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comparison to non-shedding cows**

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Note

Faecal bacterial composition in dairy cows shedding *Mycobacterium avium* subsp. *paratuberculosis* in faeces in comparison to non-shedding cows

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Abstract

The aim of this study was to determine possible differences in the faecal microbiota of dairy cows infected with *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) in comparison with not infected cows from the same herds. Faecal samples from cows in four herds were tested using real-time PCR for *Mycobacterium avium* subsp. *paratuberculosis* and faecal bacterial populations were analysed using 454 pyrosequencing of the *16S rRNA* gene. Most notable differences between shedding and not shedding cows included an increase in the genus *Psychrobacter* and a decrease in *Oscillospira*, *Ruminococcus* and *Bifidobacterium* genera in cows infected with *M. avium* subsp. *paratuberculosis*. The present study is the first to report the faecal microbial composition in dairy cows infected with *M. avium* subsp. *paratuberculosis*.

Keywords: Cattle; MAP; Pyrosequencing; Faecal microbial composition

Johne's disease (paratuberculosis) is a chronic wasting disease of cattle and other domestic and wild ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis*. Clinical signs include diarrhoea and weight loss. Johne's disease causes significant economic losses in the cattle industry due to decreased milk and meat production (Lombard 2011). Generally, animals become infected at a young age, although due to the long incubation period of the disease they develop clinical signs much later, usually due to stress conditions or malnutrition. *M. avium* subsp. *paratuberculosis* infects the host most often through the gastrointestinal tract, where it resides within the macrophages of the intestine and intestinal lymph nodes. *M. avium* subsp. *paratuberculosis* causes inflammation and changes in the intestine leading to diarrhoea, dehydration and overall weakness (Weiss and Souza 2008; Sweeney 2011).

The intestinal microbiota plays an important role in immunity and in the maintenance of overall health. In humans, studies have linked changes in microbial structure in the intestine to inflammatory bowel disease and other chronic intestinal illnesses (Chassaing and Darfeuille-Michaud, 2011). However, there is no information in the literature about the bacterial composition in the intestinal tract of diseased animals. The aim of the present study was to determine if there were differences in the faecal microbiota of dairy cows infected with and shedding *M. avium* subsp. *paratuberculosis*, in comparison with non shedding cows from the same herds.

Faecal samples were collected from 5-12 dairy cows aged between 3-6 years in each of four herds with similar management during routine check-ups for Johne's disease. Obtained samples were immediately frozen at -20 °C. The feed ratios on each farm were 28% corn silage, 23% sugar beet, 16% supplement, 11% lucerne haylage, 9% dried corn, 5% hay,

64 6% corn cob mix and 2% molasses. *M. avium* subsp. *paratuberculosis* was quantified in
65 faeces using real-time quantitative PCR (qPCR; Slana et al. 2008). Each qPCR-positive cow
66 was matched with a cow negative for *M. avium* subsp. *paratuberculosis* from the same farm.
67

68 Cows were divided into three groups consisting of cows with clinical signs of Johne's
69 disease and also shedding $> 10^6$ cells/g *M. avium* subsp. *paratuberculosis* in faeces ($n = 6$),
70 cows with subclinical *M. avium* subsp. *paratuberculosis* infection, shedding (10^3 - 10^6 cells/g
71 faeces; $n = 12$) and uninfected not shedding cows ($n = 18$).
72

73 To determine the bacterial composition of the faeces, DNA was isolated using a
74 commercially available kit (QIAamp Stool DNA isolation kit, Qiagen) as described
75 previously (Kralik et al. 2014). PCR was performed using universal primers for the V3/V4
76 regions of the *16S rRNA* gene (Nossa et al. 2010), followed by pyrosequencing using the 454
77 GS Junior platform with GS Junior Titanium sequencing chemistry (Roche Diagnostics).
78 Sequences were analysed after quality trimming and chimera elimination (chimera slayer
79 method) using the QIIME programme (Caporaso et al. 2009) and classified into operational
80 taxonomic units (OTUs) with a discrimination level of 97%, using a de novo OTU method
81 with the UCLUST algorithm (Edgar 2010). Diversity analyses (Chao, Equitability, Shannon
82 and Simpson indices) on OTU clusters were performed using all sequences available for each
83 sample. The Kruskal-Wallis and paired Mann-Whitney *U* tests were performed to compare
84 the microbial composition at the genus level in the three groups of animals.
85

86 In total, 134 311 reads were derived from 36 individual samples. Table 1 shows the
87 number of species in each group, together with Chao, Equitability, and Shannon and Simpson
88 indices. The intestinal microbiota mainly consisted of *Firmicutes* and *Bacteroidetes* phyla. At

the class level, bacteria belonging to classes *Bacteroidia* and *Clostridia* predominated in healthy cows. However, in cows with Johne's disease and cows with subclinical *M. avium* subsp. *paratuberculosis* infection, there were increased numbers of the *Actinobacteria*, *Gammaproteobacteria* and *Bacilli* classes compared to uninfected cows (Figure 1).

The relative abundance of the most frequently identified genera is presented in Table 2. There was a decrease in the abundance of the genera *Oscillospira*, *Ruminococcus* and *Bifidobacterium*- and an increase in the abundance of the genus *Psychrobacter* in cows with Johne's disease and cows with subclinical *M. avium* subsp. *paratuberculosis* infection compared to non shedding cows.

We utilized nonparametric statistics, because normality of distribution in the individual samples was tested and rejected by Shapiro-Wilks test. Using the Kruskal-Wallis test on pairs of individual animals, we found no differences between non-shedding cows from different farms ($P = 0.98$), or between non-shedding cows and cows shedding *M. avium* subsp. *paratuberculosis* ($P = 0.2$), whereas significant differences were identified between non-shedding cows and cows with clinical disease ($P = 0.01$). Thus, we decided to perform between-groups comparisons. Due to uneven number of samples in individual groups, we used Mann-Whitney test with Bonferroni correction for multiple comparisons. The test revealed statistically significant differences between non-shedding cows and cows shedding *Mycobacterium avium* subsp. *paratuberculosis* ($P = 0.0126$). The same test did not reveal any statistically significant difference between non-shedding cows and cows with clinical disease ($P = 0.3094$), whereas the *Mycobacterium avium* subsp. *paratuberculosis*-shedding cows and cows with clinical disease did show statistically significant differences from each other ($P = 0.0050$). With respect to *Gammaproteobacteria*, there were significant differences between

non-shedding and cows shedding *Mycobacterium avium* subsp. *paratuberculosis* ($P= 0.0025$) and between non-shedding cows and those with clinical disease ($P= 0.0002$), while there were no significant differences between *Mycobacterium avium* subsp. *paratuberculosis*-shedding cows and those with clinical disease ($P = 0.1074$).

The present study is the first to report the faecal microbial composition in dairy cows infected with *M. avium* subsp. *paratuberculosis*. In previous studies, the faecal microbial composition reported in cattle was similar to that found in the group of healthy animals in this study (Shanks et al. 2011, de Oliveira et al. 2013, Mao et al. 2015). Difference in faecal microbial communities may be influenced by the presence of other bacteria. For example, differences between *E. coli* O157:H7 super-shedder and non-shedder feedlot Steers have been described (Xu et al. 2014).

In humans, there have been many studies performed in order to determine changes in faecal microbiota in connection to diseases such as inflammatory bowel diseases (IBD). As reviewed by Chassaing and Darfeuille-Michaud (2011), the general trend observed in patients with IBD, is decrease of the microbiota diversity in faeces. We have obtained similar data in the number of observed species (Table 1). Decrease in the abundance of *Firmicutes* has also been observed by many authors (Bull and Plummer, 2014). However, we have not observe decrease of the *Firmicutes* phylum in cows with clinical disease.

Our study was limited by the relatively low number of animals included, as well as the fact that we utilised only sequencing of the 16S rRNA segment. Further research should be performed in order to determine specific changes in the microbiota of cows infected with *Mycobacterium avium* subsp. *paratuberculosis*, and whether these changes are caused by *Mycobacterium avium* subsp. *paratuberculosis*. Such research may facilitate the development of techniques to improve the bacterial microbiota in animals in order to reduce the impact of

disease on animal production and health. Functional analysis of the metagenome and metatranscriptome changes in the ileum and jejunum of cattle affected by paratuberculosis would be valuable for understanding the interactions of bacteria and the immune system and would be contribute to better understanding the mechanism of the disease.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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204

205 **Figure legend**

206

207 Figure 1. Distribution of the six most abundant bacterial classes in faecal samples from the
208 three groups of cows. The taxonomic affiliations were predicted using the Greengenes
209 database with a bootstrap cut-off of 80. The median relative abundance from all of the
210 examined samples within a group is shown.

Table 1

Diversity indices in operational taxonomic unit (OTU) sequences obtained from the three groups of animals.

	Observed species	Chao1	Equitability	Shannon	Simpson
Non-shedding MAP (n =18)	1415	3123.39	0.896	9.260	0.996
SD	685	2286.28	0.028	0.685	0.002
MAP-shedding (n = 12)	923	1670.78	0.841	8.525	0.991
SD	646	1519.26	0.084	1.281	0.019
Johne's disease (n = 6)	1022	2553.06	0.850	8.506	0.990
SD	325	879.69	0.093	0.990	0.026

The presented values are means of all the samples analysed.

SD, standard deviation.

Table 2

Relative abundance of the bacterial genera in the faeces of the three groups of cows.

	Non-shedding	<i>M. avium</i> <i>paratuberculosis</i> - shedding	Johne's disease
<i>Bifidobacterium</i>	3.1%	0.9%	1.6%
5-7N15	5.0%	3.7%	4.4%
BF311	1.2%	0.1%	0.0%
<i>Bacteroides</i>	2.2%	1.5%	1.5%
<i>Paludibacter</i>	1.1%	1.3%	1.9%
<i>Parabacteroides</i>	0.6%	0.1%	0.1%
<i>Prevotella</i>	0.6%	0.5%	0.8%
<i>Odoribacter</i>	2.7%	0.0%	0.0%
CF231	3.4%	2.7%	2.2%
YRC22	0.6%	0.5%	1.2%
Unclassified <i>Paraprevotellaceae</i>	0.6%	0.5%	3.5%
<i>Paenibacillus</i>	0.4%	0.1%	0.0%
<i>Solibacillus</i>	0.9%	1.0%	0.4%
<i>Sporosarcina</i>	0.4%	0.1%	0.0%
<i>Turicibacter</i>	1.1%	1.7%	0.7%
<i>Clostridium</i>	0.5%	0.9%	0.5%
<i>Butyrivibrio</i>	0.3%	0.6%	0.1%
<i>Coproccoccus</i>	0.7%	0.1%	0.0%
<i>Dorea</i>	1.3%	1.0%	0.8%
<i>Anaerofilum</i>	1.1%	0.1%	0.0%
<i>Oscillospira</i>	12.1%	2.4%	4.4%
<i>Ruminococcus</i>	22.0%	14.9%	16.3%
<i>Escherichia</i>	0.5%	1.4%	0.4%
<i>Acinetobacter</i>	0.9%	3.7%	1.3%
<i>Psychrobacter</i>	0.7%	1.7%	5.3%
Unclassified <i>Bacteroidales</i>	16.6%	9.6%	10.9%
Other	20.2%	49.1%	39.5%

Figure 1. Distribution of the six most abundant bacterial classes in faecal samples from the three groups of cows. The taxonomic affiliations were predicted using the Greengenes database with a bootstrap cut-off of 80. The median relative abundance from all of the examined samples within a group is shown.

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