

## DIGESTIVE MICROBIOTA STUDIES IN RABBITS BY REP-PCR METHOD

Pérez de Rozas A.M.<sup>1</sup>, Rosell J.M.<sup>2</sup>, Díaz J.V.<sup>2</sup>, Carabaño R.<sup>3</sup>, García J.<sup>3</sup>,  
González J.<sup>1</sup>, Aloy N.<sup>1</sup>, Badiola I.<sup>1\*</sup>

<sup>1</sup>CReSA (UAB-IRTA), Campus de Bellaterra, Edifici CReSA, 08193-Bellaterra, Barcelona, Spain

<sup>2</sup>NANTA, Canto Blanco, Madrid, Spain

<sup>3</sup>Departamento de Producción Animal, E.T.S.I. Agrónomos, Madrid, Spain

\*Corresponding author: ignacio.badiola@cresa.uab.es

### ABSTRACT

Different systems are used to discriminate the similarity degree between strains. Some methods are based on phenotypic characteristics and some others on genotypic studies. In this paper we describe a Repetitive Extragenic Palindromic-PCR (REP-PCR) method to evaluate the similarity degree between *Bacteroides* spp. strains isolated from intestinal samples of rabbits to analyse the biodiversity of one of the most abundant bacteria in the digestive system of rabbits.

**Key words:** *Bacteroides*, REP-PCR, Molecular biology methods.

### INTRODUCTION

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its microbial balance (Fuller, 1989). Probiotics have been reported to increase feed intake, growth (Lessard and Brisson, 1987), immune responses (Isolauri *et al.*, 1995; Lanning *et al.*, 2000). However, experiments have failed to show consistent and beneficial responses of probiotics, and probably a more accurate knowledge of intestinal biodiversity will help to develop new feed additives.

The intestinal microbiota in productive animals consists in a population of around  $10^{14}$  bacteria of 200 species, and 40–50 genera (Savage, 1980). In rabbits, *Bacteroides* spp. is the main bacterial species and begins to colonize the gut as soon as two days after birth (Kovács *et al.*, 2006). Its number is increased progressively and, at day ten of life arrives to concentrations of  $10^{8.5}$  bacteria per gram of intestinal content. *Bacteroidaceae* family arises to  $10^{10}$  bacteria per gram of intestinal content in adult rabbits (Yanabe *et al.*, 1999). Contrary to what happens in pigs, *Lactobacillus* spp. is not a habitual component of the microbiota of rabbits. At the beginning of life (two-days old) the lactobacilli counts are  $10^{6.0}$  bacteria per gram of intestinal content, and progressively decrease till  $10^{3.5}$  bacteria per gram of intestinal content at ten days of life (Kovács *et al.*, 2006). Other main components of the intestinal microbiota of rabbits are: Clostridia (10.0%), *Peptococcaceae* (1.0%), Eubacteria (1.0%), *Enterobacteriaceae* (<0.1%), Streptococci (<0.1%) (Yanabe *et al.*, 1999). *Escherichia coli* represents only a minor gut population at levels of  $10^{4.0}$  bacteria per gram of intestinal content before weaning, and is practically absent in adult rabbit.

Some *Bacteroides* species, as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*, play different roles on the immune response of the gut associated lymphoid tissue (Bry *et al.*, 1996; Brubaker *et al.*, 1999; Rhee *et al.*, 2004; Mazmanian *et al.*, 2005) and more knowledge on these species could be important to implement the intestinal health of rabbits.

To discriminate between *Bacteroides* spp. strains, with the aim of the selection of clearly different strains for later more exhaustive studies, diverse molecular biology methods can be used. We present the results obtained with the Repetitive Extragenic Palindromic-PCR (REP-PCR) method, a PCR

amplification based on the use of primers that hybridise with repetitive sequences along the bacterial genome, for the discrimination between *Bacteroides* spp. strains isolated from gut samples of rabbits.

## MATERIALS AND METHODS

### Microorganisms used

Twenty-three strains of *Bacteroides* spp. were isolated by culture of fresh gut samples on BBE-Amikacin (Biomedics, Madrid) at 37°C on anaerobical conditions during 24-48 h. Bacterial strains were cloned and purified by subcultures on the same media and conditions, and maintained at -70°C in skim milk plus thioglycollate broth (Biomedics).

### Nucleic acid extraction

Total DNA from *Bacteroides* strains was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90°C, and a posterior incubation step with lysozyme was added (10 mg/ml, 37°C, 30 min) in order to improve the bacterial cell rupture. The bacterial DNA was eluted in 200 µl of Qiagen buffer AE and was stored at -20°C until use. The purified DNA was stabilized with the addition of 4 µl of 40 mg/ml bovine serum albumin (Sigma-Aldrich Química SA, Madrid) plus 2 µl of ribonuclease A (Sigma-Aldrich Química SA).

### Primers and PCR conditions

To analyse the biodiversity degree of *Bacteroides* spp. strains, REP-PCR profiles were examined. The REP-PCR primers used were: 5'-NNNNcGNCgNCATCNgGc-3' and 5'-NcgNCTTATCNgGcCTAC-3'.

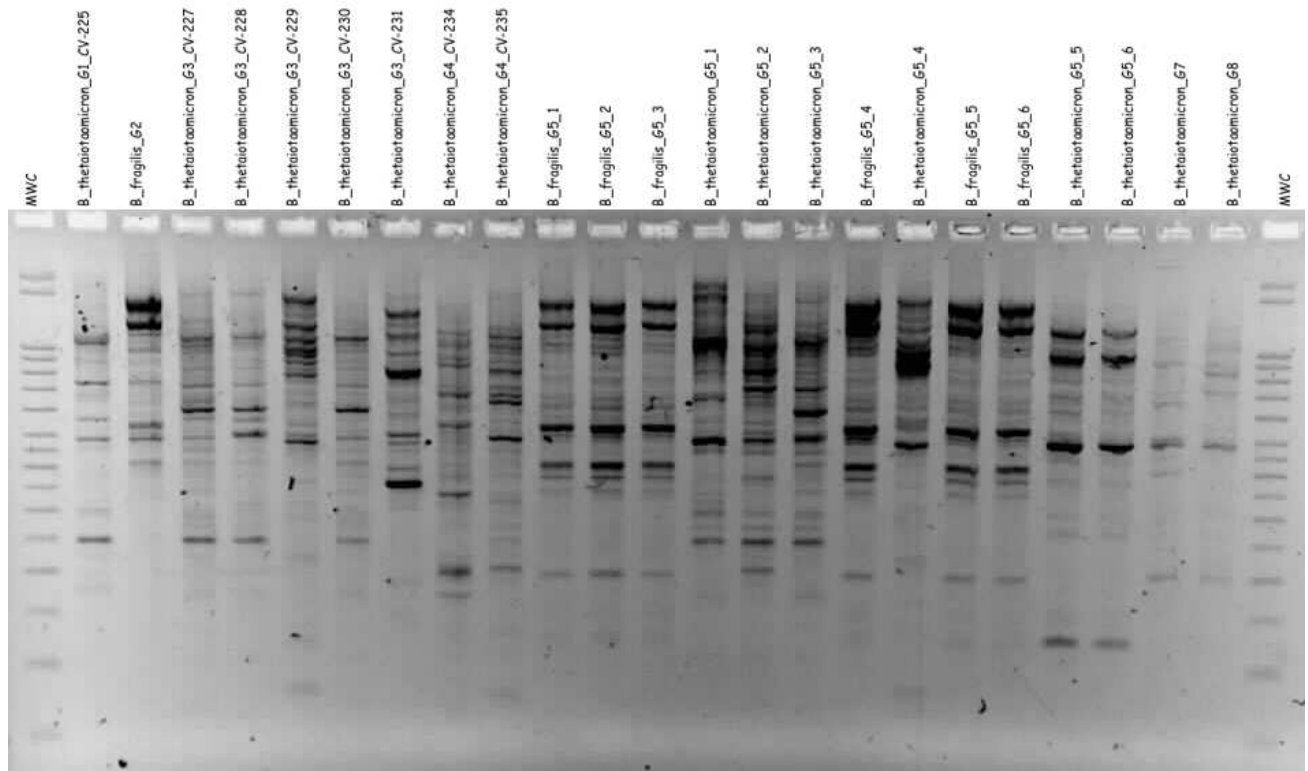
The PCR reaction was composed of 2 µl purified DNA, diluted 1:10, 2.5 µl of each primer (final concentration 2 pmol/µl), 25 µl of AmpliTaq Gold PCR Master Mix (Applied Biosystem) and 18 µl of deionised water. The REP-PCR reaction was performed using a GeneAmp PCR System 9700 thermocycler (PE, Biosystems, Warrington, UK). The DNA amplification conditions were: 95°C (5 min); 30 cycles of denaturing at 95°C (1 min), annealing at 42°C (1 min), and extension at 68°C (8 min); and a final extension step at 68°C (10 min). The reaction was maintained at 4°C until use. The REP-PCR fragments obtained were examined by DNA electrophoresis, using 1% agarose gel (Sigma-Aldrich Química SA), and applying an electric field of 150 V during 60 minutes. The size and the intensity of the bands obtained was analysed using the ChemiGenius (Syngene, Cambridge, UK) for the electronic capture of the electrophoretic profiles, and the GENE TOOLS software (Syngene). Dendrograms and similarity arrays were generated using the Manhattan distance (Kaufmann and Rousseeuw, 1990) of electrophoregrams.

## RESULTS

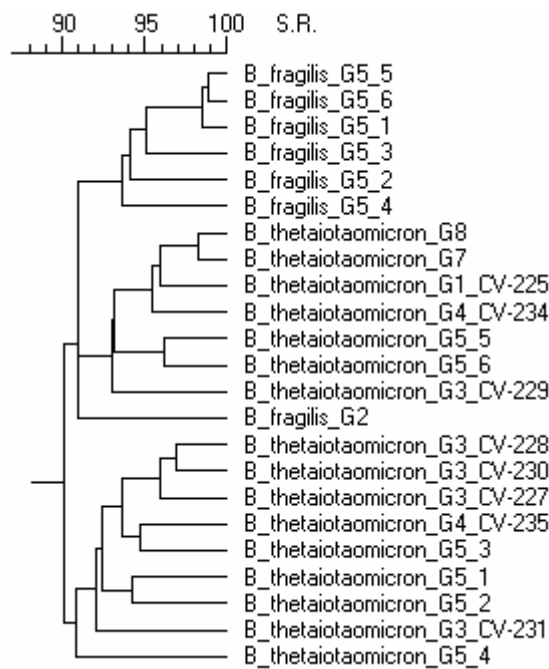
In the Figure 1 it can be seen the electrophoretic profiles obtained by REP-PCR amplification of DNAs from 23 strains of *Bacteroides* spp. isolated from the intestinal contents of rabbits.

In Figure 2 is represented the dendrogram constructed from the REP-PCR profiles of *Bacteroides* strains examined in the gel of Figure 1.

Different clusters are observed for *B. fragilis* and *B. thetaiotaomicron* strains, clusters that could represent genetic relationship between strains.



**Figure 1:** REP-PCR profile for different *Bacteroides* spp. strains isolated from rabbits



**Figure 2:** Dendrogram obtained after REP-PCR profile analysis

### CONCLUSIONS

Comparison of electrophoretic REP-PCR profiles of *Bacteroides* strains isolated from intestinal contents of rabbits should be an easy method to analyse the similarity/dissimilarity degree between strains. Because “\_Gnumber”, in the name of the strains, represents the number of farm, for *B.*

*thetaitaomicron*, different strains were isolated from the same farm (*i.e.*: B\_thetaitaomicron\_G5\_5 and B\_thetaitaomicron\_G5\_3) and similar strains were isolated from different farms (*i.e.*: B\_thetaitaomicron\_G8 and B\_thetaitaomicron\_G7). For *B. fragilis* two different strains were isolated from two different farms (six similar strains in farm 5 [B\_fragilis\_G5\_n] and one singular strain in farm 2 [B\_fragilis\_G2]).

Clearly different strains, and others selected using the same method, will be used for additional *in vitro* and *in vivo* studies that could be useful to improve the intestinal health of rabbits.

## REFERENCES

- Brubaker J.O., Li Q., Tzianabos A.O., Kasper D.L., Finberg R.W. 1999. Mitogenic activity of purified capsular polysaccharide A from *Bacteroides fragilis*: differential stimulatory effect on mouse and rat lymphocytes in vitro. *J. Immunol.*, 162, 2235–2242.
- Bry L., Falk P.G., Midtvedt T., Gordon J.I. 1996. A model of host-microbial interactions in an Open Mammalian Ecosystem. *Science*, 273, 1380-1383.
- Fuller R. 1989. Probiotics in man and animals – A Review. *J. Appl. Bacteriol.*, 66, 365-378.
- Isolauri E., Joensuu J., Suomalainen H., Luomala M., Vesikar T. 1995. Improved immunogenicity of oral D x RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. *Vaccine*, 13, 310-312.
- Kovács M., Szendrő Zs., Milisits G., Bóta B., Bíró-Németh E., Radnai I., Pósa R., Bónai A., Kovács F., Horn P. 2006. Effect of nursing methods and faeces consumption on the development of the bacteroides, lactobacillus and coliform flora in the caecum of the newborn rabbits. *Reprod. Nutr. Dev.*, 46, 205–210.
- Lanning D., Zhu X., Zhai S.K., Knight L. 2000. Development of the antibody repertoire in rabbit: gut-associated lymphoid tissue, microbes, and selection. *Immunol. Rev.*, 175, 214-228.
- Lessard M., Brisson G.J. 1987. Effect of a *Lactobacillus* fermentation product on growth, immune response and fecal enzyme activity in weaned pigs. *Can. J. Anim. Sci.*, 67, 509-516.
- Mazmanian S.K., Liu C.H., Tzianabos A.O., Kasper D.L. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*, 122, 107–118.
- Rhee K.J., Sethupathi P., Driks A., Lanning D.K., Knight K.L. 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J. Immunol.*, 172, 1118–1124.
- Rolfe R.D. 2000. The Role of Probiotic Cultures in the Control of Gastrointestinal Health. *J. Nutr.*, 130, 396S–402S.
- Savage D.C. 1980. Impact of antimicrobials on the microbial ecology of the gut. *In: The effects on human health of subtherapeutic use of antimicrobials in animal feeds, National Academy of Sciences*, pp. 130-157.
- Yanabe M., Shibuya M., Gonda T., Asai H., Tanaka T., Narita T., Sudo K., Itoh K. 1999. Establishment of Specific Pathogen-Free Rabbit Colonies with Limited-Flora Rabbits Associated with Conventional Rabbit Flora, and Monitoring of Their Cecal Flora. *Exp. Anim.*, 48, 101-106.