Title: Short communication. Effects of antibiotics (oxytetracycline, florfenicol or tulathromycin) on neonatal calves' faecal microbial diversity.

Running head: Antibiotics and calves' faecal microbiota

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Abstract

In this study, we used barcoded pyrosequencing of the 16S rRNA gene to characterise the effects of antibiotic treatment upon the faecal microbiota of neonatal calves. Eleven pre-weaned calves were treated for pneumonia or otitis using one of three antibiotics (oxytetracycline, florfenicol or tulathromycin) and were matched for age /date of birth and sex with eleven control calves. All calves were born and reared at the same farm. Faecal microbial diversity data were obtained by barcoded pyrosequencing of the 16S rRNA gene one week pre-treatment, and one and two weeks post treatment for both treated and control calves. Using multivariate discriminant analysis we were able to show that antibiotic treatment has a substantial effect on faecal samples' microbial composition one week after administration; this effect was no longer observed two weeks after administration. The effect of oxytetracycline treatment on *Lactobacillus* spp. was shown to be significant but many other important species appeared to be unaffected. The small number of calves used in the study prohibited quantitative comparisons of the effects of individual antibiotics compared to others on Chao1 richness index; despite this, however, some interesting numerical differences were apparent. In conclusion, our study serves to illustrate that change occurs in the gut microbiome of the young ruminant in response to antimicrobial administration. Given the limitations of our study we suggest that further similar studies are necessary.

Keywords: Calf; Antibiotics; Microbial diversity; Pyrosequencing; 16s rRNA genes

Uyeno and others (2010) and Oikonomou and others (2013) identified dynamic changes in the faecal microbiota of dairy calves during the first twelve and first seven weeks of life respectively; their findings suggested that diet and gut development may drive these changes. Antibiotics are commonly used in the treatment of bacterial infections in all animal species but the effects of antibacterial drugs upon the microbial communities of the gut are poorly understood. Studies in humans and other monogastric species have demonstrated changes in the gut microbiota subsequent to antimicrobial administration (Suchodolski and others 2009; Panda and others 2014). However, to the best of our knowledge, the effects of antibiosis upon the gut microbiota characterized with the use of a culture independent metagenomic approach in ruminant species and particularly neonatal calves have not been examined yet.

The data used in the study described here were collected in a prospective cohort study (Oikonomou and others 2013) that described faecal microbial diversity in 61 female pre-weaned Holstein calves during their first seven weeks of life. Faecal samples were collected at birth then weekly and kept frozen until used for bacterial DNA extraction. Farm management, sample collection, DNA extraction, PCR and pyrosequencing are described in detail by Oikonomou and others (2013). Eleven of these calves contracted pneumonia or otitis during the study, and were treated with systemic antibiotics. Seven calves were treated with oxytetracycline ("Biomycin®" Boehringer-Ingleheim, single intramuscular injection of 20 mg/kg of body weight), one calf was treated with tulathromycin ("Draxxin®", Zoetis, single subcutaneous injection of 2.5 mg/kg of body weight) and three calves were treated with florfenicol ("Nuflor®" Schering-Plough, single subcutaneous injection of 40 mg/kg of body weight). This enabled the retrospective analysis of the effects of antibiotics on calves' faecal microbial diversity after matching each treated calf to a healthy control for date of birth, all of which had been sampled on the same days as the treated calves. The relative

abundance of faecal bacterial genera by week was examined; one week before treatment, one week post treatment and two weeks post treatment (for both treated and control calves). For example, if a calf was treated with antibiotics during its fourth week of life the bacterial genera relative abundance information used in the analysis was from the samples obtained during its third week of life and during its fifth and sixth one. The same information (third, fifth and sixth week of life) obtained from this calf's control calf was also included in the analysis. No data were missing in these analyses. The data were analysed using JMP Pro 11 (SAS Institute Inc., North Carolina).

Different genera relative abundances in each sample were used as covariates in stepwise multivariate discriminant analysis models. Variables were removed in a stepwise manner until only variables with a P value < 0.1 were retained. Discriminant analysis was performed using bacterial genera relative abundances as covariates and the interaction of time with treatment/control group as a categorical variable. Multivariable mixed effects linear regression models were used to evaluate the effect of different antibiotics on the relative abundance of the 5 most prevalent bacterial genera, (*Lactobacillus*, *Faecalibacterium*, *Bacteroides*, *Parabacteroides* and *Sharpea).* Genus relative abundance was the outcome variable. Treatment group and treatment group interaction with time relative to treatment were fitted in the model. Calf id was also fitted in the model as a random effect. The same analytical approach was used to evaluate the Chao1 richness index of the faecal microbiome in antibiotic treated and control calves and to evaluate the effects of different antibiotics on Chao1 diversity index over time. This could not be done for tulathromycin though as only one calf was treated with this antibiotic. Number of sequences per sample was also offered in these models.

The Discriminant Analysis by group and time shows in Figure 1 that faecal microbiota composition pre-treatment is similar in control calves and treated calves. One

week post treatment the groups show a greater difference in their faecal microbiota composition. Two weeks post- treatment the microbiomes of the control group and treatment group are more similar and have overlapped, indicating a temporal increase in similarity of the microbiomes and showing fewer differences than the groups demonstrated pre-treatment. Among the bacteria seen to be statistically significantly affected by antibiosis in the present study was *Lactobacillus* spp. A significant interaction of treatment with oxytetracycline by time relative to treatment was observed ($P < 0.05$). Adjusted mean relative abundances for *Lactobacillus* spp. for treated with oxytetracycline and control calves by time are shown in Figure 2. There was a temporal increase in samples' richness in both control and treated calves. The control calves underwent an increased rate of change in the microbial diversity compared to treatment calves, thus showing a numerically (but not statistically, $P > 0.05$) significant divergence in Chao1 index by one week post treatment. Both groups' microbial diversity increased to two weeks post treatment at which time the Chao 1 indices were identical.

Lactobacillus spp. in control calves underwent an initial increase then a reduction in adjusted relative abundance over the three week study period (one week pre to two weeks post treatment in treated calves), which may be a natural change in response to dietary change from predominantly milk to a less milk based diet. Diseased, treated calves exhibited significantly different changes in *Lactobacilli* prevalence throughout the study which may imply either an effect of antibiotics on these bacteria or a delay in the ability of the treated calves to transition from a milk based diet to concentrates. The small number of calves used in the study prohibited quantitative comparisons of the effects of individual antibiotics compared to others; despite this, however, numerical differences were apparent. When compared to control calves at one week post treatment, florfenicol treatment reduced samples' richness by a numerically significant amount and this was reduced further by the

date of final sampling (two weeks post treatment), whereas that of the control calves had increased upon each sampling date. Conversely, oxytetracycline treated calves underwent a more rapid increase in richness than the control group and demonstrated a higher Chao1 index compared to controls at each post treatment sampling; again the difference was numerically but not statistically significant. The tulathromycin treated calf underwent the greatest reduction in Chao1 index by one week post treatment, and a degree of recovery of richness at two weeks post treatment, but this was still significantly less than the pre-treatment richness. Note that this study did not permit longer term assessment of microbial biodiversity but the results of the canine study by Suchodolski and others (2009) showed that depression of some taxa did persist for several months. Similarly antibiotic usage has been shown to reduce the Chao1 index of intestinal microbiota profiles in humans (Claesson and others 2011) and pigs (Looft and others 2012).

In conclusion, this study serves to illustrate that change occurs in the gut microbiome of the young ruminant in response to antimicrobial administration; however, any concurrent effects solely attributable to the disease necessitating the treatment were not characterised nor can a gender effect be eliminated as only female calves were used. Given the limitations of our study (small number of calves used, retrospective analysis of data collected originally for a different purpose) we suggest that further studies are necessary to identify the functions of specific bacterial phyla and genera, quantify the effects of specific antibiotics upon these, measure the effects of microbial changes upon the host and develop the possibilities of deliberate manipulation of the microbiome to the advantage of the host.

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Conflict of interest statement

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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Figures legends

Fig. 1. Discriminant analysis of faecal samples microbiome by control (C) or treatment (T) group and time (pre = one week pre-treatment, 1 = one week post treatment and 2 = two weeks post treatment). Groups are colour coded. The centre of gravity for each group is represented by a + sign and variability by a circle.

Fig. 2: Adjusted mean relative abundances (± SE) for *Lactobacillus* spp. for treated with oxytetracycline (black line) and control (grey line) calves by time (Pre =one week pre-treatment, 1 = one week post treatment and 2 = two weeks post treatment).