CHARACTERIZATION OF THE MICROBIAL DIVERSITY OF RABBIT INTESTINAL TRACT BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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ABSTRACT

Because only a small portion of the intestinal bacteria are cultivable, the development of molecular techniques, as restriction fragment length polymorphism (RFLP), allows having a better knowledge of the bacterial diversity with no culture requirements. The aim of the present study was to compare the intestinal bacterial communities present in the gut of rabbits, and its relationship with the age of rabbits and the feed formulation. The RFLP was conducted on total intestinal DNA, by the QIAamp DNA Stool Mini Kit (QIAGEN), with some modifications. A fragment of the bacterial 16S r-DNA gene was amplified by PCR with CTACGGGAGGCAGCAGT and CCGTCWATTCMTTTGAGTTT primers and digested with four different restriction enzymes (*AluI, RsaI, HpaII, CfoI*). Some changes have been observed in the gut microbiota by direct microscopic observation, associated to the age of the animals. These changes can be linked to some changes in the gut microbiota components, and related to these changes in the RFLP profiles can be expected. In the window of ages analysed (15, 25, and 35 days old), some electrophoretic bands reduce their intensities with the age of the animals at the same time that other bands increase their intensities. It seems that these band variations are independent of the type of diet used in the feeding of the animals. In other animal species, as pigs or chickens, the degree of biodiversity in intestinal microbiota is higher in the caecum than in the ileum, but in rabbits the relationship is inverted, with a higher biodiversity degree in the ileum. Caecotrophy could explain this difference with other species.

Key words: RFLP, gut bacteria, rabbit, feed, age.

INTRODUCTION

The gut microbiota, or the overall microbial species that are present in the intestinal tract of animals, is composed by more than 40 genera and some hundred species (MACKIE *et al.*, 1999). Only twenty-three per cent of the microbial species present in this complex microbiota can be cultured *in vitro*, a characteristic that difficult its knowledge by the researchers, and because that, the possibility of its control by feed composition.

In the past, the studies on changes in the gut microbiota has focused in alterations on microorganisms easily cultured as *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringens*..., but the introduction of new molecular techniques, and specially the amplification of DNA by PCR (polymerase chain reaction), allows the possibility of cut off the use of *in vitro* cultures and to extend the study of the intestinal changes to uncultured microbiota.

To conduct these molecular studies, the amplification of a universal gene is carried out, and because some interesting characteristics, the gene that codes the 16S ribosomal-RNA (16S r-DNA) has been the most frequently used. This gene is located in the chromosome of all the prokaryotic cells, and in the genetic material present in mitochondria or chloroplast of eukaryotic cells. Only some anaerobic protozoa have not any 16S r-DNA, but to analyse these microorganisms the amplification of 30S ribosomal-RNA might be used.

The 16S ribosomal-RNA molecule has two additional characteristics, related with its primary structure, that confer its potentiality in the study of complex microbiota, as gut microbiota is: it has some highly conserved areas at the same time that some hyper-variable areas. The first characteristic allows the use of some universal primers that lead to the amplification of a high percentage of microorganisms. The second characteristic enables the resolution between microorganisms, especially by changes in the recognition sequences of restriction nucleases. The combination of DNA amplification of 16S r-DNA, digestion with restriction nucleases of amplified DNA, and analysis by agarose gel electrophoresis becomes an excellent system to study the composition of gut microbial components without the necessity of *in vitro* cultures.

The aim of this article was to analyse the influence of the age of animals, feed type, or intestinal portion on the gut microbiota composition.

MATERIAL AND METHODS

Sample collection

To prevent the post collection changes in the intestinal microbiota composition, gut samples collected without any additive, and maintained at -70ºC, or gut samples collected in ethanol (1:3 w/v), and maintained at 4ºC, were previously compared, with a more satisfactory results in the case of gut samples collected in ethanol (1:3 w/v).

Approximately one gram of ileal or caecal samples were collected in a sterile plastic tube that contains 3 mL of 98% molecular biology grade ethanol. These tubes were maintained at 4ºC until use.

To analyse the parameters that centre the aim of this article: influence of the age of animals, feed type, or intestinal portion on the gut microbiota composition, ileal and caecal samples from rabbits with different ages and fed diverse diets were collected.

DNA extraction

Two hundred and twenty milligrams of gut contents were processed for total DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Chatsworth, Calif.) system, in accordance with the instructions of the manufacturer, with two additional steps of lysozyme and proteinase K that implement the DNA extraction of some microorganisms. The extracted DNA was maintained at -20ºC until use.

PCR amplification and restriction enzyme digestion

Two primers 5'-CTACGGGAGGCAGCAGT-3' and 5'-CCGTCWATTCMTTTGAGTTT-3' designed from regions of the 16S rRNA gene conserved among a wide range of microorganisms (LANE, 1991), were synthesized at Sigma-Genosys.

The PCR reactions were prepared with PCR-Master Mix with 1.25 U of Taq polymerase (Applied Biosystems), template DNA, the above primers, and distilled water in a total volume of 50 µl.

PCR mixtures were heated to 94°C for 5 minutes once, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute, and extension at 72° for 1:15 minutes. The last extension cycle was continued for 5 minutes. The PCR amplification reaction was conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems).

In general, PCR amplification products were processed just after amplification, but they were maintained at -20ºC until use in other case.

The DNA fragments amplified by PCR were digested with *Alu* I, *Rsa* I, *Hpa* II, *Sau* 3A I or *Cfo* I restriction endonucleasas (Sigma-Aldrich) in accordance with the manufacturer specifications.

DNA electrophoresis and image analysis

The endonuclease restriction fragments were resolved using a 2% wide range agarose electrophoresis, supplemented with ethidium bromide, applying 150 V during 60 minutes. The bands were visualized with the aid of an UV transilluminator, and captured in a Chemigenious Image System (SynGene) using the GeneSnap software.
The electrophoretic profile obtained is known as Restriction Fragment Length Polymorphism (RFLP) and is characteristic of microbial genera, and in some cases, of microbial species.

Two molecular weight controls were used: Step 50 (Sigma) and an in situ control prepared with the digestion products of 16S r-DNA amplified fragments of well characterized animal bacteria.

The sizes of all the bands obtained were calculated with the aid of the GeneTools software (SynGene).

With the information resulting from the relative size of the fragments, the intensity of the bands, the information stored in the "SSU_Una.gb" file from the Ribosomal Database Project (MAIDAK *et al.,* 1997), and specific software, developed in our Institute, we calculate the degree of biodiversity, among other parameters.

Biodiversity parameters

Because some bands are common to more bacterial species that other bands, we believe it is more appropriate to introduce the term "*Biodiversity Degree*", defined as the number of 16S r-DNA sequences, deposited in the Ribosomal Database Project, compatible with the RFLP profile obtained from the total DNA extracted from the gut samples.
ANOVA analysis was conducted to determine significant differences between treatments and intestinal segments for biodiversity degree.

RESULTS AND DISCUSSION

Effect of sample collection method

Gels that run twin samples collected with or without ethanol, and maintained at 4ºC or at -70ºC respectively, showed little differences between the RFLP profiles obtained. The visible differences, when observed, in general were in favour of ethanol collected samples and maintained at 4ºC.

This was an excellent result because allows the possibility of sample collection at field conditions without any negative effect on the results obtained.

Effect of the age of the animals

In the Figure 1 it can be seen the RFLP profile obtained, for two different restriction enzymes (*Alu* I and *Rsa* I), from intestinal samples collected from animals with different ages of life (15, 25, and 35 days old), and fed with three different feeds (T, X and Z).

In the Figure 1 it is possible to observe some examples of bands, and consequently the related microorganisms, which disappear or reduce their intensity (one asterisk mark) with the age of the animals. At the same time, it is possible to appreciate other bands, and consequently the related microorganisms, which appear or increment their intensity (double asterisk mark) with the age of the animals. This kind of results was not surprising because in other animal species, and by microscopic observation, morphological differences in gut microbiota between animals with different ages can be observed.

Furthermore, in this figure it is possible to observe that in all the types of feeds, with some changes in intensity, the bands that change are the same.

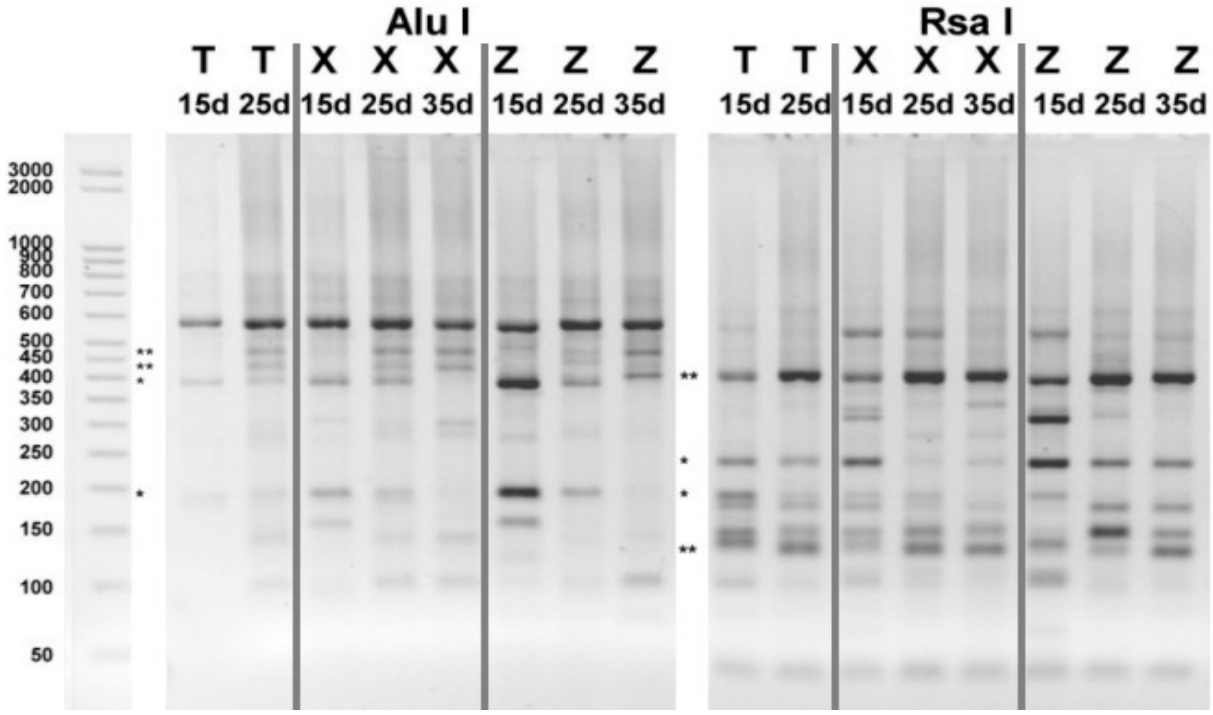

Figure 1: RFLP profile obtained, for two different restriction enzymes (*Alu* I and *Rsa* I), from intestinal samples collected from animals with different ages of life (15, 25, and 35 days old), and fed with three different feeds (T, X and Z). * Bands disappeared with the age, ** Bands appeared with the age.

Degree of biodiversity at different intestinal portions

In Figure 2 we represented the degree of biodiversity for ileal and caecal samples collected from 35 days old rabbits, six per treatment, fed with four different feeds (T, X, Y, and Z).

In this figure it is possible to observe a higher biodiversity degree at ileal level, when compared with caecal samples. ANOVA analysis showed a significant difference between intestinal segments (P<0,001). Additionally, statistically significant differences were observed, at ileum (P=0,022) and caecum (P<0,017), on the biodiversity degree for different feeds (see Table 1).

Table 1: Biodiversity degree of ileal and caecal samples collected from 35 days old rabbits fed with four different feeds. (Average of six samples. Numbers with the same letters were not statistically different).

	Ileum	Caecum
Feed T	1001^{a}	847^{a}
Feed X	1845^{b}	689^{a}
Feed Y	1631^{ab}	266^{c}
Feed Z	2347^{b}	875^{a}

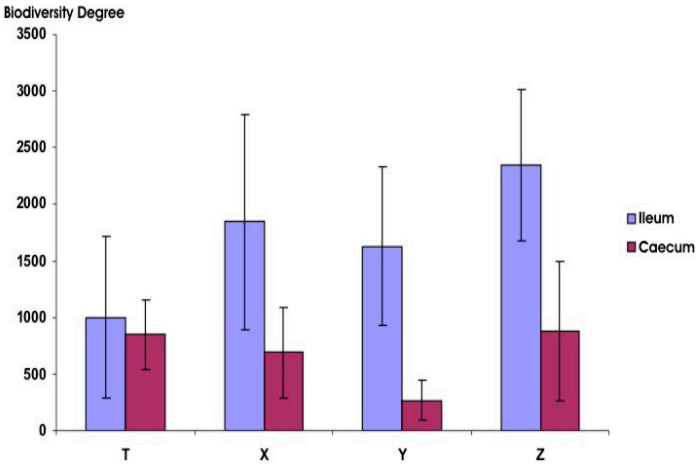

Figure 2: Biodiversity degree obtained from ileal and caecal samples collected from 35 days old rabbits, mean±S.D. obtained after the analysis of six animals per treatment.

CONCLUSIONS

RFLP profiles could be a valuable system to analyse the microbial components located in the gut of rabbits. This method is independent from the culture conditions and, because of that, useful to globally analyse the intestinal microbiota and its variations induced by changes on feed formulation.

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