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Effect on digestion and performance of dietary protein content and of increased substitution of lucerne hay with soya-bean protein concentrate in starter diets for young rabbits

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The aim of this work was to study the effect of protein source / availability on the intestinal microbiota, digestive traits and nutritional performance of early-weaned rabbits. The effects of supplemental antibiotics in the drinking water were also evaluated. Four isoenergetic and isofibrous diets were formulated: a control diet with a high protein (207 g/kg dry matter (DM)) and lucerne hay content (HPHL), a diet with low crude protein (CP) (179 g/kg DM) and high lucerne hay content (LPHL) and low protein diets in which the lucerne hay in diet LPHL was replaced partially (LPML) or totally (LPLL) with soya-bean protein concentrate. Rabbits, weaned at 25 days (52 per diet), were fed the experimental diets for a 2-week period and thereafter received a commercial diet until 56 days of age. The incidence of mortality was investigated using 70 animals per diet without supplemental medication. The profile of the ileal microbiota was studied at 35 days of age in rabbits treated (18 per diet) or not (12 per diet) with antibiotic. As expected, supplementation with antibiotics effectively reduced fattening mortality rate and microbial biodiversity. However, lowering of also the dietary CP content led to a reduction in the mortality rate ($P < 0.05$), both in animals treated with (by 80%) or without (by 39%) antibiotics. In addition, there was a reduction ($P < 0.05$) in the frequency of Clostridium perfringens in non-medicated animals. Neither jejunal morphology nor growth performance, over the whole fattening period, was affected by dietary CP content of the experimental diets. However, with HPHL, feed efficiency was higher (by 4.8%; $P < 0.01$) than with LPHL diets. Substitution of lucerne hay with soya-bean meal in low protein diets did not affect apparent faecal or ileal digestibility of DM and CP. However, the ileal digestibility of cystine, alanine, aspartic acid, and proline was lowered ($P < 0.05$) with increasing substitution by soya bean. Nevertheless, ileal CP flow, incidence of mortality and presence of C. perfringens were unaffected. Our results suggest that a reduction in dietary CP, resulting in reduced lumenal flows of nitrogen through the ileum, may be beneficial for young rabbits and limit the numbers of potentially harmful bacteria in the lower gut. Modulation of dietary CP should be contemplated as a strategy to increase the intestinal health in rabbits.

Keywords: feed efficiency, microbial flora, protein intake, protein sources, rabbits

Introduction

Around the weaning, pathologies occur in a context of incomplete development of the digestive physiology. Up to now, the use of antibiotics seems to have been the most effective way to control the high mortality associated with enteric disorders, by limiting the pathogenic microbiota. The supply of balanced diets has also been related with the prevention of disorders by means of two mechanisms: (i) promoting a lower retention time of the digesta in the digestive tract, or (ii) causing a lower flow of easily available substrates into the fermentative area. The alteration of the gut microbiota has been postulated as the possible primary cause of these pathologies. In the case of the rabbit, the dietary factors more related with the incidence of enteric disease are the levels of starch and fibre, usually inversely correlated, and the type of fibre (Blas and Gidenne, 1998; De Blas *et al*., 1999; Gidenne, 2000) but little attention has been paid up to now on the role of the protein.

Some previous studies have shown that diets with a high level of protein (>180 g/kg) promoted in rabbits an increase of *Clostridium* population (Haffar *et al*., 1988) and a higher incidence of digestive problems around weaning (De Blas

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et al., 1981; Maertens and De Groote, 1988), that also occurred when low ileal digestible protein sources were included in the diet (Gutiérrez *et al*., 2003). These results might be explained by a higher availability of substrates for microbial growth, as proteolytic activity has been proven to be relevant in the caecum of rabbits (Emaldi *et al*., 1979). Works in other non-ruminant species such as broiler chickens (Drew *et al*., 2004) and dogs (Zentek *et al*., 2004) also showed a positive relationship between level of protein and intestinal proliferation of *Clostridium perfringens* population. However, the hypothetical effect of diet (promoting changes in availability of protein at ileum and caecum) on the development of pathogenic bacteria and the outbreak of digestive disorders has not been tested in rabbits.

Otherwise, a reduction in the protein dietary supply might affect growth rate, maturation and functionality of intestinal mucosa, which acts as a first line of defence against dietary toxins and pathogens. Some studies in early-weaned piglets have shown that low protein diets led to an atrophy of intestinal mucosa and to a reduction in its absorptive and immunological capacity (Nunez *et al*., 1996; Gu and Li, 2004). There is a lack of information on the role of level of dietary protein in mucosal functionality in rabbits, although previous work indicate that the villus atrophy observed at weaning (Gutiérrez *et al*., 2002a) might be alleviated through the dietary supply of high quality animal plasma (Gutiérrez *et al*., 2000).

The aim of this work was to study the effect of a reduction of the ileal protein flow, by decreasing the dietary level of protein or by increasing protein ileal digestibility, on digestive traits, mucosal morphology and growth performance, and its interactions with the antibiotic supplementation on composition of intestinal microbiota and incidence of enteric disease.

Material and methods

Diets

Four isoenergetic and isofibrous diets were formulated to induce differences in the ileal flow of crude protein (CP) through changes in the dietary level and the apparent ileal digestibility of protein. A basal diet with a high protein and lucerne hay content (HPHL) was formulated according to De Blas and Mateos (1998) and recent works on optimal starter feed composition (Gutiérrez *et al*., 2002a and b and 2003). Another diet (LPHL; low CP, high lucerne hay) was designed by substituting dietary starch for CP, maintaining the apparent ileal CP digestibility of the diets according to an estimation made from previous works (García *et al*., 2005). Two additional low protein diets were formulated further to reduce the ileal flow of protein by replacing lucerne hay in diet LPHL with a more digestible protein source (soya-bean protein concentrate) plus a mixture of fibrous ingredients (sunflower hull, sugar beet and apple pulp), totally (LPLL) or partially (LPML). All the diets were pelleted and included 5 g/kg of lucerne hay marked with an indigestible marker (ytterbium) according to the procedure described by García *et al.* (1999). The ingredients and chemical composition of the diets are shown in Tables 1 and 2.

Faecal digestibility trial

Forty rabbits weighing 464 (s.e. 18) g at 25 days of age, were blocked by litter and assigned at random to the experimental diets (10 per diet) to determine the apparent faecal digestibility of dry matter (DM), CP and energy. Rabbits were individually caged and had *ad libitum* access to the food during the experimental period. Following a 1-week adaptation period, the feed intake and total faecal output (caecotrophy was not prevented) were recorded from 32 to 35 days of age. The faeces daily collected were stored at −20°C, dried at 80°C for 48 h and ground to pass a 1-mm sieve for analysis.

Ileal digestibility trial

Sixty four rabbits weighing 454 (s.e. 19) g at 25 days of age were blocked by litter and assigned at random to the experimental diets (16 per diet) to determine the apparent ileal digestibility of DM, CP and amino acids (AA). Following a 10-day adaptation period, animals were slaughtered by cervical dislocation weighing 788 (s.e. 33) g. Slaughter time was between 1900 and 2100 h to avoid the influence of caecotrophy on the chemical composition of the digesta (Merino and Carabaño, 2003). The last 20 cm of the ileum were taken and the ileal contents were removed, frozen and freeze-dried. The samples were then ground and, because of the small quantity available, they were pooled in groups of two rabbits of the same treatment to analyse

Table 1 *Ingredients in the experimental diets (g/kg)*

	Diets[†]			
	HPHL	LPHL	LPML	LPLL
Wheat	195	300	300	300
Wheat bran	300	140	140	140
Sunflower meal	130	50	50	50
Lucerne hay	335	369	182	0
Lucerne hay-Yb	5	5	5	5
Sunflower hulls	0	0	14	28
Soya-bean protein concentrate	0	0	37	74
Apple pulp	0	0	67	135
Beet pulp	0	0	55	110
Wheat straw	0	89	102	111
Lard	20	30	26	22
L-Lysine HCl 78	4.1	5.7	5.2	4.7
DL-Methionine 99	0.4	1.7	1.8	1.8
L-Threonine	0.8	1.6	1.8	1.8
Calcium carbonate	0	0	5	10
Sodium chloride	5	5	5	5
Vitamin/mineral pre-mix[‡]	5	5	5	5

[†] HPHL = high protein high lucerne-hay. LPHL = low protein high lucerne-hay. LPML = low protein medium lucerne-hay. LPLL = low protein low lucerne-hay.

[‡] Provided by Trouw Nutrition España S.A. (Madrid, Spain): mineral and vitamin composition (mg/kg feed): Mg, 290; Na, 329; S, 275; Co, 0.7; Cu, 10; Fe, 76; Mn, 20; Zn, 59.2; I, 1.25; choline, 250; riboflavin, 2; niacin, 20; pyridoxine, 1; phytylmetaquinone, 1; alpha-tocopherol, 13; thiamine, 1; retinol, 2.5, and cholecalciferol, 0.019.

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Table 2 *Chemical composition of experimental diets (g/kg dry matter)*

	Diets[†]			
	HPHL	LPHL	LPML	LPLL
Dry matter	911	908	909	912
Ash	82.8	80.6	75.7	63.0
Crude protein	207	179	174	176
Starch	200	227	224	220
Neutral-detergent fibre (NDF)	329	335	355	363
Acid-detergent fibre (ADF)	165	173	183	185
Acid-detergent lignin (ADL)	46.3	46.9	51.4	55.7
Gross energy (MJ/kg DM)	19.1	18.8	18.9	19.2
Digestible energy (MJ/kg DM)	14.0	13.7	13.7	14.1
Arginine	11.8	8.75	9.29	9.49
Cystine	3.85	3.91	3.60	3.22
Histidine	4.51	3.45	3.66	3.74
Isoleucine	7.13	5.79	5.98	5.97
Leucine	12.9	10.7	10.5	10.7
Lysine	11.0	10.6	10.5	10.7
Methionine	4.93	4.95	4.67	4.85
Phenylalanine	8.11	6.87	7.02	7.29
Threonine	8.07	7.59	7.39	7.40
Valine	8.90	7.11	7.25	7.15
Alanine	8.93	7.53	7.17	6.67
Aspartic acid	17.8	15.2	14.4	13.1
Glutamic acid	37.3	29.5	31.2	32.4
Glycine	9.94	7.85	7.55	7.18
Proline	13.6	12.1	11.6	9.93
Serine	8.17	5.99	6.30	6.69
Tyrosine	5.21	4.66	4.88	4.71

[†] HPHL = high protein high lucerne-hay. LPHL = low protein high lucerne-hay. LPML = low protein medium lucerne-hay. LPLL = low protein low lucerne-hay.

CP and ytterbium. To determine the AA content of the ileal digesta, a fixed amount (0.05 g) of all the samples belonging to each treatment were pooled. Ytterbium content of experimental diets and ileal digesta were analysed to calculate apparent ileal digestibility of CP and AA (CP_{id} and AA_{id} according to the following equations):

$$CP_{id} = [1 - \text{dietary ytterbium concentration} \times \text{ileal CP concentration} / \text{ileal ytterbium concentration} \times \text{dietary CP concentration})] \times 100.$$

$$AA_{id} = [1 - (\text{dietary ytterbium concentration} \times \text{ileal amino acid concentration} / \text{ileal ytterbium concentration} \times \text{dietary amino acid concentration})] \times 100.$$

Digestive traits trial

Sixty weanling mixed-sex rabbits (15 animals per diet) 25 days old and weighing 501 ± 16 g, were fed the experimental diets up to 35 days of age. Final weight was 922 ± 43 g. Rabbits were individually caged and had *ad libitum* access to the food during the experimental period. Animals were slaughtered by cervical dislocation between 1900 and 2100 h to avoid the caecotrophy period. After that, the gastro-intestinal tract was removed and weighed. Stomach and caecum were weighed separately with and without their contents. The pH of caecal and stomach contents was determined. In the stomach digesta, three measurements of pH were taken: at the pyloric and fundus areas, and in the mixture of both areas. A sample was taken from the middle part of the jejunum of each animal to determine mucosa histology. The samples were placed in a 10% buffered neutral formaldehyde solution (pH 7.2 to 7.4) and were gradually dehydrated with increasing concentrations of ethyl alcohol (50 to 100%). These dehydrated specimens were first embedded in paraffin, prepared by sectioning at 6 μm, and stained with hematoxylin and eosin. The sections were analysed under a light microscope (Olympus BX40, Olympus Optical Co., 20 097, Hamburg, Germany) to determine their morphometric index by computer-assisted image analysis (The ImageJ v 1.26. Wayne Rasband, National Institutes of Health, Bethesda, MD 20 892, USA). Villi heights (from the top of the villi to the villi crypt junction) at three cross sections were measured according to Hampson (1986) from the mean value of 30 vertically oriented villi per animal.

Finishing performance trial

Two hundred and eight New Zealand White × California weanling mixed-sex rabbits (52 per diet), 25 days old and 494 ± 13 g live weight, were blocked by litter and assigned at random to the experimental diets. After weaning, rabbits were individually caged and were fed the experimental diets through a 2-week period. Drinking water was supplemented during this period with a mixture of 100 mg/kg of apramicine sulphate and 120 mg/kg of tylosin. After 39 days of age, all the animals received a commercial feed (CUNIUNIC®, NANTA, S.A.: 170 g CP, 144 g starch, 373 g neutral-detergent fibre (NDF) and 49 g acid-detergent fibre (ADF) per kg) until they reached 56 days of age. Animals had *ad libitum* access to the feed and water throughout the whole experimental period. Feed intake, weight gain and mortality rate at day 14 after weaning and at the end of the experimental period were recorded per cage. Mortality rate was also controlled in another group of 280 rabbits (70 per diet) weighing 498 ± 13 g at 25 days of age. Animals were blocked by litter and assigned at random to the same dietary treatments, but they were not supplemented with antibiotics.

Ileal microbiota characterisation trial

One hundred and twenty 25-day-old rabbits (30 per diet) weighing 448 ± 19 g live weight, were blocked by litter and assigned at random to the experimental diets during 10 days. Within each treatment, 18 animals received antibiotic supplementation in drinking water (100 mg/kg of apramicine sulphate and 120 mg/kg of tylosin), whereas

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the other 12 rabbits were not supplemented. At 35 days of age the rabbits, weighing 860 ± 30 g, were slaughtered by cervical dislocation between 1900 and 2100 h. One g of ileal digesta was collected in a sterile plastic tube that contained 3 ml of 98% molecular biology grade ethanol and were stored at 4°C to be analysed by RFLP according to the following procedure:

A 400-mg sample of gut contents was processed for total DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Chatsworth, CA) system, in accordance with the instructions of the manufacturer with two additional steps of lysozyme and proteinase K. The purified DNA was maintained at −20°C until use. Two primers (5′-CTACGGGAGGCAGCAGT-3′ and 5′-CCGTCWATTCMTTTGAGTTT-3′) for regions I and II of the 16S rRNA gene (Lane, 1991), were used to amplify a DNA segment of 500-600 bp. PCR mixtures (PCR-Master Mix – Applied Biosystems – with 1.25 IU of Taq polymerase, 50 ng of DNA template, 0.2 μmol/l the preceding primers, and distilled water in a total volume of 50 μl) were heated to 94°C for 5 min once, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min, and DNA extension at 72°C for 1 min 15 s. The last extension cycle was continued for 5 min. Aliquots of the amplified DNA fragments were digested, in separated tubes, with Alu I, Rsa I, Hpa II, Sau 3A I or Cfo I restriction endonucleases (Sigma-Aldrich) in accordance with manufacturer specifications. The endonuclease fragments were solved in 2% wide range agarose by electrophoresis at 150 V for 60 min. The bands of DNA were visualised in an UV Chemigenious Image System (SynGene) using the GeneSnap software (SynGene). Pictures with 4.63 s exposure were stored. With the information resulting from the relative size of the restriction fragment length polymorphism (RFLP) bands, the information stored in the 'SSU_Una.gb' file from the Ribosomal Database Project (Maidak *et al.*, 1997), and a specific software developed in our Institute, we could identify the bacterial genus or species compatible with the obtained RFLP profile. From each animal a 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, was recorder. Also, the frequency of the compatibility profile of every bacteria studied defined as the presence or absence of RFLP bands compatible with the theoretical RFLP bands for a certain genus or bacterial species was studied for every animal (i.e. a combination of bands with 92 bp + 467 bp in *Alu* I, plus 120 bp + 404 bp + 35 bp bands in *Rsa* I, plus 189 bp + 58 bp + 312 bp bands in *Hpa* II, plus 82 bp + 477 bp bands in *Sau*3A, plus a band of 559 bp in *Cfo* I is compatible with *C. perfringens*. The absence of any of the above mentioned bands in the RFLP profile infer that the level of *C. perfringens* in the sample is below the detection level, as in other PCR-electrophoresis methods around 10^4-10^5 DNA copies per g of sample).

Housing

Animals were housed in wire metabolism cages measuring 250 × 600 × 330 mm. A cycle of 12 h of light and 12 h of dark was used throughout the experiment. The light was switched on at 0730 h. Heating and forced ventilation systems allowed the building temperature to be maintained between 18 and 23°C throughout the experiment. Rabbits were handled according to the principles for the care of animals in experimentation published by the Spanish Royal Decree 1201/2005 (2005). Because the trials were carried in a farm affected by epizootic rabbit enteropathy (ERE), animals were supplemented with antibiotics (100 mg/kg of apramicine sulphate and 120 mg/kg of tylosin) in drinking water. Mortality and ileal microbiota characterisation trial were additionally developed without supplementation in drinking water.

Analytical methods

Chemical analysis of diets and faeces was performed using the procedures of Association of Official Analytical Chemists (2000) for DM (930.15), ash (923.03), Dumas N (968.06) and starch (according to the alpha-amyloglucosidase method, 996.11). NDF, ADF and acid-detergent lignin were determined according to the sequential method of Van Soest *et al.* (1991). Gross energy (GE) was measured by adiabatic calorimetry. AAs were determined following acid hydrolysis using a Beckman System 6300HPA amino acid analyser (Fullerton, CA, USA). Samples were hydrolysed by reflux in 25 ml of 6 mol/l HCl with 10 g/l added phenol for 24 h at 120 °C. For the determination of sulphur amino acids (methionine and cystine), samples were oxidised with performic acid at 0 °C for 16 h and then, neutralised with 0.5 g of sodium meta-bisulphite before analysis. Tryptophan, being destroyed during acid hydrolysis, was not determined. Ytterbium content of diets and ileal digesta were analysed by atomic absorption spectrometry (Smith Hieftje 22, Thermo Jarrel Ash, MA, USA) using predosed samples to prepare common matrix standards. Previously, samples were ashed (600°C) and then digested by boiling with a solution of 1.5 mol/l HNO_3 and KCl (3.81 g/l).

Statistical analysis

Data from ileal and faecal digestibility, digestive traits and growth trials were analysed as a completely randomised-block design using type of diet as main effect and litter as block effect, by using the GLM procedure of Statistical Analysis Systems Institute (1991). Treatment sums of squares were partitioned into the effect of dietary level of protein (contrast of diets HPHL *v.* LPHL) and the linear and quadratic effects of level of substitution of lucerne hay with soya-bean protein concentrate (diets LPHL, LPML and LPLL). Weaning weight was used as a linear covariate in the growth traits analyses.

To analyse mortality, biodiversity degree and frequency of the compatibility profile, type of diet, antibiotic supplementation and their interaction were used as main effects. Mean comparisons of mortality and frequency

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of the compatibility profile traits were made using a chi-square test. Regression procedures were used to relate fattening mortality with frequency of the compatible profile of several entero-pathogenic bacteria.

Results

Digestibility trial

The effect of treatments on the ileal apparent digestibility (IAD) and faecal apparent digestibility (FAD) of experimental diets is shown in Table 3. Neither dietary protein level nor the substitution of lucerne hay with soya-bean concentrate affected FAD of DM, CP and GE (0.724, 0.829 and 0.730 on average, respectively). The effect of a decrease in dietary protein concentration (207 *v.* 179 g/kg DM) on the IAD differed among AAs, as a reduction was observed for phenylalanine ($P < 0.001$), glycine ($P < 0.05$) and serine ($P < 0.05$) by 16, 22, and 9% respectively, whereas that of cystine increased by 18% ($P < 0.01$) and no differences ($P > 0.10$) were detected for the other AAs. The substitution of lucerne hay with soya-bean concentrate led to a linear reduction of the IAD of cystine ($P < 0.01$), alanine ($P < 0.05$), aspartic acid ($P < 0.01$) and proline ($P < 0.05$) by respectively 18, 10, 13, and 6% between extreme diets, and had a quadratic effect on methionine IAD ($P < 0.05$).

Digestion traits

The effect of dietary treatments on several digestive traits and jejunal morphology is presented in Table 4. Treatments did not affect pylorus and mixed digesta pH (1.56 and 2.02 on average, respectively). Animals fed HPHL diet had a lower fundus pH than those fed LPHL diet (1.97 *v.* 2.86, $P < 0.05$). The substitution of lucerne hay with soya-bean concentrate did not affect stomach pH, but led to a linear effect on the caecal pH, which decreased from 5.51 to 5.37 between extreme diets ($P < 0.05$), whereas dietary CP concentration did not influence caecal pH (5.49, on average). No effect of treatments in the weights of stomach, caecum and their contents was detected. Neither dietary CP level nor source of protein affected villus height, crypt depth or the ratio villus height/crypt depth, which were on average 618 μm, 135 μm and 4.9 respectively.

Finishing performance

The effect of the dietary treatments on the finishing performance is shown in Table 5. During the first 2 weeks after weaning, when the animals were fed the experimental diets, neither dietary CP content nor source of protein affected weight gain or feed intake, which were on average 48.6 and 77.9 g/day, respectively. However, feed efficiency was 5% higher ($P < 0.01$) in animals fed HPHL with respect to LPHL diets. In the whole fattening period the

Table 3 *The effect of dietary treatments on the ileal and faecal apparent digestibility of experimental diet*

	Diets[†]					Significance of effects[§]		
	HPHL	LPHL	LPML	LPLL	s.e.[‡]	1	2	3
Ileal apparent digestibility								
Dry matter	0.470	0.466	0.457	0.499	0.016			
Crude protein	0.602	0.609	0.612	0.635	0.020			
Arginine	0.796	0.799	0.775	0.796	0.011			
Cystine	0.603	0.707	0.617	0.578	0.018	**	**	
Histidine	0.723	0.699	0.680	0.704	0.015			
Isoleucine	0.718	0.717	0.685	0.699	0.015			
Leucine	0.731	0.739	0.697	0.710	0.014			
Lysine	0.766	0.776	0.743	0.776	0.012			
Methionine	0.771	0.788	0.735	0.777	0.012			*
Phenylalanine	0.786	0.660	0.671	0.628	0.017	***		
Threonine	0.657	0.676	0.642	0.635	0.017			
Valine	0.684	0.683	0.654	0.681	0.016			
Alanine	0.687	0.685	0.642	0.619	0.017		*	
Aspartic acid	0.712	0.722	0.660	0.626	0.016		**	
Glutamic acid	0.812	0.805	0.789	0.799	0.010			
Glycine	0.553	0.429	0.359	0.443	0.029	*		
Proline	0.794	0.793	0.766	0.742	0.011		*	
Serine	0.678	0.614	0.603	0.642	0.019	*		
Tyrosine	0.761	0.740	0.725	0.737	0.013			
Faecal apparent digestibility								
Dry matter	0.725	0.722	0.720	0.729	0.006			
Crude protein	0.814	0.829	0.829	0.842	0.007			
Energy	0.735	0.729	0.723	0.734	0.006			

[†] HPHL = high protein high lucerne-hay. LPHL = low protein high lucerne-hay. LPML = low protein medium lucerne-hay. LPLL = low protein low lucerne-hay.
[‡] Ileal apparent digestibility: no. = 8. Faecal apparent digestibility: no. = 10.
[§] 1 = effect of level of protein (diet HPHL *v.* diet LPHL), 2 = linear effect of dietary substitution of lucerne hay with soya-bean protein concentrate. 3 = quadratic effect.

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Table 4 *The effect of dietary treatments on digestive traits and jejunal morphology*

	Diets[†]					Significance of effects[§]	
	HPHL	LPHL	LPML	LPLL	s.e.[‡]	1	2
Pylorus pH	1.52	1.58	1.62	1.52	0.07		
Fundus pH	1.97	2.86	2.79	2.42	0.22	*	
Mixed digesta pH	1.73	2.20	2.14	2.00	0.14		
Caecal pH	5.47	5.51	5.44	5.37	0.04		*
Stomach							
Organ (g/kg LW)	14.9	14.7	14.2	15.9	0.62		
Content (g/kg LW)	44.1	43.7	43.3	44.7	2.13		
Caecum							
Organ (g/kg LW)	19.8	18.7	21.1	19.9	0.79		
Content (g/kg LW)	73.0	66.0	67.1	70.9	3.13		
Jejunal morphology							
Villus height (μm)	632	606	636	600	48		
Crypt depth (μm)	134	134	137	135	4.3		
Villus height/crypt depth	5.04	4.80	4.90	4.72	0.45		

[†] HPHL = High protein high lucerne-hay. LPHL = Low protein high lucerne-hay. LPML = Low protein medium lucerne-hay. LPLL = Low protein low lucerne-hay.
[‡] no. = 15 except for jejunal morphology where no. = 7.
[§] 1 = effect of level of protein (diet HPHL *v.* diet LPHL), 2 = linear effect of dietary substitution of lucerne hay with soya-bean protein concentrate. The quadratic effects were not significant.

dietary protein content did not influence any of the finishing performance traits studied. The linear substitution of lucerne hay with soya-bean concentrate reduced linearly feed intake ($P < 0.05$) and weight gain ($P < 0.10$), by 6 and 4%, respectively between extreme diets, which resulted in a tendency to increase feed efficiency (from 0.396 to 0.404).

Ileal microbiota characterisation and mortality

The effect of dietary treatments on the biodiversity, the frequency of the compatibility profile of ileal microbiota and the mortality in the fattening period of rabbits supplemented or not with antibiotics is shown in Table 6. Neither dietary protein level nor the substitution of lucerne hay with soya-bean concentrate affected the biodiversity of ileal microbiota, whereas the antibiotic supplementation led to a reduction ($P < 0.001$) from 1431 to 473 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. A significant interaction ($P < 0.05$) between dietary CP content and antibiotic supplementation was detected in the frequency of the compatibility profile of *C. perfringens* that decreased with dietary level of protein by 70% in

Table 5 *The effect of dietary treatments on finishing performance*

	Diets[†]					Significance of effect[§]	
	HPHL	LPHL	LPML	LPLL	s.e.[‡]	1	2
First 2 weeks after weaning period[‖]							
Weight gain (g/day)	50.0	47.4	48.4	48.4	1.16		
Feed intake (g/day)	77.2	76.8	78.9	78.7	1.84		
Feed efficiency (g gain per g intake)	0.648	0.617	0.618	0.616	0.007	**	
Whole finishing period (25–56 days)							
Weight gain (g/day)	49.9	49.3	48.6	47.3	0.77		¶
Feed intake (g/day)	125	125	123	118	2.09		*
Feed efficiency (g gain per g intake)	0.399	0.396	0.397	0.404	0.003		¶

[†] HPHL = high protein high lucerne-hay. LPHL = low protein high lucerne-hay. LPML = low protein medium lucerne-hay. LPLL = low protein low lucerne-hay.
[‡] no. = 42.
[§] 1 = effect of level of protein (diet HPHL *v.* diet LPHL), 2 = linear effect of dietary substitution of lucerne hay with soya-bean protein concentrate. The quadratic effects were not significant.
[‖] Weaned at 25 days.
[¶] Approaching significance ($P < 0.10$).

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Table 6 *The effect of treatments on the biodiversity, the frequency of the compatibility profile (%) of ileal microbiota and the mortality in fattening period of rabbits supplemented or not with antibiotics*

	Diets[†]								Significance of contrasts[‡]				
	Antibiotic supplementation				No antibiotic supplementation								
	HPHL	LPHL	LPML	LPLL	HPHL	LPHL	LPML	LPLL	1	2	3	4	5
Biodiversity[§]	714	395	535	250	1400	1102	2457	764			***		
Frequency of the compatibility profile (%)[§]													
Campylobacter spp.	38.9	22.2	16.7	16.7	100	75.0	58.3	100			***		
Clostridium spp.	83.3	88.9	61.1	33.3	100	100	100	100		**	***		**
Clostridium perfringens	11.1	11.1	0.00	0.00	83.3	25.0	25.0	33.3	*		***	*	
Clostridium difficile	0.00	5.56	5.56	5.56	8.33	16.7	33.3	58.3		¶	***		¶
Escherichia coli	5.56	0	5.56	0	25.0	16.7	41.7	8.3			***		
Helicobacter spp.	44.4	27.8	22.2	27.8	100	83.3	66.7	91.7			***		
Mortality (%)[‖]													
14 days after weaning period	7.69	1.92	1.92	0	32.9	20.0	22.9	20.0	*		***		
Whole fattening period	9.61	1.92	1.92	3.84	32.9	20.0	24.3	22.9	*		***		

[†] HPHL = high protein high lucerne-hay. LPHL = low protein high lucerne-hay. LPML = low protein medium lucerne-hay. LPLL = low protein low lucerne-hay.
[‡] 1 = effect of level of protein (diet HPHL *v.* diet LPHL). 2 = linear effect of dietary substitutions of lucerne hay with soya-bean protein concentrate. 3 = effect of antibiotic supplementation. 4 = interaction level of protein × antibiotic. 5 = interaction substitutions of lucerne hay with soya-bean concentrate × antibiotic.
[§] Antibiotic supplementation: no. = 18. No antibiotic supplementation: no. = 12.
[‖] Antibiotic supplementation: no. = 52. No antibiotic supplementation: no. = 70.
[¶] Approaching significance ($P < 0.10$).

non-medicated animals, whereas it was not affected in animals supplemented with antibiotics. Another significant interaction was found between the type of protein and antibiotic supplementation on frequency of the compatibility profile of *Clostridium* spp. ($P < 0.01$), which decreased with the substitution of lucerne hay by soya-bean concentrate but only in medicated animals (by 62% between extreme diets) and on that of *C. difficile* ($P < 0.10$), which increased linearly from 16.7 to 58.3% (by 71%) between extreme diets only in non-medicated animals. The supplementation of drinking water with antibiotics decreased the frequency of the compatibility profile of each species studied (72% on average, $P < 0.001$), ranging from 33% for *Clostridium* spp. to 89% for *Escherichia coli*.

A reduction of dietary CP level decreased ($P < 0.05$) the mortality rate during the first 2 weeks after weaning (when animals received experimental diets) and in the whole fattening period, both in animals supplemented or not with antibiotics. The substitution of lucerne hay with soya-bean concentrate did not affect this trait. Medication of drinking water reduced ($P < 0.001$) the mortality rate, both during the first 2 weeks after weaning and in the whole fattening period by around 83%.

Discussion

A reduction of dietary protein content did not affect, as expected, the IAD of DM, CP and that of most of the AA. However, glycine, serine and phenylalanine IAD decreased, whereas that of cystine increased. A reduction in the protein intake might result in a disproportionate effect on the IAD of several AAs present in low proportion in the diet but preponderant in the endogenous losses as glycine and serine, as also observed in pigs (Donkoh and Moughan, 1994).

The IAD of CP did not increase with the substitution of lucerne hay with soya-bean concentrate plus a mixture of fibrous ingredients. The lack of effect of source of protein could be partially explained by an increment in the amount of endogenous material as dietary level of inclusion of pulps increased. The endogenous losses represent a high proportion of the total ileal CP flow (García *et al.*, 2004 and 2005). Dietary addition of beet pulp increased intestinal viscosity in rabbits (Volek *et al.*, 2005). It also led to an increase of endogenous secretions and to a reduction in the apparent ileal digestibility of nutrients in broilers (Smits *et al.*, 1997; Langhout, *et al.*, 1999). Other results in pigs (Mosenthin *et al.*, 1994) and rats (Ikegami *et al.*, 1990; Satchithanandam *et al.*, 1990), also indicate that dietary inclusion of viscous substances (apple and citrus pectins, guar gum, or carboxyl methyl cellulose) increased the production of different components of endogenous losses as mucins, intestinal and pancreatic secretions. There are no available data about this subject in rabbits, but the results on apparent ileal AA flow obtained in the present work could confirm this hypothesis. In this way, previous studies (García *et al.*, 2004; Llorente *et al.*, 2006) have shown that endogenous losses in rabbits have a high concentration of aspartic, proline or alanine, whose IAD decreased in our study when dietary pulps concentration increased.

A reduction of dietary CP content from 207 to 179 g/kg DM led to a decrease of ileal CP flow by 17% (from 6.00 (s.e. 0.3) to 4.98 (s.e. 0.2) g/day) and to a reduction of mortality rate ($P < 0.05$) both during the first 2 weeks after weaning and in the whole fattening period (by 44

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and 46%, respectively). A reduction of fattening mortality rate has been also reported when decreasing the dietary protein to energy ratio (De Blas *et al*., 1981) or when increasing dietary CP ileal digestibility by including highly digestible vegetable protein sources (Gutiérrez *et al*., 2003). A high ileal flow of protein has been related to a proliferation of harmful bacteria as Clostridia and *Escherichia coli* in rabbits (Cortez *et al*., 1992; Haffar *et al*., 1988). Data on microbial characterisation of ileal digesta in the present study indicate that the highest correlation observed between the mortality rate in animals not supplemented with antibiotic and the frequency of the compatibility profile of ileal bacteria was for *C. perfringens*. The presence of this bacterium has been reported in animals affected by ERE, a pathology that affects mainly to young animals aged between 3 and 10 weeks and is responsible for high mortality rates in commercial farms (Pérez de Rozas *et al*., 2005). This pathology is characterised clinically by the presence of liquid and acid stomachs, which agrees with the lower fundus pH observed in the present work in animals fed with the diet highest protein content. A positive relationship between level of dietary protein and the intestinal proliferation of *C. perfringens* populations has been also observed in chickens (Drew *et al*., 2004) and dogs (Zentek *et al*., 2004).

The supplementation with antibiotics was also effective to reduce the fattening mortality rate, the microbial biodiversity degree (by 67%) and the frequency of the compatibility profile of every species studied, including that of *C. perfringens*. Apramycin sulphate has been previously described in rabbits as an efficacious treatment against the collateral adverse effect of the ERE, reducing the mortality rate from 45 to 10% when it was added to the drinking water (Badiola *et al*., 2000). In our study the combined use of this antibiotic with tylosin reduced the mortality rate by 83%. The reduction of dietary protein content additionally contributed to decrease fattening mortality (from 9.6 to 1.9%). In this case, the reduction of mortality rate can not be explained by a lower presence of *C. perfringens*, which remained low independently of the level of CP. Instead, the reduction of the ileal flow of CP was parallel to a decrease of the frequency of the compatibility profile of other potential harmful bacteria, as *Helicobacter* spp. (from 44.4 to 27.8%) and *Campylobacter* spp. (from 38.9 to 31.0%). These differences did not reach significant levels, but the average frequency of detection of these bacteria were positively correlated ($r = 0.930$, $P < 0.001$ and $r = 0.956$, $P < 0.001$, respectively) with the average whole fattening mortality of the eight treatments studied.

Our data show that a moderate reduction of dietary CP content did not affect the intestinal morphology in the jejunum, which suggest that the supply of AAs was sufficient to ensure enteric growth. Neither did it impair the growth performance when the total fattening period was regarded, although a higher feed efficiency was observed during the first 2 weeks after weaning in diets with the highest CP content.

The substitution of lucerne hay with soya-bean concentrate plus a mixture of beet and apple pulp, did not affect the ileal flow of CP (4.8 g/day, on average), neither the frequency of the compatibility profile of *C. perfringens,* nor the mortality rate during the first 2 weeks and the whole fattening period. However, the substitution of lucerne hay with dietary pulps and soya-bean concentrate led to changes in the microbial population in the intestinal gut, affecting the frequency of the compatibility profile of several ileal bacteria not correlated with the mortality rate observed in our study, such as *Clostridium* spp and *C. difficile*. Variations in microbial population and its fermentation products might also be responsible for the reduction of the caecum pH observed when dietary pulps were included. These results agree with those obtained by García *et al.* (1993) and Carabaño *et al.* (1997) who also observed a reduction on caecal pH when lucerne hay was substituted with beet pulp.

Our results show that the use of antibiotic is an effective way to reduce the fattening mortality in rabbits by limiting the presence of several harmful bacteria. In addition, decreasing ileal flow of nitrogen compounds obtained by reducing dietary CP level from 209 to 179 g/kg, affected the composition of the ileal microbiota and especially reducing the frequency of detection of *C. perfringens*. The fattening mortality was also reduced and this might be also contemplated as a helpful strategy for increasing intestinal health and reducing the use of antibiotics. Our results also indicate that the CP requirements to maximise the feed efficiency in the post-weaning period could be higher than those needed to ensure an adequate intestinal health. However, this reduction in feed efficiency obtained during this period was compensated during the whole fattening period.

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