

EFFECT OF COMPETITIVE EXCLUSION IN RABBITS USING AN AUTOCHTHONOUS PROBIOTICCUNHA S.*; MENDES Â.*; REGO D.*; MEIRELES D.*; FERNANDES R.†; CARVALHO A.*;
MARTINS DA COSTA P.**

*Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Laboratório de Microbiologia e Tecnologia Alimentar, 4050-313 Porto, Portugal.

†Ciências Químicas e das Biomoléculas, Escola Superior de Tecnologia de Saúde do Porto, Instituto Politécnico do Porto, 4400-330 VILA NOVA DE GAIA, Portugal.

‡Centro Interdisciplinar de Investigação Marinha e Ambiental (CIAMAR), Universidade do Porto, 4050-123 Porto, Portugal.

Abstract: Animal nutrition has been severely challenged by the ban on antimicrobials as growth promoters. This has fostered the study of alternative methods to avoid colonisation by pathogenic bacteria as well as to improve the growth of animals and feed conversion efficiency. These new options should not alter the normal intestinal microbiota, or affect it as little as possible. The use of probiotics, which are live microorganisms that beneficially affect the host by improving its intestinal microbial balance, can be seen as a promising way to achieve that goal. In this study, New Zealand White rabbits were fed diets containing an autochthonous probiotic of *Enterococcus* spp., with the strains Eal, Efal and EfaD, and *Escherichia coli*, with the strains ECI 1, ECI 2 and ECD, during a 25-d trial, to evaluate the impact of the probiotic on the faecal microbiota, including population dynamics and antimicrobial resistance profiles. A control group of rabbits, which was fed a diet containing a commonly used mixture of antimicrobials (colistin, oxytetracycline, and valnemulin), was also studied. To assess the colonisation ability of the mentioned probiotic, the faecal microbiota of the rabbits was characterised up to 10 d after the administration had ended. Isolates of enterococci and *E. coli* were studied for phylogenetic relationships using enterobacterial repetitive intergenic consensus (ERIC-PCR) and pulse-field gel electrophoresis (PFGE), respectively. Although partially affected by an unexpected clinical impairment suffered by the rabbits in the experimental group, our results showed the following. The difference between the growth rate of the animals treated with antimicrobials and those fed the probiotic was not statistically significant ($P > 0.05$). The competitive exclusion product was present in the faecal samples in a large proportion, but stopped being recovered by culture as soon as the administration ended and the housing conditions were changed. Multidrug-resistant strains of enterococci and *E. coli* were more commonly recovered from faecal samples of animals fed diets containing antimicrobials, than from rabbits fed diets with our probiotic formula. The use of *E. coli* probiotics to prevent infection by enteropathogenic strains must be carefully considered due to the possible occurrence of gastrointestinal signs. On the other hand, enterococci strains may be more effective, but lack the long-term colonisation ability.

Key Words: autochthonous probiotic, *Escherichia coli*, *Enterococcus* spp., rabbits.

INTRODUCTION

Antimicrobials were widely used for several decades in animal production to control bacterial infections and improve production indicators, in particular by boosting growth and/or feed conversion rates (Michelan *et al.*, 2002). However, the indiscriminate use of these drugs has generated a public health issue, as it promoted the development of multidrug-resistant bacterial strains, harbouring resistance genes potentially transferable to the human microbiota through the food chain (Salyers *et al.*, 2004; Mathur and Singh, 2005). This concern has led to the banning of antimicrobials as growth promoters in the European Union since 2006 (European Commission, 2001; European Commission, 2003).

Correspondence: P. Martins da Costa, pmcosta@icbas.up.pt. Received August 2016 - Accepted December 2016
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Paradoxically, from that date on, a substantial increase in the use of antimicrobials for therapeutic purposes has been reported (Casewell *et al.*, 2003). This rise was very likely due to the increase in the intensification of animal production systems, which leaves no space for disease outbreaks (da Costa *et al.*, 2013). This has encouraged research into alternative methods to reduce the use of antimicrobials and enhance the natural defence mechanisms of the animals.

Probiotics, a specific dietary supplement consisting of live microorganisms that can benefit the host by balancing the intestinal flora, have been widely used in poultry, swine and ruminant feed (La Ragione *et al.*, 2004; Philippeau *et al.*, 2010; Chu *et al.*, 2011). They are inherently associated with the concept of competitive exclusion (CE), which means the introduction of live and non-pathogenic microbial cultures into the intestinal tract of animals, outcompeting opportunistic pathogens for colonisation space and reducing the risk of enteric disease (Galyean and Eng, 1998; Schneitz, 2005). CE products may be composed of a single or several strains, and they are completely defined, partially defined or undefined microbial cultures (Cox and Chung, 2000).

Besides being metabolically active, probiotic bacteria must be able to survive in the gastrointestinal tract. Although this is ensured by many of the commercially available probiotic formulas, these bacterial strains are commonly eliminated as soon as the administration stops. In humans, this may be explained by the phenomenon of colonisation resistance, whereby the resident intestinal microbiota limits the access of allochthonous bacteria (Stecher and Hardt, 2011). Even autochthonous strains of the gut microbiota may not be shared between relatives, due to individual specificities that may disable the colonisation of the gastrointestinal tract (Walter, 2008).

Several studies focusing on the effectiveness of probiotics in rabbits have reported conflicting results (Gardiner *et al.*, 1999; Jin *et al.*, 2000; Jost and Bracher-Jakob, 2000; Falcão-e-Cunha *et al.*, 2007). Despite the vast amount of knowledge developed in recent decades regarding the action mechanism of probiotics in pigs and poultry, much research is still needed to better understand the application of these products in rabbits. These animals are monogastric herbivores and have a unique intestinal microbiota. The latest techniques in molecular biology revealed that the predominant phylum of the faecal microbiota is the *Firmicutes*, whereas the *Bacteroidetes*, previously considered the most abundant, represent only 4% of the 16S rDNA of faecal content (Combes *et al.*, 2011). Genera such as *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Escherichia* are considered to be in low density or even rare in the digestive microbiota of rabbits (Combes *et al.*, 2011; Eshar and Weese, 2014). However, feed supplementation with probiotics composed of such unusual bacterial inhabitants was already reported as being successful in increasing the faecal levels of certain beneficial intestinal bacteria in healthy adult rabbits (Benato *et al.*, 2014).

As established in Regulation (EC) 1831/2003 on additives for use in animal nutrition, the term “feed additives” includes “substances, microorganisms or preparations, other than feed material and premixes, which are intentionally added to feed or water.” Probiotics are considered live microbial feed additives and their use shall thus follow the rules set out in the Community legislation. In the particular case of autochthonous probiotics, although the legal background is not clear, they have already been applied successfully in animal nutrition research (Ridha and Azad, 2015; Idoui and Karam, 2016).

This study aimed to evaluate the colonisation ability and the zootechnical effects of an autochthonous probiotic composed of *Enterococcus* spp. and *Escherichia coli* in growing rabbits. *Enterococcus* spp. is a well-recognised genus of the caecal flora of healthy adult rabbits and commonly considered in probiotic formulas (Straw, 1988; Benato *et al.*, 2014). In contrast, *E. coli* is an unusual species of the rabbit gut, but it is hypothesised that providing the animals with *E. coli* strains recovered from healthy individuals’ intestinal microbiota may protect them from dysbacteriosis.

MATERIAL AND METHODS

Study design

This study was carried out at the Department of Veterinary Sciences of the Abel Salazar Institute for the Biomedical Sciences, University of Porto, Portugal.

Two groups of 6 New Zealand White rabbits were kept in pairs inside ventilated pens at a constant temperature (22°C) and humidity (60%) from 38 to 63 d of age. Both groups were fed a commercial diet *ad libitum*, but the

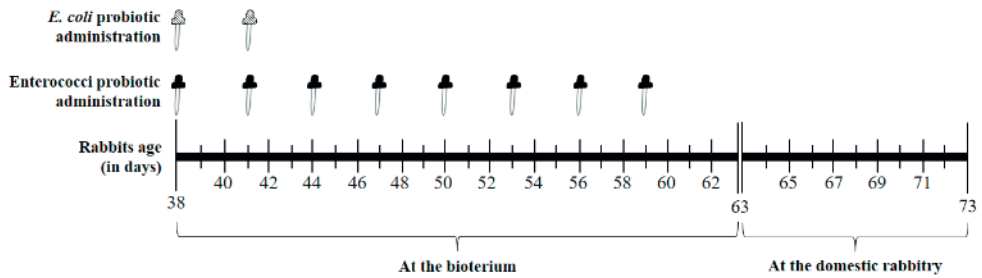


Figure 1: Timesheet of the probiotic administration according to the age of the enrolled rabbits.

control group (A) had its diet supplemented with antibiotics (colistin, oxytetracycline and valnemulin; EcoNor, 10%, 0.3 g/kg), while the P group rabbits were fed an autochthonous probiotic (1 mL/kg) of an *E. coli* and *Enterococcus* spp. suspension at 5.0 log CFU/mL (colony forming units per millilitre) every 72 h in replacement of those antimicrobial compounds in the diet. Administration of *E. coli* was performed only twice. Figure 1 shows a timesheet illustrating the oral administrations of the probiotic formula. All 12 animals were previously considered healthy after a physical examination and the occurrence of clinical signs was continuously monitored during the study period. Feed and water consumption were measured daily throughout the study. Live weight was recorded every 48 h. The ingredients and composition of the basal diet are shown in Table 1.

After the trial period of 25 d, both groups of animals were mixed and transported to a small domestic rabbitry, where they were fed the same diet but supplemented with fruits and vegetables. At the end of 10 d, faecal samples were microbiologically analysed to assess the persistence of the probiotic strains.

Probiotic preparation

The experimental probiotic was manufactured in our laboratory. We started with the selection of 4 enterococci and 3 *E. coli* strains from faeces of 2 different groups of rabbits: i) a group present at an industrial unit, the same unit from which the rabbits under trial came (*Enterococcus avium*, assigned strain Eal; *Enterococcus faecalis*, Efal; 2 *E. coli* strains, ECI 1 and ECI 2); and, ii) a group of rabbits raised at a domestic rabbitry (*Enterococcus faecalis*, EfaD; *Enterococcus faecium*, EfeD; *E. coli*, ECD). These bacterial isolates were selected using the same protocol described in more detail below on the microbiological characterisation of bacterial specimens collected from faeces of rabbits under trial. So, faeces collected from donors were suspended in buffered peptone water (1:9), homogenised, serially diluted (10-fold dilutions until 10⁻⁸) in saline broth and immediately plated and incubated in Plate Count Agar (PCA, 30°C for 72 h), Slanetz-Bartley agar (SB, 37°C for 48 h) and Tryptone Bile X-Glucuronide medium (TBX, 37°C for 24 h). The selection of four enterococci and 3 *E. coli* strains to be used as autochthonous probiotic bacteria was based on relative counts and differential colony morphologies in SB and TBX. The isolates were also submitted to Gram staining and molecular characterisation, using multiplex PCR, as described below. Pure colonies were then transferred to Mueller-Hinton agar (MH) and incubated for 18 h at 37°C. After that, fresh colonies were suspended in sterile water, adjusting its concentration to 5.0 log CFU/mL, which corresponds to a spectrophotometric absorbance between 0.01 and 0.02 at 600 nm followed by a 1:100 dilution in sterile water. All the strains were set up to the same concentration, and the duplicates kept in glycerol at -20°C and Tryptic Soya Agar (TSA) slants for future use.

Table 1: Main ingredients and composition of the basal diet.

Ingredients	Composition (%)
Wheat	30
Sunflower	19
Lucerne	15
Beet pulp	15
Citrus pulp	8
Palm kernel	5
Molasses	3
Straw	2

Microbiological analysis

Faecal samples were collected every 72 h, weighed in sterilised bags and suspended in buffered peptone water (1:9) and homogenised for 2 min in a Stomacher 400® (*Circulator*). The suspension was immediately submitted to 10-fold serial dilutions (until 10⁻⁸) in sterile saline broth. From appropriate dilutions, 1.0 mL was plated using the pour plate technique in Plate Count Agar (PCA, 30°C for 72 h), Slanetz-Bartley agar (SB, 37°C for 48 h) and Tryptone Bile X-Glucuronide medium (TBX, 37°C for 24 h) for the enumeration of total viable cells, enterococci and *E. coli*, respectively. All reagents were obtained from Biokar Diagnostics (Beauvais, France), except the SB and MH media (OXOID, Basingstoke, United Kingdom).

Antimicrobial susceptibility testing

All isolates were evaluated for their antimicrobial resistance susceptibility, according to CLSI guidelines (2007). Enterococci isolates were tested for the following antimicrobials: ampicillin (AMP, 10 µg), gentamicin (CN, 120 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), nitrofurantoin (F, 300 µg), vancomycin (VAN, 30 µg), teicoplanin (TEC, 30 µg), erythromycin (E, 15 µg), azithromycin (AZM, 15 µg), rifampicin (RD, 5 µg) and quinupristin/dalfopristin (QD, 15 µg). For *E. coli* isolates, the antimicrobials used were the following: cephalothin (KF, 30 µg), cefoxitin (FOX, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), amoxicillin/clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), tobramycin (TOB, 10 µg), amikacin (AK, 30 µg), streptomycin (S, 10 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), nitrofurantoin (F, 300 µg) and sulfamethoxazole/trimethoprim (SXT, 25 µg).

Molecular characterisation

Determination of *E. coli* phylogroups

The *E. coli* strains administered in the probiotic formula and those recovered from faecal samples were subjected to a DNA extraction using the InstaGene Matrix (BioRad, California, USA), following the manufacturer's instructions, and to a further phylogenetic characterisation according to the multiplex PCR technique described by Clermont *et al.* (2000). The polymerase chain reaction (PCR) was performed under the following conditions: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; final extension step of 7 min at 72°C. Each reaction was performed in the presence of the following 3 primer pairs: ChuaA.1 (5'-GACGAACCAACGGTCAGGAT-3') and ChuaA.2 (5'-TGCCGCCAGTACCAAAGACA-3'); YjaA.1 (5'-TGAAGTGCAGGAGACGCTG-3') and YjaA.2 (5'-ATGGAGAATGCGTTCCTCAAC-3'); TspE4C2.1 (5'-GAGTAATGTCGGGGCATTCA-3') and TspE4C2.2 (5'-CGCGCCAACAAAGTATTACG-3'). The amplified products were further detected by electrophoresis at 150 mV for 45 min on a 1.5% agarose gel (Seakem Agarose, Lonza, Rockland, USA) in Tris/Borate/EDTA buffer 1×, containing 0.5 µg/mL of ethidium bromide. A *GeneRuler 1kb plus DNA Ladder* (0.1 µg/µL; Thermo Scientific) was used as molecular weight marker.

Assessment of the genetic relatedness of *E. coli* isolates

The pulsed-field gel electrophoresis (PFGE) was performed as designed by Ejmaes *et al.* (2006), with some modifications. Briefly, *E. coli* colonies were grown for 18 h in MH and incorporated into agarose plugs to protect the DNA against breakage and allowing the free flow of lytic solutions. The plugs were washed three times for 30 min in sterile distilled water and twice for 30 min in washing buffer (20 mmol/L Tris [pH 8.0] and 50 mmol/L EDTA). Then, each plug was digested with 40 U of the restriction enzyme XbaI (10 U/µL, Thermo Scientific) for 18 h, according to the manufacturer's instructions. PFGE was carried out in a CHEF-DRIII System (BioRad) using a 1% pulsed field certified agarose in 0.5× TBE-buffer. The electrophoresis was performed under the following conditions: gradient of 6 V/cm, angle of 120°, ramped pulse time of 2 to 35 s; run time of 21 h. The gels were stained with a 0.5 µg/mL ethidium bromide solution for 45 min and photographed in a Molecular Imager Gel Doc XR® (BioRad).

Identification of *Enterococcus* species

Extraction of genomic DNA from enterococci isolates was performed using an enzymatic treatment with 0.04 mg/mL of lysostaphin (Sigma-Aldrich) for 2 h at 37°C, followed by treatment with 0.25 mg/mL of lysozyme (AppliChem GmbH, Darmstadt, Germany) and 0.1 mg/mL of proteinase K (Bioron, Ludwigshafen, Germany) for another 2 h at the same temperature.

Identification of enterococcal species was performed with a multiplex PCR, amplifying species-specific genes, as described previously (Jackson *et al.*, 2004). PCR products were analysed by electrophoresis on a 1.5% agarose gel (Seakem Agarose), at 150 V for 45 min. Gels were stained and photographed as described above.

Determination of enterococci fingerprinting by enterobacterial repetitive intergenic consensus (ERIC)-PCR

The ERIC-PCR reaction mixture (25 µL) contained 150 ng of the extracted DNA, 200 µmol/µL of each primer (ERIC-1R [5'-ATG TAA GCT CCT GGG GAT TCA C] and ERIC 2 [5'-AAG TAA GTG ACT GGG GTG AGC G]), 2.5 µL of reaction buffer 10×, 0.2 mmol/L of each dNTP, 250 µmol/L of MgCl₂ and 3.75 U of DFS-Taq DNA polymerase (Bioron). The PCR was performed under the conditions described by Wei *et al.* (2004).

ERIC-PCR patterns of each isolate were visualised after electrophoresis as described above.

Analysis of DNA fingerprinting patterns

The DNA band patterns were analysed using the FPQuest 4.5 software (Bio-Rad). Gels were normalised using the molecular weight marker and levels of similarity between profiles were calculated using the Dice correlation coefficient. The dendrogram was generated by UPGMA (unweighted pair group method using arithmetic averages) (Dice, 1945; Alam *et al.*, 1999; Hur and Chun, 2004).

Statistical analysis

Data analysis was carried using the SPSS version 21.0. Differences in growth and food intake between both groups (A and P) were assessed with the Mann-Whitney test. Chi-square test was used to analyse resistance and genetic phenotypes. *P*-values were considered significant if below 0.05.

RESULTS

Health status and growth performance

During the study period, clinical signs of disease were not observed among the animals in group A, while those in group P showed diarrhoeic faeces between the second and fifth day of the trial. During that period, the gastrointestinal signs went along with a decrease in feed intake (Figure 2b) and an increase in water consumption (data not shown), but specific antimicrobial treatment was not required due to the mild nature of the illness and fast recovery of the feed intake. Nevertheless, the administration of *E. coli* through the probiotic was permanently interrupted after the second inoculation.

Although not statistically significant, when compared to group A, a lower mean weight gain and slightly lower final weight were noticed among the animals fed the probiotic supplemented diet (group P), despite the improvement in the last stage of the study (Figure 2a). Excluding the period of illness, both groups showed normal and comparable feed intake values (Figure 2b).

Microbiological examination of faecal microbiota

Table 2 shows the faecal enumeration of total bacteria, *E. coli* and *Enterococcus* spp. in both groups A and P.

E. coli and *Enterococcus* spp. were isolated from every sample, *E. coli* counts being higher than enterococci in both groups throughout the study. Rabbits fed a diet containing the probiotic showed higher numbers of *E. coli* than group A (*P*<0.05).

The enumeration of total bacteria and enterococci did not differ significantly between both groups.

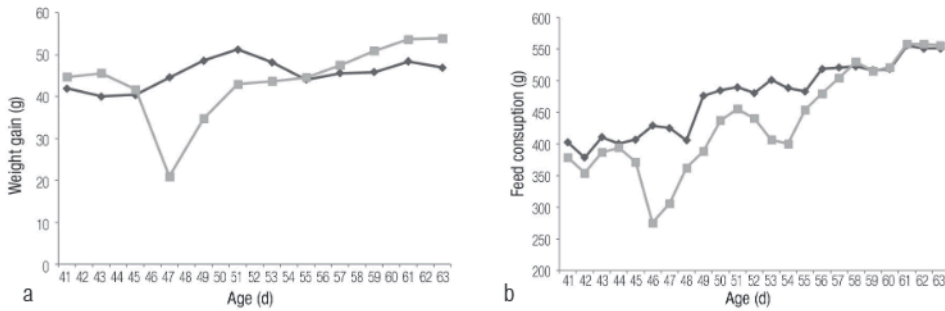


Figure 2: Graphs representing the (a) evolution of rabbits' average weight gain and (b) the average feed consumption, in the 2 study groups. ◆ Group A, ■ Group B.

Antimicrobial susceptibility and molecular fingerprinting of bacterial isolates

E. coli profiles

Antimicrobial resistance profiles of probiotic strains (ECI 1, ECI 2 and ECD) and faecal isolates of *E. coli* are shown in Table 3. ECI 1 and ECI 2 revealed very similar multidrug resistance phenotypes, while the strain collected from a domestic rabbitry (ECD) was only resistant to tetracycline. During the 35 d of the study, 15 different antimicrobial resistance profiles were found, but only in faecal samples from group P it was possible to recognise the same phenotypes of those inoculated in the probiotic formula. At the 35th day of study, after the animals had been transferred to the small domestic rabbitry, all the 12 studied isolates harboured a new resistance profile.

The 15 resistance profiles that were obtained were split into 3 classes (R1, R2 and R3), taking into account the number of resistances, allowing us to perceive that R3 (with more resistant strains) was predominantly collected from group A ($P < 0.05$).

Regarding the molecular characterisation, all *E. coli* strains, including probiotic and faecal isolates, belonged to the B1 phylogenetic group. Figure 3 shows the DNA fingerprinting results through PFGE, and highlights the clusters in which probiotic strains are included. Probiotic strains ECI 1 and ECD were only found in faecal samples from the group P, while ECI 2 was also found in group A. *E. coli* strains from the same clusters of those inoculated as probiotics were mostly recovered from the group P ($P < 0.05$). Finally, all the strains collected from the mixed group at the end of the study were included in the same genetic cluster, which was completely different from the others previously found.

Table 2: Counts of *E. coli*, *Enterococcus* spp. and total microorganisms every 72 h in the 2 study groups - group A (antibiotic) and group P (probiotic). Registration started when animals were 41 d old and ended when they were 62 d old. Values represent daily means.

Age (d)	Counts (log colony forming units per gram of faeces)					
	<i>E. coli</i>		<i>Enterococcus</i> spp.		Total microorganisms	
	Group A	Group P	Group A	Group P	Group A	Group P
41	5.64	7.36	4.63	5.11	7.69	7.51
44	6.04	7.80	5.04	6.26	7.57	7.93
47	6.83	9.00	5.94	6.41	7.71	9.04
50	7.23	8.78	6.61	6.68	7.62	8.94
53	7.04	8.32	6.97	7.72	7.81	8.63
56	6.36	7.11	6.87	6.41	7.36	7.83
59	5.96	5.41	5.81	5.99	7.11	6.94
62	6.20	6.52	5.11	6.11	6.85	7.15
Mean	6.71	8.38	6.45	6.92	7.56	8.52
Standard deviation	6.78	8.56	6.57	7.25	7.30	8.64

Table 3: *E. coli* resistance phenotypes identified during the study period (days marked from 1 to 32). A, P and AP represent the antibiotic and probiotic groups and the joining of the 2 groups, respectively. The values in the table represent the number of isolates found with each phenotype on each day in groups A, P and AP. The phenotypes were distributed in the groups in a ratio form in which the denominator value represents the total isolates collected each day in each group. Blanks show the absence of isolates with the respective phenotype. *Mark the resistance phenotypes of probiotic strains (1-ECD; 8-ECI 1; 9-ECI 2).

Group	Phenotype n°	Resistance Phenotype	Days of study														
			1		9		12		16		19		22		32		
			P	A	P	A	P	A	P	A	P	A	P	A	AP		
R1	1	TE*			1:6		3:6					1:6		3:6			
	2	TE;S						2:6									
	3	CIP;NA											2:6				
	4	AMP;SXT													12:12		
	5	CIP;TE;NA				3:6	3:6		1:6	1:6				3:6			
R2	6	CIP;TE;NA;SXT	1:6						2:6		3:6						
	7	AMP;TE;S;SXT			1:6												
	8	AMP;CIP;TE;NA;SXT*	3:6	3:6	1:6		1:6		2:6		1:6						
	9	AMP;CIP;TE;NA;TOB;SXT*	2:6	1:6	1:6		2:6		1:6								
	10	AMP;CIP;TE;NA;SXT;K			1:6												
R3	11	AMP;CIP;TE;NA;C;SXT					1:6										
	12	AMP;CIP;TE;S;NA;C;SXT				2:6	2:6		1:6		2:6						
	13	AMP;CIP;TE;NA;C;SXT;K		6:6													
	14	AMP;CIP;TE;NA;C;TOB;SXT							1:6	2:6		1:6		3:6			
	15	AMP;CIP;TE;S;NA;C;TOB;SXT					1:6			1:6							

Enterococcus spp. profiles. Antimicrobial resistance profiles of inoculated probiotic strains (Eal, EfaI, EfaD and EfeD) and faecal isolates are shown in Table 4. During the entire trial, we found 11 different resistance phenotypes and those equal to probiotic strains were all recovered from group P, with the exception of one that was also discovered in group A, on the ninth day of study. From the last sampling, at the domestic rabbitry, four faecal isolates showed the same resistance phenotype as EfaD, while the other four strains had a different profile, equal to one found on the 16th day of the study in group P.

The 11 resistance phenotypes of enterococcal isolates were divided into 2 groups (R1 and R2) according to the resistance recorded and, as described for *E. coli*, the group with more resistances comprised phenotypes of isolates predominantly collected from group A ($P < 0.05$).

Regarding the molecular characterisation, firstly it was verified that most of the enterococcal faecal isolates were *E. faecalis* (41 out of 56), whereas *E. faecium* (7/56), *E. gallinarum* (7/56) and *E. avium* (1/56) were found in a lower proportion. Secondly, it was possible to verify that, except for Eal, all the remaining probiotic strains were found only in group P. Finally, none of the 8 strains collected at the end of the study from faecal samples of the mixed group had a similar genetic profile to any probiotic strain, but can be characterised as follows: i) four strains were *E. faecalis*, showing an equal genetic profile to each other and to an isolate collected at the 19th day of trial; and, ii) the other four isolates were new in the study and probably the same strain of *E. gallinarum*. Figure 4 shows the results of DNA fingerprinting, through ERIC-PCR, and highlight clusters in which probiotic strains are included.

DISCUSSION

This article describes a preliminary trial focused on the colonisation ability of an autochthonous probiotic composed of *Enterococcus spp.* and *E. coli* in growing rabbits, comparing microfloritic and zootechnical features of animals fed a probiotic-supplemented diet to animals fed a diet containing a commonly used mixture of antimicrobials. To the best

Table 4: *Enterococcus* spp. resistance phenotypes identified during the study period (days marked from 1 to 32). A, P and AP represent the antibiotic and probiotic groups and the joining of the 2 groups, respectively. The values in the table represent the number of isolates found with each phenotype on each day in groups A, P and AP. The phenotypes were distributed in the groups in a ratio form in which the denominator value represents the total isolates collected each day in each group. Blanks show the absence of isolates with the respective phenotype. *Mark the resistance phenotypes of probiotic strains (1-EfeD; 2-EfaD; 8-Eal; 10-Efal).

Group	Phenotype n°	Resistance phenotype	Days of study												
			1		9		12		16		19		22		32
			P	A	P	A	P	A	P	A	P	A	P	A	AP
R1	1	No resistance*			2:4		1:4		1:4		1:4				
	2	TE*					2:4				1:4		1:4		4:8
	3	F											1:4		
	4	TE;CIP		2:4											
	5	CN;CIP	1:4												
R2	6	QD;TE							1:4						
	7	TE;RD									1:4				
	8	TE;E;AZM*				1:4		1:4				1:4			
	9	TE;RD;E;AZM						1:4							4:8
	10	QD;TE;E;AZM;C;CIP*	3:4		2:4	2:4		2:4			1:4		1:4		
	11	QD;TE;RD;E;CN;AZM;CIP		2:4		2:4		2:4		4:4		4:4		4:4	

of our knowledge, this is the first experiment with rabbits in which a probiotic formula with *E. coli* has been tested. In recent decades, intestinal health problems have been the leading cause of impaired performance in growing rabbits, especially due to the increased incidence of Epizootic Rabbit Enteropathy (ERE), in which enteropathogenic *E. coli* strains may be involved (From *et al.*, 2005). If we were able to enhance the intestinal colonisation by "preferred" *E. coli* strains through treatment with a CE product, these *E. coli* related enteric disorders could be prevented, reducing the consumption of antimicrobials. However, our attempt revealed unsuccessful results, as the P group, which was inoculated with the probiotic, revealed signs of enteric illness and more oscillatory growth rates when compared to group A. On the other hand, after the permanent interruption of *E. coli* administration, faecal pellets became harder and drier, and a phase of compensatory growth occurred. Interestingly, in comparison to the control group, the periods in which the faecal counts of *E. coli* were lower in the experimental group (at the beginning and at the end of the trial) coincided with higher growth rates. However, we must bear in mind that the inoculation of enterococci was not interrupted. As previously reported, the use of an enterococcal probiotic showed beneficial effects on both intestinal health and growth rates (Linaje *et al.*, 2004; Simonová *et al.*, 2009; Benato *et al.*, 2014).

Several molecular tests were performed to characterise the colonisation ability of the tested probiotic formula. Then, 40 out of 60 bacterial isolates (36 of *E. coli* and 24 of enterococci) recovered from faecal samples of group P showed a similar genetic profile to the inoculated strains. This evidence may corroborate the competitive exclusion ability of our probiotic formula, including probiotic strains with few resistances collected from a domestic rabbitry (ECD, EfaD and EfeD). These results should be further explored, due to the advantageous effects of having less resistant bacteria in the rabbit's intestinal lumen (Kudva *et al.*, 1997). Indeed, intestinal colonisation by less resistant microbial strains before slaughter is a recommended and desired achievement, as it lowers the probability of environmental dissemination of multidrug-resistant strains through the spread of rabbit's faecal waste as manure, as well as the transmission of resistance and virulence determinants to slaughterhouse workers and meat consumers (Kudva *et al.*, 1997).

Although consistently found in faecal isolates throughout the study period, probiotic bacterial strains did not remain in the gastrointestinal tract longer than one week after the administration ended. This suggests that the (i) lack of (re) inoculation, (ii) a dietary change, (iii) a different water source, and/or (iv) a new household may have impaired probiotic persistence in the rabbits' intestinal microbiota.

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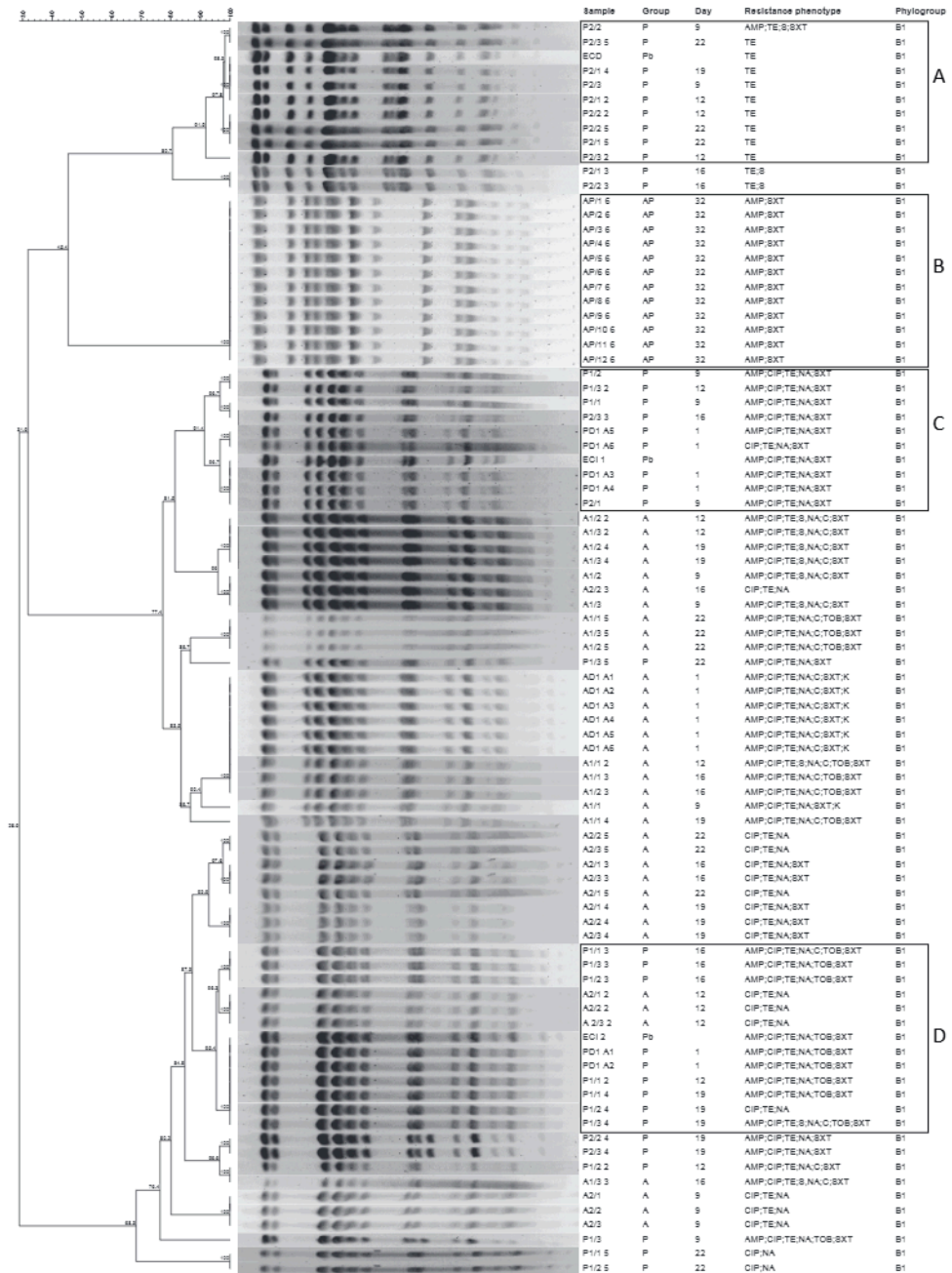


Figure 3: Dendrogram representing the genetic relationships between *E. coli* strains based on PFGE fingerprints. Dice coefficient was used to measure similarity among isolates and the matrix was clustered by the UPGMA method. Additional information is shown for each sample (sample name, study group from which it was collected, day of collection, resistance phenotype and phylogenetic group). Marked clusters represent the isolates similar to probiotic strains ECD (A), ECl 1 (C) and ECl 2 (D), and isolates collected on last day of study (B).

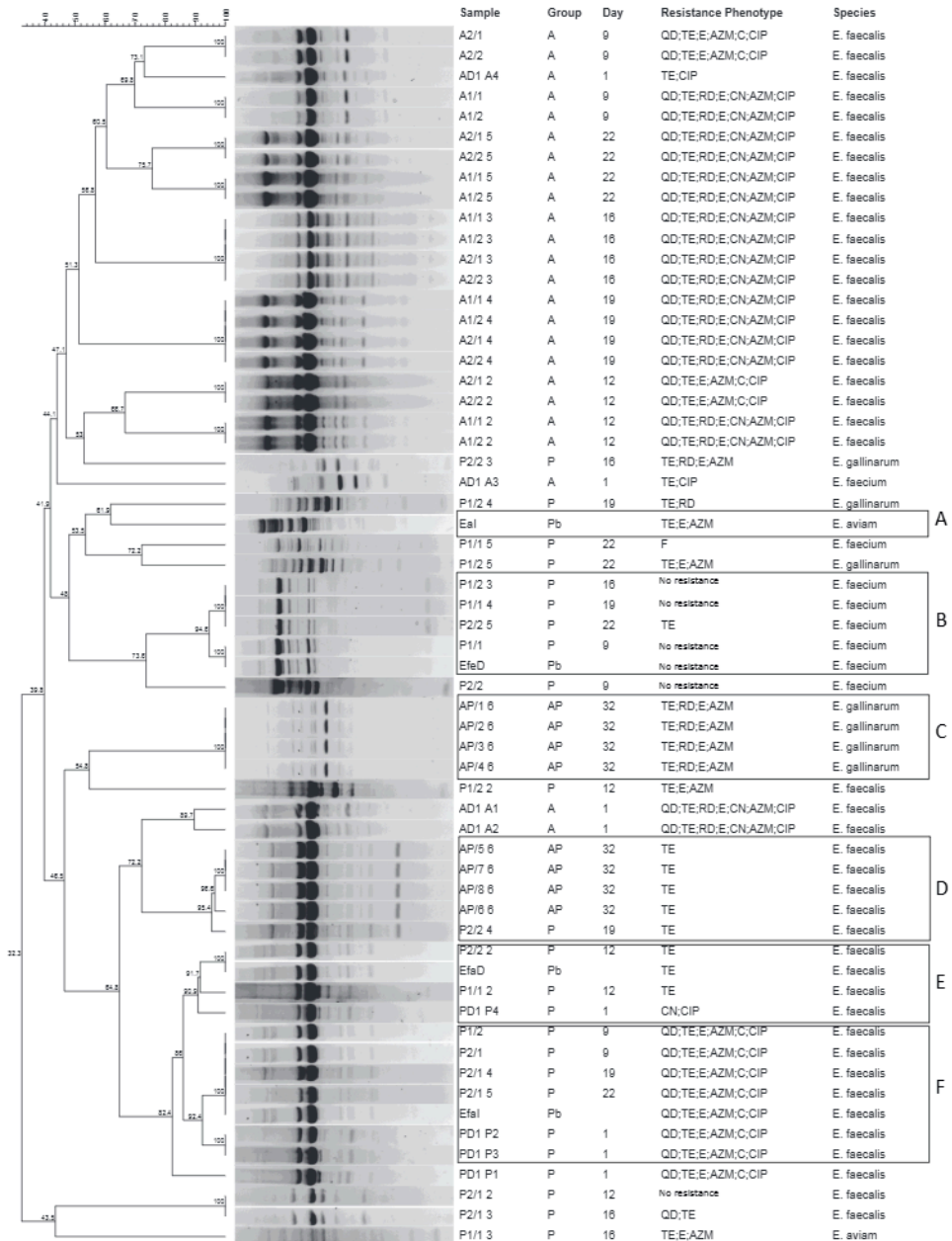


Figure 4: Dendrogram representing the genetic relationships between *Enterococcus* spp. strains based on ERIC-PCR fingerprints. Dice coefficient was used to measure similarity among isolates and the matrix was clustered by the UPGMA method. Additional information is shown for each sample (sample name, study group from which it was collected, day of collection, resistance phenotype and species). Marked clusters represent the isolates similar to probiotic strains Eal (A), EfeD (B), EfaD (E) and EfaI (F), and isolates collected on last day of study (C, D).

In the first stage of bacterial clustering analysis, phenotypic resistance profiles were assessed to find possible clusters, using Dice correlation coefficient. However, with the described fingerprinting, it was possible to verify that different resistance profiles may be found in the same cluster, and that the same resistance profile may be shared among the various strains.

Finally, the probiotic strain Eal (*E. avium*) was isolated from the faeces of healthy adult rabbits, which were raised in the industrial unit from where the rabbits used in this experiment also came. So, this species was expected to be a good choice for inclusion in the probiotic formulation, but only one isolate out of the 56 collected throughout the study was *E. avium*. The explanation may lie in a possible effect of competitive exclusion exerted by other strains included in the probiotic. Furthermore, several studies focusing on intestinal microbiota in rabbit also found that the predominant enterococci were *E. faecalis* (Linaje *et al.*, 2004) and *E. faecium* (Simonová and Lauková, 2004, Simonová *et al.*, 2005) and, in a smaller proportion, *E. durans* (Linaje *et al.*, 2004; Simonová *et al.*, 2005), *E. gallinarum* and *E. casseliflavus* (Simonová and Lauková, 2004). Our study corroborates the hypothesis of high prevalence of *E. faecalis* in rabbit intestinal microbiota and the lower amount of *E. faecium* and *E. gallinarum*.

CONCLUSIONS

Even under the constraint of illness that affected the probiotic group at the beginning of the trial, the following must be highlighted: i) no statistically different final growth rates must be expected between animals treated with antimicrobials or probiotics; ii) the use of probiotics containing *E. coli* to prevent infection by enteropathogenic strains must be carefully considered due to the possible occurrence of gastrointestinal signs; and, iii) although found in faecal samples throughout the period of inoculation with probiotics, enterococcal strains may not have a long-term colonisation ability of the rabbit's gastrointestinal tract when household and management conditions are changed.

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