycobacterium* in sheep with and without OJD. Other *Mycobacterium* strains may provide competitive exclusion of *MAP*.

Acknowledgements

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Biographies

Andy Leu graduated from the Australian National University (ANU) with a Bachelor of Medical Science (Honours I) in 2013. His Honours project examined the relative abundance of *Mycobacterium avium paratuberculosis* (MAP) in Johne's disease tissues. Andy was recently awarded an Australian Postgraduate Award scholarship to pursue a PhD under the supervision of Dr Gene Tyson.

Paul Pavli is a gastroenterologist with clinical and basic scientific research interests in the inflammatory bowel diseases. He was on the steering committee of the multicentre Australian study using long-term anti-mycobacterial agents in Crohn's disease (Selby *et al.*, *Gastroenterology*, 2007). Current research interests are in

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David Gordon received his BSc from King's College London and his PhD from McGill University, Montreal. His research concerns the ecology, population genetics and evolution of the *Enterobacteriaceae*, especially *E. coli*.

Jeff Cave graduated from Murdoch University in 1988 and spent two years in mixed private practice in South Australia before working in the Pacific for much of the 1990s. For the past 16 years he has worked as the District Veterinary Officer in Wodonga, Victoria.

Jacek Gwozdz is a Veterinary Pathologist at the State veterinary laboratory in Victoria. He received his PhD in Veterinary Science from the Massey University in New Zealand and worked over the past 16 years in research and diagnostic microbiology and pathology.

Nick Linden is a research scientist with the Victorian Department of Primary Industries and Environment, he has a particular interest in the lamb supply chain and is passionate about linking on farm production through to carcass appraisal and consumer acceptance. His recent projects have been focused on lamb feed conversion efficiency as well as on-line grading of lamb carcasses.

Grant Rawlin is Research Leader of Veterinary Pathobiology at the State veterinary laboratory in Victoria. His career has spanned veterinary diagnostics as a pathologist, research in veterinary and human microbiology and research into control of infectious diseases of animals at a population level.

Dr Gwen Allison received her PhD in Microbiology from North Carolina State University. Her research interests have included molecular biology of lactic acid bacteria and microbial ecology of the gastrointestinal tract and environment. The current research was conducted when Dr Allison was a Senior Lecturer at the ANU.

Dr Claire O'Brien received her PhD from the Australian National University Medical School (ANUMS) in 2012. Her main research interest is the gut microbiome, particularly in Crohn's disease. Claire is an NHMRC Peter Doherty Fellow at the ANUMS and Canberra Hospital.