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RECENT ADVANCES IN ERE IN GROWING RABBITS
(Invited paper)...

Full text of the communication

RECENT ADVANCES IN «ERE» IN GROWING RABBITS

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ABSTRACT

During the last years the IRTA-CReSA and ETSIA-Madrid teams, together other European groups, especially from France and Belgium, have demonstrated the role of intestinal microbiota imbalance (specific dysbiosis) in the pathogenesis of Epizootic Rabbit Enteropathy (ERE). Additionally, these teams have hypothesized the role of different bacterial components (*Clostridium perfringens*, *Bacteroides* spp., *Akkermansia muciniphila*...), in combination with the concentration and the type of fiber, and the concentration of protein in the feed on the pathogenesis of ERE.

The experimental inoculation of rabbits with specific strains of *Clostridium perfringens* permitted us the experimental reproduction of ERE symptomatology (caecal impaction) with a mortality rate of 45%. The comparative analysis of the ERE-related and ERE-not-related *Clostridium perfringens* strains detected some changes in the proteins profile of the bacterial culture supernatant, singularly proteins with molecular mass of 189587 Da and 241521 Da, and the identification of a plasmid with more than 65 kbp.

In addition to the aim implicit in the above paragraphs –the experimental reproduction of ERE–, our team has been interested in the development of new ways to control the negative effects of ERE, especially mortality. Inoculating different *Bacteroides* spp. to naturally ERE infected rabbits we obtained a significant reduction on mortality when compared with non-treated animals. The additional studies conducted with these *Bacteroides* strains allowed us to infer positive effects on different immunological functions of rabbits.

The design of new molecular tools that could help in the diagnostic of ERE, and the development of vaccines and probiotics tools that could help to the prevention of ERE and the reduction of the use of antimicrobials in rabbit farms, are the principal objectives of a new research project funded by INIA and INTERCUN in our country.

Key words: Epizootic rabbit enteropathy, ERE, intestinal microbiota, clostridium perfringens, bacteroides spp, probiotics.

INTRODUCTION

The physiology of a complex macro-organism, like rabbit, is the association of their own physiology and the physiology of a large number of microorganisms that live associated to them, mostly at the internal mucous membranes. These microorganisms are the endogenous microbiota, and reach its maximum expression in the digestive system, with the intestinal microbiota. From birth to death, the host is colonized by a vast, complex and dynamic consortium of microorganisms (Tuohy *et al.*, 2003). The relationship between the microbiota and the host can be from commensal to pathogenic, depending on the strains of microorganisms, the animal physiological conditions, and host tolerance reduction to certain microbiota's microorganisms (Hooper and Gordon, 2001), which may cause chronic intestinal inflammation followed by significant metabolic alterations (Pakandl, 2009)

Under normal conditions, during the first weeks of life significant changes of intestinal microbiota of rabbits are observed with an initially higher amount of *Bacteroides* spp., which decreases around weaning, and an initially lower amount of *Firmicutes* that increases after weaning. In animals with ERE, the decrease of

Bacteroides spp. produced after weaning is not observed, and one of the pathognomonic microbiota profile in animals with ERE is the increase of *Bacteroides* population (Pérez de Rozas *et al.*, 2005).

In this multifactorial disease, *Clostridium perfringens* plays a principal role in the pathogenesis of ERE (Dewree *et al.*, 2003; Le Normand *et al.*, 2003; Nicodemus *et al.*, 2004; Marlier *et al.*, 2006; Huybens *et al.*, 2009; Romero *et al.*, 2009). The comparative studies conducted by our team with a *Clostridium perfringens* strain that induce caecal impaction and high mortality, and other *Clostridium perfringens* strains that don't produce caecal impaction or mortality resulted that the principal difference between them is related with the production of soluble proteins with mucinase activity by ERE-related *Clostridium perfringens* strain. Additionally, the mucinase activity has been observed in other bacteria that are over-represented in animals with ERE (*Bacteroides thetaiotaomicron* or *Akkermansia muciniphila*). This over-representation of some intestinal microbiota species observed by our team (Pérez de Rozas *et al.*, 2005; Delgado *et al.*, 2012; Pérez de Rozas *et al.*, 2013) has been also observed by other groups (Monteils *et al.*, 2008; Combes *et al.*, 2011; Combes *et al.*, 2013; Huybens *et al.*, 2013; Bäuerl *et al.*, 2014).

Antimicrobials have been the principal instrument to control the clinical signs of ERE, including mortality, but the interest to reduce the antibiotic use in livestock has increased the researches on alternative measures like the increase of fiber and the reduction of protein amount in the feed (Garcia *et al.*, 2005; Gomez-Conde *et al.*, 2007), the restriction of feed (Martignon, 2010; Martignon *et al.*, 2010) or some bacterial species (Pérez de Rozas *et al.*, 2005). Related to the use of some bacterial species as probiotics, our team is working on the role of different *Bacteroides* spp strains in the control of ERE, working line derived from the observation of a significant reduction of the mortality ratio in animals naturally infected with ERE and treated with specific strains of *Bacteroides fragilis* (Pérez de Rozas *et al.*, 2005).

MATERIALS AND METHODS

Animals and experimental design
Samples for microbiota profile in rabbits with different ages

At ETSIA-Madrid facilities, conventional rabbits (not-SPF) were fed with four different diets (two types of fibre with omega-3 or omega-6 fatty acids supplementation) from weaning until sacrifice. Sequential euthanasia was conducted when selected rabbits (four by treatment and period) had 25, 39 or 70 days of life. Ileal, caecal, and mesenteric lymph nodes samples were collected in Eppendorf vials with 1 mL of alcohol (to avoid changes in microbiota) and were stored at 4° C until processing.

Samples for gut microbiota profile in rabbits with or without ERE signs

Caecal samples from rabbits with or without ERE signs from five field farms were collected in Eppendorf vials with 1 mL of alcohol and were stored at 4° C until processing. The animals had 28-30 days of life (just after weaning) or older (slaughterhouse age with 60-75 days of life). *Effect of Bacteroides spp. strains inoculation in kits* Ten young rabbit per female from 10 pregnant females were used to analyze the *in vivo* effect of some *Bacteroides* spp. strains. At 8 days postpartum *Bacteroides* inocula were directly injected into the stomach of rabbits, via trans-abdominal wall. Each rabbit received 0.5 mL of the appropriate bacterial suspension adjusted to 107 CFU/mL.

All the kits of two females were inoculated with CV-0183 (*Bacteroides dorei*) strain, the kits of the two females in the second group were inoculated with CV-0293 (*Bacteroides fragilis*) strain, the kits of two females in the third group were inoculated with CV-0315 strain (*B. fragilis*), and the kits of two females in the fourth group were inoculated CV-0607 strain (*Bacteroides acidifaciens*). The kits of the two does in the fifth group were inoculated only with Buffered Peptone Water (BPW) to constitute the negative control group.

At the end of the lactation period (28 days), five kits per female were randomly euthanized to analyze and sampling. From each rabbit the following samples were collected: (a) mucosal scrapings in RNA later for RNA extraction (to study immunological markers); (b) mucosal scrapings in alcohol for DNA extraction (to

study the variable part of immunoglobulins at DNA level (VDJ segment); (c) ileal content in alcohol for DNA extraction (to study microbiota and to make bacterial counts); and (d) caecum content from ileo-caecal valve in alcohol to extract DNA (to study microbiota and to make *Bacteroides* spp. quantification).

Study of intestinal mucosa immunological markers

The mRNA expression of γIFN, IL-2, IL-10, TNF, TLR2, MCH-I, MCH-II and the GAPDH as housekeeping gene were quantified by QRT-PCR performed on a 7500-Fast real-time PCR equipment (Applied Biosystems, Cal., USA) using the following temperature-time profile: one cycle of 50ºC for 30 min, 95ºC for 15 min, and 45 cycles of 94ºC for 30 s, 60ºC annealing temperature for 30 s followed by 72ºC for 30 s. The primers used are in Table 1. Quantitec kit SYBR-Green QRT-PCR kit (Qiagen) was used, following the manufacturer's instructions for the amplification of mRNA.

Table 1: Primers used for the evaluation of mRNA expression degree of immunological markers.

Primer	Sequence (3'-5')	Reference
Rab_GAPDH-F	TGACGACATCAAGAAGGTGGTG	Schnupf and Sansonetti, 2012
Rab_GAPDH-R	GAAGGTGGAGGAGTGGGTGTC	
Rab_GIFN-F	TGCCAGGACACACTAACCAGAG	Schnupf and Sansonetti, 2012
Rab_GIFN-R	TGTCACTCTCCTCTTTCCAATTCC	
Rab_IL2-F	GCCCAAGAAGGTCACAGA	Schnupf and Sansonetti, 2012, mod I.Badiola
Rab_IL2-R	GATTCTCTGGTATTTCCCCC	
Rab_IL-10-F	GAGAACCACAGTCCAGCCAT	Godomes *et al.*, 2007
Rab_IL-10-R	CATGGCTTTGTAGACGCCTT	
Rab_TNFa-F	CTGCACTTCAGGGTGATCG	Schnupf and Sansonetti, 2012
Rab_TNFa-R	CTACGTGGGCTAGAGGCT	
Rab_TLR2-F	GAGAACCACAGTCCAGCCAT	Kajikawa *et al.*, 2005
Rab_TLR2-R	CATGGCTTTGTAGACGCCTT	
Rab_MHCI-F	ACCAGACCCAGGACACGGA	This study
Rab_MHCI-R	TGTATCTCTGCTCCTCCCCA	
Rab_MHCII-F	TGGAACAGCCAGAAGGAC	This study
Rab_MHCII-R	TCACAGAGCAGACCAGGAG	

The method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) was used to quantify and compare the expression degree of immunological markers. Applying this method, in a first step, the expression degree of the different genes of each sample is relativized to the expression level of the GAPDH gene from the same sample; and in a second step, the degree of gene expression is relativized to the average of the expression degree of the same gene in the animals from the negative control group.

***Bacteroides* spp. quantification in gut contents**

For the *Bacteroides* quantification we used a standard curve obtained by extracting the DNA, suspensions with different concentrations (108 to 102) of *Bacteroides* spp., using the QIAmp DNA Stool Mini kit (Qiagen). The elements of the standard curve of *Bacteroides* were included in each of the plates used for the study. ABI-Prism translates the Ct of each sample given bacterial concentration C*t* values of the standard curve. The primers used for Q-PCR were Bac-F 5'- CTATGGGTYGTAAACTTCTT-3' and Bac-R 5'-CGCTCCCTTTAAACCCAATAAA-3'. QRT-PCR was performed on a 7500-Fast real-time PCR equipment (Applied Biosystems, Cal., USA).

Study of the diversity of immunoglobulin genes

DNA from mucosal scrapings of the caecal appendix, fixed in alcohol and kept at 4 ° C until processing, was extracted using the DNeasy Blood & Tissue Kit (Qiagen). The primers used for the amplification step were: VDJ-Rabbit-F 3'-ACCCTGGTCACCRTCTCYTCA-5' and VDJ-Rabbit-R 3'-TGARGAGACGGTGACCAGGGT-5'. The amplicons were purified by DNA electrophoresis and extracted from the gel using the Nucleo Spin kit Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG).

The purified DNA-VDJ amplicons were sent to the CRAG (Centre for Research in Agricultural Genomics, UAB, Barcelona, Spain) for sequencing by Ion Torrent system. Qiime tools were used for the analysis of DNA sequences.

Microbiota profiles of ileal, caecal and mesenteric lymph node samples

Deep sequencing

DNA extraction was made with the QIAmp DNA stool mini kit (Qiagen) using 400 mg of the original sample and following the manufacturer's instructions, with some modifications: (a) the recommended lysis temperature was increased to 90ºC; (b) an incubation step with lysozyme was added (10 mg / mL, 37ºC, 30 minutes) in order to improve the breakdown of certain bacterial populations (Gram-positive); (c) The DNA obtained was stabilized by the addition of 4 μL of a 40 mg / mL BSA (bovine serum albumin, Sigma) and 2 μL of ribonuclease A (Sigma) and stored at -20°C until use.

The microbiota analyses were conducted by deep sequencing (IonTorrent system) and using a collection of 40 primers that amplify fragments of 16S rRNA gene resulting in 350-400 bp length. The forward primers are based on the primer R0512 5'- AGCAGCCGCGGTAATA- 3' fused to the specific IonTorrent sequence plus the sequences associated to bar codes. As reverse primer R0907-R 5'-CCTCTCTATGGGCAGTCGGTGATCCGTCWATTCMTTTGAGTTT-3' was used. The reaction was performed using a GeneAmp PCR System 9700 thermocycler (PE, Biosystems, Warrington, UK). The DNA amplification conditions were 94 ºC (4 min); 35 cycles of denaturation at 94 ºC (1 min), annealing at 45 ºC (1 min) with an increment of 0.1 ºC per cycle, extension at 72 ºC (1 min 15 s); and a final extension at 72 ºC (15 min).

Microbiome profiles were examined using different Qiime bioinformatics pipelines (alpha diversity, beta diversity, OUT distribution, Taxa Summary by factors, Principal components analysis …)

PCR-RFLP and randomly sequencing

The DNA was extracted and purified from 250 mg of ileum and cecum digesta by using the QIAmp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The lyse temperature was increased to 90 ° C and an additional incubation step with lysozyme was included (10mg/mL, 37ºC, 30 min) to improve the bacterial cell rupture. The bacterial mucosa associated DNA was extracted and purified from ileum and caecum mucosa using the QIAmp DNA Tissues and Blood Mini Kit (Qiagen, West Sussex, UK). In order to analyze the total bacteria in the digesta, a 580-bp fragment of 16S rRNA gene were amplified from DNA extracts by PCR using primers specific to conserved sequences flanking variable regions V3, V4 and V5: 5'-CTACGGGAGGCAGCAGT-3' (forward) and 5'- CCGTCWATTCMTTTGAGTTT-3' (reverse). The reaction was performed using a GeneAmp PCR System 9700 thermocycler (PE, Biosystems, Warrington, UK). The DNA amplification conditions were 94 ºC (4 min); 35 cycles of denaturation at 94 ºC (1 min), annealing at 45 ºC (1 min) with an increment of 0.1 ºC per cycle, extension at 72 ºC (1 min 15 s); and a final extension at 72 ºC (15 min). Following visual confirmation of PCR products with agarose gel electrophoresis, five independent enzymatic restrictions were carried out (AluI, RsaI, HpaII, Sau 3AI, CfoI; F. Hoffmann-LaRoche Ltd. Group, Basel, Switzerland). The digestions were performed as recommended by the manufacturer; with the appropriate restriction buffer at 37 ºC for 3 h. Fragments were separated using a 2% high- resolution agarose gel. Bands with significant differences between samples were cloned and randomly sequenced to identify the mayor bacterial species present in samples (Sanger *et al.*, 1977). Dendrogram with homology relationship between sequences were constructed to evaluate the bacterial species related or not related with ERE.

Analysis of the supernatant proteins and plasmid profiles of *Clostridium perfringens* strains

The supernatants of forty-eight hours culture of L-100 and L-101 strains of *Clostridium perfringens* under anaerobic conditions in Brain Heart Infusion Broth medium (BHI, Oxoid, Thermo Fisher Scientific Inc., UK) were collected to analyse the proteins' electrophoretic profile using precasting NuPAGE™ Novex™ 3-8% Tris-Acetate Protein Gels (Thermo Fisher Scientific Inc, UK) and Coomassie Blue (Bio-Rad Laboratories, Inc., UK) for staining.

For plasmid profile, overnight cultures of L-100 and L-101 strains of *Clostridium perfringens* under anaerobic conditions in Brain Heart Infusion Broth medium (BHI, Oxoid, Thermo Fisher Scientific Inc., UK) were centrifuged to collect bacterial bodies and plasmidic DNA was purified using NucleoBond® PC (Macherey-

Nagel GmbH & Co., KG). DNA electrophoresis using high-resolution agarose (Sigma-Aldrich, USA) gels at 0.5% was used to obtain the plasmids' profiles and the plasmid purification for subsequent steps in DNA sequencing.

PageRuler Broad Range Unstained Protein Ladder (Thermo Scientific, UK) was used as molecular weight marker for protein electrophoresis, and Lambda DNA Mixed Digest (Sigma-Aldrich, USA) was used as molecular weight marker for DNA electrophoresis.

RESULTS AND DISCUSSION

Changes on the intestinal microbiota profiles associated with the age of rabbit

In Table 2 and Figure 1 it can be seen the changes on intestinal microbiota profiles at different days of life.
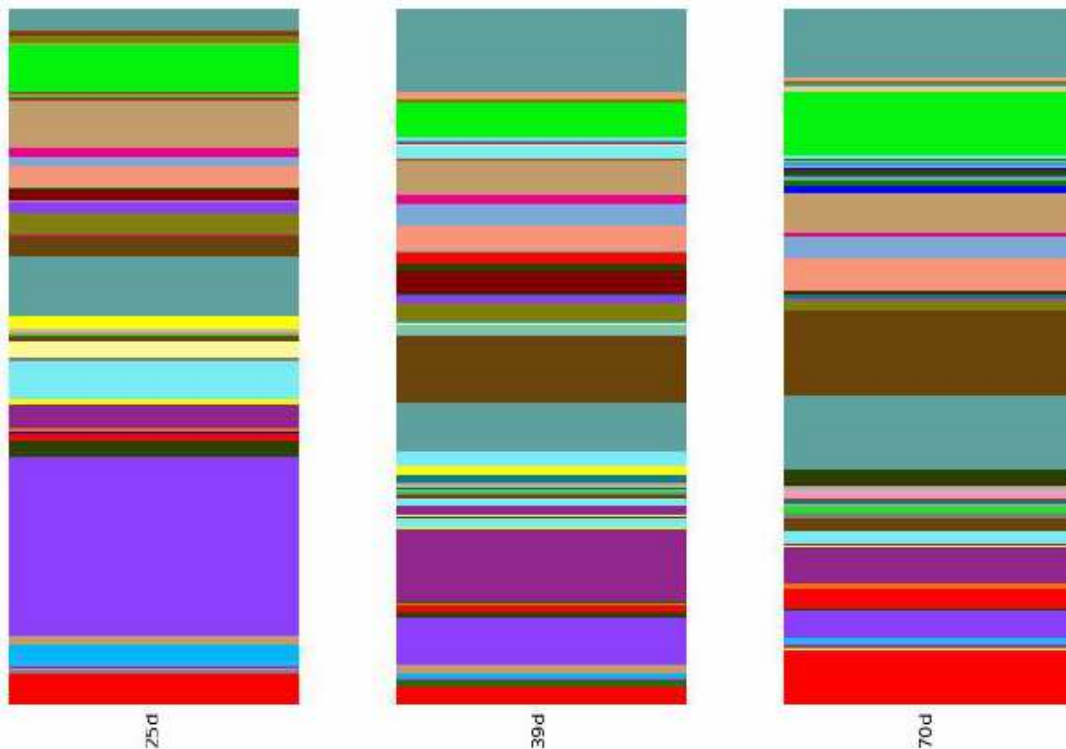

Figure 1: Microbiota profiles obtained from samples of rabbits with 25, 39 or 70 days old.

The genus *Bacteroides* was the most frequent OTU (Operational Taxonomic Unit) in the animals with 25 days of life, representing 25.8% of all sequences. This OTU continuously decreased to 6.9% on day 39 of life, and to 3.8% on day 70 of life. This continuous reduction of genus *Bacteroides* in older animals seems to be correlated with physiological conditions, but *Bacteroides* genus is characteristically increased in animals with ERE (Pérez de Rozas *et al.*, 2005).

The Mollicutes class was present at low level (3.2%) in animals with 25 days of life, but increases four times in the animals with 39 days of life (11.9%), and remains constant till 70 days of life (10.0%).
In the case of Clostridiales order, the global distributions of OTUs were similar into all the examined ages. Nevertheless, significant differences were observed at higher OTUs levels (family and genus). The highest *Clostridium* genus amount was observed on day 39 of life, the age with the highest incidence of ERE symptomatology. Other unidentified genera into Clostridiales order increased from 3.0% on day 25 of life to 9.4% on day 39 of life, and to 11.9% on day 70 of life.

Table 2: Percentage of OTUs with some of the analysed age (25, 39 or 70 days old rabbits) with relative abundance ≥0.5% (k: Kingdom, p: Phylum, c: Class, o: Order, f: Family, g: Genus).

Legend	Taxonomy (OTUs)	25d	39d	70d
	k__Bacteria;p__Other;c__Other;o__Other;f__Other;g__Other	4.4%	2.6%	7.8%
	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__ACK-M1;g__	0.0%	0.6%	0.0%
	p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__*Corynebacterium*	0.5%	0.0%	0.2%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other;Other	3.2%	0.6%	0.9%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__	1.2%	1.3%	0.2%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__*Bacteroides*	25.8%	6.9%	3.8%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__*Parabacteroides*	2.3%	0.6%	0.3%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;Other	1.1%	1.0%	2.7%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__*Alistipes*	0.3%	0.1%	0.8%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__	3.3%	10.6%	5.3%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];Other	0.6%	0.2%	0.0%
	p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];g__	5.5%	1.3%	0.1%
	p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__*Flavobacterium*	2.2%	0.3%	0.0%
	p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__	0.0%	1.2%	0.1%
	p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	0.0%	1.1%	1.8%
	p__Firmicutes;Other;Other;Other;Other	0.7%	0.4%	2.0%
	p__Firmicutes;c__Bacilli;Other;Other;Other	0.1%	0.4%	0.8%
	p__Firmicutes;c__Bacilli;o__Bacillales;Other;Other	0.2%	0.2%	0.8%
	p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__*Staphylococcus*	0.3%	0.3%	1.4%
	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__*Enterococcus*	0.0%	1.1%	0.1%
	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__*Lactobacillus*	0.0%	0.0%	2.2%
	p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__*Streptococcus*	1.8%	1.2%	0.0%
	p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__*Turicibacter*	0.0%	2.1%	0.0%
	p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	8.6%	7.0%	10.7%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	3.0%	9.4%	11.9%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__*Clostridium*	0.0%	1.4%	0.0%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	3.1%	2.8%	1.4%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	1.7%	1.0%	0.3%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Blautia*	0.1%	0.2%	0.5%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Clostridium*	1.5%	3.3%	0.2%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Coprococcus*	0.0%	1.0%	0.2%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__*Epulopiscium*	0.0%	1.5%	0.0%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	3.1%	3.5%	4.7%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	1.5%	3.2%	3.2%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__*Oscillospira*	1.2%	1.3%	0.4%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__*Ruminococcus*	6.8%	4.9%	5.7%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__*Anaerococcus*	0.0%	0.0%	1.0%
	p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__*Peptoniphilus*	0.0%	0.0%	0.9%
	p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	0.0%	1.9%	0.0%
	p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;Other	0.0%	0.0%	0.8%
	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other	6.8%	5.0%	9.3%
	p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__*Delftia*	0.0%	0.0%	0.8%
	p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__	1.0%	0.3%	0.6%
	p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__*Escherichia*	0.1%	0.9%	0.0%
	p__Tenericutes;c__Mollicutes;o__RF39;f__;g__	3.2%	11.9%	10.0%

The Mollicutes class was present at low level (3.2%) in animals with 25 days of life, but increases four times in the animals with 39 days of life (11.9%), and remains constant till 70 days of life (10.0%).
In the case of Clostridiales order, the global distributions of OTUs were similar into all the examined ages. Nevertheless, significant differences were observed at higher OTUs levels (family and genus). The highest *Clostridium* genus amount was observed on day 39 of life, the age with the highest incidence of ERE symptomatology. Other unidentified genera into Clostridiales order increased from 3.0% on day 25 of life to 9.4% on day 39 of life, and to 11.9% on day 70 of life.

Detection of DNA of bacterial species associated with animals that present signs of ERE

The analysis of randomly cloned and sequenced DNA fragments (Figure 2) resulted in significant relationship between ERE and *Clostridium perfringens* (data not included in Figure 2), *Bacteroides thetaiotaomicron*, *B. fragilis* and *Akkermansia muciniphila*.

Using the pyrosequencing technique, the IATA team has found that animals with ERE showed a marked dysbiosis, with a decrease of Alistipes and Ruminococcus *species, accompanied by a significant increase of the genera Bacteriodes, Akkermansia, Rikenella*, *Clostridium* and gamma- Proteobacteria, specifically the genus *Escherichia* (Bäuerl *et al.*, 2014).

Unfortunately, the analysis of positive correlation between ERE symptomatology and different genus or species has been insufficient to confirm the pathogenesis of ERE. Nevertheless, our experience with specific strains of *Bacteroides fragilis*, which significantly reduce the mortality of animals natural ERE, and with specific strains of *Clostridium perfringens*, which induce caecal impaction and mortality rates of 45% on experimentally infected animals (Pérez de Rozas *et al.*, 2005) could result in the necessity to talk about bacterial strains rather than genera or species.

At present, our team is centred on this type of strains, the first as possible probiotic and the second as one of the bacteria involved in the pathogenesis of ERE.
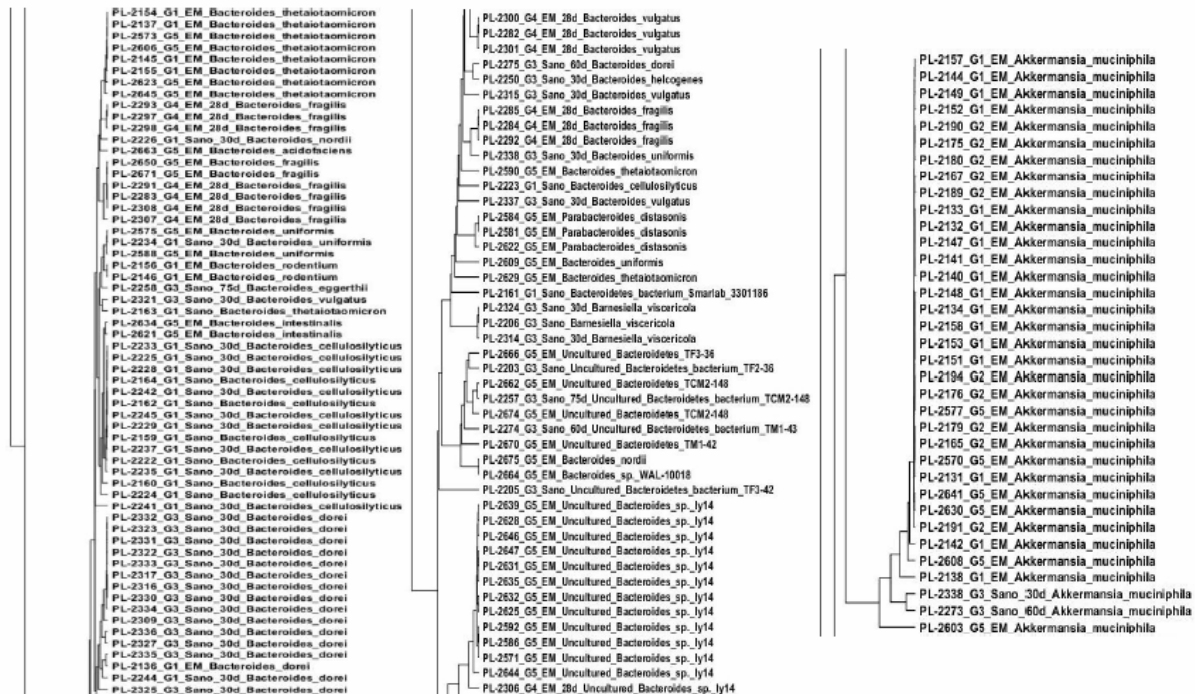

Figure 2: Relationship between amplicons randomly obtained from gut samples of rabbits with (EM) or without (Sano) signs of ERE at productive farms. The animals had 28-30 days of life (just after weaning) or older (slaughterhouse age with 60-75 days of life).

Identification of supernatant proteins and plasmid profiles of *Clostridium perfringens* strains involved in ER

The electrophoretic profile of supernatant proteins of different *Clostridium perfringens* strains (Figure 3-A, L-101) associated with the reproduction of ERE symptomatology permitted the identification of high molecular weight proteins that are not present in *Clostridium perfringens* strains (Figure 3-A, L100) without ERE association.

The electrophoretic profiles of plasmids isolated from the *Clostridium perfringens* strain (Figure 3-B, L-101) associated with the reproduction of ERE symptomatology and the *Clostridium perfringens* strains (Figure 3-B, L100) not associated with ERE, together different plasmid profiles of conjugants between L-100 and L-101 strains are in Figure 3-B. These profiles show a high molecular weight plasmid that is present in the L-101

strain, the strain able to reproduce the ERE symptomatology, together some other conjugant strains and which is absence in the L-100 strain, a non-ERE *Clostridium perfringens* strain.
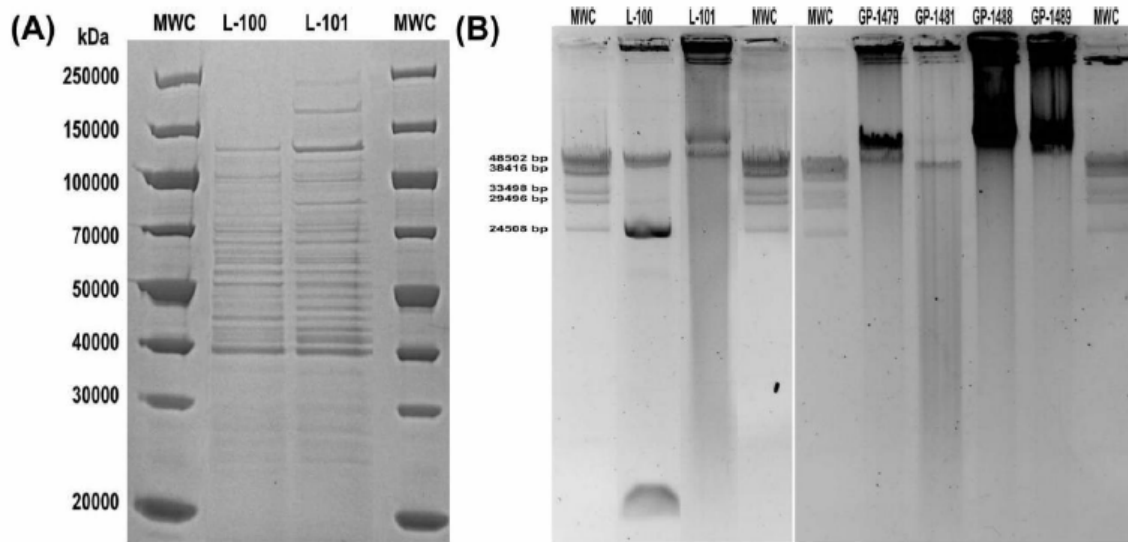

Figure 3: Supernatant protein profiles (A) and plasmid profiles (B) of Clostridium perfringens strains involved in ERE symptomatology (L101) or not involved in the reproduction of ERE symptomatology (L-100), together plasmid profiles of different conjudants between L-100 and L-101 strains (Gp-nnnn). MWC=molecular weight controls.

The MALDI-TOF UltrafleXtreme (Bruker Daltonics) analysis of the purified proteins, carried out in SePBioEs (Universitat Autònoma de Barcelona, 08193-Bellaterra, Spain), resulted in high homology with Peptidase M60-like family proteins (Figure 4). This family of peptidases contains a zinc metallopeptidase motif (HEXXHX(8,28)E) and possesses mucinase activity. These proteins have 1687 residues and a molecular weight of 189587 Da (Figure 4-A) and 2142 residues and a molecular weight of 241521 Da (Figure 4-B).
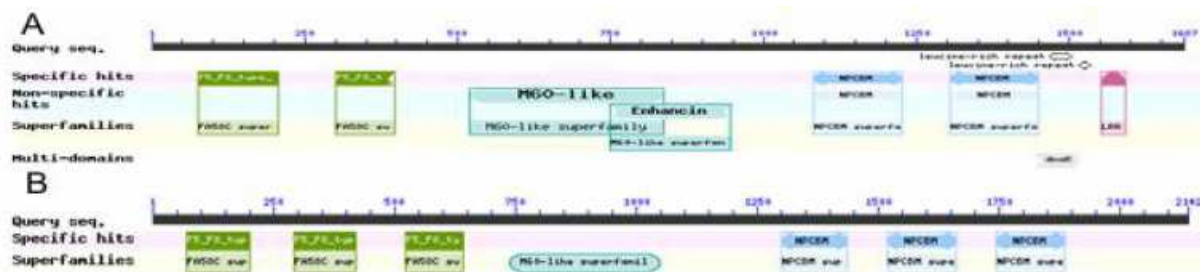

Figure 4: Schematic structure of high molecular weight proteins that are present in the supernatant of different *Clostridium perfringens* strains associated with the reproduction of ERE symptomatology.

At present, we are sequencing the plasmid located into the *Clostridium perfringens* strains involved in ERE. We are examining if the mucinase- type protein genes are located into this plasmid.

Effect of *Bacteroides* spp. strains inoculation in kits

In the Figure 5 the significant reduction of the amount of *Bacteroides* spp can be observed when the kits were inoculated with the *Bacteroides dorei* strain CV-0183, the *Bacteroides fragilis* strain CV-0293, and the *Bacteroides fragilis* strain CV-0315, when compared with notinoculated animals.

Examining together this significant reduction on the amount of *Bacteroides* spp. produced by specific strains together the increase of *Bacteroides* spp. in animals with ERE (Pérez de Rozas et al., 2005), the positive effect of the strain CV-315 on ERE mortality (50% mortality rate reduction) could be correlated with this effect of reduction on the amount of *Bacteroides* spp.
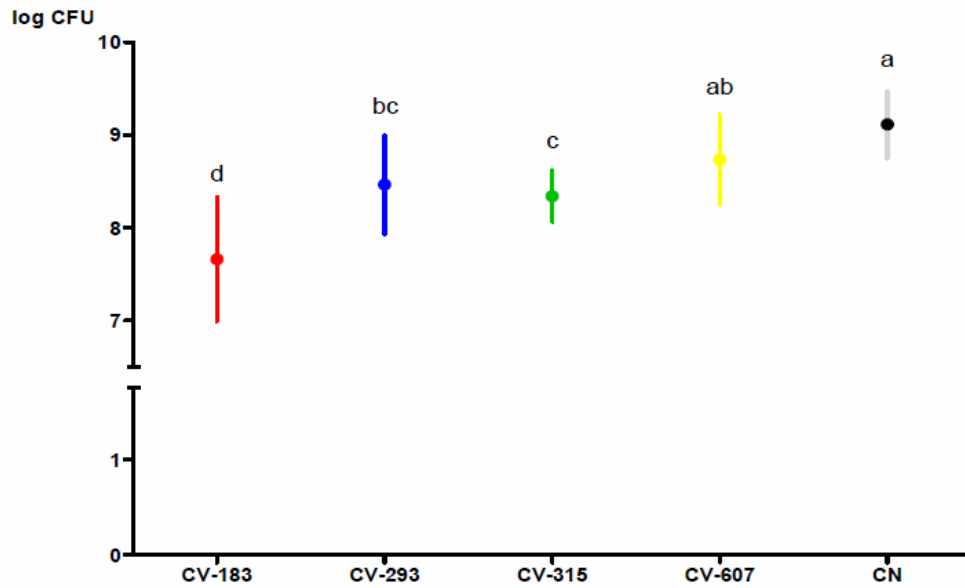
Figure 5: Mean ± SD of *Bacteroides* spp. examined by quantitative PCR of the caecal contents of kits inoculated with different strains of *Bacteroides* spp. (*Bacteroides dorei* strain CV-0183, *Bacteroides fragilis* strain CV-0293, *Bacteroides fragilis* strain CV-0315 and *Bacteroides acidifaciens* strain CV-0607) or inoculated with buffered peptone water (CN). Different letters on data bars represent statistically significant differences between the strains of *Bacteroides* spp. by Tukey test (*P* <0.05).

At present, we are working with CV-0183, CV-293, and CV-315 strains to analyze their possible probiotic effect on animals with ERE.

Table 3: Geometric mean ± standard deviation of different immunological markers (IFN-γ, IL-2, IL- 10, TNF-α, TLR 2 MHC I and MHC II) of the intestinal mucosa determined by quantitative PCR and the algorithm $2^{-\Delta\Delta Ct}$ of 10 rabbits per group (n) inoculated with different strains of *Bacteroides* spp (CV-0183, CV-0293, CV-0315 and CV-0607) or inoculated with BPW (CN). (In the last two columns statistical *P* of *Bacteroides* spp strains effect obtained by Kruskal-Wallis test and GLM analysis. Different letters represent statistically significant differences between the strains of *Bacteroides* spp., ns = no significant difference).

	Inocula					Kruskal-Wallis	GLM
	CV-183	CV-293	CV-315	CV-607	CN		
Num. animals	10	10	10	10	10		
IFN-γ	0,44±0,29[b]	0,37±0,35[b]	0,77±0,56[a]	0,66±0,79[ab]	1,00±0,60[a]	$P_{K\text{-}w}$=0,040	P_{GLM}=0,044
IL-2	0,830±0,64[ns]	0,893±0,56[ns]	0,81±0,53[ns]	1,19±0,71[ns]	1,00±0,40[ns]	$P_{K\text{-}w}$=0,721	P_{GLM}=0,640
IL-10	1,23±1,84[ns]	1,29±0,73[ns]	1,08±0,65[ns]	1,11±0,66[ns]	1,00±0,58[ns]	$P_{K\text{-}w}$=0,832	P_{GLM}=0,640
TNFα	0,55±0,56[bc]	0,56±0,35[c]	0,65±0,41[c]	1,78±1,02[a]	1,00±0,33[b]	$P_{K\text{-}w}$=0,023	P_{GLM}=0,001
TLR 2	0,85±0,50[ns]	1,11±0,52[ns]	0,76±0,47[ns]	1,41±0,56[ns]	1,00±1,03[ns]	$P_{K\text{-}w}$=0,369	P_{GLM}=0,344
MCH I	1,55±0,62[ab]	1,28±0,58[bc]	0,97±0,39[c]	1,65±0,57[ab]	1,00±0,31[c]	$P_{K\text{-}w}$=0,035	P_{GLM}=0,010
MCH II	2,44±0,94[a]	2,36±1,62[ab]	1,97±1,06[ab]	1,47±0,64[bc]	1,00±0,56[c]	$P_{K\text{-}w}$=0,015	P_{GLM}=0,008

Deepening on the analysis of the positive effects of specific *Bacteroides* spp. strains the analysis of intestinal mucosa immune-stimulation resulted in the data summarized in Table 3. In this table we can see that, using parametric (GLM) or non-parametric (Kruskal-Wallis) analysis, the expression degree of the genes IFN-γ, TNF-α, MHC I, and MHC II was significantly correlated with the *Bacteroides* spp. strains used.

Finally, the evaluation of the percentage of singular sequences (sequences without repetitions) in the VDJ region amplicons obtained from the DNA extracted from the appendix of the inoculated kits with different strains of *Bacteroides* spp, resulted in higher immunoglobulin diversity in the animals inoculated with the *Bacteroides dorei* strain CV-0183 and the *Bacteroides fragilis* strain CV-0315 (Table 4).

In general, *Bacteroides dorei* strain CV-0183, *Bacteroides fragilis* strain CV-0293, *Bacteroides fragilis* strain CV-0315 reduced the expression of the pro-inflammatory cytokines IFN-γ and TNF-α. Additionally, these

strains increased the expression degree of the gene MHC II, a surface antigen that is present in the antigen presenting cells (macrophages, dendritic cells, and under especial conditions in enterocytes). These effects could be positives on ERE reducing the inflammation at intestinal level and activating or stimulating the chemotaxis to macrophages and dendritic cells.

Table 4: Percentage of singular sequences of nucleotides (diversity) obtained by massive sequencing of the amplicons obtained by PCR using primers of the VDJ region of immunoglobulin genes in the mucosa of caecal appendix rabbits inoculated with the different strains of *Bacteroides* spp. (CV-0183, CV-0293, CV-0315 and CV-0607) or inoculated with BPW (CN). In the last column, the statistical effect of the inoculums by GLM analysis. Different letters indicate significant difference between inoculums.

	Inoculum Inóculo					
	CV-183	CV-293	CV-315	CV-607	CN	
	25,35%	22,99%	25,82%	22,95%	24,48%	
	25,63%	22,87%	25,37%	22,27%	24,39%	
	25,07%	22,52%	25,85%	22,67%	24,50%	
	25,94%	22,54%	26,33%	22,53%	24,37%	
	25,73%	23,10%	25,77%	22,53%	24,33%	
	24,66%	34,76%	26,07%	22,52%	22,77%	
	24,82%		25,93%	22,82%	22,69%	
	24,44%		26,19%	22,76%	23,02%	
	24,19%		26,04%	22,79%	23,09%	
	24,77%		25,78%	22,92%	23,22%	
N	10	6	10	10	10	
Average	25,06%[a]	24,80%[ab]	25,91%[a]	22,68%[b]	23,69%[b]	P_{GLM}=0,002

The proliferation and the initial diversification of rabbit's lymphocytes are produced between birth and weaning (Fortun-Lamothe and Boullier, 2007; Hanson and Lanning, 2008; Mage *et al.*, 2006), with the participation of the intestinal microbiota (Lanning *et al.*, 2000). The observed effect in this *in vivo* assay may represent that *Bacteroides dorei* strain CV-0183 and *Bacteroides fragilis* strain CV-0315, stimulate an increase in the initial repertoire of lymphocytes from rabbits during the first weeks of life, resulting in the increased variability of the VDJ fragment, and could represent a desirable property in potential probiotic strains.

CONCLUSIONS

Epizootic Rabbit Enteropathy is a high mortality disease of rabbits characterized by a specific intestinal disbiosis with significant increase of mucinase producing bacteria like *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and other *Bacteroides* spp., *Clostridium perfringens* and *Akkermansia muciniphila*.

Nevertheless, specific strains of different bacterial species could play a principal role in the pathogenesis of ERE, like specific strains of *Clostridium perfringens*, or in the control of the negative effects of ERE, like specific strains of *Bacteroides fragilis* or *Bacteroides dorei*.

We will continue our research on these two bacterial genera, the first to develop a future vaccine against ERE, and the second to develop a probiotic that could help to the control of ERE, reducing the use of antimicrobials, and improving the global health of rabbits.

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