

## CHALLENGING THE GROWING RABBIT WITH A MODERATELY PATHOGENIC *E. COLI* UNDER *AD LIBITUM* OR LIMITED FEED INTAKE CONDITIONS: IMPACT ON DIGESTIVE PHYSIOLOGY, BACTERIAL COMMUNITIES, AND ON POST-WEANING GROWTH

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**Abstract:** The impact of a challenge with moderately pathogenic *Escherichia coli* O128:C6 on the digestive physiology and gut bacterial community of growing rabbits under two feeding programmes was analysed. Upon weaning (28 d old), 180 rabbits were allocated to four groups (9 cages of 5 rabbits per group) for two weeks: group C100 was non-inoculated and fed *ad libitum*; C70 was non-inoculated and feed intake was limited to 70% of C100; I100 and I70 were inoculated and fed *ad libitum* or restricted to 70%, respectively. At the age of 31 d (D0), rabbits were orally inoculated with *E. coli* (2.2×10<sup>8</sup> colony forming units/rabbit). The effects of inoculation spiked on D4, with a 28% lower growth rate for I100 than for C100. Limited feed intake reinforced the inoculation's effects on growth: I70 had a 66% lower growth rate than C70. The morbidity rate peaked at 42% between D4 and D7 for inoculated groups, without significant effect of the feed intake level. *E. coli* concentration peaked on D5/D6 in the caecum of the I100 and I70 groups. Inoculation reduced by 30% ( $P<0.05$ ) the villus height/crypt depth and villus/crypt area ratios in the ileum, with no significant effect of the intake level. Inoculation was associated with a tenfold increase in serum haptoglobin ( $P<0.001$ ) for both *ad libitum* and restricted rabbits. On D5, the inoculation modified the structure of the ileal bacterial community ( $P<0.05$ ), but not that of the caecum. The feed intake level did not affect either the structure or diversity of the bacterial community, both in the ileum and caecum.

**Key Words:** growing rabbit, *Escherichia coli* O128:C6, caecal and ileal bacterial ecosystem, feed restriction, ileal histometry, haptoglobin.

### INTRODUCTION

Digestive disorders are the most prevalent cause of mortality among growing rabbits (Marlier *et al.*, 2003; Licois, 2004; Agnoletti, 2012). With epizootic rabbit enteropathy (ERE), colibacillosis is one of the two main digestive pathologies for growing rabbits. The *E. coli* involved in these disorders are enteropathogenic (EPEC) and can attach to the epithelial brush borders (ileum, caecum and colon), inducing villous atrophy (Peeters *et al.*, 1988; Licois *et al.*, 1991) and specific digestive lesions known as attaching and effacing lesions (Milon *et al.*, 1990). The severity of clinical signs and disease depends on the *E. coli* strain. The *E. coli* O128:C6 strain is known to be moderately pathogenic, most frequently inducing retarded growth and slight digestive disorders (diarrhoea) without mortality (Camguilhem and Milon, 1989; Milon *et al.*, 1990). Moreover, the sensitivity of growing rabbits to EPEC decreases a little after six weeks of age, and is greatly reduced in adults (Licois *et al.*, 1992). The specific sensitivity of young

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rabbits to digestive disorders is thus probably linked to the incomplete maturation of their digestive and immune systems (Fortun-Lamothe and Boullier, 2007).

To tackle the digestive pathologies of growing rabbits, metaphylactic antibiotherapy is often used (Agnoletti *et al.*, 2012), although alternative strategies have been developed (Maertens, 2011). A high fibre intake, for instance, increases resistance to an EPEC challenge (Gidenne and Licois, 2005). The main alternative mostly used in France in the past decade consists of applying a short-term post-weaning limitation of feed intake, as it has been shown to be beneficial to the digestive health of young rabbits (Gidenne *et al.*, 2012), and especially of growing rabbits challenged with ERE (Boisot *et al.*, 2003). Similarly, a post-weaning feed restriction was found to be beneficial to the digestive health of piglets, resulting in a lower faecal score of haemolytic *E. coli* (Rantzer *et al.*, 1996). However, the effect of a feed intake limitation on the gut bacterial ecosystems of growing rabbits challenged with an EPEC had never been previously studied.

We thus investigated the impacts of a challenge with enteropathogenic *E. coli* O128:C6 on the digestive physiology of growing rabbits, including their caecal and ileal bacterial communities, and the effect of a limitation in feed intake following an EPEC challenge. A moderate feed restriction (70% of free intake) was chosen, as several studies show that this level of intake could be efficient to control digestive troubles (Gidenne *et al.*, 2012).

## MATERIAL AND METHODS

Animals were treated following the guidelines for animals used in experiments, according to EU 2010/63/EU and in accordance with French legislation (NOR:AGRG1238753A 2013). The local ethics committee (ANSES, Ploufragan) also approved the inoculation protocol.

### ***Experimental design, health status and performance***

The study was carried out on 180 hybrid commercial breed rabbits (Hycol®) at the ANSES experimental farm (Ploufragan, France). At weaning (28 d old, D-3), rabbits were identified and allocated to four groups of 45 rabbits (5 cages of nine rabbits per group, cage dimension: 0.50m<sup>2</sup>, 1.00×0.50 m), in a 2×2 factorial design: C100 was a non-inoculated control group fed *ad libitum*; C70 was a non-inoculated group whose feed intake was limited to 70% of the *ad libitum* group's intake; I100 was an inoculated group fed *ad libitum*; and I70 was an inoculated group with limited feed intake to 70%. The rabbits were randomly caged for two weeks, with a mean initial live weight of 588±48 g. On D0 (31 d old), the I100 and I70 rabbits were orally inoculated with *E. coli* O128:C6 (2.2×10<sup>8</sup> colony forming units [CFU]/animal), while the two non-inoculated groups received a dose of saline sterilised water. The animals were housed in two separate rooms, one for the two control groups and the other for the two inoculated groups. Half of the rabbits in each room were fed *ad libitum*, whereas the other half was submitted to a feed restriction. All the rabbits had unrestricted access to drinking water. No antibiotics were provided during the experiment. The feed-restricted groups were fed daily at around 10:00, and the feed remained freely accessible after distribution. The quantity of feed distributed was adjusted twice a week according to the average intake of groups C100 and I100. The housing units were kept at a temperature of 20°C (±2°C) and under a 09:00 to 19:00 lighting schedule alternated with 14 h of darkness. The experimental diet (Table 1) was formulated to cover the nutritional requirements of growing rabbits (Gidenne *et al.*, 2015).

Each group's feed intake and each rabbit's live weight were checked twice a week. The refusal of feed by feed-restricted rabbits was verified daily when the feed was distributed (no refusal was found). Morbidity and mortality were checked daily. Morbid rabbits were observed to be prostrate, bloated and/or having diarrhoea (Bennegadi *et al.*, 2001). In addition, animals without visible digestive disorders but with severely retarded growth (<20 g/d for *ad libitum* rabbits and <10 g/d for feed-restricted groups) were counted as morbid.

### ***Sampling and analysis of the ileum, caecum and blood***

Faecal samples were collected for 24 h ending at 10:00 on D-2, D2, D4, D6 and D11 using containers under the cages. Each analysed sample thus corresponded to one pool of faecal excretions of rabbits in the same group (five pools/group).

**Table 1:** Ingredients and chemical composition of the diet.

Ingredients	%	Chemical composition	g/kg
Wheat bran	21.60	Dry matter	914
Sunflower meal	17.70	Crude ash	87
Dehydrated alfalfa meal	17.70	Crude protein	158
Dehydrated sugar beet pulp	13.70	Crude fibre	170
Wheat middlings	6.50	aNDFom <sup>a</sup>	367
Barley	6.10	ADFom <sup>b</sup>	209
Apple pomace	2.80	ADL <sup>c</sup>	54
Sugar cane molasses	2.16		
Oats	2.00		
Rapeseed meal	2.00		
Citrus pulp	2.00		
Kaolin	2.00		
Grape pulp	1.20		
Soybean oil	1.20		
Premix	1.00		
Lysine	0.22		
Choline	0.08		
DL Methionine	0.04		

<sup>a</sup>Neutral detergent fibre expressed exclusive of residual ash.

<sup>b</sup>Acid detergent fibre expressed exclusive of residual ash.

<sup>c</sup>Acid detergent Lignin.

On D-2, D5 and D11, five rabbits from each group were sacrificed according to their health status: unhealthy rabbits were preferentially selected, and blood was sampled for haptoglobin analysis, to evaluate the reliability of the haptoglobin level to assess the inflammatory status. On D5, ileal segments (15 cm upstream of the ileo-caecal junction) were sampled for histological analysis. The caecal and ileal contents were also sampled on D5 to perform molecular bacteriology analysis by capillary electrophoresis using single strand conformation polymorphism (CE-SSCP).

Histometric measurements were performed on ileum mucosa. First, the ileal segment samples were rinsed with a saline solution of NaCl (9 g/L), then opened longitudinally and immersed in buffered formalin for 12 to 24 h. They were stored in 90% ethanol before analysis according to the method of Goodlad *et al.* (1991). Samples were then stained with Feulgen reagent. The villi and crypts were first carefully separated under a dissecting microscope. The preparations were then slide-mounted with a few drops of an aqueous agent for microscopy purposes. The length and area of villi and crypts (20 of each sample) as well as the ratio of villus height compared with crypt depth and of villus area compared with crypt area, were measured using an optical microscope (Nikon Eclipse E600), a camera (Sony XC77E) and image analysis software (Visilog 6, Noesis).

Serum was isolated from blood samples. Serum haptoglobin concentration was measured using a Phase<sup>TM</sup> Haptoglobin kit (AbCys, Paris, France) following the manufacturer's instructions. Optical density was read at 620 nm on a Sunrise microplate reader (Tecan, AES Chemunex). This measurement was converted to a concentration (mg/mL) using a calibration curve.

### **Gram-bacillus culture and *E coli* counts**

Fresh faecal samples (15 g) and caecal samples (1 g) were diluted ( $10^{-1}$ ) in peptone buffer. The samples were tenfold serially diluted in tryptone salt and three dilutions of each sample were plated on MacConkey agar no. 3 (CM0115B, Oxoid, England) for Gram-bacillus numeration. Purple colonies were counted after incubation at 37°C for 24 h. The bacterial concentration in the original sample was calculated. The results are expressed as CFU/g±sd (standard deviation) from five cages per group. Fifteen of all the bacterial colonies growing on MacConkey plates were serotyped using an *E. coli* O128 coagglutination reagent (Labocea, Ploufragan, France).

### **Analysis of caecal and ileal bacterial communities by CE-SSCP**

Total DNA from about 0.2 g of caecal sample was extracted and purified with a QIAamp® DNA Stool Mini kit (Qiagen Ltd, West Sussex, England) as previously described (Michelland *et al.*, 2011). The V3 region of the 16S rRNA genes was used as a bacterial diversity marker with the primers w49 and 5'-6FAM-labelled w34. Polymerase chain reaction assays were performed as described previously (Michelland *et al.*, 2011). The CE-SSCP was performed on an ABI Prism 3100 genetic analyser (Applied Biosystems, Branchburg, New Jersey, USA). The CE-SSCP profiles were aligned and normalised using the StatFingerprints program version 2.0 (Michelland *et al.*, 2009) running on R version 2.8.3 (R development Core Team, 2008). The Simpson diversity index was estimated on each CE-SSCP profile with  $-\log \sum (a_i)^2$  the relative area under the peak (Rosenzweig, 1995).

### **Data and statistical analysis**

To study the structure of the bacterial communities involved, we analysed the size of the various peaks throughout the CE-SSCP profiles using StatFingerprints (Michelland *et al.*, 2009). All the quantitative variables (live weight, feed intake, weigh gain, feed conversion ratio, diversity, histometric measurements, serum haptoglobin) were analysed according to a two-way ANOVA including inoculation, feed intake level and their interaction as main effects. All the variables were also analysed using a model with a one-way ANOVA to look at the group effect (four levels): C100, C70, I100, I70. These quantitative variables were analysed using R software. The profiles of bacterial communities from groups were compared using pairwise ANOSIM. The variability within each group was assessed using pairwise "maximum method" procedures. These methods of analysis are included in the "Statfingerprints" application by R software (Michelland *et al.*, 2009). The morbidity rate was analysed using the CATMOD procedure of SAS (SAS online guide). Differences were considered significant at  $P \leq 0.05$  and tendency was discussed at  $P \leq 0.10$ .

## **RESULTS**

### **Feed intake, growth and health status of animals**

As expected, for the initial period (D-3 to D0) the feed intake was 32% lower in restricted groups, leading to a 47% reduction in growth ( $P < 0.001$ ). However, from D0 to D11 the feed restriction programme led to a 42% reduction in feed intake and growth among non-inoculated rabbits (C100 vs. C70). During the four days after inoculation, the intake for rabbits fed *ad libitum* was 9% lower in the inoculated group ( $P < 0.05$ , Table 2), but the growth rate was reduced by 28%. In parallel, the inoculated and feed-restricted group (I70) showed a 66% lower growth rate than the feed-restricted control group (C70). At the end of the experiment, inoculated rabbits fed *ad libitum* (I100) had a 10% lower live weight than non-inoculated rabbits (C100). The growth impairment due to inoculation was accentuated in I70 rabbits, leading to a 19% lower live weight on D11 post inoculation (p.i.). Over the whole trial, the inoculation impaired the feed conversion (+10%) in both restricted- and unrestricted-intake rabbits. Our feed restriction programme (40% reduction in intake) tended to improve feed conversion, especially in inoculated rabbits (-6%: I70 vs. I100).

No mortality was observed during the experiment whatever the group. Before inoculation, almost no morbidity was recorded, regardless of the group considered (Table 3). As expected, diarrhoea was mostly observed between 3-6 d p.i. Morbidity peaked between 4 and 7 d p.i., with the highest level of clinical symptoms (diarrhoea, prostration and/or bloating) spread over D4 and D5 (data not shown). The morbidity rate for inoculated rabbits ( $P = 0.002$ ) reached 12% for group I100 and 35% for group I70 between D0 and D4. At this stage, morbidity was mainly due to growth disorders. During the whole post-inoculation period, the morbidity rate rose to 50% for inoculated rabbits ( $P < 0.001$ ), the feed intake level did not affect the overall morbidity rate of inoculated rabbits, and no influence of inoculation was detected. However, a monofactorial analysis (four groups) detected a transitory higher morbidity rate (60%,  $P < 0.05$ ) for inoculated feed-restricted rabbits during the four days p.i.

### ***E. coli* analysis and bacteriological communities in the ileum and caecum**

Before inoculation, no colonies of the *E. coli* isolated belonged to the O128 serogroup. This observation remained true for control group samples until the end of the experiment. On the other hand, all the *E. coli* isolated from the caecal and faecal samples from inoculated rabbits were identified as O128 (Figures 1A and 1B).

**Table 2:** Growth and feed intake pattern of the rabbit according to EPEC inoculation (control, C vs. inoculated, I) and feed intake level “IL” (*ad libitum*, 100 vs. restricted, 70).

Period	Groups <sup>4</sup>				SEM <sup>1</sup>	P-value <sup>2</sup>		
	C100	C70	I100	I70		Infection	IL	Inf. × IL
D-3 to D0 (28-31 d old)								
Initial live weight (D-3) (g)	585	594	582	590	3.6	0.64	0.23	0.99
Feed intake (g/d rabbit)	58.4 <sup>i</sup>	40.1	61.4 <sup>i</sup>	41.1	1.8 <sup>3</sup>	ND	ND	ND
Weight gain (g/d rabbit)	44.9 <sup>b</sup>	25.5 <sup>a</sup>	48.8 <sup>b</sup>	24.3 <sup>a</sup>	1.1	0.36	<0.001	0.09
D0 to D4 (31-35 d old)								
Feed intake (g/d rabbit)	88.0 <sup>j</sup>	44.0	79.8 <sup>k</sup>	44.0	2.5 <sup>3</sup>	ND	ND	ND
Weight gain (g/d rabbit)	49.5 <sup>d</sup>	18.2 <sup>b</sup>	35.6 <sup>c</sup>	12.8 <sup>a</sup>	1.4	<0.001	<0.001	<0.01
D4 to D7 (35-38 d old)								
Feed intake (g/d rabbit)	113.2	58.7	98.4	58.7	4.0 <sup>3</sup>	ND	ND	ND
Weight gain (g/d rabbit)	55.5 <sup>c</sup>	38.8 <sup>a</sup>	48.5 <sup>bc</sup>	40.7 <sup>ab</sup>	1.4	0.31	<0.001	0.081
D7 to D11 (38-42 d old)								
Final live weight (D11) (g)	1368 <sup>e</sup>	1060 <sup>a</sup>	1236 <sup>b</sup>	1007 <sup>a</sup>	18.1	<0.001	<0.001	0.082
Feed intake (g/d rabbit)	131.8 <sup>i</sup>	88.8	115.8 <sup>k</sup>	84.5	3.7 <sup>3</sup>	ND	ND	ND
Weight gain (g/d rabbit)	64.3 <sup>c</sup>	47.4 <sup>ab</sup>	54.4 <sup>b</sup>	41.8 <sup>a</sup>	1.4	<0.001	<0.001	0.33
D0 to D11 (31-42 d old)								
Feed intake (g/d rabbit)	110.8 <sup>i</sup>	64.3	101.9 <sup>k</sup>	62.7	3.6 <sup>3</sup>	ND	ND	ND
Weight gain (g/d rabbit)	49.1 <sup>c</sup>	29.3	39.3	26.9 <sup>a</sup>	1.4	<0.001	<0.001	0.33
Feed conversion ratio	2.26 <sup>ab</sup>	2.20 <sup>a</sup>	2.50 <sup>c</sup>	2.34 <sup>b</sup>	0.07	<0.01	0.099	0.15

<sup>1</sup>SEM: standard error of the mean.

<sup>2</sup>P-values for a bifactorial model, with effect of contamination (control, C vs. infected, I) and of intake (*ad libitum*, AL vs. restricted, R).

<sup>3</sup>SEM calculated for C100 and I100 groups (only), and corresponding means having a common superscript (i,k) did not differ at the level  $P < 0.05$ .

<sup>4</sup>Number of replicates for live weight=45 per group from D-3 to D0, 40 per group from D0 to D7, 25 per group from D7 to D11.

ND: not determined,  $\sigma^2 = 0$  for feed-restricted rabbits.

<sup>a,b,c</sup>Within a row, means without a common superscript differ ( $P < 0.05$ , for a monofactorial model: group effect).

Before inoculation (29 days old, D-2), the variability of total *E. coli* caecal concentration between animals reached about 2 log CFU/g ( $n=20$ ), illustrating that some rabbits harboured no *E. coli* while others had up to 4.8 log CFU/g. On D5, all four groups showed a strong increase in *E. coli* ( $>3$  log,  $P < 0.05$ ), without a significant effect of inoculation (average 5.8 log CFU/g on D5; Figure 1A). On D11, the total caecal *E. coli* concentration for both inoculated groups stabilised at 5 log higher ( $P < 0.001$ ) than non-infected groups (7.0 vs. 1.9 log CFU/g). Feed restrictions did not significantly modify *E. coli* levels in the rabbits' caecal contents.

Similarly, the concentration of total *E. coli* in hard faeces did not differ among the four groups on D-2, and was established at a mean level of  $5.2 \pm 1.2$  log CFU/g. This *E. coli* level remained steady till D6 for non-inoculated rabbits. In contrast, a 2 log higher *E. coli* level ( $P < 0.05$ ) was detected on D11 for C70 compared with the C100 group. As

**Table 3:** Effects of EPEC inoculation (control, C vs. inoculated, I) and feed intake level “IL” (*ad libitum*, 100 vs. restricted, 70) on post-weaning morbidity<sup>1</sup> in the rabbit.

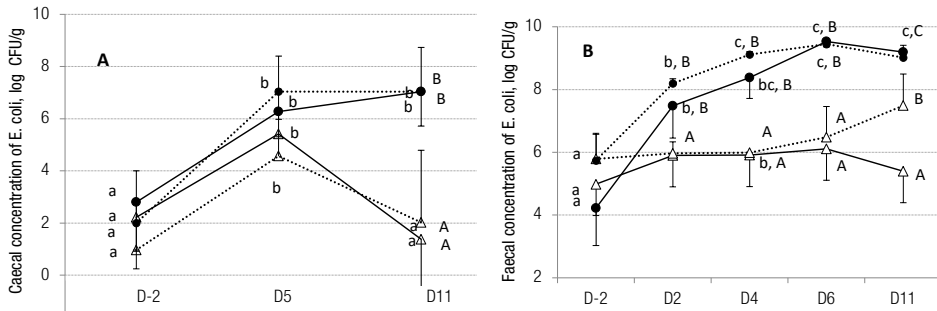
	Groups				P-level		
	C100	C70	I100	I70	Infection	IL	Inf. × IL
D-3 to D0 <sup>2</sup>	1/45	0/45	0/45	0/45	0.98	0.94	0.93
D0 to D4	1/40 <sup>a</sup>	1/40 <sup>a</sup>	5/40 <sup>a</sup>	14/40 <sup>b</sup>	<0.01	0.70	0.18
D7 to D11	0/30 <sup>ab</sup>	0/30 <sup>a</sup>	8/30 <sup>b</sup>	5/30 <sup>ab</sup>	0.025	0.33	0.90
D0 to D11	2/45 <sup>a</sup>	1/45 <sup>a</sup>	21/45 <sup>b</sup>	25/45 <sup>b</sup>	<0.001	0.58	0.56

<sup>1</sup>Morbidity: prostrate/bloated animals or with diarrhoea, and/or stunted growth compared with rabbits in the same group.

<sup>2</sup>D0: inoculation with EPEC; D-3: weaning (28 d old)

<sup>3</sup>NS: not significant, with a  $P$ -value  $> 0.15$ .

<sup>a,b</sup>Within a row, means having a common superscript did not differ ( $P < 0.05$ , for a monofactorial model: group effect).



**Figure 1:** Post inoculation kinetics of total *E. coli*\* concentration in the caecal content “A” (n=5/d group) and faeces “B” (n=5/d group), according to inoculation (control, C vs. inoculated, I) and feed intake level (*ad libitum*, 100 vs. restricted, 70). —△—: C100; ---△---: C70; —●—: I100; ---●---: I70.

<sup>a,b,c</sup>Within a group, means without a common superscript differ ( $P < 0.05$ ); <sup>A,B,C</sup>Within a day, means without a common superscript differ ( $P < 0.05$ , for a monofactorial model: group effect).

\*At D-2, no *E. coli* belongs to the O128 serogroup, and from D2 all *E. coli* belong to the O128 serogroup.

expected, the faecal *E. coli* concentration strongly increased by 4 log ( $P < 0.05$ ) in both inoculated groups (with or without feed restrictions), as early as four to six days after inoculation.

Intake levels did not significantly modify the diversity of the bacterial communities in the ileum and caecum (Table 4). During the two weeks of the trial, bacterial diversity remained steady, whatever the group and organ considered. However, five days after inoculation, diversity in the ileum was lower (−0.6 units,  $P = 0.04$ ) and also tended to be lower in the caecum (−0.3 units,  $P = 0.06$ ). The structure of the ileal and caecal bacterial communities was unaffected by the feed intake level, whatever the group and time after inoculation (data not shown). In contrast, five days after inoculation, the ileal bacterial structure was modified in both the inoculated and control groups (Table 5). The ileal bacterial structure of non-inoculated rabbits did not further evolve between D5 and D11, whereas by D11 the ileal bacterial structure of inoculated rabbits had changed significantly from their structure on D5.

### Ileum histometry and serum haptoglobin

No significant effect of feed intake level was illustrated with respect to the histometry of the ileal mucosa (on D5, Table 6). However, five days after inoculation, villi height was one third lower than that of non-inoculated animals,

**Table 4:** Diversity of the ileal and caecal bacterial community<sup>1</sup> throughout rabbit growth, according to EPEC inoculation (control, C vs. inoculated, I) and feed intake level “IL” (*ad libitum*, 100 vs. restricted, 70).

	Groups				SEM	P-value <sup>2</sup>		
	C100 (n=5)	C70 (n=5)	I100 (n=5)	I70 (n=5)		Inoculation	IL	Ino.×IL
<b>Ileal bacterial diversity</b>								
D-2	5.68	5.46	5.52	5.14	0.145	0.43	0.31	0.80
D5	6.01	6.10	5.45	5.59	0.126	0.04	0.61	0.92
D11	5.99	5.61	5.62	5.67	0.156	0.68	0.65	0.53
<b>Caecal bacterial diversity</b>								
D-2	3.99	4.35	4.34	4.27	0.139	0.64	0.63	0.47
D5	4.15	4.33	3.84	3.95	0.093	0.06	0.43	0.85
D11	4.17	4.22	4.28	4.18	0.114	0.91	0.92	0.77

<sup>1</sup>Modified Simpson index (Michelland *et al.*, 2011).

<sup>2</sup>P-values for a bifactorial model, with effect of contamination (control, C vs. inoculated, I) and of feed intake (*ad libitum*, AL vs. restricted, R).

SEM: standard error of the mean.

**Table 5:** Structure of ileal and caecal bacterial community throughout rabbit growth, according to feed intake level (*ad libitum*, 100 vs. restricted, 70) and for non-inoculated (control, C) and inoculated rabbits (I).

	Not infected		Infected	
	P-value	R <sup>1</sup>	P-value	R <sup>1</sup>
Ileum				
D-2 vs. D5	0.01	0.425	0.01	0.444
D5 vs. D11	>0.05	/	0.03	0.080
D-2 vs. D11	0.02	0.255	0.02	0.165
Caecum				
D-2 vs. D5	>0.05	/	>0.05	/
D5 vs. D11	>0.05	/	>0.05	/
D-2 vs. D11	>0.05	/	>0.05	/

<sup>1</sup>R: Degree of proximity (Ramette, 2007).

P-value<0.05, for a monofactorial model.

while villi area tended to be reduced by inoculation. Inoculation did not significantly modify crypt characteristics. Accordingly, the villus height/crypt depth and villus area/crypt area ratios following inoculation were lower by 1.4 ( $P<0.001$ ) and 6.3 ( $P=0.04$ ), respectively.

Throughout the experiment, the serum haptoglobin concentration remained unaffected by the feed intake level (Table 6). Haptoglobin concentration in non-inoculated rabbits rose with age, as within the two weeks of the trial (between D-2 and D11) it increased by 270% ( $P=0.009$ ) and by 64% ( $P=0.02$ ) for C100 and C70, respectively. In inoculated animals, the level of haptoglobin was found to be five- to sevenfold higher on D5, but then decreased to be only two- to threefold higher than in control rabbits on D11.

## DISCUSSION

### *Challenging the growing rabbit with a moderately pathogenic E. coli strain*

As expected, the EPEC challenge impaired growth and feed intake as early as four days after inoculation. The peak of inoculation effects was between four and 11 days p.i., with a high morbidity rate without mortality and a high *E. coli*

**Table 6:** Effects of EPEC inoculation (control, C vs. inoculated, I) and feed intake level “IL” (*ad libitum*, 100 vs. restricted, 70) on ileal mucosa histometry and serum haptoglobin concentration.

	Groups <sup>1</sup>				SEM	P-value <sup>2</sup>		
	C100	C70	I100	I70		Inoculation	IL	Ino.xIL
Ileum								
Histometric parameters (D5)								
Villus height (µm)	306 <sup>b</sup>	303 <sup>b</sup>	229 <sup>ab</sup>	209 <sup>a</sup>	13	<0.001	0.57	0.66
Villus area, µm <sup>2</sup>	31790	31189	27441	21677	2113	0.107	0.45	0.54
Crypt depth, µm	77	66	78	78	2	0.092	0.15	0.16
Crypt area, µm <sup>2</sup>	1819	1353	1822	1922	99	0.16	0.36	0.15
Villus height/crypt depth	4.0 <sup>b</sup>	4.5 <sup>b</sup>	3.0 <sup>a</sup>	2.7 <sup>a</sup>	0.2	<0.001	0.62	0.16
Villus/crypt area	18.0	22.5	16.2	11.8	1.5	0.037	0.98	0.11
Serum Haptoglobin concentration (mg/L)								
D-2	95 <sup>a,A</sup>	163 <sup>ab,A</sup>	199 <sup>b,A</sup>	130 <sup>ab,A</sup>	12	0.09	0.96	0.003
D5	221 <sup>a,AB</sup>	215 <sup>a,AB</sup>	1215 <sup>b,B</sup>	1560 <sup>b,B</sup>	157	<0.001	0.33	0.31
D11	351 <sup>B</sup>	268 <sup>B</sup>	787 <sup>AB</sup>	726 <sup>A</sup>	103	0.032	0.70	0.95

<sup>1</sup>5 replicates per group.

<sup>2</sup>P-values for a bifactorial model, with effect of contamination (control, C vs. inoculated, I) and of feed intake (*ad libitum*, AL vs. restricted, R).

<sup>a,b,c</sup>Within a row, means without a common superscript differ ( $P<0.05$ , for a monofactorial model: group effect).

<sup>A,B,C</sup>Within a column, means without a common superscript differ ( $P<0.05$ ).

SEM: standard error of the mean.

level in both the caecum and faeces. Our results were consistent with previous publications (Camguilhem *et al.*, 1989; Milon *et al.*, 1990, 1992) referencing *E. coli* strain O128:C6 as a moderately pathogenic strain inducing weight loss and diarrhoea without mortality. Similar delays in growth were also observed post inoculation by Skrivanová *et al.* (2009). Moreover, the inoculation effects peak was associated with marked changes in intestinal physiology: the threefold higher serum haptoglobin levels strongly suggested increased inflammation of the intestine that could be related to the sharp drop in ileal villi height. Serum haptoglobin could thus be considered as a good marker of the systemic inflammation level, as described in pigs (Le Floch *et al.*, 2014) or in rabbits (Georgieva *et al.*, 2009; Kimsé *et al.*, 2011).

Our histometric results were consistent with the attaching and effacing phenomena associated with EPEC, leading to the destruction of absorptive epithelial cells and villous atrophy (Peeters *et al.*, 1985, 1988; Licois *et al.*, 1991). Therefore, digestive and resorptive intestinal capabilities were reduced, inducing diarrhoea, poor feed conversion and retarded growth.

With respect to bacterial communities in the intestine, the inoculation significantly reduced the diversity of the ileal bacterial community only five days after inoculation. At this stage, the structure of the ileal bacterial community was also affected in both inoculated and control rabbits. This is in keeping with the EPEC colonisation process in growing rabbits, mostly affecting the distal part of the digestive tract of the ileum, caecum and colon (Cantey and Inman, 1981; Peeters *et al.*, 1984, 1988). In suckling rabbits, on the other hand, the whole small and large intestines were colonised by EPEC (Coussement *et al.*, 1984; Peeters *et al.*, 1984, 1988). Therefore, the resilience of the ileal bacterial community structure observed five days after inoculation was related to the high *E. coli* levels measured in the caecum and faeces between four and six days after inoculation. These EPEC colonisation kinetics have been classically described in several animal species (Allison and Martiny, 2008; Antonopoulos *et al.*, 2009). However, our moderate EPEC colonisation was no longer able to affect the global diversity and richness of the gut bacterial ecosystem over five days, either in the ileum or caecum. Similarly, using an SSCP approach, Combes *et al.* (2009) did not detect significant modifications in the structure, richness and density of total bacterial community for caecal or faecal samples during an ERE experimental inoculation.

### **Consequences of limiting feed intake during an EPEC challenge**

Over two weeks, the 40% feed restriction reduced the growth rate in both inoculated and control animals, as highlighted by Gidenne *et al.* (2012). However, this restriction impaired growth more markedly (–60%) during the first week after weaning, highlighting the sensitivity of young rabbits during the post-weaning period. In parallel, the EPEC challenge reinforced the negative impact of feed restriction on growth during this period (significant negative interaction), leading to a 60% reduction in growth of animals that were both inoculated and feed-restricted, although their intake was equal to the non-inoculated feed-restricted group. Accordingly, this acute phase of the infection occurred four to seven days after inoculation was associated with the highest morbidity rate and haptoglobin level. On the contrary, feed restrictions during an ERE challenge led to increased growth and lower morbidity and mortality (Boisot *et al.*, 2003; Foubert *et al.*, 2008) compared with rabbits fed *ad libitum*. Therefore, it appears that a short-term feed intake limitation strategy after weaning differentially interacts with the pathogenic model, for instance ERE vs. EPEC. Accordingly, French veterinary surveys reported that limiting feed intake is a more effective strategy for ERE outbreaks than EPEC outbreaks (Le Bouquin *et al.*, 2009).

In agreement with Martignon *et al.* (2010), limiting feed intake after weaning did not induce modifications either in the global diversity and structure of the bacterial community or in the intestinal morphometry and systemic inflammation. There was no interaction with the EPEC challenge. Likewise, systemic inflammation was similar in piglets fed *ad libitum* or submitted to a feed restriction (Le Floch *et al.*, 2014), but the bacterial community profile was affected by both the intake level and a poor hygiene challenge. More recently, using high-throughput sequencing of 16S rRNA, Combes *et al.* (2017) were also able to detect an impact of both feed intake level and hygiene challenge on the caecal bacterial community, especially for dominant genera belonging to the *Ruminococcaceae* family, without any significant interaction between the challenge and feed intake level.



## CONCLUSIONS

The challenge with a moderately pathogenic *E. coli* clearly impairs the intestinal mucosal morphometry and is associated with marked systemic inflammation and a high morbidity rate, but has little impact on the gut bacterial community. Limiting the post-weaning feed intake level was not beneficial to rabbits after *E. coli* inoculation and even impaired growth. Feed intake limitation neither impacted the gut bacterial community nor the mucosa morphometry.

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