MOLECULAR PROFILING OF THE MAJOR BACTERIAL SPECIES IN THE RABBIT CAECUM AS AFFECTED BY THERAPEUTICAL DOSES OF ANTIBIOTICS

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ABSTRACT

Intensive rabbit production is hampered by pathologies that restrict animal growth and often cause important death rates. The use of therapeutical doses of antibiotics added to feed is a common practise in such situation. However, to what extent these treatments may affect bacterial population and consequently nutrient utilisation by growing rabbits is as yet poorly understood. This paper makes use of Denaturing Gradient Gel Electrophoresis (DGGE), a molecular profiling technique, to study population shifts within the major bacterial species in the caecum of the rabbit following dietary supplementation with three of these antibiotics (bacitracin, chlortetracycline and tiamulin). The resulting profiles were interpreted by carrying out pair wise comparisons and measuring Hamming Distances between profiles. The dendogram resulting from this analysis demonstrates that there is a relatively large variation in the major bacterial species present in control animals (as assessed by the long branches in the dendrogram). Branches of a similar length were also observed for samples collected from animals that had been fed bacitracin. However, less variation was observed in the samples from animals fed either chlortetracycline or tiamulin. These observations suggest that the biodiversity levels of the major bacterial species present in the caecum of the rabbits is less following feeding with either chlortetracycline or tiamulin, and that the effects of these antibiotics is more profound than that seen in the bacitracin.

Key words: bacteria, biodiversity, rabbit caecum, DGGE, antibiotics

INTRODUCTION

The level of intensification of production systems for growing rabbits demands for a high growth rate, which is at the edge of the physiological capacity of animals. In such situations, the incidence of pathologies increases, restricting animal growth and often causing important death rates. When these problems arise, the use of medicated feeds is a common practise. In this regard antibiotics may control pathogens, but to what extent these treatments may affect indigenous bacterial population and consequently nutrient utilisation is as yet poorly understood. This topic is especially important in animals such as rabbits, since up to 40 % of the maintenance energy needs of growing

rabbits are satisfied by microbial metabolism in the caecum (PARKER, 1976; MARTY and VERNAY, 1984).

In order that this approach may become most useful it is essential to obtain a better understanding of which microbes are being affected by the antibiotics before being able to elucidate their consequences on nutrient utilisation. In the rabbit gut ecosystem it is not known which bacterial species are being affected by the addition of different antibiotic supplementation regimes, or even if the same bacterial species are being targeted by the different antibiotics being used.

In other words, the approaches that have been conducted so far to study rabbit caecal population have used traditional anaerobic culture methods (GIDENNE and FORTUN-LAMOTHE, 2002; ABECIA *et al.,* 2002), with the inherent problems derived from them, such as the wide margin of error and the high number of bacterial species that do not grow *in vitro* (AMANN *et al.,* 1995).

In this work molecular profiling was performed to study the population shifts in the major bacterial species following antibiotic supplementation of the diet with one of three different antibiotics.

MATERIALS AND METHODS

Sixteen growing New Zealand rabbits (667 ± 25.5 g initial weight) were used. Animals were weaned at 28 days and allocated to 4 groups of 4 rabbits each, that were housed in an environmentally-controlled barn. Diets consisted of a common mixed feed to which several doses of antibiotics commonly used in practice were added: 100 ppm bacitracin, 400 ppm chlortetracycline or 100 ppm tiamulin were added. These treatments were compared with the untreated diet as a control. Rabbits were given the experimental diets *ad lib.* for 28 days, being weighed individually every 7 days. After 28 days, rabbits were slaughtered, their caecum excised. Samples of caecum contents were obtained and immediately frozen in liquid nitrogen until their analysis by molecular techniques. Other samples and parameters obtained from this experiment have been already presented (ABECIA *et al*., 2003, 2004).

DNA was extracted from digesta from four rabbits from each dietary regime using a stool extraction kit (Qiagen), following the manufacturer's instructions. PCR amplification across the 16S *rDNA* V3 region was carried out using the primers designed for DGGE analysis by (MUYZER *et al.*, 1993), (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG–3') and 534r (5'- ATT ACC GCG GCT GCT GG-3'). Sample DNA (50 ng) was added to a 50 µl reaction mix containing 1 µM of each primer, 0.8 mM of dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, and 2.5 U of Taq DNA polymerase in 10 mM Tris-HCl (pH 9.0). Amplification conditions were 35 cycles with 1 min at 94 ^{o}C for denaturation, 1 min at 55 ^{o}C for annealing and 2 min at 72 ^{o}C for extension, except for 5 min denaturation in the first cycle and 7 min extension in the last cycle. PCR amplification products were visualised on 2 % (w/v) TBE agarose gels prior to DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE) was performed using the BioRad Dcode Universal Mutation Detection System, following the manufacturer's guidelines. PCR products (20 µl) were loaded onto 8% (w/v) polyacrylamide gels in 1x TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.3), which contained a 35-55% denaturant gradient (100% denaturant, 7 M Urea and 40% (v/v) deionised formamide). Electrophoresis was performed at a constant voltage and temperature of 130 V and 60 ^{o}C for 5 h. Gels were then stained for 30 min with Silver stain and the gel image saved with a BioRad GelDoc 2000 gel documentation system.

DGGE profiles within the same gels were compared using similarity comparisons. Each band position present in the gel was binary coded for its presence or absence within a lane, and each lane was compared using a similarity matrix. Trees were constructed using the Hamming Distance values generated for each comparison (indicating the number of bands that differed between lanes) as an input for the NEIGHBOR program (PHYLIP version 3.6; FELSENSTEIN, 2002).

RESULTS AND DISCUSSION

A typical gel obtained following DGGE electrophoresis of PCR products obtained using the MUYZER *et al*. (1993) primers is shown in Figure 1. Among the various bands appearing in the different lanes, the one labelled as 1 (at the bottom of the gel) as well as band 2 (at the top) appear in all samples, although at different intensity, whereas bands 3 to 5 were only detected from control animals. Band 6 to 8 have the same mobility, and appear in all samples except for those from bacitracin, and band 9 was absent from the control. Even at a casual glance it is clear that there are differences between the various lanes. However, it is not obvious which lanes are most similar to each other. In order that this comparison may be made pair wise Hamming Distance values were calculated. The results of this analysis are shown in Figure 2.

Figure 2 shows that both chlortetracycline and tiamulin affect similarly the caecal rabbit population as compared with animals from the control treatment, and therefore their biodiversity is very similar, as it is shown by the short length of their branches and their proximity. There is less variation between the samples from chlortetracycline and tiamulin than there is in either the control or bacitracin samples, as evidenced by the length of the branches on the tree; longer branch lengths being associated with greater biodiversity. This suggests that the effects of bacitracin are relatively limited in terms of the number of major bacterial species which are being affected by this antibiotic. Conversely the branch lengths of the samples from bacitracin fed rabbits are almost as long as those from the control animals, suggesting that the number of major bacterial species being affected by this antibiotic is relatively minor.

Previous studies on digestive performances with therapeutical doses of antibiotics (ABECIA *et al*., 2002; MAERTENS, 1992) have shown that dietary inclusion of bacitracin increased diet digestibility compared to chlortetracyclin.

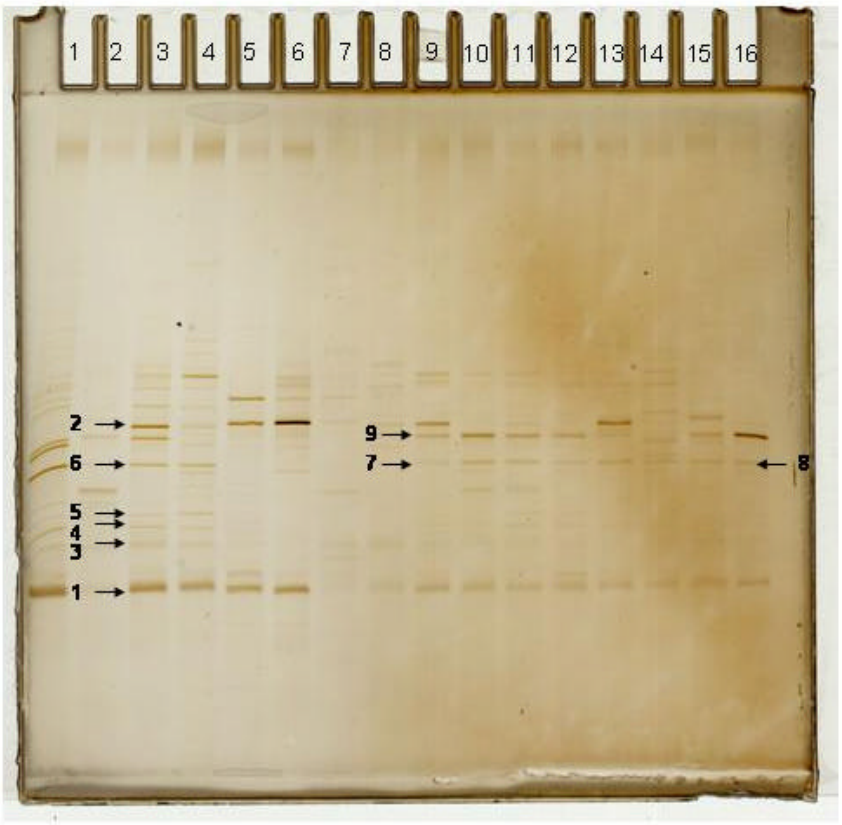

Figure 1. DGGE gel obtained using samples from four rabbits on each of the four antibiotic supplementation regimes (control, lanes 1 to 4; bacitracin, lanes 5 to 8; chlortetracycline, lanes 9 to 12; tiamulin, lanes 13 to 16).

CONCLUSIONS

From these results it can be concluded that therapeutical treatment with antibiotic affects bacterial caecal population in rabbits, which supports possible differences in nutrient digestive utilisation in this organ. Furthermore, this effect varies with the type of antibiotic, chlortetracyclin and tiamuline modifying caecal flora to a higher extent than bacitracin. Further studies may be conducted in order to specify which indigenous organisms are affected to a higher exte nt by each antibiotic.

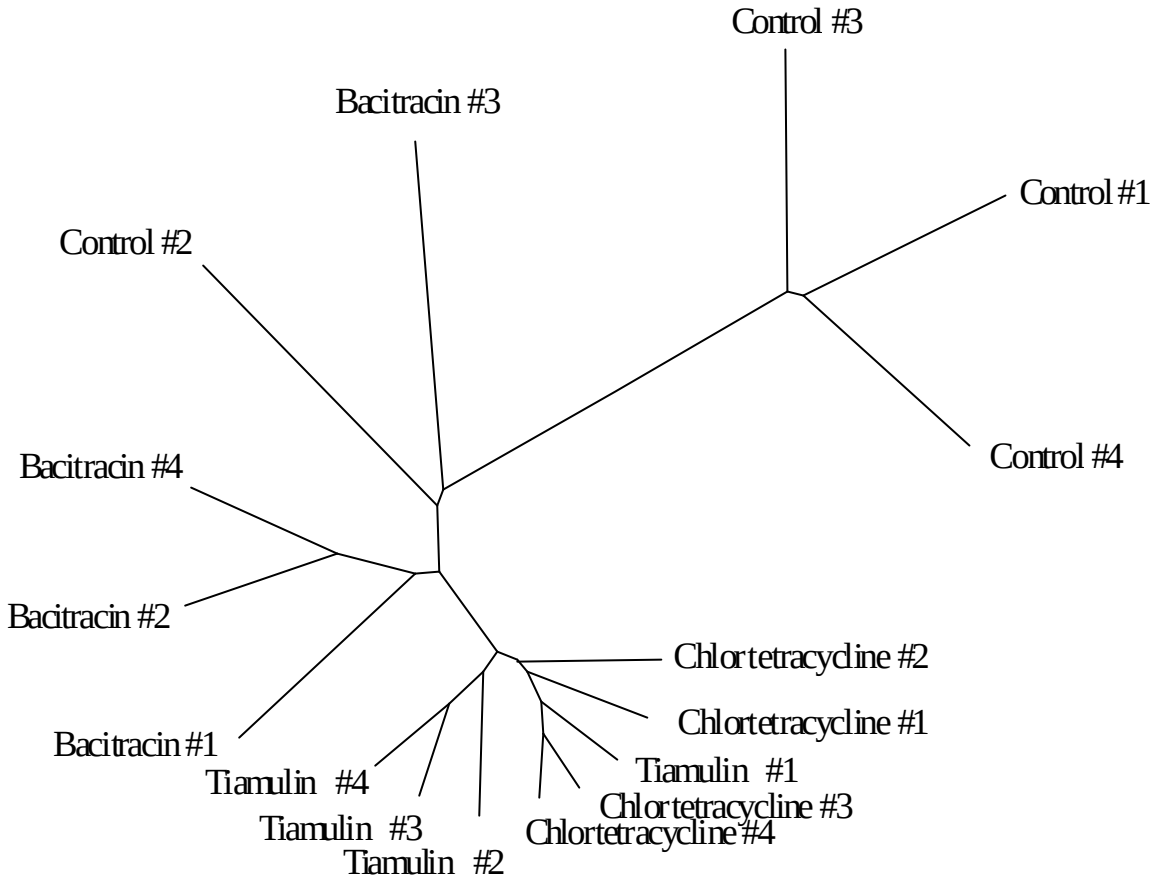

Figure 2. Hamming Distance analysis of the similarity between lanes on the gel shown in Figure 1.

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