THE USE OF SOFT FAECES FOR THE PREDICTION OF THE CAECAL CONTENTS CONCENTRATION OF *CLOSTRIDIUM PERFRINGENS* IN RABBITS WEANED AT TWO AGES

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ABSTRACT

Since its emergence in 1997, the Epizootic Rabbit Enteropathy (ERE) has become a severe disease affecting Europe almost as a whole. The aetiological agent remains to be identified, even if several researchers have shown the bacterium Gram-positive *Clostridium perfringens* to be involved in ERE development. The average count of *Clostridium perfringens* in the caecal contents of young rabbits was found to be highly correlated with average diarrhea mortality in the fattening period (R^2=0.92; P<0.001), whereas high counts (>2 x 10^6 cfu/g) of *Clostridium perfringens* were related with the appearance of the clinical signs of ERE. Accordingly, the caecal enumeration of *Clostridium perfringens* might be used as a good indicator of ERE. However, previous slaughter of rabbits is required to make this determination. Slaughter might be avoided by using samples of caecotrophs. In this context, the main aim of this work was to develop a method leading to estimate the caecal contents counts of *Clostridium perfringens* through its concentration in the rabbit soft faeces. Forty-four rabbits weaned at 28 or 42 d of age were used. Animals were fed a common commercial diet, not supplemented with antibiotics, and housed in pairs in flat-deck cages. Nine days after weaning, rabbits were fitted neck plastic collars during four hours (from 08:00 to 12:00). Afterwards, they were slaughtered and both their soft faeces excretion and caecal contents were sampled in order to determine the concentration (cfu/g) of *Clostridium perfringens*. This concentration was determined by plate counting according to the standard ISO 7937 (1997). Values were transformed to a logarithmic scale to homogenize variances. Eight out of the 44 animals used did not excrete soft faeces; five of them showed clinical symptoms of ERE. Otherwise, concentration of *Clostridium perfringens* in the caecal contents tended to be higher in animals weaned at 28 than at 42 days of age (5.46 vs. 5.13 log cfu/g), although differences did not reach significance levels. Regression procedures were used to relate *Clostridium perfringens* quantification in the soft faeces (CPsf, log cfu/g) with that of the caecal contents (CPc, log cfu/g) for each rabbit. The regression equation obtained was: CPc=0.622+0.858 CPsf (r=+0.885, RSD=0.529; P<0.001). In conclusion, collecting and analyzing soft faeces could be used as an alternative method to predict the content of *Clostridium perfringens* in the caecal contents of rabbits before presenting symptoms of ERE.

Key words: *Clostridium perfringens*, ERE, Caecotrophs, Caecum.

INTRODUCTION

Appeared in 1997, Epizootic Rabbit Enteropathy (ERE) is still nowadays one of the main severe intestinal disorders in rabbit farms in Europe. This digestive disease has increased the mortality in fattening rabbits up to more than 50%. Even if the aetiological agent remains unknown, it has been possible to reproduce experimentally the ERE symptoms in specific pathogen free rabbits by means of inocula originated from intestinal contents of ill animals, in which bacterium *Clostridium perfringens* was detected (Licois *et al.*, 2006; Szalo *et al.*, 2007). Moreover, several authors (Le Normand *et al.*, 2003; Dewrée *et al.*, 2003; Marlier *et al.*, 2003; Marlier *et al.*, 2006) have also shown that *Clostridium perfringens* may play a major role in this pathology because of its important proliferation in digestive tract when animals are affected by ERE. Also Romero *et al.* (2007) found that the caecal contents

concentration of colonies of *Clostridium perfringens* was closely related (r = + 0.96; P<0.001) to the fattening mortality rate due to ERE and that high counts (>2 x 10^6 cfu/g) of *Clostridium perfringens* were related with the appearance of the clinical signs of ERE.

As a result of its peculiar feeding behaviour, rabbits excrete both soft and hard faeces in a circadian rhythm. As a consequence of the mechanical separation of the digesta at the caecum, soft faeces have a similar chemical composition to that of the caecal contents but different to that of hard faeces (Emaldi *et al.*, 1978; Ehrlein *et al.*, 1982; Carabaño and Piquer, 1998). Caecotrophs 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 *Clostridium perfringens*.

The aim of this work was to establish a relation between the concentration of *Clostridium perfringens* colonies in caecum and soft faeces of rabbits which might be used in further studies requiring quantification of this bacterium in the gut.

MATERIALS AND METHODS

Animals and experimental design

Forty-four mixed-sex rabbits from eleven litters of New Zealand x Californian does (originating from strains genetically improved at the Universidad Politécnica of Valencia, Spain) were chosen at random for this study. Two rabbits of each litter were weaned at 28 days of age whereas the rest stayed with the mother for another two weeks. Samples of the caecal contents and soft faeces were taken nine days after weaning. Rabbits weighed at slaughter 861 ± 167 g and 1520 ± 232 g on average when weaned at 28 or 42 days, respectively. Nine days after weaning, animals were fitted a neck collar which avoided caecotrophs re-ingestion. Collars were made on transparent plastic (33.0 g and 330 mm of diameter on average). They were put from 8:00 a.m. to 12:00 a.m. After that, animals were slaughtered in a CO_2 chamber. Then, samples of caecotrophs and caecal contents were collected and analysed the same day. *Clostridium perfringens* enumeration 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 37ºC.

Rabbits were kept under controlled environmental conditions (room temperature between 16 and 24ºC; 12 daily hours of light) and housed in pairs in flat-deck cages measuring 60 cm x 25 cm x 33 cm. This trial was carried out at the Universidad Politécnica of Madrid facilities according to the principles of the Spanish Royal Decree 1201/2005. Animals were fed the same diet (Tables 1 and 2) and had *ad libitum* access to the feed and water throughout the whole experimental period. The experimental diet was formulated according to the nutrient recommendations of De Blas and Mateos (1998). Neither feed nor drinking water was medicated with antibiotics. However, a coccidiostat (robenidine) was given in the feed.

Chemical analyses

Chemical analyses were performed at the Poultry and Rabbit Research Center of Nutreco using the procedures of Association of Official Analytical Chemists (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, ADF and acid-detergent lignin were determined according to the sequential method of Van Soest *et al.* (1991).

Statistical analysis

Data were analyzed as a complete random design with weaning age as main factor. A Levene´s test showed lack of homogeneity of variance; accordingly, values were transformed to a logarithmic scale. Regression procedures (Statistical Analysis Systems Institute, 1991) were used to relate *Clostridium perfringens* colonies enumeration in soft faeces with its value in caecal contents for each rabbit.

Table 1: Ingredient composition of the experimental diet (%)

Barley	31.0
Alfalfa meal, 17% CP	28.3
Sunflower meal, 30% CP	19.7
Beet pulp	15.0
Soybean oil	2.10
Sodium chloride	0.50
Calcium carbonate	1.15
Monocalcium phosphate	0.50
L-lysine	0.15
L-threonine	0.10
Sepiolite	1.00
Mineral and vitamin premix[1]	0.50

[1] 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 B_6, 1; Vitamin K, 1; Vitamin E, 20 IU/kg; Thiamine, 1; Vitamin A, 8375 IU/kg,, Vitamin D_3, 750 IU/kg, Robenidine, 60

Table 2: Chemical composition and nutritive value of experimental diet (% as fed)

Dry matter	90.9
Crude protein	15.5
Ether extract	4.00
Ash	8.60
Starch	14.9
Crude fibre	15.7
NDF	33.0
ADF	20.3
ADL	4.70
Sugars	3.50
Soluble fibre[1]	11.4
Digestible energy (MJ/kg)[2]	10.1

[1]Estimated as (100 – moisture – ash – CP – ether extract – NDF – starch – sugars)
[2]Value estimated according to FEDNA (2003)

RESULTS AND DISCUSSION

Eight of the 44 animals did not excrete soft faeces. Five of them 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. Only another rabbit with ERE symptoms produced caecotrophs. Concentration of *Clostridium perfringens* in the caecal contents and soft faeces of this animal were respectively 42 x 10^6 and 48 x 10^6 cfu/g, which were well above average values obtained in healthy animals. Otherwise, the average concentration of *Clostridium perfringens* in the caecal contents of rabbits with or without ERE symptoms were 6.81 ± 1.83 (SD) and 5.03 ± 1.08 log cfu/g, respectively.

Animals weaned at 28 days of age (n = 20) contained 5.46 ± 1.57 log cfu/g of *Clostridium perfringens* in caecal contents. On the contrary, the enumeration was lower (5.13 ± 1.12 log cfu/g) if weaning occurred later (n = 20), although this difference did not reach statistical significance levels. However, when removing ill animals from the data the average values became the same: 5.03 log cfu/g.

The number of colony forming units of *Clostridium perfringens* found in soft faeces (CPsf, log cfu/g) was linearly related (r = +0.885; RSD = 0.529; P<0.001; n = 36; see Figure 1) with the enumeration established in the caecal contents (CPc, log cfu/g). The regression equation obtained was:
CPc = 0.621 (± 0.419) + 0.858 (± 0.0809) CPsf

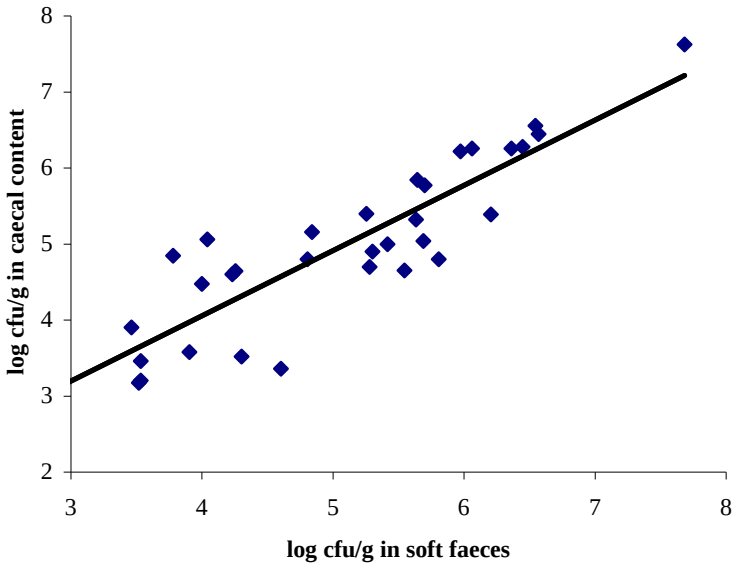

Figure 1: Regression between counts of *Clostridium perfringens* (log cfu/g) in samples of soft faeces and caecal contents obtained from the same animals

These results are relevant in the scope of a previous work (Romero *et al*., 2007) that has shown that a reduction of the proliferation of *Clostridium perfringens* in the gut (below 100.000 cfu/g caecal contents) allowed to decrease fattening mortality (below 10%) in animals not supplemented with antibiotics housed in a farm affected by ERE. In this context, the use of samples of caecotrophs to predict caecal concentration of *Clostridium perfringens* would avoid the need of slaughter the animals. It would also permit to follow the evolution of this concentration throughout the time in parallel to the presentation of the symptoms of intestinal disorders.

CONCLUSIONS

The results from this study indicate that count of colonies of *Clostridium perfringens* in soft faeces can be used as a reliable indicator of the number of colonies of *Clostridium perfringens* present in the caecal contents of rabbits.

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