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The use of soft faeces for the prediction of the caecal contents concentration of *Clostridium perfringens* in relation with epizootic rabbit enteropathy

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Abstract

Two experiments were conducted to investigate the use of *Clostridium perfringens* concentration in soft faeces (CPsf) as predictor of *C. perfringens* proliferation in the caecal contents (CPc) and the development of epizootic rabbit enteropathy (ERE). In Exp. 1, 44 rabbits weaned at 28 or 42 days were used. Animals were fed a commercial diet, not supplemented with antibiotics. Nine days after weaning, rabbits were fitted neck collars from 08:00 to 12:00 am. Afterwards, they were slaughtered and both their soft faeces excretion and caecal contents were sampled in order to determine the concentration (cfu g^{-1}) of *C. perfringens*. In Exp. 2, 38 rabbits weaned at 31 days of age, followed the same procedure to determine the evolution of *C. perfringens* counts in soft faeces up to 48 days of age. A total of 15 animals showed ERE symptoms. In both experiments, diseased animals presented a greater ($P < 0.001$) concentration of *C. perfringens* in gut samples than healthy animals. A regression analysis performed in Exp. 1 showed a significant correlation ($R^2 = 0.66$; $P < 0.001$) between CPsf and CPc concentrations measured for each rabbit. The regression model also included a significant effect of age at weaning ($P < 0.001$), as enumeration of CPc was higher in animals weaned at 28 than at 42 days of age. Weight gain decreased ($P = 0.06$) in Exp. 2 with concentration of CPsf, especially ($P = 0.03$) in older animals, whereas the presence of ERE symptoms impaired growth and increased weight variability at each age.

Additional key words: gut microbiota, intestinal health, weaning age, weight gain.

Resumen

Uso de los cecotrofos para la predicción de la concentración de *Clostridium perfringens* en el contenido cecal en un contexto de enteropatía epizoótica del conejo

Se realizaron dos experimentos para investigar el uso de la concentración de *Clostridium perfringens* en las heces blandas (CPsf) para predecir la concentración de *C. perfringens* en el ciego (CPc) y el desarrollo de la enteropatía epizoótica del conejo (ERE). En el Exp. 1 se usaron 44 gazapos destetados a 28 ó 42 días. Los animales recibieron un pienso comercial sin antibiótico. Nueve días después del destete, se les colocó un collar desde las 8:00 hasta las 12:00 para medir y muestrear la producción de cecotrofos. Posteriormente fueron sacrificados para tomar muestras del contenido cecal. Las muestras de contenidos digestivos se analizaron para determinar su concentración de células de *C. perfringens*. En el Exp. 2, se usó el mismo procedimiento para determinar la evolución de los conteos de CPsf con la edad. Quince animales mostraron síntomas de ERE. En ambos experimentos, los animales enfermos mostraron una concentración de *C. perfringens* en las muestras digestivas superior ($P < 0{,}001$) que en animales sanos. Un análisis de regresión realizado en el Exp. 1 mostró una correlación ($R^2 = 0{,}66$; $P < 0{,}001$) entre las concentraciones de CPc y CPsf determinadas en cada animal. El modelo incluyó también un efecto de la edad al destete ($P < 0{,}001$), ya que los conteos de CPc fueron superiores en gazapos destetados precozmente. La ganancia de peso tendió a reducirse ($P = 0{,}06$) con la concentración de CPsf en el Exp. 2, especialmente ($P = 0{,}03$) en animales de mayor edad, mientras que la existencia de síntomas de ERE empeoró el crecimiento y aumentó la variabilidad del peso a cada edad.

Palabras clave adicionales: edad al destete, flora digestiva, ganancia de peso, salud intestinal.

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Introduction

Appeared in 1997, Epizootic Rabbit Enteropathy (ERE) is still nowadays one of the main severe intestinal disorders in rabbit farms in Europe. Mortality caused by ERE peaks at 8-12 days after weaning, irrespectively of weaning age (Garrido *et al*., 2009). High mortality rates are not the only problem caused by the ERE in intensive rabbit production. Some animals affected by ERE survive to the syndrome, but show a decrease in food consumption, which results in lower weight gain (Licois *et al.*, 2006; Szalo *et al.*, 2007).

Although losses due to the syndrome are considerable, the aetiological agent remains to be identified, as it has not been possible to reproduce the disease from pathogens isolated or given in combination (Marlier *et al*., 2006). However, several works have reproduced experimentally the ERE symptoms in specific pathogen free rabbits by means of inocula originated from intestinal contents of ill animals or from dust collected in contaminated farms, in which bacterium *Clostridium perfringens* was detected in high numbers (Licois *et al*., 2003; Marlier *et al.*, 2006; Szalo *et al*., 2007). Also, Romero *et al*. (2009) found that the average concentration of colonies of *C. perfringens* in the caecal contents was highly correlated to the fattening mortality rate due to ERE and that high counts of *C. perfringens* were closely related to the appearance of the clinical signs of ERE.

As a result of its peculiar feeding behaviour, rabbits excrete both hard and soft faeces in a circadian rhythm. As a consequence of the mechanical separation of the digesta at the caecum, soft faeces have a similar chemical and microbiological composition to that of the caecal contents but different to that of hard faeces (Emaldi *et al.*, 1979; Ehrlein *et al.*, 1983; García *et al.*, 1995). Soft faeces also have a high microbial nitrogen concentration (more than double than that of hard faeces; García-Ruiz *et al.*, 2005). Consequently, sampling of soft faeces contents could be useful in order to detect and quantify bacteria present in caecum, such as *C. perfringens*, avoiding the slaughter of the animals.

The aims of this work were i) to establish the relationship between the concentration of *C. perfringens* colonies in the caecum and soft faeces of rabbits nine days after weaning, which might be used in further studies requiring quantification of this bacterium in the gut, and ii) to follow the evolution with age of *C. perfringens* concentration in the digestive contents by means of the soft faeces excreted by rabbits, as well as the evolution of the weight gain, in relation with non-induced ERE symptoms.

Material and methods

Animals and housing

All the experiments were carried out with litters of New Zealand × Californian does originating from strains genetically improved at the Universidad Politécnica de Valencia (Spain). In experiment 1, 44 mixed-sex rabbits from 11 litters were chosen at random. Two rabbits of each litter were weaned at 28 days of age whereas the rest stayed with the mother for another two weeks. In experiment 2, 38 mixed-sex rabbits from ten litters were chosen at random and weaned at 31 days of age.

In both experiments, rabbits were kept under controlled environmental conditions (room temperature between 16 and 24°C; 12 daily hours of light) and housed individually in flat-deck cages measuring 60 cm · 25 cm · 33 cm. These trials were carried out at the Universidad Politécnica of Madrid facilities according to the principles of the Spanish Royal Decree 1201/2005 (BOE, 2005).

Diets

The experimental diets were formulated according to the nutrient recommendations of De Blas and Mateos (1998). The ingredient and chemical composition of the diet used in each experiment is shown in Tables 1 and 2, respectively. Animals had *ad libitum* access to the feed and water throughout the whole experimental period. Neither feed nor drinking water was medicated with antibiotics. However, a coccidiostat (robenidine) was given in the feed.

Experimental procedures

In experiment 1, samples of caecal contents and soft faeces were taken nine days after weaning at both

Abbreviations used: ADF (acid detergent fibre), ADL (acid detergent lignin), CP (crude protein), CPc (*Clostridium perfringens* proliferation in the caecal contents), CPsf (*Clostridium perfringens* concentration in soft faeces), DM (dry matter), ERE (epizootic rabbit enteropathy), GEE (generalized estimating equation), NDF (neutral detergent fibre).

Table 1. Ingredient composition of the experimental diets (%)

Ingredients	Experiment 1	Experiment 2
Barley	31.0	6.0
Wheat bran	—	15.0
Wheat straw	—	10.0
Alfalfa meal, 17% CP	28.3	28.3
Sunflower meal, 30% CP	19.7	19.7
Beet pulp	15.0	15.0
Soybean oil	2.1	2.1
Sodium chloride	0.5	0.4
Calcium carbonate	1.15	1.40
Monocalcium phosphate	0.50	0.57
L-lysine	0.15	0.15
L-threonine	0.10	—
Sepiolite	1.00	0.88
Mineral and vitamin premix[1]	0.50	0.50

[1] Premix provided by Trouw Nutrition España S.A. (Madrid, Spain): mineral and vitamin composition (mg kg^{-1} 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 B_6, 1; Vitamin K, 1; Vitamin E, 20 IU kg^{-1}; Thiamine, 1; Vitamin A, 8375 IU kg^{-1}, Vitamin D3, 750 IU kg^{-1}, Robenidine, 60.

weaning ages. To collect soft faeces, animals were fitted a neck collar which avoided soft faeces re-ingestion. Rabbits weighed at slaughter 861 ± 167 g and 1,520 ± 232 g on average when weaned at 28 or 42 days, respectively. Collars were made on transparent plastic (33.0 g and 330 mm of diameter on average) and were put from 8:00 to 12:00 am. After that, animals were slaughtered in a CO_2 chamber and samples of caecal contents were also collected. *C. perfringens* enumeration was determined the same day both in soft faeces and caecal contents and it was performed according to the standard ISO 7937 (1997). The cultural medium used was agar tryptose sulphite added with antibiotic D-cycloserine. Later on, the plates were incubated during 18 hours at 37°C.

In experiment 2, rabbits were weighed at 34, 38, 41, 45 and 48 days of age at 8 am. After that, they were fitted with a neck collar to collect soft faeces and determine *C. perfringens* enumeration following the same protocols than in experiment 1.

Samples of dust were collected over the air extractors of the experimental farm at the start, in the middle and at the end of both experimental periods. All these samples were analyzed 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 environmental concentration of spores.

Table 2. Chemical composition and nutritive value of experimental diets

Nutrients (% dry matter)	Experiment 1	Experiment 2
Dry matter	90.9	90.8
Crude protein	15.5	14.7
Ether extract	4.0	4.0
Ash	8.6	9.9
Starch	14.9	6.70
Crude fibre	15.7	19.9
Neutral detergent fibre	33.0	42.5
Acid detergent fibre	20.3	24.9
Acid detergent lignin	4.70	5.50
Sugars	3.50	0.90
Soluble fibre[1]	11.4	12.1
Digestible energy[2], MJ kg^{-1}	10.1	8.67

[1] Estimated as (100 – moisture – ash – crude protein – ether extract – neutral detergent fibre – starch – sugars). [2] Value estimated according to FEDNA (2003).

Analytical methods

All chemical analyses were conducted in duplicate. Procedures of the AOAC (2000) were used to determine dry matter (930.15), ash (923.03), crude protein (954.01), ether extract (920.39), crude fibre (978.10), sugars (974.06) and starch (996.11). Contents of NDF, ADF and acid-detergent lignin were determined according to the sequential method of Van Soest *et al*. (1991).

Statistical analysis

The relationship between the concentration of *C. perfringens* colonies in the caecal contents and soft faeces was analysed using a negative binomial regression, which served to account for overdispersion found when data were analyzed using the Poisson regression (Cameron and Trivedi, 1998). Weaning age was also included as a factor in the model to estimate different regressions at each age. The model was fitted with the GENMOD procedure of SAS (SAS Inst, 1990).

The evolution with the age of the *C. perfringens* counts in the soft faeces was analyzed using a negative binomial regression model. The explanatory variables were age and the presence/absence of symptoms of ERE at the particular age. The count responses for individual rabbit were assumed to be correlated with an autoregressive correlation structure. The Generalized

Estimating Equation (GEE; Liang and Zeger, 1986) was used for estimating the regression parameters of the model. The model was fitted with the GENMOD procedure of SAS (SAS Inst, 1990) using the repeated statement to invoke the GEE method.

In order to quantify the effect of the *C. perfringens* concentration in the soft faeces and the ERE symptoms on the weight gain of rabbits, mixed effect models were used. The analysis strategy employs a linear growth curve model for the rabbits as well as a variance-covariance model that incorporates correlations for all of the observations arising from the same rabbit. The explanatory variables were the age of rabbit, the average natural logarithm of the *C. perfringens* counts in soft faeces (from 34 to 48 days of age), and the presence/absence of ERE symptoms in the rabbit at the day of weighing. The model allows for different regressions of the weights on the age for each rabbit, assuming that the intercept and slope are random coefficients (Littell *et al*., 1996). Correlation of weights within rabbits was accounted with a first-order continuous autoregressive structure. The model was fitted with the mixed procedures of SAS (SAS Inst, 1990). Finally, the heterogeneity of residual variance associated to the presence/absence of ERE symptoms was incorporated.

Results

Experiment 1

Five out of the 44 animals studied presented clinical signs of ERE as bloat, relatively low body weight, distension of both stomach and small intestine, and either liquid or compacted caecal contents. Four of them were weaned at 28 and the other at 42 days of age. The average number of colony forming units (cfu) of *C. perfringens* in the caecal contents was 84.2 times higher ($P < 0.001$) in rabbits showing ERE symptoms ($46 \cdot 10^6$ cfu g^{-1}; $n = 5$), than in healthy animals ($0.55 \cdot 10^6$ cfu g^{-1}; $n = 39$). The threshold level above which symptoms appeared was $12 \cdot 10^6$ cfu of *C. perfringens* per gram of caecal contents. Otherwise, only one rabbit with ERE symptoms produced soft faeces. The concentration of *C. perfringens* in the caecal contents and soft faeces of this animal was respectively $42 \cdot 10^6$ and $48 \cdot 10^6$ cfu g^{-1}, both of them well above the average values obtained in healthy animals ($0.57 \cdot 10^6$ cfu g^{-1} in the case of soft faeces).

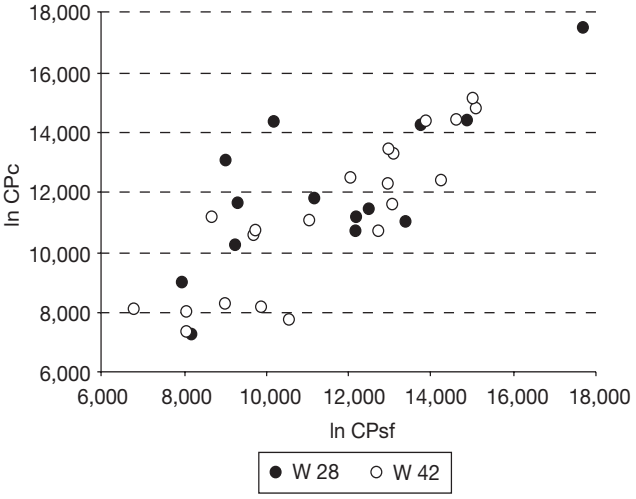

Figure 1. Relationship between counts of *Clostridium perfringens* (ln cfu g^{-1}) in samples of soft faeces (CPsf) and caecal contents (CPc) obtained from the same animals weaned at 28 (W28) or 42 (W42) days of age.

The concentration of *C. perfringens* found in the caecal contents (CPc, ln cfu g^{-1}) increased by 0.64 units per each unit of increment of the enumeration established in the soft faeces (CPsf, ln *C. perfringens*) at both ages of weaning ($R^2 = 0.66$; $P < 0.001$; $n = 40$; see Fig. 1). Furthermore, the age at weaning influenced caecal *C. perfringens* counts, which were higher ($P < 0.001$) in rabbits weaned at 28 than at 42 days of age, but did not modify significantly the relationship between CPc and CPsf. The regression equations obtained between CPc and CPsf for animals weaned at 28 (W28) or 42 (W42) days of age were:

$$\text{W28: ln (CPc)} = 5.93\ (\pm 1.31, \text{SE}) + 0.64\ (\pm 0.12)\ \text{ln (CPsf)}$$
$$P < 0.001 \qquad P < 0.001$$

$$\text{W42: ln (CPc)} = 4.27\ (\pm 1.32) + 0.64\ (\pm 0.12)\ \text{ln (CPsf)}$$
$$P = 0.0012 \qquad P < 0.001$$

Experiment 2

None of the thirty eight experimental animals died during the current trial. Soft faeces were excreted by respectively 100%, 97%, 100%, 95% and 97% of the animals at 34, 38, 41, 45 and 48 days of age. Mean body weights at the successive controls were 706 ± 153 (SD), 874 ± 153, $1{,}021 \pm 152$, $1{,}205 \pm 152$ and $1{,}348 \pm 152$ g, and average daily weight gains 42.9 ± 21.3, 45.2 ± 12.1, 45.9 ± 9.60 and 45.8 ± 6.94 g d^{-1} for the 34-38, 38-41, 41-45 and 45-48 d age-periods respectively.

Enumeration of *C. perfringens* in soft faeces was higher in experiment 2 with respect to experiment 1, in correspondence to a higher spore counting in the air extractors (0.57 *vs* $0.25 \cdot 10^6$ cfu g^{-1}). Ten among the 38 rabbits presented clinical ERE symptoms throughout the experimental period. These animals had a higher *C. perfringens* concentration in soft faeces than healthy animals ($13.8 \cdot 10^6$ *vs* $0.16 \cdot 10^6$ cfu g^{-1}; $P < 0.001$), with values in diseased animals ranging from $0.40 \cdot 10^6$ to $282 \cdot 10^6$ cfu g^{-1}. Counts made in soft faeces from healthy rabbits were always below $0.89 \cdot 10^6$ cfu g^{-1}, whereas all the animals showing ERE symptoms, except two, were above 10^6 cfu g^{-1}. *C. perfringens* enumeration in the soft faeces was not affected significantly by age of rabbits, averaging 12.0, 11.4, 11.3, 12.0, 11.7 ln cfu g^{-1} at 34, 38, 41, 45 and 48 days of age, respectively.

Weight of animals throughout the experimental period (W, g) depended on the number of days past after weaning (D), the presence/absence of ERE symptoms when the control was made, and on the average count of *C. perfringens* in soft faeces through the whole experimental period (CPsf, ln cfu g^{-1}). The regression equations obtained were:

a) *Rabbits with no ERE symptoms*

$$W = 1{,}061\ (\pm 188, SE; P < 0.001) + 65.3\ (\pm 8.07; P < 0.001)\ D - 31.3\ (\pm 15.9; P = 0.06)\ lnCPsf - 1.50\ (\pm 0.69; P = 0.03)\ D \cdot lnCPsf$$

b) *Rabbits with ERE symptoms*

$$W = 1{,}113\ (\pm 196; P < 0.001) + 55.8\ (\pm 9.74; P < 0.001)\ D - 31.3\ (\pm 15.9; P = 0.06)\ lnCPsf - 1.50\ (\pm 0.69; P = 0.03)\ D \cdot lnCPsf$$

The model predicts a lower average weight of rabbits affected by ERE 10 days after weaning (909 *vs* 1,158 g in healthy animals), when particularizing the equations for the average lnCPsf counts determined in this study. The results also indicate that weight at each control tended to decrease ($P = 0.06$) by 31.3 g as average per each increase in one unit of lnCPsf. Furthermore, the effect of lnCPsf was greater (by 1.5 g) per each day past after weaning. Otherwise, the standard deviation of weight of rabbits increased (by 5.76 ± 1.24 (SE) times) when animals showed ERE symptoms.

Discussion

The results of the current study show that *C. perfringens* cells were present in variable amounts in all the gut samples analyzed. They also confirm previous works (Marlier *et al.*, 2006; Szalo *et al.*, 2007; Romero *et al.*, 2009) that had shown that the proliferation of *C. perfringens* in the gut was closely linked with the appearance of ERE symptoms. Our study also allows to quantify the effects of the appearance of ERE symptoms and of the *C. perfringens* concentration in soft faeces on weight loss and weight variability throughout the fattening period. The higher variability associated to ERE symptoms has economical importance, especially in the case of rabbits reared in batches, because of depreciation of light carcasses at the slaughterhouse.

A decrease of *C. perfringens* counts in caecal and soft faeces samples of rabbits weaned later (at 42 instead of 28 days of age) was observed in the current study. Moreover, the effect of age at weaning was independent of maternal effects, as it was determined by comparing animals belonging to the same litter. Recent works have shown that a delay of weaning age might have a protective effect on rabbit viability by reducing *Escherichia coli* O103 (Gallois *et al.*, 2007) and *C. perfringens* (Romero *et al.*, 2009) proliferation in faeces and caecal contents, respectively. This effect might be explained by a better adaptation of the digestive and absorptive capability of young rabbits to the consumption of solid feed and/or by the presence in rabbit's milk of significant amounts of compounds with bactericide effect, as medium-chain fatty acids (Skrivanova *et al.*, 2005; Maertens *et al.*, 2006).

The current results indicate that concentration of *C. perfringens* in soft faeces might be a good indicator of the prevalence of the ERE in rabbits, because of its high correlation with the enumeration of *C. perfringens* in the caecum. These results are relevant in the scope of a previous work that has shown that a reduction of the proliferation of *C. perfringens* in the caecal contents (below $2 \cdot 10^6$ cfu g^{-1}), allowed decreasing ERE mortality (to less than 10%) in fattening animals not supplemented with antibiotics (Romero *et al.*, 2009). In this context, the use of samples of soft faeces might serve to test for different nutritional and management strategies aiming to control caecal *C. perfringens* proliferation in the gut and ERE incidence, without need of slaughtering the animals.

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