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INFLUENCE OF TWO ISO-ENERGETIC DIETS (STARCH *vs* FAT) ON EXPERIMENTAL COLIBACILLOSIS (EPEC) AND IOTA-ENTEROTOXAEMIA IN EARLY WEANED RABBITS.

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SUMMARY :
Rabbits weaned at 32 days of age were fed one of two iso-energetic (10.6 MJ ADE/kg) and iso-protein (17 %) feeds *ad libitum* : feed S with 25.8 % starch, 3.2 % fat and 16.2 % acid detergent fibre (ADF) and feed F with 13.1 % starch, 6.7 % fat and 17.9 % ADF. Both groups were inoculated orally with a toxinogenic *Clostridium spiroforme* strain NCTC 11493 and/or with a moderately enteropathogenic *Escherichia coli* (EPEC) strain belonging to sero/biotype O132/2+. The results suggest that colibacillosis favours iota-enterotoxaemia and vice-versa. Also the feed shows a distinct influence. Feed S favoured iota-enterotoxaemia. The effect was most pronounced in rabbits suffering from simultaneous EPEC infection. On the other hand the same feed inhibited saprophytic *E. coli*-proliferation. In case of experimental EPEC infection, feed S did not inhibit the huge faecal *E. coli* output, nor the associated diarrhoea and mortality. Yet, histological lesions were less pronounced in the S-group. Moreover, entero-adherent EPEC were still present in one rabbit out of five in group F 28 days p.i., while they were not detected in any of the S-rabbits. In case of mixed infection (*C. spiroforme* + EPEC), mortality was lower in group S (2/10) than in group F (5/10). So, feed F might favour colibacillosis. It was hypothesised that feeds of this type may contribute to the persistence of healthy carriers of enteropathogenic *E. coli*.

RÉSUMÉ : Influence de deux aliments iso-énergétiques (amidon vs graisses) sur la colibacillose (EPEC) expérimentale et sur l'entérotoxémie-iota chez des lapereaux après sevrage précoce

Deux aliments iso-énergétiques (10.6 MJ ADE/kg) et iso-protéiques (17 %) ont été administrés ad libitum *à des lapereaux sevrés à l'âge de 32 jours : un aliment S contenant 25,8 % d'amidon, 3,2 % de graisses et 16,2 % d'acid detergent fiber (ADF) et un aliment F contenant 13,1 % d'amidon, 6,7 % de graisses et 17,9 % ADF. Les deux groupes ont été inoculés par voie orale avec la souche toxinogène NCTC 11493 de* Clostridium spiroforme *et/ou avec une souche* d'Escherichia coli *modérement entéropathogène (EPEC) du séro/biotype O132/2+. Les résultats suggèrent que la colibacillose favorise l'entérotoxémie-iota et vice-versa. L'aliment exerce aussi un effet significatif. L'aliment S favorisait l'entérotoxémie-iota. L'effet était le plus prononcé chez les lapereaux atteints simultanément d'une infection à EPEC. Par contre, le même aliment a inhibé la prolifération d'* E. coli *saprophytes. Dans le cas d'une infection expérimentale avec des EPEC, l'aliment S n'était pas en mesure d'inhiber l'excrétion fécale massive des* E. coli, *ni la diarrhée et la mortalité associées. Pourtant, les lésions histologiques étaient moins prononcées dans le groupe S. Des EPEC attachées à la muqueuse intestinale étaient toujours présentes 28 jours après l'infection chez un lapereau sur cinq dans le groupe F, alors qu'elles étaient absentes dans le groupe S. Dans le cas de l'infection mixte (*C. spiroforme *+ EPEC), la mortalité était plus basse dans le groupe S (2/10) que dans le groupe F (5/10). L'aliment F pourrait donc favoriser la colibacillose, d'où l'hypothèse que des aliments de ce genre pourraient contribuer à la persistance de porteurs sains d'* E. coli *entéropathogènes.*

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) and *Clostridium spiroforme* iota-entero-toxaemia cause significant losses in commercial rabbitries (LICOIS, 1992, CARMAN and EVANS, 1984). *C. spiroforme* produces a toxin that destroys caecal epithelial cells and provokes caecal haemorrhage. The bacillus affects mainly early weaned rabbits and rabbits medicated with antibiotics. Moreover, *C. spiroforme* complicates EPEC induced disease. According to the classification of PEETERS *et al.* (1988a) different pathotypes occur among EPEC. Strains belonging to sero/biotypes

O15/3–, O26/4+ and O103/8+ provoke high mortality, whereas less pathogenic strains such as O128/2+ and O132/2+ are involved with enteric problems in rabbitries with a high occupation density and/or low hygienic standards (PEETERS, 1994).

In contrast to most other mammalian species, only low numbers of *Escherichia coli* are present in the gut of weaned rabbits (MATTHES, 1969, GOUET and FONTY, 1973). PROHASZKA (1980) showed *in vitro* that caecal volatile fatty acids (VFA) and low caecal pH may inhibit *E. coli* proliferation. As caecal pH and VFA levels are determined by the composition of the feed and the speed of the intestinal transit, it is probable that the diet influences the impact and outcome of colibacillosis. This hypothesis is supported by the fact that concurrent infections as coccidiosis and rotavirus infection, which cause a temporary increase of caecal pH, favour *E. coli* proliferation (LICOIS and GUILLOT, 1980, PEETERS *et al.*, 1988b).

On the other hand, there are indications that dietary starch is involved with the genesis of iota–entero–toxaemia. In normal conditions, starch is completely hydrolysed and absorbed in the small intestine by the action of pancreatic amylase and does not reach the hindgut (GIDENNE & PEREZ, 1993). However, amylase production is very low at 28 days of age and only reaches maximal levels at 42 days (CORRING *et al.*, 1972). The increase of amylase production with age occurs independently of the starch levels in the feed. As a consequence of low amylase activity, unaltered starch may reach the hindgut of early weaned rabbits fed a diet rich in starch (BLAS *et al.*, 1988). Starch will be hydrolysed into glucose by the action of the caecal microflora. As glucose is necessary for the production of iota-toxin *in vitro* (BORRIELLO and CARMAN, 1983), CHEEKE and PATTON (1980) hypothesised that diets rich in starch favour enterotoxaemia. The relationship between high dietary starch content and early post–weaning disorders has already been confirmed under practical conditions (LEBAS and MAITRE, 1989). Moreover, experimental *C. spiroforme* infection causes only iota–enterotoxaemia in 4–week–old rabbits and not in 5–12–week–old animals (CARMAN and BORRIELLO, 1984, PEETERS *et al.*, 1986a).

Therefore we tried to confirm the hypothesis of CHEEKE and PATTON by substituting a high dietary level of starch by fat in iso–energetic and iso–protein diets and examined the influence of experimental infection with *C. spiroforme* in early weaned rabbits. As the presence of undigested starch in the caecum may contribute to an increase of VFA–levels and to a decrease of caecal pH and thus may inhibit *E. coli* proliferation, we also tested the influence of both diets on experimental infection with a moderately pathogenic EPEC O132/2+ strain.

MATERIALS AND METHODS

Animals and husbandry.

Before weaning, the rabbits received a doe ration formulated according to the actual recommendations (MAERTENS, 1992). All rabbits were weaned at 32 days of age at a mean weight of 768 g (coefficient of variation = 12 %) and transferred to the National Institute of Veterinary Research. They were housed individually in heat–sterilised, wire–floored metal cages at 22°C ambient temperature. Full metal walls between cages prevented any contact between rabbits. The first day after arrival, 40 faecal samples were taken for screening pathogens : 12 out of 40 samples contained low numbers of saprophytic *E. coli* and nine low numbers of *C. spiroforme*. Rotaviruses nor *Eimeria spp.* were detected in any sample.

Feed

Rabbits were fed one of two iso–energetic (10.6 MJ ADE/kg) and iso–protein (17 %) diets (Table 1) *ad libitum* from weaning age : a diet S with 16.2 % acid detergent fibre (ADF), 25.8 % starch and 3.2 % fat and a diet F with 17.9 % ADF, 6.7 % fat and only 13.1 %

Table 1 : Ingredients and chemical composition of the diet.

	Diet S	Diet F
Ingredients (%)		
Corn	40.00	5.75
Wheat shorts	–	37.25
Alfalfa meal 18	31.25	31.25
Soybean meal 44	11.75	–
Full–fat soybeans	–	10.75
Sunlower meal 29	9.50	3.50
Flaxchaff	–	2.75
Molasses	4.00	4.00
Animal fat	–	2.00
Calcium phosphate	1.00	–
$CaCO_3$	–	0.25
DL–Methionine	0.13	0.15
Vit.–Min. mix	2.50	2.50
Diclazuril	1ppm	1ppm
Chemical composition (%)		
Dry matter	88.2	88.3
Crude protein	16.9	17.1
Crude fat	**3.2**	**6.7**
Crude fiber	13.0	15.2
NDF	27.6	31.0
ADF	16.2	17.9
Starch	**25.8**	**13.1**
ADE (MJ/kg)*	10.7	10.6
ME ((MJ/kg)*	10.2	10.1
P**	0.60	0.72
Ca**	1.15	1.01

* caculated according to MAERTENS *et al.*, 1990.

** calculated values

starch. The dietary energy content was calculated using the values and methods proposed by MAERTENS *et al.* (1990). Prior to the formulation, the most important raw materials have been analysed in order to increase the reliability of the calculations. Subsequently, the energy content of the raw materials was calculated based on these results and on the digestibility coefficients. These energy values were taken into account by the computer in order to calculate both iso-energetic diets. Diclazuril was used as anticoccidial drug.

Experimental design.

A total of 120 conventional hybrid rabbits of mixed sexes was used. The rabbits were divided into eight experimental groups of 15 animals and fed one of the experimental feeds (Table 2). To reduce variability, only litters with eight or four weanlings weighing between 600 and 900 g were divided each over the experimental groups. The rabbits received drinking water and the pelleted ration *ad libitum*. Two groups remained uninfected, whereas the six other groups were infected with EPEC and/or *C. spiroforme*. They were inoculated orally with 2 ml of bacterial suspension. All groups were housed in the same room. Special care was taken to prevent cross contamination of animals. The individual weight gain, food and water consumption were recorded once a week during four weeks. Animals were observed daily. Diarrhoea was assessed as follows : 0 = no diarrhoea; 1 = increased water content of faecal pellets; 2 = pulpy diarrhoea ; 3 = liquid stools. *E. coli* output was evaluated semi-quantitatively after streaking rectal swabs on G2S, a selective medium for *enterobacteriaceae* (POHL and THOMAS, 1966) : 0 = no colonies; 1 = isolated widely spaced colonies ; 2 = isolated closely spaced colonies ; 3 = confluent growth. Faecal output of *E. coli* belonging to serotype O132/biotype 2+ was checked with specific antisera. *C. spiroforme* output was evaluated semi-quantitatively after gram-stain of faecal smears : 0 = no bacteria ; 1 = 4-10 fields with bacteria ; 2 = multiple fields with bacteria arranged in helices ; 3 = multiple fields with microcolonies. One and four weeks after the experimental infection five rabbits of each group were sacrificed by cervical dislocation between 9 and 11 a.m. Immediately after necropsying the animals, the caecum was weighed and caecal pH was determined with a glass electrode at two places through a small slit in the body of the caecum. Aliquots were taken for evaluation of numbers of *E. coli* and *C. spiroforme*, NH3 and VFA-content. Rabbits were also checked on the presence of rotaviruses and *Eimeria spp.* Moreover, portions of ileum and caecum were processed for histology.

Infective material.

Enteropathogenic *E. coli*, recently isolated from experimentally infected rabbits and belonging to serogroup O132:K-:H2/biotype 2+were used (PEETERS *et al.*, 1988a). The inoculum was prepared from second-passage organisms grown on blood agar by inoculating colonies into nutrient broth and incubating for 6 h aerobically at 37°C in Penassay broth. Total number of *E. coli* present in the inoculum was 8.2 x 10^6 cells per ml.

A strain of *C. spiroforme* NCTC11493 obtained from the National Collection of Type Cultures (London, England) was grown on Columbia base (Oxoid Ltd, London, England) 10 % sheep blood agar plates. After 48 h of incubation in strict anaerobiosis at 37°C on Columbia agar, bacteria were suspended in PBS-pH 7.2 and used for the experimental infection. The suspension contained 2.4 x 10^6 cells per ml.

Bacteriology.

Caecal samples were diluted 1:10 in cold PBS-pH 7.2, shaken for one minute with a Heidolph shaking device and stored within 10 minutes after sacrificing the animals, at 4°C until tested 2 hours later. Further decimal dilutions were made in cold sterile PBS and 1 ml aliquots were streaked on petrifilm 6410 (3M, St Paul, Minnesota, USA). After 18 hours of aerobic incubation at 37°C, the number of lactose positive colony forming units was evaluated. O:K:H serotypes were determined by standard methods (ØRSKOV and ØRSKOV, 1984). Biotyping was performed according to the modified scheme of Okerman and Devriese (PEETERS *et al.*, 1988a). Isolation of *C. spiroforme* from caecal contents was done according to BORRIELLO and CARMAN (1983). Numbers of spores were evaluated after culture of 10-fold dilutions of caecal contents on Columbia base 10 % sheep blood agar plates. Caecal contents were analysed for the presence of iota-like toxin by intraperitoneal administration of caecal filtrates to mice and neutralisation by *C. perfringens* antitoxins (Wellcome Research Laboratories, Beckenham, Kent, England) as described elsewhere (STERNE and BATTY, 1975).

Virology.

Rotaviruses were screened by immunodiffusion (VANOPDENBOSCH *et al.*, 1978)

Parasitology.

Faecal samples were examined by the MCMASTER egg counting technique for *Eimeria spp.* and helminths (MAFF, 1977). A portion of the screened faecal suspensions was allowed to sporulate for subsequent differentiation in species.

Histology.

Within 20 minutes after sacrifying the animals, specimens of intestine were rinsed in and fixed with 10 % (v/v) formalin in phosphate-buffered saline and processed routinely for paraffin sections. Sections were cut at 5 μm. Coliform bacteria attached to the intestinal mucosa were traced in haematoxylin and eosin-stained sections at magnifications of 500 x and 1000 x with a Leitz Laborlux 12 microscope. A

Figure 1 : *E. coli* and diarrhoea scores in non infected rabbits

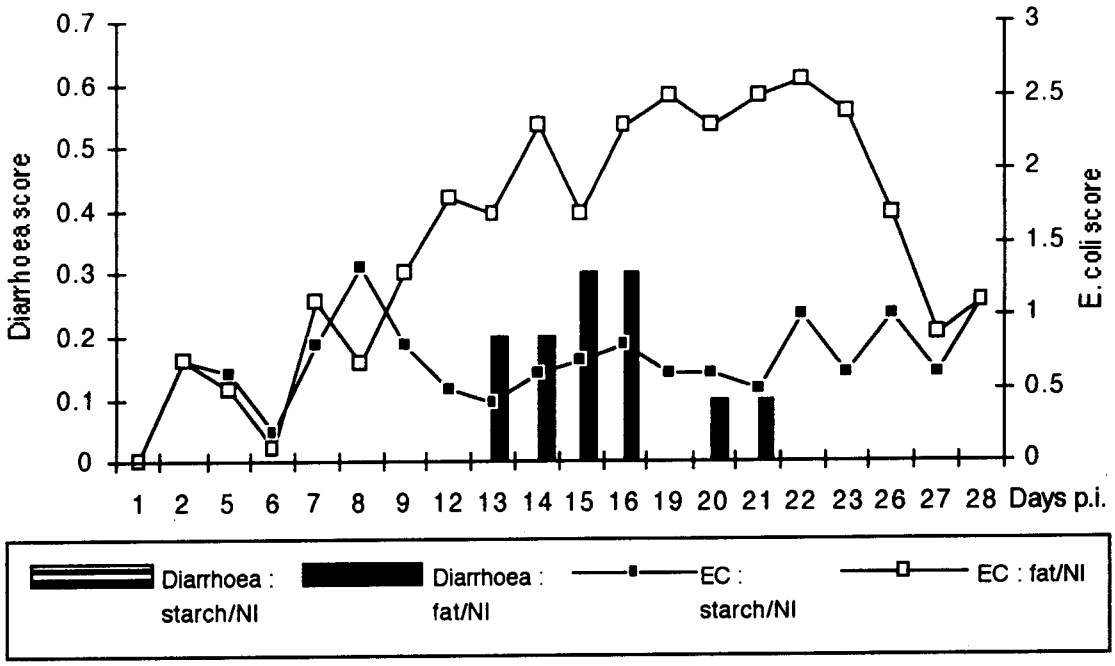
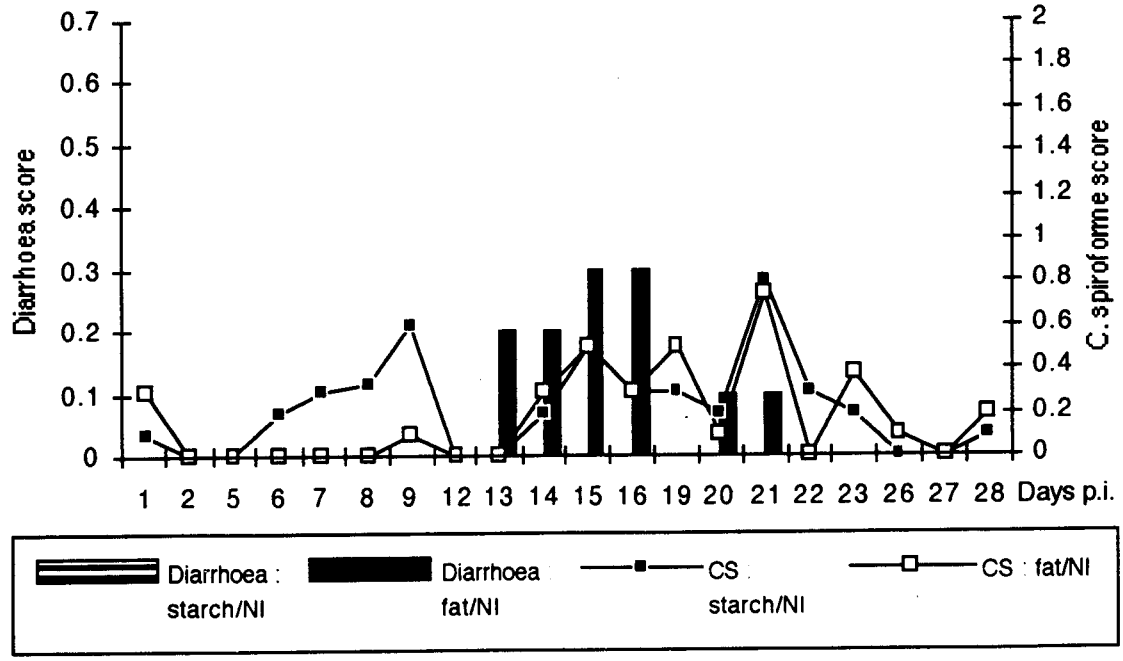


Figure 2 : *C. spiroforme* and diarrhoea scores in non infected rabbits



bacterium was considered attached to an epithelial cell if it was immediately next to the surface of the cell and if there was no mucus or other material between the bacterium and the cell surface. Infection scores reflecting the density of adherent bacteria on the intestinal mucosa was assigned for the ileum and for the apical, central and distal portion of the caecum as follows : 0 = no bacteria; 1 = isolated adherent bacteria; 2 = isolated sites colonised by microcolonies; 3 = extensive colonisation. The sum of individual scores for the four different intestinal regions was calculated and may vary between zero (absence of infection) and 12 (maximal infection). A similar evaluation method was used to estimate the impact of iota-enterotoxaemia : 0 = no epithelial desquamation; 1 = isolated cells which loose contact ; 2 = moderate desquamation and congestion of mucosal capillaries; 3 = extensive desquamation and congestion.

Biochemistry.

Caecal VFA concentrations were determined after diluting caecal samples 1:1 in distilled water. Three drops of toluene were added as protection against freezing before storing at – 20°C. After thawing, aliquots of 5 ml were acidified with 1 ml of a mixture of 75 ml meta-phosphoric acid (25 %) and 25 ml of formic acid and centrifuged twice at 12,000 r.p.m. for 15 minutes. The concentration of VFA in the resulting supernatant was determined by gas chromatography (Perkin Elmer type 8500 gas chromatograph) using a chrompack WCOT fused silica column type (25 m x 0.22 mm) with a FFAP liquid phase. NH_3 concentration was measured according to the microdiffusion method with Conway dishes (VOIGHT and STEGER, 1967).

Statistical analysis.

Differences between caecal parameters were tested by the non parametric Mann-Whitney test (SOKAL and ROHLF, 1969). Weight gain, food and water intake were evaluated by analysis of variance.

RESULTS

Non infected rabbits

Administration of both feeds was not followed by significant differences in daily weight gain, feed- and water intake nor feed conversion ratio for the whole 4-weeko observation period. Only during the third week daily weight gain dropped significantly in group F (Table 2). This was associated with a significant increase of faecal saprophytic *E. coli* scores between day +8 and +27 post-weaning in all animals sampled (Fig. 1). One animal of the F-group showed liquid diarrhoea for six days and died 19 days post-weaning with lesions of colibacillosis. Two more F-rabbits showed discrete signs of diarrhoea. In the S-group on the contrary no clinical signs were observed and *E. coli*-levels remained low. A discrete increase of faecal *C. spiroforme*-scores was established between day +5 and +12 p.i.(Fig. 2).

Eight days after administering the experimental feeds, microbiological and chemical analysis of caecal content did not reveal any significant difference. After 28 days of feeding a significant increase of caecal *E. coli*-scores was noticed in the F-group, associated with a significant lower caecal weight (-19.5 %) (Table 3). Histology 8 days p.i. did not reveal significant lesions in the S-group, whereas entero-adherent *E. coli* were detected in the caecum of one animal of the F-group. No lesions were detected 28 days p.i.

C. spiroforme NCTC11493 infected rabbits

The experimental infection of rabbits receiving diet S caused a distinct but non significant reduction of weight gain in comparison with non infected controls 8 to 21 days p.i. Such effect was not observed in the F-group (Table 2). For the whole 4-weeks observation period no significant differences between daily weight gain, feed- and water intake nor feed conversions were noticed.

The infection with *C. spiroforme* was associated with a significant increase of faecal saprophytic *E. coli* scores between day +8 and +23 post-weaning in both groups (Fig. 3), whereas *C. spiroforme*-scores increased between 16 and 26 days p.i. (Fig. 4). One animal out of the S-group showed liquid diarrhoea for six days and died 23 days post-weaning with lesions of iota-enterotoxaemia. Two more S-rabbits showed discrete signs of diarrhoea. In the F-group on the contrary no clinical signs were observed.

Microbiological and chemical analysis of caecal content revealed only significantly higher NH_3 levels in group F, 8 days p.i. After 28 days of feeding, a significant increase of caecal pH and a significant decrease of total VFA was noticed in the F-group (Table 3). Histology 8 days p.i. revealed discrete lesions of iota-enterotoxaemia in one out of five animals of the F-group and more pronounced lesions in four out of five animals of the S-group. At day 28 p.i. there were still lesions in four out of five animals of the S-group.

EPEC O132/2+ infected rabbits

The experimental infection was followed by diarrhoea from day +5 till day +19 p.i. in eight out of 10 rabbits receiving diet S and in three out of 10 rabbits receiving diet F. Diarrhoea lasted for 13 days in group S and eight days in group F. The infection was followed by a significant increase of faecal EPEC O132/2+ scores in both groups between day +1 and +21 p.i. (Fig. 5). Diarrhoea was also associated with a significant rise of faecal *C. spiroforme* scores in

Table 2 : Zootechnical, clinical and histological parameters.

Group	S/NI	F/NI	S/CS	F/CS	S/O132	F/O132	S/CS+O132	F/CS+O132
1 - **Treatment**								
Feed	Starch	Fat	Starch	Fat	Starch	Fat	Starch	Fat
Infection	No	No	*C.Spiroforme*	*C.Spiroforme*	*E. coli O132*	*E. coli O132*	*O132+C.sp.*	*O132+C.sp.*
2 - **Zootechnical parameters (X ± SD)°**								
Live weight at day 0 (g)	774 ± 114	789 ± 101	763 ± 81	774 ± 109	814 ± 91	776 ± 82	741 ± 70	713 ± 88
Daily weight gain between d 0–7 (g)	42.9 ± 9.7	43.4 ± 7.7	43.4 ± 8.5	46.4 ± 11.2	26.6 ±14.4	31.9 ± 17.3	21.1 ± 16.8	12.7 ± 19.4
Daily weight gain between d 8–14 (g)	44.1 ± 7.0	39.0 ± 17.8	39.0 ± 9.1	40.5 ± 10.0	9.5 ± 20.5a	35.7 ± 17.2b	11.1 ± 28.7	19.5 ± 30.7
Daily weight gain between d 15–21 (g)	43.4 ± 4.2a	33.9 ± 8.2b	33.8 ± 24.1	42.1 ± 12.6	35.6 ± 15.8	42.3 ± 6.0	41.2 ± 11.7	*
Daily weight gain between d 22–28 (g)	42.1 ± 8.9	42.7 ± 8.1	46.0 ± 4.5	37.8 ± 14.9	42.6 ± 8.2	46.0 ± 11.3	38.5 ± 9.8	*
Daily weight gain between d 0–28 (g)	43.9 ± 4.1	41.2 ± 5.4	42.5 ± 3.5	41.8 ± 10.4	28.1 ± 9.5a	40.2 ± 7.6b	29.7 ± 10.5	*
Daily feed intake between d 0–28 (g)	105 ± 9	96 ± 21	105 ± 10	114 ± 19	76 ± 17a	104 ± 14b	83 ± 12	*
Daily water intake between d 0–28 (ml)	195 ± 42	215 ± 68	210 ± 56	227 ± 69	155 ± 63	183 ± 34	151 ± 34	*
FCR d 0–28	2.41 ± 0.28	2.45 ± 0.16	2.48 ± 0.20	2.57 ± 0.24	2.84 ± 0.52	2.62 ± 0.23	2.57 ± 0.33	*
3 - Clinical parameters								
Mortality	0/10	1/10	1/10	0/10	2/10	0/10	2/10	5/10
Associated with :								
– EPEC	–	1	–	–	–	–	–	4
– iota–enterotoxaemia	–	–	1	–	–	–	–	–
– EPEC and iota–enterotoxoaemia	–	–	–	–	2	–	2	1
4 - Histology								
Mean EPEC adhesion score								
– day 8	0.0	0.6	1.2	0.0	1.0	6.0	4.6	5.8
– day 28	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Mean iota–enterotoxoaemia score								
– day 8	0.0	0.0	1.8	0.2	0.1	1.3	1.6	3.6
– day 28	0.2	0.2	1.1	0.4	0.0	0.0	0.0	0.0

° means followed by a different character (a, b) within the same group on infection are significantly different ($P<0.05$)

* insufficient data

Table 3 : Caecal biochemistry and microbiology.

Group	S/NI	F/NI	S/CS	F/CS	S/O132	F/O132	S/CS-O132	F/CS+O132
1 - Treatment								
Feed	Starch	Fat	Starch	Fat	Starch	Fat	Starch	Fat
Infection	No	No	*C.Spiroforme*	*C.Spiroforme*	*E. coli O132*	*E.coli O132*	*O132+ C.sp.*	*O132+C.sp.*
2 - Caecal parameters - 8 days p.i. (X ± SD)								
Mean caecal weight (g)	86 ± 10	80 ± 20	80 ± 19	74 ± 12	87 ± 24a	72 ± 8b	85 ± 17	78 ± 16
Mean *E. coli* number (log 10)	3.93 ± 0.12	4.42 ± 0.69	4.80 ± 1.64	3.91 ± 0.64	6.21 ± 1.39	6.79 ± 1.12	6.65 ± 1.57	7.04 ± 1.06
Mean *C. spiroforme* spore numb. (log 10)	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5
pH	5.85 ± 0.24	5.90 ± 0.19	5.91 ± 0.24	6.06 ± 0.26	6.17 ± 0.57	6.47 ± 0.58	6.06 ± 0.25	6.28 ± 0.64
NH3 (mmol/kg)	17.4 ± 3.9	17.0 ± 4.7	13.0 ± 3.5a	18.2 ± 3.6b	14.6 ± 2.7	13.8 ± 1.3	18.6 ± 3.4a	11.1 ± 3.8b
Total volatile fatty aids (mmol/kg)	56.3 ± 21.6	68.0 ± 7.6	74.6 ± 24.5	63.2 ± 9.8	63.7 ± 13.5	55.8 ± 13.6	63.0 ± 10.2	67.3 ± 15.3
Acetic acid (mmol/kg)	44.0 ± 16.2	54.5 ± 6.6	57.9 ± 19.1	51.0 ± 8.4	50.4 ± 12.6	46.4 ± 11.3	49.2 ± 9.4	54.9 ± 13.4
Propionic acid (mmol/kg)	3.0 ± 1.0	3.5 ± 0.9	3.1 ± 0.9	2.7 ± 0.3	3.6 ± 0.9	3.3 ± 0.6	3.4 ± 0.5	3.4 ± 0.4
Butyric acid (mmol/kg)	8.0 ± 4.3	9.1 ± 2.1	11.8 ± 4.6	8.7 ± 1.8	8.4 ± 2.7	5.5 ± 2.9	9.1 ± 2.5	8.2 ± 3.7
3 - Caecal parameters - 28 days p.i. (X ± SD)								
Mean caecal weight (g)	150 ± 11a	121 ± 15b	128 ± 17	121 ± 17	138 ± 21a	113 ± 17b	129 ± 27	114 ± 17
Mean *E. coli* number (log 10)	2.61 ± 1.30a	4.41 ± 1.71b	4.17 ± 1.35	3.85 ± 1.64	3.64 ± 1.20	3.29 ± 1.38	2.54 ± 1.19a	4.24 ± 0.42b
Mean *C. spiroforme* spore number (log 10)	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5	<10^5
pH	6.06 ± 0.26	5.97 ± 0.22	5.91 ± 0.07a	6.24 ± 0.24b	5.90 ± 0.11a	6.17 ± 0.45b	6.19 ± 0.19	6.24 ± 0.25
NH3 (mmol/kg)	10.9 ± 2.0	9.7 ± 2.0	9.4 ± 1.4	10.7 ± 3.3	13.0 ± 1.7	12.7 ± 3.6	15.7 ± 4.7	13.0 ± 3.2
Total volatile fatty acids (mmol/kg)	62.5 ± 8.4	72.9 ± 12.3	70.0 ± 5.9a	55.6 ± 5.4b	71.8 ± 8.8a	60.0 ± 10.7b	50.1 ± 9.9	55.1 ± 10.9
Acetic acid (mmol/kg)	46.0 ± 5.9	56.8 ± 9.0	51.9 ± 5.3a	43.2 ± 3.5b	53.6 ± 6.2a	47.2 ± 9.0b	37.0 ± 6.0	42.0 ± 9.1
Propionic acid (mmol/kg)	3.1 ± 0.4	3.4 ± 0.7	3.4 ± 0.3a	2.7 ± 0.3b	3.2 ± 0.4	2.9 ± 0.3	2.8 ± 0.6	2.9 ± 0.4
Butyric acid (mmol/kg)	12.0 ± 2.7	11.7 ± 2.8	13.4 ± 1.5	8.7 ± 2.0	13.4 ± 2.3a	9.1 ± 1.9b	9.1 ± 3.2	9.3 ± 1.8

* means followed by a different character within the same group of infection are significantly different (P<0.05)

Figure 3 : *E. coli* and diarrhoea scores in rabbits after experimental infection with *C. spiroforme*

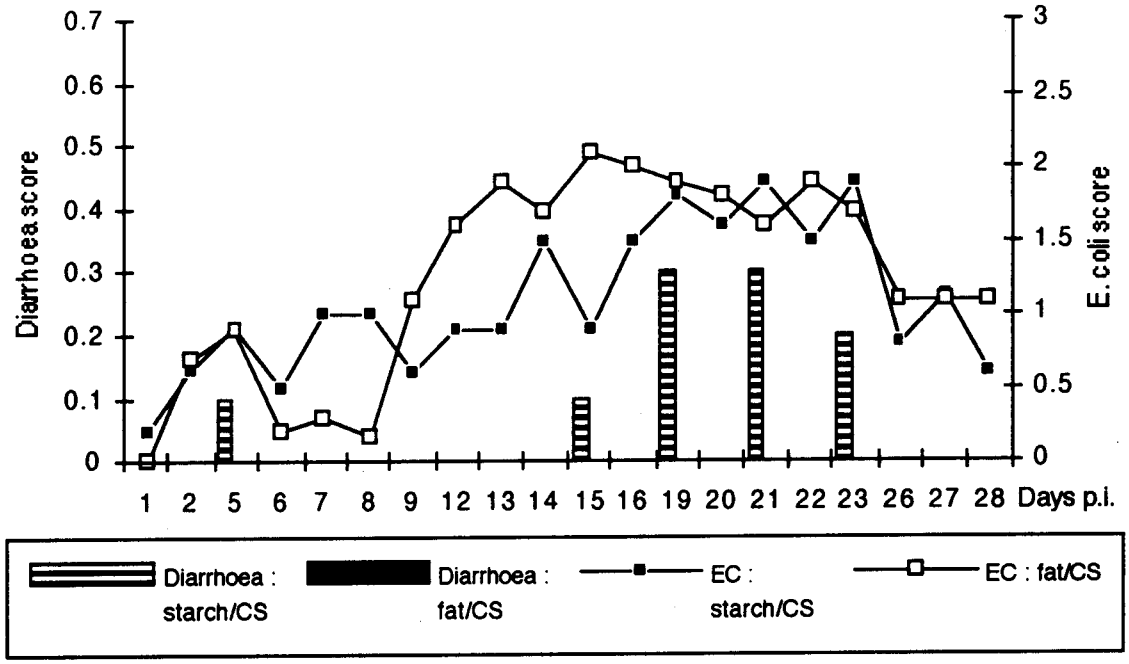
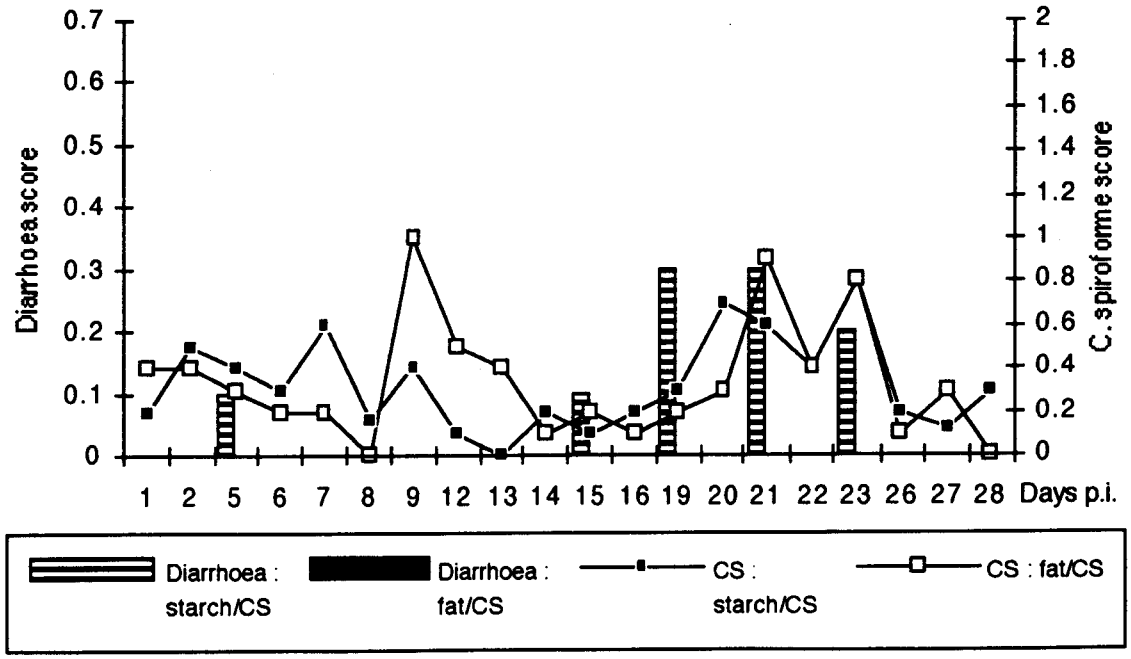


Figure 4 : *C. spiroforme* and diarrhoea scores in rabbits after experimental infection with *C. spiroforme*



rabbits receiving diet S between days 8 and 19 p.i. (Fig. 6). In the same group, two animals died on respectively day +16 and +19 p.i. from a mixed infection of colibacilosis and iota-enterotoxaemia. Moreover, weight gain and feed intake were significantly lower than in group F during the whole four-week observation period (Table 2). In group F no mortality was established.

A significant increase of caecal *E. coli* numbers was established in both groups in comparison with uninfected controls eight days p.i. Yet, between both infected groups no significant difference of pH, NH_3 or VFA-content, *E. coli* or *C. spiroforme*-numbers was found, although pH tended to be higher and butyric acid content to be lower in the F-group. However, only caecal weight was significantly lower in the F-group. After 28 days of feeding a significant increase of caecal pH and a significant decrease of total VFA was noticed in the F-group, while caecal weight was significantly lower (Table 3). Eight days p.i. histology revealed discrete lesions of EPEC in three out of five animals of the S-group and extensive lesions in four animals of the F-group (Table 2). At day 28 p.i. entero-adherent EPEC were still detected in one out of five animals of group F.

Mixed EPEC O132/2+ and *C. spiroforme* NCTC11493 infection

The experimental infection was followed by diarrhoea between day +5 and +16 p.i. (significantly higher scores than after monofactorial infection) in four out of 10 rabbits receiving diet S and in five out of 10 rabbits receiving diet F. Diarrhoea lasted for nine days in group S and 10 days in group F. The infection was followed by a significant increase of faecal EPEC O132/2+ scores in both groups between day +1 and +20 p.i. (Fig. 7). Diarrhoea was also associated with a rise of faecal *C. spiroforme* scores in both groups between days +8 and +20 p.i. Numbers were higher in group S than in group F (Fig. 8). In group S, two animals died on respectively day +16 and +26 p.i. from a mixed infection of colibacilosis and iota-enterotoxaemia, whereas in group F five animals died : one on day 16 p.i. from a mixed infection of colibacilosis and iota-enterotoxaemia and four between day 14 and 16 p.i. from colibacillosis. Weight gain and feed and water intake were significantly reduced in both groups during the first two weeks p.i. (Table 2).

Autopsy 8 days p.i. revealed a significant increase of caecal *E. coli* numbers in both groups in comparison with uninfected controls. Yet, between both infected groups no significant difference between microbiological and biochemical parameters were found. Only NH_3 was significantly higher in group S. After 28 days, caecal numbers of *E. coli* were significantly higher in the F-group in comparison with group S (Table 3). Histology 8 days p.i. revealed discrete to severe lesions of EPEC in four out of five animals of group S and mostly extensive lesions in all five animals of group F. At day +28 p.i. no lesions were detected in any of the groups. Discrete lesions of epithelial desquamation were found in four out of five animals of group S and severe lesions in three out of five animals of group F.

DISCUSSION

C. spiroforme is commonly present in the intestinal content of diarrhoeic rabbits. During a survey of 29 commercial rabbitries, the organism was detected in 52.4 % of 149 caecal samples from 24 different rabbitries (PEETERS *et al.*, 1986b). *C. spiroforme* starts colonising the gut of healthy rabbits around weaning age (PEETERS *et al.*, 1986a). Also in the experiment outlined above a colonisation rate of 23 % (9/40) was established at weaning. Most *C. spiroforme* strains were shown to produce *C. perfringens* iota-like toxin, that induces necrosis and desquamation of epithelial cells and haemorrhages in the hindgut, besides watery diarrhoea and sometimes high mortality. Although *C. spiroforme* is common in most commercial rabbitries, losses by iota-enterotoxaemia are not always observed. This suggests that favouring conditions are needed.

Different factors may be involved in the genesis of iota-enterotoxaemia. Administration of some antibiotics (ampicillin, lincomycin), which destroy partly the gram-positive flora, is followed by high *C. spiroforme*-associated mortality (CARMAN and EVANS, 1984). So, normal intestinal flora seems to protect against iota-enterotoxaemia. It is known that this mainly gram-positive flora is stabilised at the age of five to six weeks (MATTHES, 1969 ; GOUET and FONTY, 1973). This could explain why early weaned rabbits are less resistant against experimental iota-enterotoxaemia (CARMAN and BORRIELLO, 1984 ; PEETERS *et al.*, 1986a).

On the other hand also the maturity of the enzymatic system may play a role : production of trypsin, necessary for the enzymatic degradation of proteins (SANCHEZ *et al.*, 1985) and production of amylase, necessary for the digestion of starch (CORRING *et al.*, 1972) are rather low in 28-day-old rabbits and only reach maximal activity at the age of 42 days. This explains why undigested starch may reach the hindgut of early weaned rabbits (BLAS *et al*, 1990), whereas starch is normally absent from the caecum of 6-week-old rabbits. Therefore CHEEKE and PATTON (1980) put forward the hypothesis that an overload of the caecum by starch and subsequent degradation into glucose by the caecal flora may favour iota-enterotoxaemia. Glucose is indeed necessary for the production of iota-toxin *in vitro* (BORRIELLO and CARMAN, 1983). The fact that

Figure 5 : *E. coli* and diarrhoea scores in rabbits after experimental infection with *E. coli*

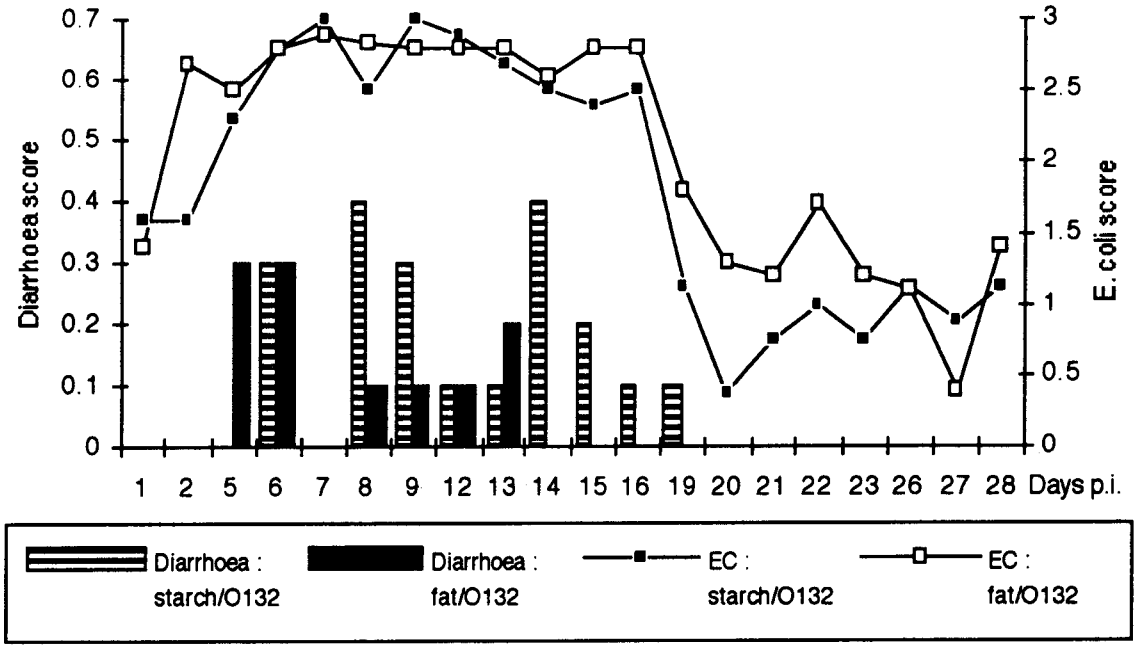


Figure 6 : *C. spiroforme* and diarrhoea scores in rabbits after experimental infection with *E. coli*

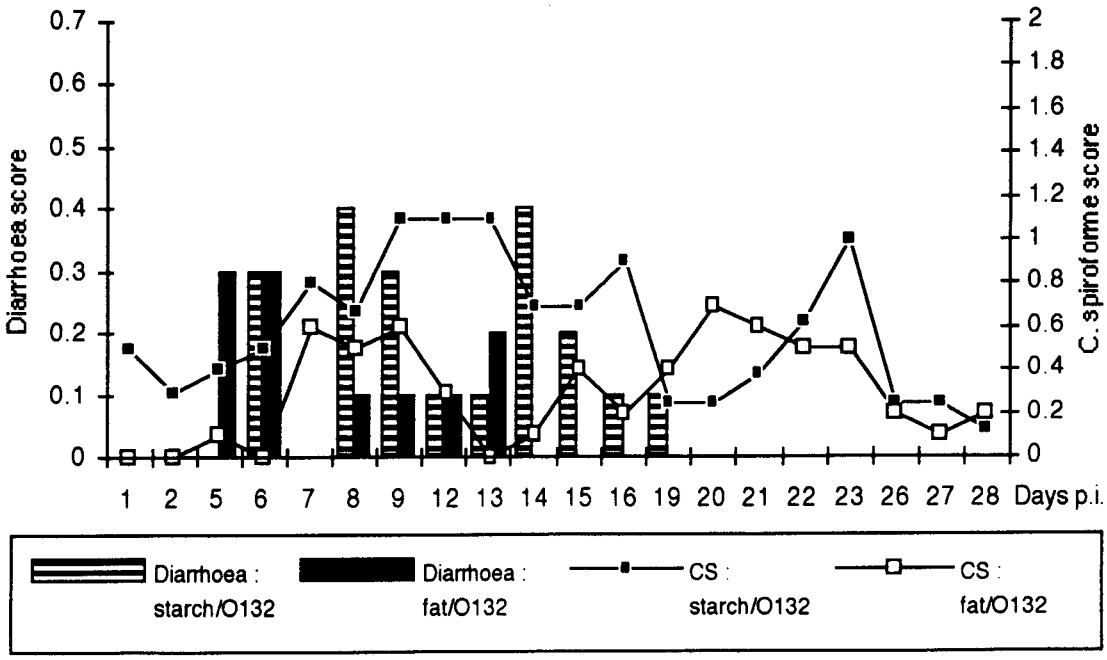


Figure 7 : *E. coli* and diarrhoea scores in rabbits after a mixed infection with *C. spiroforme* and *E. coli*

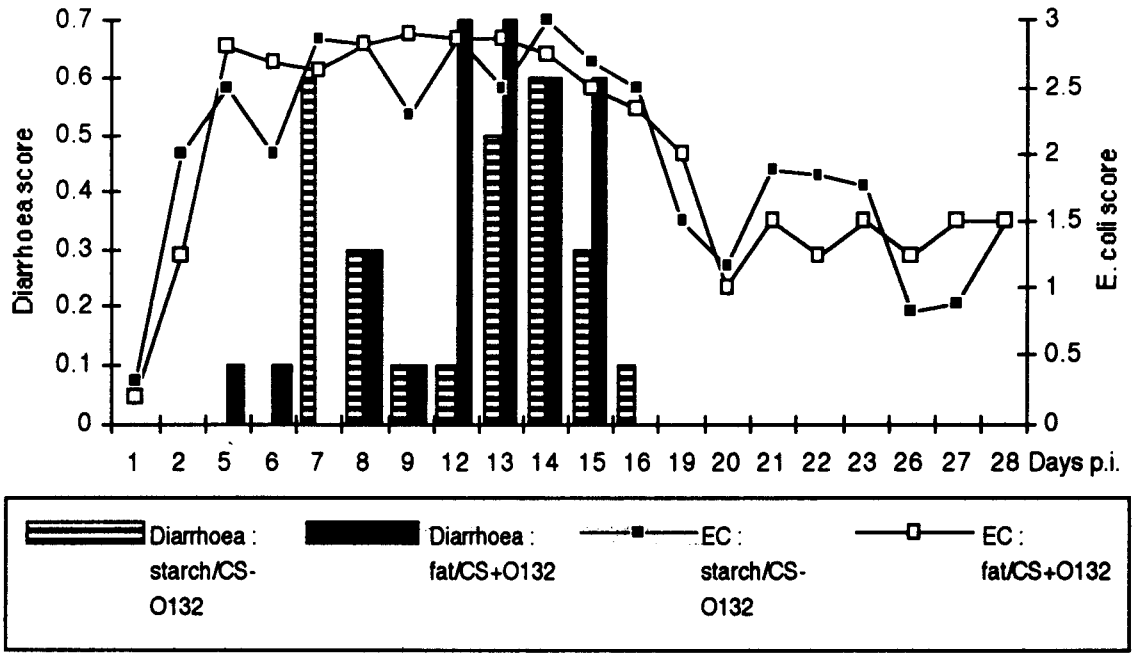
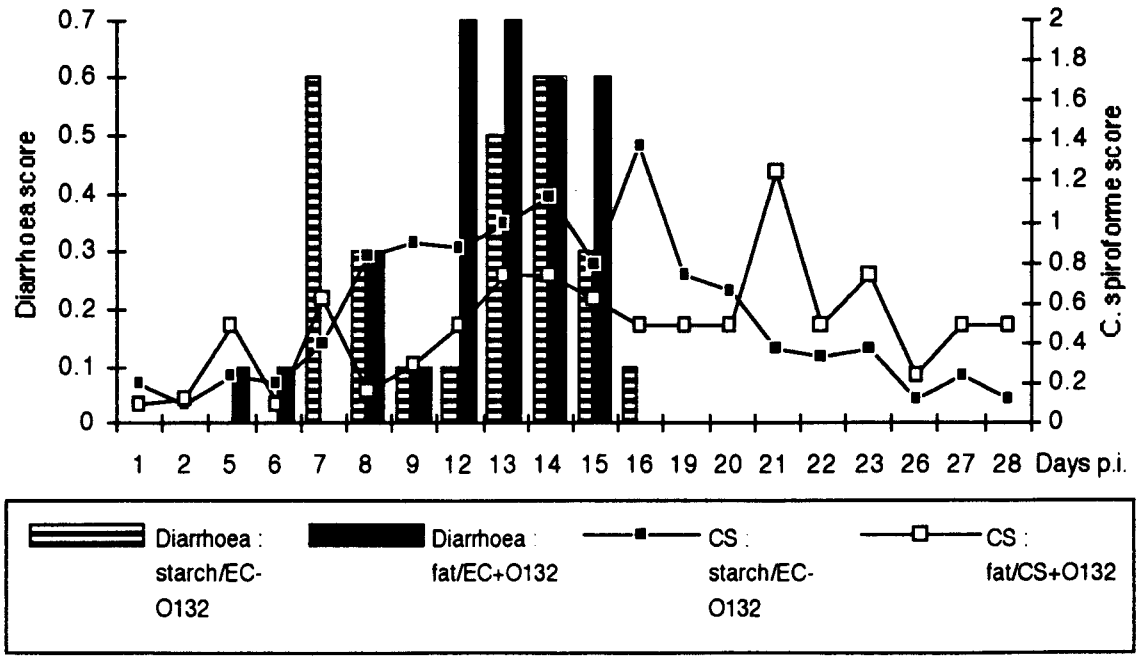


Figure 8 : *C. spiroforme* and diarrhoea scores in rabbits after a mixed infection with C. spiroforme and *E. coli*



experimental infection with *C. spiroforme* is only followed by iota-enterotoxaemia in 4-week-old rabbits and not in 5-12-week old rabbits (CARMAN and BORRIELLO, 1984 ; PEETERS *et al.*, 1986a) and that feeds containing as much as 27 % of starch do not favour iota-enterotoxaemia in experimentally infected 9- and 12-week-old rabbits (PEETERS *et al.*, 1986b) may sustain this hypothesis.

Therefore we fed rabbits diets with low (13 %) and high (26 %) starch content and infected them with *C. spiroforme* at weaning. The feeds contained corn as main source of dietary starch because of its less digestibility (BLAS *et al.*, 1990). Energy and protein content were kept constant, whereas resp. ADF, NDF and crude fibre content of diet F was only 10, 12 and 15 % higher than in diet S. Although energy content was not determined directly, the FCR of the control groups (resp. 2.41 and 2.45) provide indirect proof that both diets were in fact iso-energetic.

Our data confirm that feeds rich in starch promote iota-enterotoxaemia in early weaned rabbits. Experimental infection caused only clinical signs (depressed weight gain, diarrhoea and low mortality [1/10]) in the group receiving the feed rich in starch. Also histological lesions suggesting iota-enterotoxaemia were more pronounced in the S-group and so were *C. spiroforme* scores in most infected groups on diet S. This stresses the importance of undigested starch as favouring factor. The fact that feed S contained 10 % less ADF may have exerted a synergistic action by prolonging caecal retention of feed stuffs. This is suggested by the higher caecal weight as reported earlier (DE BLAS *et al.*, 1986).

Also concurrent infections with EPEC favour iota-enterotoxaemia : Fig. 6 shows an increase of faecal *C. spiroforme*-score between six and 20 days p.i., when lesions induced by EPEC are most pronounced. EPEC destroy the microvillous border of the small intestinal enterocytes producing disaccharidases, which is followed by a drop in disaccharidase production. This may contribute to increased caecal levels of disaccharides and consequently to higher glucose levels. Also the reverse is true : iota-enterotoxaemia favours *E. coli* proliferation (Fig. 3). This may be explained by the toxin-induced destruction of the epithelial lining, which leads to excessive protein levels in the caecum and to an increase of caecal pH by bacterial degradation. PROHASZKA (1980) showed *in vitro* that at higher pH-values higher VFA levels are needed to inhibit *E. coli* proliferation. As diseased animals take less feed, caecal VFA production will be lower and therefore intestinal alteration by enterotoxaemia will enhance indirectly the pathological effects of colibacillosis. Unfortunately, we were not able to confirm this hypothesis with the data of VFA-analysis as variation among samples was too high. This may be explained by individual variations in incubation period for the experimental infections.

The data suggest that dietary starch concentration should be limited, e.g. by using other sources of dietary energy such as fat in order to limit the risk of iota-enterotoxaemia after early weaning. Also a highly digestible starch source as for instance barley (GIDENNE & PEREZ, 1993) could be used. On the other hand, feed composition has to guarantee a sufficient caecal VFA production, as VFA and caecal pH seem to govern *E. coli*-proliferation.

From this point of view, feeds leading to excess starch levels in the caecum should inhibit caecal proliferation of saprophytic *E. coli* or moderately pathogenic EPEC . The data support this hypothesis (Fig. 1): uninfected rabbits fed a diet rich in fat and low in starch showed a significant increase of faecal *E. coli*-scores, associated with moderate diarrhoea and low mortality (1/10). Also the higher dietary fibre content of feed F may have played a significant role by reducing caecal retention time.

Although the feed rich in starch was shown to prevent proliferation of saprophytic *E. coli*, it did not inhibit the huge faecal EPEC O132 output after experimental infection, nor the associated diarrhoea and mortality. This may be due to the high experimental infection rate, which differs from the lower, but continuous infection pressure observed in practical conditions. Yet the feed rich in starch was able to inhibit EPEC-colonisation to a certain extent : 8 days p.i. histological lesions were less pronounced in the S-group than in the F-group.

Moreover, entero-adherent EPEC were still present 28 days p.i. in one rabbit out of five in group F, while they were not detected any more in group S. Also the data of the mixed infection group suggest a partial beneficial effect of the S-diet on colibacillosis associated mortality : 5/10 rabbits died in the F-group against 2/10 in the S-group and caecal *E. coli*-levels were still significantly higher in the F-group 28 days p.i. Possibly the S-feed contributes only to an inhibition of *E. coli* bacteria present in the intestinal lumen and not of bacteria attached to the mucosal surface. This could be important, as in normal conditions infection by EPEC is followed by the production of specific secretory antibodies, which inhibit further attachment of the bacteria to the mucosal surface. Yet, it has been shown that the same antibodies do not inhibit persistence of EPEC in the intestinal lumen (MCQUEEN *et al.*, 1992). This means that a feed such as diet F might enhance the persistence of healthy carriers of EPEC.

The data of the experiments do not allow the conclusion that VFA-levels and pH were indeed responsible for the inhibitory effect of feed S on *E.*

coli-proliferation. Although caecal pH was consistently lower in the S-groups in comparison with the F-groups (Table 3), the coefficient of variation among individual VFA, NH_3 and pH-values of each group was too high and the numbers of animals sampled too low to allow statistical analysis. Moreover, caecal parameters were only determined eight and 28 days p.i., whereas analysis of faecal *E. coli* output clearly indicates a kinetic process with not always peak values for all animals at the moment of necropsy. This explains the important variation within the biochemical parameters for the samples taken at autopsy. Therefore it would be advisable to use fistulated animals and to check caecal parameters continuously *in vivo* and *in situ*, in order to relate caecal biochemical and microbiological changes.

In conclusion, there exists a complex relationship between feed composition, colibacillosis and iota-enterotoxaemia. Colibacillosis promotes iota-enterotoxaemia and vice-versa. Feeds rich in starch favour iota-enterotoxaemia. The effects are most pronounced in early weaned rabbits and in rabbits suffering from simultaneous EPEC infection. The same diet inhibits *E. coli*-proliferation to a various extent : the effect is most pronounced in case of saprophytic *E. coli* or when infection rate is only low. The data suggest that feeds rich in fat and poor in starch favour *E. coli* proliferation. It can be hypothesised that such feeds also favour the persistence of healthy carriers of enteropathogenic *E. coli*. Healthy carriers contribute to new outbreaks within the rabbitries and to the spread of colibacillosis among rabbitries.

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