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Evidence for Clostridial Implication in Necrotizing Enterocolitis through Bacterial Fermentation in a Gnotobiotic Quail Model

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ABSTRACT

Despite extensive research, the pathogenesis of neonatal necrotizing enterocolitis (NEC) remains elusive. The aim of our work was to investigate the role of bacterial strains involved in NEC in gnotobiotic quails as experimental model. Six groups of germ-free quails that were fed a lactose diet were associated with *Klebsiella pneumoniae*, *Clostridium perfringens*, *C. difficile*, *C. paraputrificum*, or *C. butyricum* (two strains). Implantation level, incidence of cecal lesions, production of short-chain fatty acids, and histologic lesions of the cecal wall were investigated. Whatever the strain, the implantation level was high (~10^9 UFC/g). Neither *K. pneumoniae* nor *C. difficile* induced any cecal lesions. In contrast, the four other clostridial strains led to cecal NEC-like lesions with a variable occurrence: four of 12 quails for *C. perfringens*, eight of 12 quails for *C. paraputrificum*, and the same highest value, nine of 12 quails and eight of 10 quails for both *C. butyricum* strains. Gross aspects of the lesions may be linked to the short-chain fatty acid profiles and/or concentrations: thickening of the cecal wall (*C. butyricum* and *C. perfringens*) with high proportion of butyric acid, hemorrhages (*C. paraputrificum*) with high proportion of iso-butyric acid, and presence of other iso-acids. In addition, *C. butyricum* was characterized by pneumatosis, linked to a high gas production. Microscopic aspects confirmed the presence of edemas and intramucosa hemorrhages. Clostridia species, whose role is controversial, seem to be strongly implicated in NEC through excessive production of butyric acid as a result of colonic lactose fermentation. These results call for anaerobe detection in feces of infants who have NEC. **(*Pediatr Res* 58: 629–635, 2005)**

Abbreviations
NEC, neonatal necrotizing enterocolitis
SCFA, short-chain fatty acid

Necrotizing enterocolitis (NEC) is a devastating disease with high morbidity and mortality and the most common gastrointestinal emergency encountered in the neonatal period, affecting predominantly preterm infants. Despite extensive research, the exact pathogenesis remains incompletely understood. Three major factors—bacterial colonization, enteral feeding, and gut immaturity—are recognized to coalesce for promoting an inflammatory cascade that leads to the disease (1–3). Digestive bacterial colonization seems to be a prerequisite. In fact, NEC occurred neither before colonization of the intestine by bacteria nor in germ-free animal models. Furthermore, if NEC usually occurs sporadically, then reports of outbreaks have encouraged speculations about specific transmissible agents. Implication of bacteria is thought to be due to fermentation of nonhydrolyzed lactose, a consequence of the immaturity of the intestinal lactasic equipment in preterm infants (2). High production of bacterial metabolites (hydrogen, butyric, and iso-butyric acids) may be responsible for the onset of the intestinal lesions and for pneumatosis (4–6).

Various microorganisms are involved in NEC, including bacteria and virus [see Foglia (3) for review]; however, no specific pathogens have been found (7). The most often implicated bacteria are enterobacteria, particularly *Klebsiella pneumonia* and *Escherichia coli*, but also anaerobes species, specifically clostridia species. The most common clostridia species involved are *C. butyricum* and *C. perfringens*. *C. paraputrificum* was scarcely identified. Recently, a novel *Clostridium species*, *C. neonatale*, was associated with a NEC outbreak (8). All of these bacteria belong to the endogenous intestinal microflora, and the aberrant gut colonization observed in preterm infants (9,10) may be a risk factor (2).

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Analyses of the premature infant's fecal flora by molecular techniques were seldom used. Millar *et al.* (11), who compared preterm infants with and without NEC, did not observed any difference. In contrast, De La Cochetière *et al.* (12) described recently a significant relationship between early colonization by *C. perfringens* and the later development of NEC. However, the causative role of clostridia remains controversial.

Gnotobiotic quails that were fed a lactose diet were previously used as a suitable experimental model to mimic human premature neonates in terms of gastrointestinal and physiologic characteristics, because they are a natural alactasic species and their ceca, a pair of blind ending ducts, favors bacterial stasis (13,14). Development of cecal NEC-like lesions in quails requires the combination of two major factors that are present in preterm human infants: lactose in diet and colonization by lactose-fermenting bacteria. Indeed, experimental infection of germ-free quails with either *C. butyricum* strains (monobiotic quails) or fecal specimens (polybiotic quails), both originating from preterm infants with NEC, reproduced many aspects of the pathology, such as thickening of the cecal wall with gas cysts, hemorrhagic ulcerations, necrotic areas, and intestinal pneumatosis (13–16). Up to now, only the key role of *C. butyricum* was clearly demonstrated in monobiotic quails associated with strains from various origins (13,14). Polybiotic quails were associated with fecal specimens that comprise several bacterial species implicated in NEC: *C. perfringens*, *C. paraputrificum*, *C. difficile*, and *K. pneumoniae* (15,16). However, these species were also isolated from healthy preterm infants.

The aim of the present study was to examine and compare the role of these bacterial species isolated from NEC cases—*C. perfringens*, *C. paraputrificum*, *C. difficile*, and *K. pneumoniae*—in our experimental model. With the use of monobiotic quails, the involvement of these bacterial species was studied through the bacterial implantation, the incidence of intestinal lesions, and the production of short-chain fatty acids (SCFAs) and compared with two *C. butyricum* strains isolated from infants with NEC. In case of cecal lesions, histologic examinations were performed.

METHODS

Bacterial strains. Six bacterial strains that were isolated from preterm infants who had NEC and were hospitalized in different French pediatric units were studied (Table 1). All infants had definite or advanced NEC (Bell's stage IIc, IIIa, or IIIb). *K. pneumoniae* was isolated from an infant (NEC-stage IIIa) during an outbreak of NEC (12 cases). *C. perfringens*, *C. difficile*, and *C. paraputrificum* were isolated from fecal specimens of two preterm infants who had NEC (stage IIc) (15). *C. butyricum* MPP 195 and *C. butyricum* CB 1002–5 were isolated from two fatal NEC cases (stage IIIb). *C. butyricum* CB 1002–5 was included as a reference strain that produced NEC-like lesions in quails (13).

Experimental design. Germ-free quails (*Coturnix coturnix* subspecies *japonica*) were obtained and maintained in germ-free isolators as already described (13). Quails were fed an experimental semisynthetic diet that contained lactose 7% (wt/wt) to mimic the proportion of lactose in human milk. The diet was sterilized by gamma irradiation at 40 kGy. After their germ-free status was checked, 2-wk-old germ-free quails were transferred in six experimental isolators ($n = 7$ to 12 quails per group) and mono-associated with the different bacterial strains. Each group was inoculated orally with 100 μL of cultures that contained 10^8 viable cells of one of the various strains. Bacterial establishment was checked in fresh droppings at weekly intervals. All quails were killed 3 wk after the bacterial inoculation.

Sampling. Ceca were collected immediately after death. After macroscopic examinations, the cecal contents were removed for pH measurement and bacteriologic counts. For biochemical determinations, samples of cecal contents were immediately frozen with saturated mercuric chloride solution (10%, vol/vol) before analysis. Cecal wall samples were placed in a 10% formalin solution for histologic examinations.

Bacterial counts. Cecal contents were homogenized and diluted in peptone broth for bacterial counts. Using the Spiral System (F-35270; AES Laboratoires, Combourg, France), dilutions were spread on Columbia agar media with 5% sheep blood for anaerobic bacteria and incubated for 48 h at 37°C in jars under an anaerobic gas phase (H_2:CO_2, 95:5, vol/vol). For *K. pneumoniae*, the dilutions were spread on Trypcase agar and incubated in aerobic atmosphere for 24 h. Bacterial counts were expressed as the $\log_{10}$ CFU/g of wet feces or cecal content.

Biochemical determination. SCFA determinations and dosage were performed on *in vitro* cultures of the six bacterial strains and on the cecal contents. In case of *in vitro* experiments, bacteria were grown in TYH broth that contained lactose 1% (vol/vol) for 48 h in anaerobic or aerobic atmosphere, depending on the strain. SCFA analysis were carried out on a Perkin-Elmer Autosystem Gas Chromatography (St Quentin en Yvelines, France) fitted with a capillary (15 m × 0.53 mm) Nukol column (Supelco, St Germain-en-Laye, France) and a flame ionization detector as previously described (15). 2-Ethyl butyric acid (Sigma Chemical Co., L'Isle d'Abeau, France) was used as internal standard, and total SCFA concentrations were calculated in μmol/L or μmol/g of wet cecal content. The relative distribution of acetic (C_2), propionic (C_3), butyric (C_4), valeric (C_5), caproic (C_6), and iso-acids (iC_4, iC_5, and iC_6) was calculated as the percentage of the total concentration. pH was measured when the fecal specimen quantity was sufficient (>300 mg).

Macroscopic and Histologic Examinations of the Cecal Wall

Gross findings. Ceca were categorized as normal, thickening, pneumatosis, and hemorrhagic contents. The weight of the cecal wall was expressed as the cecal wall weight:body weight ratio. Quails with normal ceca were referred to as healthy quails, and quails with one or more cecal modifications (thickening, pneumatosis, or hemorrhagic content) were referred to as sick quails.

Microscopic findings. All of the specimens were fixed in formalin, then embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin. The elementary histologic lesions were studied and classified as follows: erosion/ulceration, edema, foci of necrosis, infiltrates, gas cysts, and peritonitis. Biochemical determinations and macroscopic and histologic examinations were performed blinded to the investigators.

Statistical analysis. Data presented as the mean ± SEM were evaluated statistically by the *t* test (cecal pH, cecal wall weight) and the Mann-Whitney nonparametric test (SCFA concentrations and profiles).

Table 1. *Characteristics of the bacterial strains*

Bacteria strains	References	Isolates origin	Toxin detection
K. pneumoniae	MPP 196*	General Hospital Center, Cayenne, Guyane	
C difficile	MPP 193	Hospital Gatien de Clocheville, Tours	Tox A +, Tox B +
C perfringens	MPP 192	Hospital Gatien de Clocheville, Tours	Alpha toxin + (type A), enterotoxin −
C paraputrificum	MPP 194	Hospital Gatien de Clocheville, Tours	
C butyricum	MPP 195	General Hospital Center, Lens	
C butyricum	CB 1002-5†	Maternity Beaudeloque, CHU Cochin, Paris	

* MPP = collection of the Microbiology Unit, Pharmacie, Paris, France.

† from Institut Pasteur, Dr Popoff

Table 2. *In vitro SCFA concentrations and profiles of the 6 bacterial strains grown in TYH + lactose 1% (TLYH)*

Bacteria strains	Total SCFAs mM/L (mean ± SEM)	Bacterial counts $\log_{10}$cfu/ml (means ± SEM)	Gas production
K. pneumoniae	20.9 ± 4.3	9.0 ± 0.1	++†
C difficile	67.6 ± 6.4	7.3 ± 0.2	−
C perfringens	25.5 ± 0.7	8.0 ± 0.1	+++
C paraputrificum	11.2 ± 0.6	7.0 ± 0.4	++++
C butyricum MPP 195	21.2 ± 2.0	8.5 ± 0.3	+++
C butyricum CB 1002-5	25.8 ± 1.3	8.4 ± 0.2	++++

	SCFA molar ratio (%)			
Bacterial strains	acetate	propionate	*iso*butyrate	butyrate
K pneumonia	95	2.4	0.6	2
C difficile	39	2	10	13
C perfringens	70	7	nd	23
C paraputrificum	76	nd	nd	24
C butyricum MPP 195	51	nd	nd	49
C butyricum CB 1002-5	67	nd	nd	33

	SCFA molar ratio (%)			
Bacterial strains	*isovalerate*	valerate	*iso*caproate	caproate
K pneumoniae	nd*	nd	nd	nd
C difficile	7	1	28	nd
C perfringens	nd	nd	nd	nd
C paraputrificum	nd	nd	nd	nd
C butyricum MPP 195	nd	nd	nd	nd
C butyricum CB 1002-5	nd	nd	nd	nd

* nd = not detected.

† negative (−) to abundant (++++) on a − to ++++ scale in TLYH agar deep culture.

RESULTS

In vitro bacterial SCFA profiles. SCFA concentrations varied from 11 to 68 mM/L, depending on the strain (Table 2). The production of SCFAs by *K. pneumoniae* consisted mainly of acetate, and gas production was moderate. *C. difficile* differed from the other species by a high production of SCFAs, a complex SCFA profile, and an absence of gas production. The four other clostridia strains shared a similar butyric acid profile and an abundant gas production. Among them, only *C. perfringens* produced propionic acid in a small amount.

In vivo experimental data. The strains were established at a high level in the six groups of the monobiotic quails, ranging from 8.5 ± 0.2 to 9.3 ± 0.4 CFU/g of cecal content (Table 3). *K. pneumoniae* did not induce any cecal lesions. Among clostridia species, only *C. difficile* did not induce cecal lesions. In contrast, the four other clostridia strains led to cecal NEC-like lesions in quails with a variable occurrence: four (50%) of eight quails for *C. perfringens*, eight (67%) of 12 quails for *C. paraputrificum*, and the same highest value nine (75%) of 12 quails and eight (80%) of 10 quails for both *C. butyricum* strains.

Cecal contents in non–lesion-inducing strains. The two strains differed from one another in cecal pH and SCFAs. With *K. pneumoniae*, SCFA concentrations were twice as high than with *C. difficile* and associated with a lower pH (Table 3). The *C. difficile* profile was more complex than the *K. pneumoniae* profile, with a significantly higher percentage of propionic, valeric, isocaproic, and caproic acids (Table 4).

Cecal contents in NEC-like lesion–inducing strains. pH values were not significantly different between groups whatever the bacterial status or pathologic state (Table 3). Total SCFA concentrations were higher with *C. perfringens* and *C. paraputrificum* groups than with both *C. butyricum* groups. The relative contribution in the total SCFA amounts differed with the strain. As far as the profile was concerned, percentages of butyric acid were significantly higher in both *C. butyricum* groups as compared with the other groups, and percentages of iso-butyric acid were significantly higher in both *C. perfringens* and *C. paraputrificum* groups. Small amounts of other SCFAs were detected in these last two groups (Table 4). There was no significant difference in SCFA profiles between sick (*i.e.* with cecal lesions) and healthy quails (*i.e.* without lesions) within the same bacterial association.

Pathologic findings were hemorrhagic fluid, predominant in the lumen of *C. paraputrificum* group (seven of the eight sick quails), and to a lesser extent in the three other clostridia groups (Fig. 1*A*).

Cecal wall in non–lesion-inducing strains. In quails associated with *K. pneumoniae* or *C. difficile*, the cecal wall weight to body weight ratio did not exceed 4.6 and 3.6, respectively (Table 3).

Cecal wall in NEC-like lesion–inducing strains. Within the same bacterial status, cecal wall weight was significantly enhanced in sick quails as compared with healthy ones for the *C. perfringens* group ($p = 0.01$) and both *C. butyricum* groups ($p = 0.03$; Table 3). No thickening of the cecal wall was observed in the *C. paraputrificum* group.

The macroscopic pathologic findings were thickening and pneumatosis of the mucosa. Both *C. butyricum* strains led to the most dramatic effects, from thickening to the association of thickening and pneumatosis with or without hemorrhagic contents (Fig. 1*A*). In the case of *C. perfringens*, because of the small number of sick animals, a specific profile of lesions was difficult to identify. Thickening was very high, except for one sick quail, and associated with either pneumatosis or hemorrhage. In quails associated with *C. paraputrificum*, only one quail displayed thickening and pneumatosis.

Microscopic findings were necrotic areas, hemorrhages, and edema with infiltrate of mononuclear cells as macrophages, lymphocytes, and heterophils combined with polynuclear cells, and alteration in the brush border (Fig. 1*B*). *C. butyricum* led to the most dramatic alterations of both cecal mucosa and cecal brush border. Brush border was characterized by edema and villus atrophy. A hypertrophy of the cecal wall was observed to be associated with a large inflammatory infiltrate in the mucosa, submucosa, musculosa, and serous membrane. Gas cysts represented another common finding (Fig. 2*A* and *B*). In quails associated with *C. paraputrificum*, hemorrhages predominated (Fig. 2*C* and *D*), and necrotic areas that were observed in four quails were very scarce as compared with the *C. perfringens* and *C. butyricum* groups.

SCFA profiles and pathologic aspects. Monobiotic quails associated with NEC-like lesion–inducing strains were characterized by significantly higher levels of either butyric or butyric

and iso-butyric acids (Table 5). Moreover, the trend of major macroscopic aspects of the lesions may be linked to the major differences in SCFA profiles. Thickening (*C. butyricum* and *C. perfringens*) was linked with a higher proportion of butyric acid, whereas hemorrhages (*C. paraputrificum*) were linked with high proportion of iso-butyric acid and presence of other iso-acids (Table 4). In addition, the *C. butyricum* group was characterized by pneumatosis, linked to the high gas production by this strain.

DISCUSSION

The important role of enteral feeding and bacterial colonization in the pathogenesis of NEC has been well recognized for several years. However, no specific pathogens were found. Using an experimental model of NEC, *i.e.*, monobiotic quails associated with bacterial species belonging to preterm neonates with NEC, we demonstrated the implication of clostridial species through the bacterial fermentation, except for *C. difficile*, and showed that *K. pneumoniae* did not interfere.

Klebsiella species is the most frequent aerobic species implicated in NEC, but most microbiologic analyses of NEC cases involving *Klebsiella* did not include anaerobic microflora cultures (17,18). Westra-Meijer *et al.* (19), comparing aerobic and anaerobic fecal flora in healthy preterm infants and infants with NEC, observed a higher colonization with *Klebsiella* species in infants with NEC but no difference in clostridia populations. Nevertheless, a careful analysis of the published results showed that the proportion of infants who were colonized with high levels of clostridia ($\geq 10^8$/g of feces) was higher in the two NEC groups (54%) than in the healthy group (25%). Moreover, the number of clostridia species, except *C. difficile*, that were isolated in the group with proved NEC (12 species isolated in 24 infants) was higher than in the suspected NEC groups (three species in 12 infants) and in the healthy group (14 species in 41 infants). In a clinical report of 12 cases of NEC, mild to moderate clinical course was associated with isolation of *Klebsiella* species, whereas severe disease was associated with the presence of clostridia (20).

Concerning *C. difficile*, our results with monobiotic quails were in accordance with our previous studies on polybiotic quails, which did not allow implication of *C. difficile* (15). In these studies, in quails associated with fecal specimens of premature neonates comprising *C. difficile*, a marked protective role by bifidobacteria through a sharp decrease in clostridia levels was demonstrated; however, both *C. difficile* counts and its toxins were not modified (mean 2.5×10^5 UFC/g). Our present results improve the understanding of the controversial role of this species commonly found in neonates, even in preterm infants (21). Indeed, in contrast to adults, high levels of toxins A and B may be detected in asymptomatic neonates (21). In studies involving *C. difficile* in NEC (22,23), the microbiologic methods performed were unable to isolate anaerobic bacteria other than *C. difficile*. Moreover, several authors did not find any relation between the isolation of *C. difficile* and/or detection of its toxins and NEC (21,24,25). In Westra-Meijer's study (19), *C. difficile* colonization was similar in the NEC groups and the healthy group (~50%). However, in very rare cases, *C. difficile* might be involved in severe enterocolitis in infants, the most obvious relationship being in regards to enterocolitis in Hirschsprung's disease (26).

The three other clostridial species studied led to NEC-like digestive lesions in quails. The effects varied in terms of occurrence and seriousness of the cecal lesions according to the species, even implanted at the same high level. The most deleterious effects were observed with *C. butyricum* strains. The key role of *C. butyricum* in NEC development has been once again suggested through our experimental model. Cecal lesions, levels of bacterial implantation, and SCFA profile between quails that were infected with either the strain of *C. butyricum* (VPI 3266) that was isolated from the autochthonous microflora of pigs or various *C. butyricum* strains isolated from healthy neonates (three strains) or from neonates with NEC (three strains) in previous studies (13,14) were similar. In the present study, a new isolate of *C. butyricum* (MPP195), originating from a fatal NEC case, led to the same cecal lesions in monobiotic quails. Lesions were macroscopically and microscopically similar to that in neonates with NEC (3). Grossly, the lesions were a mural thickening of the bowel, with ulcerations, necrosis, and pneumatosis, which is a characteristic finding in NEC. Microscopically, an inflammatory reaction that consisted of lymphocytes and neutrophils was observed in the mucosa, with hemorrhages and edema of the submucosa. In severe lesions, necrotic areas and ulcerations were observed. Last, gas-filled cysts were often present in the bowel wall.

The role of this autochthonous nontoxigenic species was largely discussed 25 y ago [reviewed by Szylit *et al.* (14)]. Our experimental findings in previous studies showed its primary role in the onset of NEC-like lesions in quails through the carbohydrate fermentation products and the production of butyric acid. When quails were fed a diet deprived of lactose, butyric acid production was very low, whereas clostridia implantation was at a similar level and no digestive lesions were observed (13,14). Numerous gas cysts were always observed in the cecal wall of quails monoassociated with *C. butyricum* (13). The pathogenesis of the gas cysts probably consists of an accumulation of gas produced by bacteria in the cecal lumen through rupture of the epithelium integrity or through spaces between epithelium cells. Gas cysts in the mucosa were shown by other authors to consist largely of hydrogen produced by the proliferation of clostridia organisms (27).

C. perfringens, which also belongs to the autochthonous microflora in adults and neonates, was often associated with NEC [see Foglia (3) for review]. Moreover, NEC cases associated with *C. perfringens* had more severe clinical course (20). In our model, the main difference between *C. perfringens*– and *C. butyricum*–induced lesions was the lower occurrence of pneumatosis and intramucosal edema with *C. perfringens*. The intestinal damage and hemorrhages could be the result of the production of the numerous *C. perfringens* toxins. However, NEC was not linked to the production of the alpha toxin (28). Moreover, compared with *C. butyricum*, butyric acid is also a major end product of lactose fermentation by *C. perfringens*, and when quails were fed a diet deprived of lactose, no lesion was observed (16).

Table 3. *Occurrence of cecal lesions and characteristic of cecal contents in monobiotic quails associated with the 6 bacterial strains*

	non-NEC inducing strains		NEC-inducing strains			
Bacterial strains	*K. pneumoniae*	*C. difficile*	*C. perfringens*	*C. paraputrificum*	*C. butyricum* MPP 195	*C. butyricum* CB 1002-5
Outcome of cecitis/no quails	0/7	0/9	4/8	8/12	9/12	8/10
Bacterial counts (mean $\log_{10}$cfu/g ± SEM)	8.9 ± 0.1	8.9 ± 0.2	9.3 ± 0.4	9.3 ± 0.2	8.7 ± 0.2	8.5 ± 0.2
Cecal wall weight/body weight × 10^3 (± SEM)						
Healthy quails	4.1 ± 0.4	3.1 ± 0.2	2.9 ± 0.4	2.7 ± 0.5	3.2 ± 0.5	2.8 ± 0.3
Sick quails			5.5 ± 1.0*	2.9 ± 0.6	5.3 ± 1.3*	8.6 ± 2.3*
Cecal pH (mean ± SEM)						
Healthy quails	5.6 ± 0.1	6.9 ± 0.1	6.6 ± 0.3	6.5 ± 0.3	6.8‡	6.3 ± 0.8
Sick quails			6.7 ± 0.2	6.6 ± 0.1	6.6 ± 0.4	6.4 ± 0.2
Total SCFAs (μmol/g, mean ± SEM)						
Healthy quails	10.5 ± 2.7	5.3 ± 1.2	12.5 ± 2.6†	12.1 ± 5.1†	5.1‡	5.8 ± 0.2
Sick quails			13.4 ± 3.7†	19.9 ± 11.4†	6.1 ± 1.1	6.8 ± 0.1

* Significantly different from healthy quails within the same bacterial status ($p < 0.05$).
† Significantly different from quails associated with *C difficile* and both *C butyricum* strains ($p < 0.05$).
‡ Individual value.

Table 4. *SCFA profiles in monobiotic quails associated with the 6 bacterial strains*

	SCFA molar ratio (%, mean ± SEM)							
Bacterial strains	Acetic acid	Propionic acid	Iso-butyric acid	Butyric acid	Iso-valeric acid	Valeric acid	Iso-valeric acid	Caproic acid
K. pneumoniae	88.0 ± 3.4	0.3 ± 0.2	3.3 ± 1.0	5.0 ± 1.0	3.0 ± 1.0	0.3 ± 0.2	nd	nd
C difficile	68.5 ± 5.0	5.0 ± 0.5	5.5 ± 1.5	9.0 ± 2.0	3.5 ± 1.0	3.0 ± 1.0*	2.5 ± 0.5*	3.0 ± 0.5
C perfringens	76.9 ± 5.8	nd	14 ± 3.4*	8.1 ± 2.2	nd	0.5 ± 0.1	nd	0.5 ± 0.3
C paraputrificum	64.5 ± 6.5	6.9 ± 0.4	18.2 ± 4.5*	6.5 ± 1.5	2.5 ± 1.9	0.2 ± 0.1	nd	1.2 ± 0.8
C butyricum MPP 195	84.0 ± 4.0	nd	nd	16.0 ± 4.5*	nd	nd	nd	nd
C butyricum CB 1002-5	78.0 ± 5.0	nd	3.5 ± 2.5	18.5 ± 3.2*	nd	nd	nd	nd

* Significantly higher than the other groups ($p < 0.05$). nd = not detected.

C. paraputrificum, like *C. butyricum*, may be found in healthy neonates (19,29,30) and does not produce any toxin. It is a lactose-fermenter species that produces acetic and butyric acids. Contrary to *C. butyricum* and to *C. perfringens*, *C. paraputrificum* did not induce any cecal wall thickening but led to intramucosa and intraluminal hemorrhages. Up to now, *C. paraputrificum* was scarcely studied as a NEC agent. One reason could be that usual bacteriologic methods do not allow growth of all anaerobes, which require specific methods that often are technically tedious. Recently, because a point-prevalence survey using rectal swabs was conducted, a NEC associated with *C. paraputricum* was diagnosed in one infant (8).

Our study confirms the role of bacterial fermentation end products, *i.e.* SCFAs and in particular butyric acid, in the onset of NEC-like lesions observed in our experimental model. However, implication of butyric acid in NEC is controversial. In fact, SCFAs and especially butyric acid are essential for the integrity of the colonic epithelium. Butyric acid stimulates the proliferation of the epithelial colonic cells and is the major source of energy for the enterocytes (31), but high concentrations can lead to deleterious effects. *In vivo*, high concentrations were shown to lower the pH of small intestine, causing mucosal breakdown and increasing susceptibility to bacterial translocation (32). Furthermore, gut luminal administration of acetic or butyric acid in newborn rats produced dose-dependant intestinal mucosal injury, resembling human NEC clinically and histologically (33). Deleterious effect of butyric acid may be a result of an inflammatory response to this bacterial fermentation end product. Indeed, butyric acid was demon-

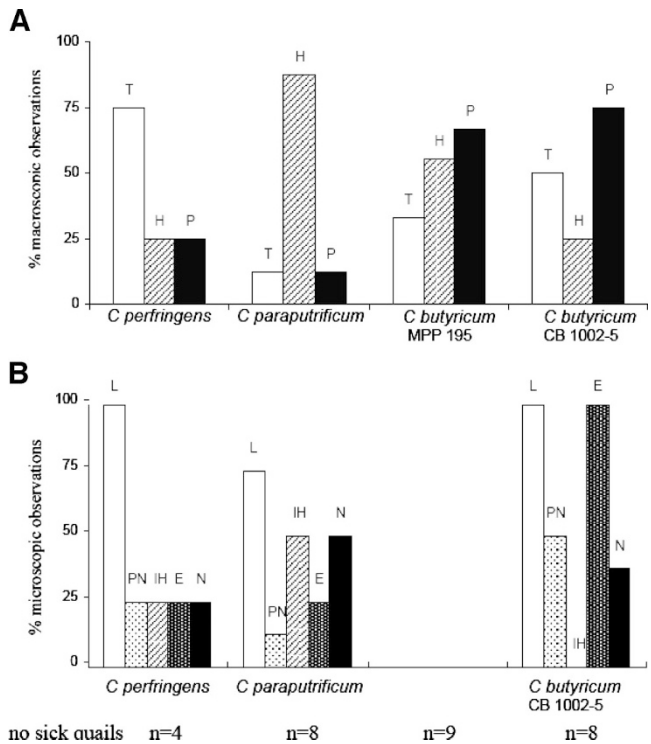

Figure 1. Macroscopic (*A*) and microscopic (*B*) observations in quails associated with NEC-like lesion–inducing strains and displaying cecal lesions. T, thickening; H, hemorrhages; P, pneumatosis; L, lymphocytes; PN, heterophils and polynuclear cells; IH, intramucosal hemorrhages; E, edema; N, necrotic areas. Histologic examinations were not performed with *C butyricum* MPP195.

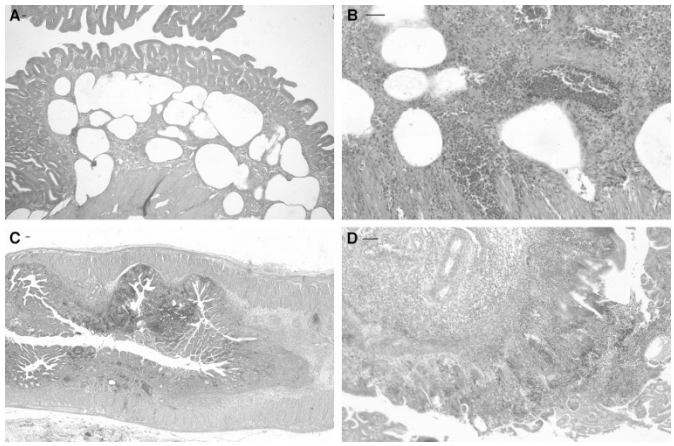

Figure 2. Various histologic examinations of the ceca of monobiotic quails (hematoxylin-eosin stain). (*A* and *B*) Cecum of quails monoassociated with *C. butyricum* CB 1002–5. Numerous gas cysts and infiltrates are in the submucosa of the cecum. (*C* and *D*) Cecum of quails monoassociated with *C. paraputrificum*. Pleomorphic infiltrates are in the cecal wall and hemorrhages. Bars = 100 μ. Magnification: $\times$100 in *A* and *C*; $\times$ 400 in *B* and *D*.

strated to increase IL-8 secretion in cultured enterocytes (34). In human pathology, IL-8 production has been observed in numerous digestive infections such as *Shigella* in colon (35). As an α-chemokine, IL-8 is a potent chemotactic factor for neutrophils and may be important in recruiting leucocytes into the gastrointestinal tract during inflammation (36). IL-8 also stimulates superoxide radical release in neutrophils and increases the permeability of vascular endothelium leading to tissue edema (36). In infants, high production of SCFAs associated with abnormal C_4 ratio (above 40%) has previously been demonstrated as potentially deleterious (5). In premature infants, because of their potential deficiency in intestinal lactase, butyric acid can be overproduced in the distal ileum and colon and thus cause intestinal injury as hypothesized by Lin (6). Furthermore, SCFA concentrations in healthy extremely premature infants were very low as compared with premature infants and full-term infants, and butyric acid concentrations varied from 0 to 1.2 μmol/g of feces, suggesting a different relationship between SCFAs and the intestinal mucosa in preterm infants (37).

Furthermore, our results pointed out a relationship between SCFA production and the aspects of the lesions: thickening was associated with a high level of butyric acid and hemorrhages with a high level of iso-butyrate. Fermentation into butyric acid and/or iso-butyric acid seemed to be a prerequisite to the pathologic change. Moreover, in a previous study, the protective role of bifidobacteria through a decrease in clostridia level was associated with a dramatic decrease or a disappearance in butyric acid (15). The presence of iso-butyric acid showed also that these clostridia species were implicated in proteolysis, the end products of bacterial protein degradation known to be implicated in the intraluminal pathogenesis of NEC (38).

Last, the fermentation ability is likely to predominate over the characteristic of the strain itself. In fact, Carbonaro *et al.* (32) suggested that the increased ability for lactose fermentation of a *K. pneumoniae* strain and the ensuing production of SCFAs may be a factor in the onset of NEC. Such a peculiar strain was isolated from an infant with NEC, but other investigators never found a difference in β-galactosidase activity in Gram-negative bacteria that were isolated from 23 neonates with NEC (39).

Anaerobic research in fecal specimen of premature neonates, when performed, is frequently limited to *C. difficile*. The present microbiologic results, strengthened by the recently described outbreak of NEC associated with *C. neonatale* (8) and the relationship observed between early colonization by *C. perfringens* and NEC (12), call for the development of clostridia detection in stools of infants who are suspected of having NEC. This finding is in accordance with the conclusions of Duffy *et al.* (40) that *Clostridium* species were the most common bacterial pathogen associated with NEC in their study ($p < 0.05$). Thus, endogenous digestive flora, even in the absence of any known pathogenic factor, plays an ambiguous role toward the host. Excessive concentration of bacterial metabolites released during lactose fermentation—a consequence of the immaturity of the lactasis equipment—may have tremendous effects on mucosal integrity. The SCFA detection in feces might help notice a change in colonic microflora leading to the onset of the disease.

Table 5. *Relationship between* iso*butyric and butyric acids concentrations and cecal lesions in monobiotic quails*

Bacterial strains	SCFA concentrations (μmol/g, mean $\pm$ SEM)		Type of cecal lesions		
	*iso*butyric acid	butyric acid	Thickening	Hemorrhages	Pneumatosis
Non-lesion inducing strains					
K pneumoniae	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1	0%	0%	0%
C difficile	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2	0%	0%	0%
NEC-like lesions inducing strains					
C perfringens	1.9 $\pm$ 0.5*	1.2 $\pm$ 0.3†	37.5%	25%	25%
C paraputrificum	3.5 $\pm$ 0.7*	1.0 $\pm$ 0.3†	8%	58%	8%
C butyricum MPP 195	nd‡	1.0 $\pm$ 0.5†	25%	42%	50%
C butyricum CB 1002-5	0.3 $\pm$ 0.1	1.2 $\pm$ 0.4†	42%	20%	60%

* Significantly higher than *K pneumoniae*, *C difficile* and both *C butyricum* groups ($p < 0.05$).
† Significantly higher than *K pneumoniae* and *C difficile* groups ($p < 0.05$).
‡ nd = not detected.

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