Dietary level of fibre and age at weaning affect the proliferation of *Clostridium perfringens* in the caecum, the incidence of Epizootic Rabbit Enteropathy and the performance of fattening rabbits

C. Romero [a], N. Nicodemus [a], P. García-Rebollar [a], A.I. García-Ruiz [b], M.A. Ibáñez [c], J.C. De Blas [a,*]

[a] *Departamento de Producción Animal, Universidad Politécnica de Madrid, ETS Ingenieros Agrónomos, 28040, Madrid, Spain*
[b] *NUTRECO Poultry & Rabbit Research Center, 45950, Casarrubios del Monte, Toledo, Spain*
[c] *Departamento de Estadística, Universidad Politécnica de Madrid, ETS Ingenieros Agrónomos, 28040, Madrid, Spain*

ARTICLE INFO

Keywords:
Epizootic Rabbit Enteropathy
NDF level
Weaning age
Fattening mortality
Caecum microbiota
Rabbits

ABSTRACT

An experiment was conducted to investigate the effects of dietary fibre content and weaning age on *Clostridium perfringens* proliferation in the caecum and fattening mortality in growing rabbits farmed in a facility having Epizootic Rabbit Enteropathy. The experiment consisted in a 2 × 2 factorial arrangement with two weaning ages (28 days vs. 42 days) and two levels of dietary neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom; 330 g/kg vs. 425 g/kg). Controls were made during two consecutive experimental periods that differed in hygienic environmental conditions by modifying the intensity of cleaning and disinfection in the farm previous to the trial. An interaction (P<0.001) was detected among the independent variables studied on *Cl. perfringens* enumeration in the caecal contents, as minimal values for this trait were obtained in non-medicated animals reared in a clean environment, and especially when they were weaned at a later age and fed the diet with the lower fibre content. The treatments studied also led to a variation in fattening mortality (from 4.7% to 34.0%), which was highly and positively correlated (P<0.001) to the average *Cl. perfringens* caecal counts in each combination of treatments. The results of the current study indicate

* Corresponding author. Tel.: +34 14524850; fax: +34 15499763.
E-mail address: c.deblas@upm.es (J.C. De Blas).

that high counts of *Cl. perfringens* in the caecum can be used as an indicator of Epizootic Rabbit Enteropathy, and suggest that strategies designed to control its proliferation in the caecum might help to limit fattening mortality in rabbit fed diets not-medicated with antibiotics.

1. Introduction

Since its emergence in early 1997, the condition referred to as Epizootic Rabbit Enteropathy (ERE) has caused an increase in the fattening mortality rate in European intensive farms up to average values of 15% (Licois et al., 2006), and hence has been an important cause of economic loss. Even if the aetiology remains unknown and poorly understood, several works have pointed out that proliferation of spore-forming bacterium *Clostridium perfringens* could be a consequence of ERE and may be associated to mortality caused by this disease (Marlier et al., 2006; Dewrée et al., 2007; Szalo et al., 2007). Currently, the harmful effects of ERE have broadly been controlled by adding some antibiotics to the feed (Duperray et al., 2003; Boisot et al., 2003b; Bostvironnois and Morel, 2003; Chamorro et al., 2007b). However, the use of antibiotics raises human food safety concerns, which emphasizes the need for the design of alternatives to control ERE.

As ERE is a digestive disorder, appropriate nutrition in the post-weaning period might have a significant role as a prevention factor. Insoluble fibre (NDF) supply decreases caecal retention time (Gidenne et al., 1998), thereby reducing caecal microbial growth (García et al., 2000). On the other hand, an excess of NDF in the diet also has a detrimental effect on mucosal integrity of jejunum at 45 days of age (Álvarez et al., 2007). A delay of age at weaning might also be a useful strategy in order to improve intestinal health in fattening rabbits, as milk intake seems to confer a transitory protection against gut pathogens, such as enteropathogenic *E. coli* O103 strain (Gallois et al., 2007). However, neither the effect of NDF supply nor that of weaning age on *Cl. perfringens* enumeration in digestive contents and mortality rate due to ERE have been studied so far in young rabbits.

The aim of this study was to evaluate in two consecutive fattening periods the effect of two different concentrations of dietary neutral detergent fibre (aNDFom; 330 and 425 g/kg), and two weaning ages (28 days vs. 42 days) on the enumeration of *Cl. perfringens* colonies in gut contents at 0 or 14 days after weaning and on the mortality throughout the fattening period.

2. Material and methods

2.1. Animals and housing

A total of 80 New Zealand × Californian rabbit does (originating from strains genetically improved at the Universidad Politécnica of Valencia, Spain) were used. Half of them were artificially inseminated 4 days after parturition and their litters were weaned at 28 days of age (early weaning, EW), whereas the other half was inseminated 14 days post-partum and weaned at 42 days (late weaning, LW). Data were obtained in two consecutive parturitions, in which animals stayed in the same reproductive rhythm. Average litter size at weaning was 8.9 rabbits.

Trials were conducted in a farm sited at the University experimental facilities. Rabbit does and litters up to weaning were kept in the same building. A cycle of 16 h of light and 8 h of dark was used throughout the experiment. Heating and forced ventilation systems allowed the building temperature to be maintained between 16 and 24 °C. Weaned rabbits were housed in a separate building. They were kept under controlled environmental conditions (room temperature between 16 and 24 °C; 12 h of light per day) and housed in pairs in wire flat-deck cages measuring $600 \times 250 \times 330\,mm^3$. Before the first experimental period, faeces were removed from the pits. Afterwards, cages, walls, the ceiling and the floor were cleaned using high pressure water containing a detergent (RM 806 ASF, Alfred Kärcher GmbH & Co. KG). Once dry, walls were whitewashed and finally buildings were disinfected spraying a disinfectant active against Gram(+) and Gram(−) bacteria, spores, virus, fungi and micoplasms (SANIVIR

Table 1
Ingredient composition of experimental diets.

Ingredients (g/kg)	Diet HF[a]	Diet LF[a]
Wheat bran	150	–
Barley	60	310
Alfalfa meal (17% CP)	281	283
Sunflower meal (30% CP)	197	197
Beet pulp	150	150
Wheat straw	100	–
Soybean oil	21	21
Sodium chloride	4	5
Calcium carbonate	14	11.5
Monocalcium phosphate	5.7	5.0
L-Lysine	1.5	1.5
L-Threonine	–	1.0
Sodium bicarbonate	2.0	–
Sepiolite	8.8	10.0
Mineral and vitamin premix[b]	5.0	5.0

[a] HF: 425 g/kg aNDFom and 67 g/kg starch; LF: 330 g/kg aNDFom and 149 g/kg starch.

[b] Premix provided by Trouw Nutrition España S.A. (Madrid, Spain): mineral and vitamin composition (mg/kg diet): 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; Vitamin B6, 1; Vitamin K, 1; Vitamin E, 20 IU/kg; Thiamine, 1; Vitamin A, 8375 IU/kg,, Vitamin D3, 750 IU/kg, Robenidine, 60.

PLUS®, BIOPLAGEN, S.L., containing: 15% gluteraldehide, 10% didecilmetyl ammonium chloride, 10% cipermetrine, solvents and excipients). Between the first and the second experimental periods, fattening farm was not disinfected in order to impair hygiene conditions in the second with respect to the first period.

Animals were handled according to the principles for the care of animals in experimentation published by the Spanish Royal Decree 1201/2005 (2005).

2.2. Experimental diets

Two diets containing a high (HF) or low (LF) level of fibre were formulated according to the nutrient recommendations of De Blas and Mateos (1998). They were made by the substitution of a mixture of 150 g/kg of wheat bran and 100 g/kg of wheat straw with 250 g/kg of barley grain, which led to a replacement of insoluble fibre in diet HF with starch in diet LF. Diets were designed to have a similar content of protein (150 g/kg), soluble fibre (120 g/kg) and degree of lignification of the NDF (130 g/kg). The ingredient and chemical composition of the diets is shown in Tables 1 and 2, respectively.

Among the 40 litters initially assigned to each reproductive rhythm, half of them were fed with the diet HF whereas the other half received the diet LF. Diets were given from 21 days of lactation until the end of the experimental period (56 days of age). Rabbit does were fed the rest of the lactation and dry period with a non-medicated commercial diet containing 175 g/kg CP and 318 g/kg aNDFom (CUNILACTAL®, NANTA, S.A.). Rabbits had ad libitum access to feed and water during the whole trial. Neither feed nor drinking water was medicated with antibiotics. However, a coccidiostat (robenidine) was added to all feeds.

2.3. Measurements

2.3.1. Enumeration of *Cl. perfringens*

2.3.1.1. Digestive contents. In each of the two experimental periods, 16 litters were chosen at random, with four of them allotted to each combination of treatments (HF-EW, HF-LW, LF-EW and LF-LW) as shown in Table 3. Rabbits were slaughtered in a hermetically closed CO_2 chamber (5 min) at 0 or 14 days after weaning (three rabbits from each litter at each age). Samples of gut contents were immediately put in sterile polystyrene tubes and analysed the same day. All the samples were tested for caecal count of *Cl. perfringens*. However, the amount collected of ileal contents was insuf-

Table 2
Chemical composition and nutritive value of experimental diets (g/kg as fed).

	Diet HF	Diet LF
Dry matter	908	909
Crude protein	147	155
Ether extract	40.0	40.0
Ash	99.0	86.0
Starch	67	149
Crude fibre	199	157
aNDFom[a]	425	330
ADFom[a]	249	203
ADL	55	44
Sugars	9	35
Lysine[b]	7.2	7.7
Sulphur amino acids[b]	5.5	5.5
Threonine[b]	6.8	6.9
Calcium[b]	4.5	11
Phosphorous[b]	5.0	5.1
Energy digestibility (%)[c]	55.6	61.8
Digestible energy (MJ/kg)[c]	8.67	10.1

[a] Samples for sequentially analysed NDF were assayed with a heat stable amylase and expressed exclusive of residual ash.
[b] Values calculated from FEDNA (2003).
[c] Value estimated according to De Blas et al. (1992).

ficient in some cases to perform the analysis, so that only 98 out of 192 animals could be sampled for ileal concentration. *Cl. perfringens* enumeration was performed according to the Standard ISO 7937 (1997). This technique analyses all the toxinotypes of *Cl. perfringens*. Sampling was achieved by blending each 1-g sample of digestive content with 9 ml of peptone water. All blended samples were vortexed and further diluted. All dilutions (5 dilutions per sample) were plated to determine the population of the suspensions. The cultural medium used was agar tryptose sulphite added with antibiotic D-cycloserine (Byrne et al., 2008). Agar plates were incubated at 37 °C in anaerobic jars with carbon dioxide generating envelopes for 18 h. *Cl. perfringens* reduced sulphite to sulphide and, in the presence of iron, black colonies developed. These colonies were observed and counted.

2.3.1.2. Environment. Samples of dust were collected over the air extractors of the fattening farm. Moreover, samples of feed and drinking water were also taken. All these samples were analysed

Table 3
Experimental design and number of animals used for the microbiological analyses and the fattening mortality control.

Experimental period	Age at weaning	Diet	Total number of litters	Microbiological trial			Fattening trial	
				Number of litters	Number of rabbits at weaning[a]	Number of rabbits 14 day after weaning[a]	Number of litters	Number of weaned rabbits
First	EW[b]	HF[c]	20	4	12	12	16	139
		LF[c]	20	4	12	12	16	136
	LW[b]	HF	20	4	12	12	16	127
		LF	20	4	12	12	16	145
Second	EW	HF	20	4	12	12	16	147
		LF	20	4	12	12	16	156
	LW	HF	20	4	12	12	16	144
		LF	20	4	12	12	16	151

[a] Three rabbits were chosen at random from each litter.
[b] EW: rabbits weaned at 28 days; LW: rabbits weaned at 42 days.
[c] HF: 425 g/kg aNDFom and 67 g/kg starch; LF: 330 g/kg aNDFom and 149 g/kg starch.

according to the Standard ISO 7937 (1997). In addition, the 10-fold diluted solution was previously heated to 75 °C for 15 min to estimate the concentration of spores in the samples.

2.3.2. Fattening performance and health status

Litters that were not chosen for the microbiological study were fattened from weaning to 56 days of age in both experimental periods (see Table 3). Feed intake and daily weight gain were recorded for the whole fattening period. All the experimental animals were controlled daily for mortality. Dead rabbits were necropsied to check for the appearance of clinical symptoms of ERE: bloated abdomen, relatively low body weight, presence of mucus under the cages, distension of both stomach and small intestine, absence of visible inflammation and either liquid or compacted caecal contents (Licois et al., 2006; Dewrée et al., 2007).

2.4. Chemical analysis

Chemical analyses of experimental diets were performed using the procedures of AOAC (2000) for dry matter (930.15), ash (923.03), Dumas N (968.06), ether extract (920.39), crude fibre (978.10), sugars (974.06) and starch (996.11). Contents of NDF (aNDFom), ADF (ADFom) and acid-detergent lignin were determined according to the sequential method of Van Soest et al. (1991), after assaying samples for NDF with a heat stable amylase and expressed exclusive of residual ash.

2.5. Statistical analysis

Cl. perfringens counts and fattening mortality results were analysed using generalized linear models (McCullagh and Nelder, 1989) with the GENMOD procedure of SAS (SAS Institute, 1990). Litter was the experimental unit for all the analyses. For bacterial counts, where rabbits were the observational units, the litter effect was accounted in the analysis using the repeated statement of the GENMOD procedure. Generalized linear models allow the mean of the response variable to depend on a linear predictor through a non-linear link function and also allow the response probability distribution to be different than that of the normal distribution. It was assumed that *Cl. perfringens* caecal counts possessed a negative binomial distribution, which is a method for handing overdispersed count data (McCullagh and Nelder, 1989). Overdispersion is a phenomenon that sometimes occurs when count data appear more dispersed than expected for a particular distribution, the Poisson distribution in this case. The link function was the natural log function which related the natural logarithm of the mean of *Cl. perfringens* caecal counts with the linear combination of the explanatory variables used in these analyses. Mortality results were carried out using a binomial distribution. The link function was the logit transformation, $\ln(\mu/1-\mu)$, where μ was the mean value of the mortality rate. The effects of the period, weaning age, diet and their interactions were included as explanatory variables in both analyses. A linear polynomial regression model using the GLM procedure of SAS was made to relate ileal and caecal counts of *Cl. perfringens* and to test the linear and quadratic effects of the *Cl. perfringens* caecal counts 14 days after weaning on fattening mortality. Data of feed intake and daily weight gain from the performance trial were analysed as a factorial structure by the GLM procedure of SAS including in the model period, type of diet, weaning age and their interactions.

3. Results

Among the 192 rabbits of the microbiological study, 14 of them showed all the described ERE symptoms when they were slaughtered. None of the animals with a caecal count of *Cl. perfringens* lower than 10^6 were affected by ERE. Otherwise, caecal and ileal (when available) counts of *Cl. perfringens* in rabbits affected by ERE were always above 1.90×10^6 and 1.58×10^5 cfu/g, respectively (Table 4). Another seven animals had caecal counts higher than 10^6 cfu/g, but did not show symptoms of ERE. Five were still unweaned. Animals with ERE symptoms were not included in the analysis of variance of the treatment effects, as they were considered as a different population.

Individual caecal counts of *Cl. perfringens* ranged from 1×10^1 to 500×10^6 cfu/g. The distribution of values is presented in Fig. 1. Caecal concentrations of *Cl. perfringens* at weaning were lower at the

Table 4
Caecal and ileal counts (log cfu/g) of *Cl. perfringens* and ERE symptoms of ill animals from the microbiological study.

Period	Weaning[a]	Feed[b]	Caecal count	Ileal count[c]	Caecal symptoms
1	LW	HF	6.58	–[d]	Liquid caecal contents
1	LW	HF	8.70	–	Compacted caecal contents[e]
1	LW	LF	8.00	5.20	Liquid caecal contents
1	LW	LF	8.30	–	Liquid caecal contents
1	EW	HF	6.90	7.11	Liquid caecal contents
1	EW	HF	7.40	–	Liquid caecal contents
1	EW	HF	7.40	8.68	Compacted caecal contents
1	EW	LF	6.46	5.89	Liquid caecal contents
1	EW	LF	8.28	7.86	Liquid caecal contents
2	LW	LF	8.51	–	Liquid caecal contents
2	LW	LF	6.30	–	Liquid caecal contents
2	LW	LF	7.83	6.90	Liquid caecal contents
2	EW	LF	7.38	–	Compacted caecal contents
2	EW	LF	7.28	5.43	Compacted caecal contents

[a] LW: rabbits weaned at 42 days; EW: rabbits weaned at 28 days.
[b] HF: 425 g/kg aNDFom and 67 g/kg starch; LF: 330 g/kg aNDFom and 149 g/kg starch.
[c] Only 98 out of 192 animals could be sampled for ileal concentration.
[d] Insufficient amount of sample to perform analysis.
[e] Proximal colon of this rabbit also contained mucus.

first than at the second experimental period (1.23×10^5 cfu/g vs. 4.57×10^4 cfu/g, as average; P<0.001), but were not affected by weaning age or type of diet (data not shown). Caecal counts 14 days after weaning were also higher (P=0.001) at the second (2.26×10^5) than at the first period (1.68×10^4 cfu/g; see Table 5). This result was parallel to a higher enumeration of *Cl. perfringens* spores at the second period in dust collected at the middle of the fattening trial over the air extractors (8.32×10^5 cfu/g vs.700 cfu/g) and in samples of feeds taken from the feeders (40 cfu/g vs. 0 cfu/g, in the second and the first experimental period, respectively). Neither spores nor vegetative cells were found in samples of feeds taken from sacks or in samples of drinking water. A significant effect (P<0.001) of the interaction among age at weaning, type of diet and the experimental period on *Cl. perfringens* counts 14 days after weaning was detected, as rabbits weaned later had lower *Cl. perfringens* counts only at the second experimental period and especially when they were fed LF diet.

The results of the fattening trial are shown in Table 5. An interaction type of diet x weaning age on average feed intake was observed (P=0.04), as the increase in consumption observed for the diet with the higher aNDFom concentration was superior in animals weaned later (from 106 to 128 g/day) than in early weaned rabbits (from 99 to 102 g/day). Average daily gain was higher in rabbits weaned later (40.1 g vs. 46.8 g; P<0.001). An interaction between period × diet × weaning age on feed intake (P=0.13)

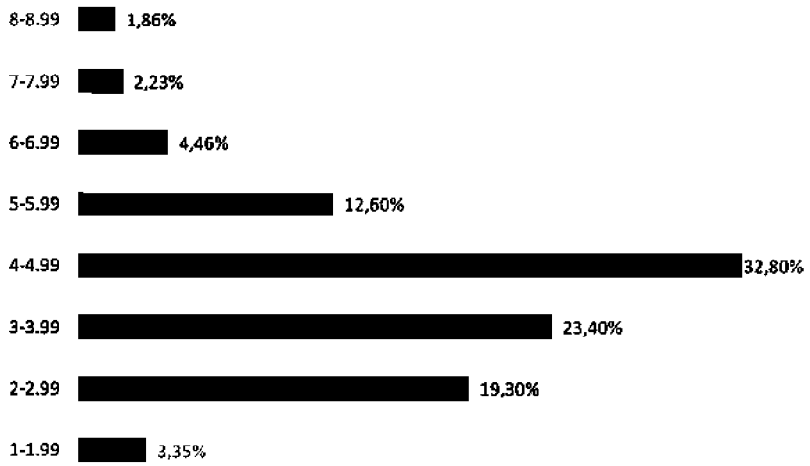

Fig. 1. Distribution of individual values of caecal concentrations of *Cl. perfringens* (log cfu/g).

Table 5
Effect of experimental period, age at weaning and dietary aNDFom content on feed intake, fattening performance and *Cl. perfringens* caecal count 14 days after weaning.

Experimental period	Age at weaning	Diet	Feed intake (g/day)	Daily weight gain (g/day)	Fattening ERE mortality (%)	CP counts (cfu/g)
First	EW[a]	HF[b]	112	39.9	5.21	6085
		LF[b]	111	41.4	4.17	27784
	LW[a]	HF	145	49.9	7.91	32862
		LF	113	42.4	6.64	508
Second	EW	HF	93	40.2	19.4	257102
		LF	87	38.7	34.0	313407
	LW	HF	111	46.4	17.5	212679
		LF	100	48.2	10.7	120230
Significance of effects	SEM (n = 16)		5.6	1.8		
Experimental period (P)	<0.001	NS[c]	<0.001	<0.001		
Age at weaning (W)	<0.001	<0.001	NS	<0.001		
Diet (D)	0.004	NS	NS	0.01		
$P \times W$	NS	NS	0.068	NS		
$P \times D$	NS	NS	NS	0.043		
$W \times D$	0.038	NS	NS	<0.001		
$P \times W \times D$	0.13	0.035	NS	<0.001		

[a] EW: early weaning (28 days); LW: late weaning (42 days)
[b] HF: 425 g/kg aNDFom and 67g/kg starch; LF: 330 g/kg aNDFom and 149 g/kg starch
[c] NS: non-significant (P>0.15).

and daily gain (P=0.035) was also observed, as these traits decreased at the second experimental period, especially in rabbits weaned at 28 days fed diet LF, whereas the maximal values were observed at the first period in animals weaned at 42 days fed the diet HF.

Total fattening mortality averaged 13.8%. Most of the dead animals (96 out of 101) presented ERE clinical symptoms. An interaction (P=0.068) between experimental period and age at weaning on fattening ERE mortality was detected (see Table 5), as mortality at the first period was not affected by age at weaning (6%, as average), whereas it decreased with weaning age at the second experimental period (26.7% vs. 14.1% in rabbits weaned at 28 or 42 days of age, respectively). Neither type of diet nor their interactions with the other independent variables in the model had a significant influence on fattening ERE mortality. The distribution of rabbit losses throughout the fattening period showed that ERE mortality increased up to 14 days after weaning when rabbits were weaned early, whereas the distribution was more homogeneous in the case of rabbits weaned later (see Fig. 2).

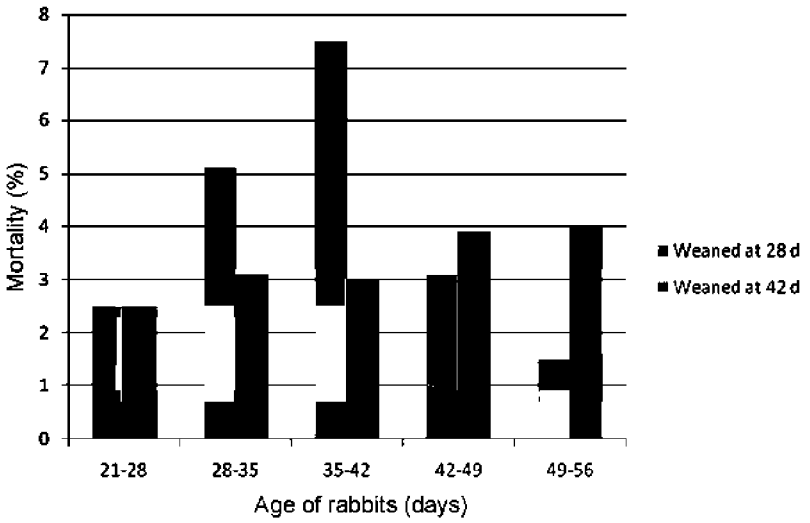

Fig. 2. Distribution with age of rabbit losses caused by ERE (expressed as % over the initial number of rabbits weaned at each age).

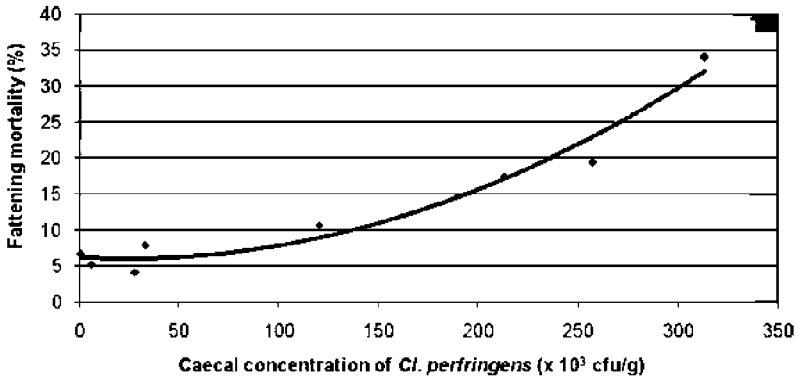

Fig. 3. Quadratic regression between fattening ERE mortality and caecal concentration of *Cl. perfringens* 14 days after weaning. $M = 6.25(\pm 1.40) - 0.0155(\pm 0.030) \times CP_{14} + 0.000312(\pm 0.00010) \times CP_{14}^2$, RSD = 5.49, $R^2 = 0.961$, $P<0.001$, and $n = 8$.

Average values of mortality caused by ERE at the fattening period (M, %) per each of the eight combinations of treatments (two experimental periods × two types of diet × two ages at weaning) were related to the average values per treatment of caecal counts of *Cl. perfringens* in samples taken 14 days after weaning (CP_{14}, cfu × 10^3/g). The covariance analysis showed a significant linear (P<0.001) and quadratic (P=0.026) effect (see Fig. 3).

4. Discussion

Cl. perfringens is a ubiquitous Gram-positive anaerobic bacterium, widely spread in the environment and commonly found in soil, dust, decaying vegetation, sewage and raw foods, and in the normal flora of the intestinal tract of healthy men and animals (Petit et al., 1999; EFSA, 2005). Data from the current study show that *Cl. perfringens* was present in variable amounts in all the digestive samples studied, even in those obtained from healthy animals. They also suggest a correspondence between high *Cl. perfringens* counts (>2 × 10^6 cfu/g) and the appearance of ERE symptoms. These results agree with the positive correlations found between the frequency of detection of *Cl. perfringens* in digestive contents assessed by RFLP techniques with ERE mortality in some studies (Chamorro et al., 2007a,b; Gómez-Conde et al., 2007). Other authors (Marlier et al., 2006; Szalo et al., 2007) observed that *Cl. perfringens* was frequently encountered in faeces from animals that died with ERE symptoms (>10^4 cfu/g in 83% of the samples). Moreover, *Cl. perfringens* positive for α, αβ2 and the enterotoxin have been isolated in Belgian, Dutch and Italian rabbit farms from animals affected by the disease (Marlier et al., 2006; Cocchi et al., 2008). A high concentration of colonies (>10^6 cfu/g) is a predisposing factor for the release of toxins that causes damage to the host (Hatheway, 1990; Songer, 1996; EFSA, 2005). Therefore, the overgrowth of potentially pathogenic strains in the rabbit intestine might contribute to the increase in mortality observed. Nevertheless, no experimental reproduction of ERE has been obtained after inoculation with strains of *Cl. perfringens* (Licois et al., 2003; Marlier et al., 2006). Accordingly, it is still a subject of debate whether proliferation of *Cl. perfringens* in the caecum is a consequence rather than a cause of ERE.

A delay of weaning age from 28 up to 42 days reduced fattening mortality during the second experimental period, where the ERE incidence was higher. This effect was correlated to a decrease in *Cl. perfringens* caecal counts 14 days after weaning in this period. Furthermore, at 42 days of age, caecal counts were lower for animals being weaned than for animals that had been weaned 14 days before (1.32×10^5 vs. 3.21×10^5; P=0.03). Rabbit milk contains antimicrobial compounds, such as medium-chain fatty acids (caprylic and capric acids; Maertens et al., 2006), that have been proved to have a significant bactericide effect against *Cl. perfringens* in vitro (Skrivanova et al., 2005). Early substitution of solid feed for milk in young rabbits also implies a decrease of digestibility of nutrients and an increase in the flow of nutrients reaching the ileum. An increase in the flow of ileal protein has been correlated with a higher frequency of detection of *Cl. perfringens* in the ileal contents and with a higher fattening mortality in rabbits (Chamorro et al., 2007b). In the current study, starch and NDF intake were not significantly correlated either with fattening mortality or with *Cl. perfringens* counts in the

caecal contents. Instead, digestible energy intake decreased linearly with an increase in ERE mortality ($r = -0.68$; P=0.014; $n = 8$) and with *Cl. perfringens* caecal counts ($r = -0.78$; P=0.003; $n = 8$). A decrease of feed and energy intake has also been described as one of the symptoms of ERE (Licois et al., 2006; Dewrée et al., 2007).

A decrease of dietary NDF concentration from 425 to 330 g/kg in late weaned rabbits additionally reduced *Cl. perfringens* caecal counts 14 days after weaning. Fattening mortality was not significantly affected by type of diet, although the minimal mortality in the second experimental period was reached in the combination late weaning/low fibre diet. A minimal content of insoluble fibre (300–320 g/kg NDF) in the growing rabbit diets seems to be required to decrease total and caecal mean retention time of digesta, dilute dietary and ileal starch and protein content and reduce total microbial growth (Gidenne et al., 1998; García et al., 1995, 2000). It also decreases fattening mortality with respect to diets with an insufficient insoluble fibre concentration (<250–270 g/kg NDF, Gidenne et al., 2004a,b; Nicodemus et al., 2004). Nevertheless, several workers have showed that an increase in dietary NDF content (from 300–320 to 360–385 g/kg) caused more fattening rabbit mortality (Gutiérrez et al., 2002), and an increased sanitary risk (mortality + morbidity; Feugier et al., 2006) and to an impairment of the structure of the mucosa (Álvarez et al., 2007).

The results from the current study indicate that adequate cleaning and disinfecting processes to improve environmental safety might be effective in reducing both the incidence of ERE without using antibiotics and caecal *Cl. perfringens* counts. This effect agrees with the reduction of ERE mortality observed in field conditions after changes in management, like the use of the batch breeding system (Licois et al., 2006). Previous works (Licois et al., 2003; Marlier et al., 2006; Szalo et al., 2007) have also demonstrated that it was possible to reproduce experimentally the ERE symptoms in specific-pathogen-free rabbits by means of inocula originated from dust collected in contaminated farms or from intestinal contents of ill animals, in which bacterium *Cl. perfringens* was detected. The accumulation of spores and vegetative cells of *Cl. perfringens* in the environment could contribute to explain the highly variable incidence of ERE observed in the field among commercial farms.

5. Conclusion

These preliminary results indicate that high counts of *Cl. perfringens* in the hindgut are associated with clinical symptoms of ERE and mortality. However, the present study does not elucidate whether the high counts are the cause or the effect. Limiting dietary NDF to around 330 g/kg and a delay of weaning age may also help to control *Cl. perfringens* proliferation and also to decrease fattening mortality in poor hygienic conditions.

Acknowledgement

This work was supported by Eureka Project E! 3854.

References

Álvarez, J.L., Margüenda, I., García-Rebollar, P., Carabaño, R., De Blas, J.C., Corujo, A., García-Ruiz, A.I., 2007. Effects of type and level of fibre on digestive physiology and performance in reproducing and growing rabbits. World Rabbit Sci. 15, pp. 9–17.

AOAC, 2000. Official methods of analysis (seventeenth ed). Association of Official Analytical Chemists, Washington, DC.

Boisot, P., Licois, D., Gidenne, T., 2003b. Evaluation de l'efficacité de la bacitracine soluble (Bacivet S®) dans l'eau de boisson lors d'une reproduction expérimentale de l'entéropathie épizootique (EEL) chez le lapin en croissance. In: 10èmes Journées de la Recherche Cunicole, INRA, Paris, pp. 275–278.

Bostvironnois, C., Morel, A., 2003. Intérêt et positionnement de la tylosine dans la maîtrise de l'entéropathie épizootique (EEL) du lapin de chair. In: 10èmes Journées de la Recherche Cunicole, INRA, Paris, pp. 279–281.

Byrne, B., Scannell, A.G.M., Lyng, J., Bolton, D.J., 2008. An evaluation of *Clostridium perfringens* media. Food Control 19, 1091–1095.

Chamorro, S., Carabaño, R., Badiola, I., García, J., De Blas, J.C., 2007a. Effet d'une supplémentation alimentaire en glutamine et en arginine sur la croissance, quelques paramètres de la flore iléale et caecale et la santé intestinale chez le lapereau, sevré précocement et atteint par le syndrome de l'EEL. In: 12èmes Journées de la Recherche Cunicole, INRA, Le Mans, pp. 89–92.

Chamorro, S., Gómez-Conde, M.S., Pérez de Rozas, A.M., Badiola, I., Carabaño, R., De Blas, J.C., 2007b. 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. Animal 1, 651–659.

Cocchi, M., Drigo, I., Bacchin, C., Bano, L., Marcon, B., Agnoletti, F., 2008. Toxin-genotyping of *Clostridium perfringens* strains isolated from rabbits with enteric disease. In: 9th World Rabbit Congress, Verona, Italy, pp. 921–924.

De Blas, J.C., Wiseman, J., Fraga, M.J., Villamide, M.J., 1992. Prediction of the digestible energy and digestibility of gross energy of feeds for rabbits 2. Mixed diets. Anim. Feed Sci. Technol. 39, 39–59.
De Blas, J.C., Mateos, G.G., 1998. Feed formulation. In: De Blas, J.C., Wiseman, J. (Eds.), The Nutrition of the Rabbit. Commonwealth Agricultural Bureau, Wallingford, UK, pp. 241–253.
Dewrée, R., Meulemans, L., Lassence, C., Desmecht, D., Ducatelle, R., Mast, J., Licois, D., Vindevogel, H., Marlier, D., 2007. Experimentally induced epizootic rabbit enteropathy: clinical, histopathological, ultrastructural, bacteriological and haematological findings. World Rabbit Sci. 15, 91–102.
Duperray, J., Boisot, P., Guyonvarch, A., Richard, A., 2003. Persistance de l'efficacité de la bacitracine pour lutter contre l'entéropathie épizootique du lapin (EEL) après quatre années d'utilisation sur le terrain. In: 10èmes Journées de la Recherche Cunicole, INRA, Paris, pp. 271–274.
EFSA, 2005. Opinion of the Scientific Panel on Biological Hazards on the request from the Commission related to Clostridium spp. in foodstuffs. EFSA J. 199, pp. 1–65.
FEDNA, 2003. Normas FEDNA para la formulación de piensos compuestos. In: De Blas, J.C., Mateos, G.G., García, P. (eds). Fundación Española para el Desarrollo de la Nutrición Animal, Madrid.
Feugier, a., smit, M.N., Fortun-Lamothe, L., Gidenne, T., 2006. Fibre and protein requirements of early weaned rabbits and the interaction with weaning age: effects on digestive health and growth performance. Anim. Sci. 82, 493–500.
Gallois, M., Gidenne, T., Tasca, C., Caubet, C., Coudert, C., Milon, A., Boullier, S., 2007. Maternal milk contains antimicrobial factors that protect young rabbits from enteropathogenic *Escherichia coli* infection. Clin. Vac. Immunol. 14, 585–592.
García, J., De Blas, J.C., Carabaño, R., García, P., 1995. Effect of type of lucerne hay on cecal fermentation and nitrogen contribution through caecotrophy in rabbits. Reprod. Nutr. Dev. 35, 267–275.
García, J., Carabaño, R., Perez Alba, L., De Blas, J.C., 2000. Effect of fiber source on cecal fermentation and nitrogen recycled through cecotrophy in rabbits. J. Anim. Sci. 78, 638–646.
Gidenne, T., Carabaño, R., García, J., De Blas, J.C., 1998. Fibre digestion. In: De Blas, J.C., Wiseman, J. (Eds.), The Nutrition of the Rabbit. Commonwealth Agricultural Bureau, Wallingford, UK, pp. 69–88.
Gidenne, T., Mirabito, L., Jehl, N., Perez, J.M., Arveux, P., Bourdillon, A., Briens, C., Duperray, J., Corrent, E., 2004a. Impact of replacing starch by digestible fibre at two levels of lignocellulose on digestion, growth and digestive health of the rabbit. Anim. Sci. 78, 389–398.
Gidenne, T., Jehl, N., Lapanouse, A., Segura, M., 2004b. Inter-relationship of microbial activity, digestion and gut health in the rabbit: effect of substituting fibre by starch in diets having a high proportion of rapidly fermentable polysaccharides. Br. J. Nutr. 92, 95–104.
Gómez-Conde, M.S., García, J., Chamorro, S., Eiras, P., Rebollar, P.G., Pérez de Rozas, A., Badiola, I., De Blas, J.C., Carabaño, R., 2007. Neutral detergent-soluble fiber improves gut barrier function in 25 d old weaned rabbits. J. Anim. Sci. 85, 3313–3321.
Gutiérrez, I., Espinosa, A., García, J., Carabaño, R., De Blas, J.C., 2002. Effect of levels of starch, fiber and lactose on digestion and growth performance of early-weaned rabbits. J. Anim. Sci. 80, 1029–1037.
Hatheway, C.L., 1990. Toxigenic clostridia. Clin. Microbiol. Rev. 3, 66–98.
Licois, D., Franchet, C., Persillon, C., 2003. Obtention d'échantillons d'air, prélevés dans des locaux experimentaux à l'aide de biocollecteurs, chez des lapins sains et des lapins après reproduction expérimentale de l'entéropathie épizootique. In: 10èmes Journées de la Recherche Cunicole, INRA Paris, pp. 259–262.
Licois, D., Coudert, P., Marlier, D., 2006. Epizootic rabbit enteropathy. In: Maertens, L., Coudert, P. (Eds.), Recent Advances in Rabbit Sciences. Institute for Agricultural and Fisheries Research, Melle, Belgium, pp. 163–170.
Maertens, L., Lebas, F., Szendro, Z., 2006. Rabbit milk: a review of quantity, quality and non-dietary affecting factors. World Rabbit Sci. 14, 205–230.
Marlier, D., Dewrée, R., Lassence, C., Licois, D., Mainil, J., Coudert, P., Meulemans, L., Ducatelle, R., Vindevogel, H., 2006. Infectious agents associated with epizootic rabbit enteropathy: isolation and attempts to reproduce the syndrome. Vet. J. 172, 493–500.
McCullagh, P., Nelder, J.A., 1989. Generalized Linear Models, Second Edition. Chapman and Hall, New York.
Nicodemus, N., Pérez-Alba, L., Carabaño, R., De Blas, J.C., Badiola, I., Pérez de Rozas, A., García, J., 2004. Effect of level of fibre and level of ground of fibre sources on digestion and ileal and caecal characterization of microbiota of early weaned rabbits. In: 8th World Rabbit Congress, 1, Puebla (México), pp. 928–929.
Petit, L., Gibert, M., Popoff, M.R., 1999. *Clostridum perfringens*: toxinotype and genotype. Trends Microbiol. 7, 104–110.
SAS Institute, 1990. SAS/STAT® User's Guide, Version 6, 4th ed. SAS Institute, Cary, NC.
Skrivanova, E., Marounek, M., Dlouha, G., Kanja, J., 2005. Susceptibility of *Clostridium perfringens* to c-2-c-18 fatty acids. Lett. Appl. Microbiol. 41, 77–81.
Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9, 216–234.
Spanish Royal Decree 1201/2005. Sobre protección de los animales utilizados para experimentación y otros fines científicos. Boletín Oficial del Estado 252, 34367–34391.
Standard ISO, Standard ISO 7937/1997. http://www.iso.org/iso/iso_catalogue/catalogue_ics/ catalogue_detail_ics.htm?csnumber=14908&ICS1=07&ICS2=100&ICS3=30.
Szalo, I.M., Lassence, C., Licois, D., Coudert, P., Poulipoullis, A., Vindevogel, H., Marlier, D., 2007. Fractionation of the reference inoculum of epizootic rabbit enteropathy in discontinuous sucrose gradient identifies aetiological agents in high density fractions. Vet. J. 173, 652–657.
Van Soest, J.P., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.