BACTERIAL ECOLOGY AND PATHOGENICITY: MODELS OF INFECTION

Clostridial pathogenicity in experimental necrotising enterocolitis in gnotobiotic quails and protective role of bifidobacteria

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The pathogenesis of neonatal necrotising enterocolitis (NEC) remains unclear. Gnotobiotic quails fed a lactose diet have been used to investigate the role of clostridial strains originating from faecal specimens of neonates through the intestinal lesions, the changes in microflora balance and the production of bacterial metabolites, i.e., short-chain fatty acids and hydrogen. Bifidobacteria are thought to exert various beneficial effects on host health, including interaction with the colonic microflora. Therefore, it was hypothesised that a protective role could be exercised through bifidobacterial colonisation. A *Clostridium butyricum* strain (CB 155-3) and a whole faecal flora including three clostridial species (*C. butyricum*, *C. perfringens*, *C. difficile*), each from premature infants suffering from NEC, caused caecal lesions in quails similar to those observed in man, i.e., thickening of the caecal wall with gas cysts, haemorrhagic ulceration and necrotic areas. Conversely, a whole faecal flora including bifidobacteria (identified as *Bifidobacterium pseudo-catenulatum*) and no clostridia, isolated from a healthy premature infant, was unable to produce NEC-like lesions. When the two clostridial groups were associated with a *Bifidobacterium* strain (*B. infantis-longum*, CUETM 89-215, isolated from a healthy infant), bifidobacterial colonisation suppressed all pathological lesions. This study is the first demonstration of a protective role for bifidobacteria against NEC via the inhibition of growth of *C. butyricum* or the disappearance of *C. perfringens*. *C. difficile* was not found to be responsible for the aetiology of the caecal lesions in quails. The main effect of bifidobacteria on lactose fermentation was either a dramatic decrease or a disappearance of butyric acid. The protective role was not associated with changes in H₂ production. Therefore, a new step between colonic colonisation and its relevance to NEC is thought to involve the fermentation of unabsorbed lactose into butyric acid at the onset of the disease.

Introduction

Bifidobacteria are dominant in the faeces of full term neonates fed breast milk and this feature is associated with a low colonisation by Enterobacteriaceae, enterococci and anaerobic bacteria such as members of genera *Bacteroides* and *Clostridium* [1–4]. Various bifidobacteria are reported to have an inhibitory effect on different bacterial pathogens both *in vitro* [5, 6] and

in a rat model [7] involving bifidobacteria and *C. perfringens*, *Escherichia coli* and, to a lesser extent, several other enteropathogens. Moreover, bifidobacteria are considered to exert various biological activities related to host health [7].

Colonisation by bifidobacteria is delayed in premature infants as compared with full-term infants [8–10]. Necrotising enterocolitis (NEC), which predominantly affects premature infants, is a very severe illness characterised by abdominal distension, gastrointestinal bleeding, mucosal ulcerations and intestinal pneumatosis [11]. The pathogenesis remains unclear and several factors are involved, including injury to the

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intestinal mucosa, enteral feeding and colonisation by enteric bacteria [11]. The bacteria most often associated with the disease are *Klebsiella* spp. [12], *E. coli* [13] and several clostridia [14–18]. Among clostridia, the most commonly isolated species are *C. butyricum* [19–22] and *C. perfringens* [14, 16, 17].

Studies in gnotobiotic animals have helped to define a role for *C. butyricum* in the onset of NEC. Gnotobiotic chickens [23] and quails [24] colonised with *C. butyricum* strains displayed NEC-like lesions in the caeca when fed a lactose diet. Like premature babies, birds exhibit a low endogenous lactase activity and their caeca present a physiological stasis favouring overgrowth with lactose-fermenting bacteria.

Enhanced colonic bacterial fermentation and its possible relevance to NEC have been reviewed [25–27]. According to Clarks *et al.* [27] the rapid onset of lactose fermentation and the immediate production of organic acids may be responsible for the onset of NEC. Hydrogen (H₂)-producing bacteria are responsible for H₂ found in the NEC gas cysts [28]. Among bacterial metabolites, short-chain fatty acids (SCFAs) excretion reflects intracolonic fermentation of carbohydrates and proteins which are involved in different aspects of colonic functions and interfere with intraluminal events, absorption and mucosal metabolism [29].

NEC is 6-to-10 times less common in babies fed only breast milk than in those fed only formula, which suggests a protective role for breast milk [30]. Fresh human milk has been shown to promote the growth of bifidobacteria [1, 3, 9]. As these bacteria are known to change the proportion of organic acids such as acetic and lactic acids [5], it was hypothesised that the protective role can be exerted through bifidobacterial colonisation.

The aim of the study was to use whole faecal flora originating from premature human infants to determine whether clostridia were involved in NEC and if colonisation with bifidobacteria results in a reduction of the prevalence or severity of the disease, or both. This was investigated in gnotobiotic quails by studying the incidence of intestinal lesions, the changes in the

microflora balance and the production of bacterial metabolites.

Materials and methods

Bacterial strains and flora

Three different microflora were investigated: one *C. butyricum* strain (strain CB155-3 isolated from a premature human infant suffering from NEC and used in a previous study [24]) and two faecal flora which came from premature dizygotic twins, born at a gestational age of 33 weeks at the Centre of Pédiatrie Gatien de Clocheville, France. No antibiotic treatment was given at birth and enteral feeding started at day 2 with breast milk. One twin had no digestive problem whereas the other developed NEC a few hours after stool collection with bloody and mucous stools, so that the corresponding faecal flora were referred to as normal flora and NEC flora, respectively. Stools were collected on day 4 in glycerol-brain-heart broth in an anaerobic bag (Anaerocult Merck) and immediately frozen (–80°C). The quantitative studies of the aerobic and anaerobic faecal flora were performed by the techniques described below. *Bifidobacterium infantis-longum* (abbreviated as *B.inf.*; strain CUETM 89-215, collection de l'Unité d'Ecotoxicologie Médicale, INRA, Villeneuve d'Ascq, France) was isolated from the stool of a healthy premature infant.

Maintenance and inoculation of quails

Germ-free quails (*Coturnix, coturnix* subsp. *japonica*) were obtained by the method of Reynier and Sackteder [31] with the modifications described by Bousseboua *et al.* [24]. Six groups of 2-week-old germ-free quails were transferred to the various experimental isolators and colonised with known flora (gnotobiotic quails, Table 1). Group A was associated with *C. butyricum* CB155-3 alone (monobiotic quails) and group B was associated with the NEC flora (polybiotic quails). Group C was associated with the normal flora (polybiotic quails). To investigate the influence of *B. infantis-longum*, two other groups were associated with either *C. butyricum* plus *B. infantis-longum* (group A + *B. inf.*, dibiotic quails) or the NEC flora plus *B. infantis-longum* (group B + *B. inf.*,

Table 1. Characteristics of six groups of germ-free quails inoculated with various flora

Group of quails	Number of quails	Inoculum		Status of gnotobiotic quails
		Infant flora	Strain added	
A	11	<i>C. butyricum</i> *	...	Monobiotic
B	9	NEC flora	...	Polybiotic
C	8	Normal flora	...	Polybiotic
A + <i>B. infantis-longum</i>	11	<i>C. butyricum</i> *	<i>B. infantis-longum</i> †	Dibiotic
B + <i>B. infantis-longum</i>	8	NEC flora	<i>B. infantis-longum</i> †	Polybiotic
C + <i>C. butyricum</i>	12	Normal flora	<i>C. butyricum</i> *	Polybiotic

*Strain CB 155-3.

†Strain CUETM 89-215.

polybiotic quails). To investigate the influence of clostridia, one group was associated with the normal flora plus *C. butyricum* CB 155-3 (group C + *C. but.*, polybiotic quails). The germ-free status of quails was checked before each experiment. Quails were inoculated orally with cultures containing 10^8 viable cells of *C. butyricum* or *B. infantis-longum*/ml or a 10-fold dilution of the faecal flora (volume 100 μ l), or both. Bacterial establishment was checked in fresh droppings at weekly intervals.

Diets

Quails were fed an experimental semi-synthetic diet [24] containing lactose (6–8% w/w) to mimic the proportion of lactose in milk. The diet was sterilised by γ -irradiation at 40 kGy.

Sampling, bacterial counts, and biochemical determinations

At the end of the experiments, the quails were transferred to a respiratory chamber and hydrogen production was measured with a Quintron apparatus [32]. Quails were then killed at between 2 and 3 weeks for group A and on day 21 for the other groups. Caeca were collected immediately after death and the contents were removed for pH measurement, and bacteriological and biochemical determinations. The weight of the caecal wall was expressed as the caecal wall weight/body weight ratio. Caeca were examined macroscopically and categorised as normal (N), thickening (T), pneumatosis (P) and haemorrhagic contents (H). As there is a good correlation between macroscopic and microscopic observations [24], histological aspects were checked on only two healthy animals and half of the sick animals. Samples were fixed in a Bouin solution, embedded in paraffin, serially sectioned and stained with haematoxylin and eosin.

The caecal contents were weighed and homogenised in a peptone liquid medium and repeatedly diluted in 100-fold steps from 10^2 to 10^6 for bacterial counts. The dilutions were spread on several agar media with the Spiral System (AES Laboratoire, F-35270 Combourg, France). The count threshold was 10^4 cfu/g of fresh faeces. When counts of clostridia were below this threshold, undiluted samples were examined and counts listed as <4.0 . Aerobic cultures were performed on Drigalski agar (Diagnostics Pasteur) for enterobacteria. Enterococcosel (bioMérieux) for enterococci and Chapman 110 Agar (Merck) for staphylococci; all were incubated at 37°C. For anaerobic bacteria, the dilutions were plated on different media, i.e., cysteine (160 mg/L) Columbia agar base supplemented with sheep blood 5% and neomycin 100 mg/L for total anaerobes; whole milk 5%, colistin 10 mg/L and neutral red 40 mg/L for clostridia [33]; *C. difficile* supplement (bioMérieux) for *C. difficile*, and trimethoprim 16 mg/L. D-cycloserine 10 mg/L and

polymyxin B sulphate 20 mg/L for *C. butyricum* [34]. For bifidobacteria, Wilkins Chalgren agar base containing D-glucose 10 g/L, kanamycin 7.5 mg/L, L-cysteine 0.5 g/L and Tween 80 0.5% v/v was used. Plates were incubated at 37°C in anaerobic jars in an anaerobic gas phase ($H_2:CO_2$, 95:5 v/v). Aerobic cultures were examined on days 1 and 2 and anaerobic ones on days 2 and 5. Anaerobic bacteria were identified according to Holdeman *et al.* [35]. Bacterial numbers were expressed as the $\log_{10}$ cfu/g of faeces or caecal content.

SCFA concentrations were determined by gas chromatography [36]. A saturated mercuric chloride solution (10% v/v) was added to the caecal content samples which were then frozen. Before the analysis, samples were thawed and centrifuged and the supernates were deproteinised with phosphotungstic acid (saturated solution 0.4 ml/g of contents) for 16 h at 0°C.

Statistical analysis

Data presented as the mean and SD were evaluated statistically by Student's *t* test.

Results

Composition of the two newborn faecal flora and their establishment in quails

Differences were apparent between the normal flora inoculum for group C and the NEC flora inoculum for group B (Table 2). Both contained enterococci and *Proteus mirabilis*, but *E. coli* was present only in the normal flora. The major differences observed were the occurrence in the NEC flora of three different *Clostridium* species: *C. butyricum* at high level and *C. difficile* and *C. perfringens* at low levels. In the normal flora, clostridia were not detected and a high level of bifidobacteria was found. Bifidobacteria were identified as *B. catenulatum* or *B. pseudocatenulatum* on the basis of phenotypic characters (biochemical and enzymatic characters; bioMérieux API, F-69280 Marcy l'Etoile, France) with the numerical identification system developed by F. Gavini (personal communication) and further listed as *B. pseudo-catenulatum*.

The implantation of the inoculum was checked in the faeces of the quails on day 3 after inoculation. The faecal flora showed some modifications compared with the inoculum (data not shown). In group B, *C. butyricum*, the dominant *Clostridium* sp. in the faeces from the premature sick twin, was no longer detected 1 week after inoculation, although *C. perfringens* became established at a relatively high level ($\log_{10}$ 5.0–7.0) in the quails, by 2 weeks after inoculation. In group C, *P. mirabilis* and enterococci which were at low levels in the healthy twin faeces were established at high levels (*c.* $\log_{10}$ 8.0 and 7.0, respectively)

Table 2. Influence of bifidobacteria on bacterial counts in the inoculum and in the caecal content of germ-free quails inoculated with various flora

Groups of quails (n)	Bifid. status	Outcome	Number of		Mean (SD) log ₁₀ bacterial counts*															
					Bifid.				C. but.				C. perf.				C. diff.			
			quails	analyses	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C
A (11)	-	healthy	3	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
		sick	8	6	...	...	...	...	9.1 (0.2)	7.4** (0.5)	...	...	...	...	...	...	...	...	...	...
B (9)	-	healthy	3	3	...	...	...	...	8.4	<4	<4	7.1 (0.5)	4.1	8.6	9.8 (0.2)	...	...	...	5.8	8.7 (0.1)
		sick	6	4	...	...	...	...	<4	7.2 (0.1)	...	...	...	...	10.0 (0.1)	...	...	...	...	8.5 (0.2)
C (8)	+ [†]	healthy	8	6	8.1	8.9 (0.5)	...	...	...	...	...	...	...	4.2	7.1 (0.2)	10.1	<4	7.8 (0.3)	...	...
		healthy	11	11	...	9.2 (0.2)	...	...	6.1 (0.1)	...	...	...	...	...	...	...	...	...	...	...
A + B. inf. (11)	+ [‡]	healthy	8	4	...	8.6 (0.0)	...	...	<4	...	...	...	...	5.4 (0.6)	8.6 (0.4)	...	...	...	8.1 (0.5)	...

Bifid., *Bifidobacterium*; C. but., *C. butyricum*; C. perf., *C. perfringens*; C. diff., *C. difficile*; Entero., *Enterococcus*; E. coli, *Escherichia coli*; P. mir., *Proteus mirabilis*.

I, inoculum; C, caecal contents.

*I, cfu/ml; C, cfu/g.

[†]*B. pseudo-catenulatum* initially present.

[‡]*B. infantis-longum* CUETM 89-215 added.

[§]*C. butyricum* CB 155-3.

^{||}Similar data were obtained with group C + *C. butyricum*.

**p < 0.05 versus healthy quails with the same bacteriological status.

^{††}p < 0.01 versus healthy quails with the same bacteriological status.

whereas a decrease was observed in *E. coli* counts (from $\log_{10}$ 10.1 to *c.* 8.0). Other bacteria isolated from both twin faeces were found at a similar level in quails of groups B and C.

In-vitro SCFA formation by *Clostridium* spp.

The SCFA profile comprised acetic, butyric and propionic acids (the latter in small amounts) for *C. perfringens*; acetic and butyric acids for the isolates of *C. butyricum*; acetic, butyric, isocaproic, and, in small amounts, propionic, isobutyric, isovaleric and valeric acids for *C. difficile*. For the *Bifidobacterium* strains, only acetic acid was detected.

Caecal changes in quails inoculated with the non-bifidobacterial flora (groups A and B)

Caecal bacterial counts. In group A, *C. butyricum* was established at a high level (Table 2). A significant decrease in this population was observed when lesions were found as compared with healthy animals. In group B, *C. butyricum* was not detected and *C. perfringens* appeared at the same level in both healthy and sick animals (*c.* $\log_{10}$ 7.0). The only difference was the significant increase in the *C. difficile* counts in quails displaying intestinal lesions ($\log_{10}$ 6.4 versus 4.6).

Occurrence of caecal lesions. In group A, caecal lesions were observed in eight of the 11 quails (Table 3). The caecal wall weight was significantly enhanced in the eight sick animals as compared with the three healthy ones. In group B, caecal lesions were observed in six of the nine quails and the occurrence of lesions led to a significant increase in the caecal wall weight. Moreover, the caecal wall in polybiotic quails appeared significantly thicker than in monobiotic quails, both in healthy and sick animals. Thickening was associated with haemorrhagic contents in six cases and pneumatosis in five cases.

Bacterial fermentation metabolites. Total caecal SCFA concentrations were about six times higher in group B than in group A, but in both groups, the caecal lesions were not associated with a modification in the concentrations (Table 4). Acetic, butyric and isobutyric acids were the three SCFAs detected in group A,

whereas the profiles were more complex in group B, as propionate, valerate and isovalerate and caproate were also detected.

Caecal pH was not different in groups A and B in the healthy quails. Caecal lesions in group A were associated with a pH increase in comparison to group B.

Caecal changes in quails inoculated with Bifidobacterium-containing flora

Caecal bacterial counts. The *Bifidobacterium* strain was established at a high level in quails (*c.* $\log_{10}$ 9.0) when it was added in groups A and B or initially present in group C (Table 2). The colonisation with bifidobacteria led to a fall of *C. butyricum* numbers in quails of groups A + *B. inf.* and of *C. perfringens* in quails of group B + *B. inf.* *C. difficile* level did not significantly differ between groups B and B + *B. inf.* when compared to either healthy or sick animals. The other population levels (enterobacteria, enterococci) were not modified.

In group C + *C. but.* the levels of the strains of group C were the same as was found in group C, and *C. butyricum* failed to colonise the quails (data not shown in the table).

Occurrence of caecal lesions. No caecal lesions were detected 21 days after inoculation whatever the group studied (Table 3). The caecal wall weights did not significantly differ between the four groups (mean value = 3.5).

Bacterial fermentation metabolites. With the exception of sick animals of group A, there were no differences in caecal pH between groups with and without bifidobacteria (Table 4). However, total SCFA concentrations and profiles were strongly affected. With group A + *B. inf.*, the total SCFA concentration doubled as compared with group A, but only acetic acid was detected. With group B + *B. inf.*, the total SCFAs concentration was decreased by a factor of 2 compared with group B and proportions of butyric acid and SCFAs of protein origin decreased. Consequently, in

Table 3. Influence of bifidobacteria on caecal changes in germ-free quails inoculated with various flora

Group of quails (n)	<i>Bifid.</i> status	Outcome	Number of quails	Mean (SEM) caecal wall wt/body wt (10^3)	Number of quails with macroscopic observation							
					N	T	H	P	T+H	T+P	P+H	T+P+H
A (11)	—	healthy	3	2.97 (0.09)	3							
		sick	8	4.05 (0.18)*	0	2	0	0	4	2	0	0
B (9)	—	healthy	3	3.88 (0.11)	3							
		sick	6	4.99 (0.42)*	0	0	2	1	0	0	1	2
C (8)	+†	healthy	8	3.61 (0.18)	8							
A + <i>B. inf.</i> (11)	+‡	healthy	11	3.24 (0.16)	11							
B + <i>B. inf.</i> (8)	+‡	healthy	8	3.65 (0.28)	8							

N, normal; T, thickening; H, haemorrhagic; P, pneumatosis.

**p* < 0.05 versus healthy quails with the same bacteriological status.

†,‡ See footnote to Table 2.

Table 4. Influence of bifidobacteria on caecal pH and SCFA concentrations in germ-free quails inoculated with various flora

Group of quails (n)	Bifid. status	Outcome	Number of quails	Mean (SEM) pH value	SCFA concentration		SCFA molar ratio (%)						
					number analysed	mean (SEM) $\mu\text{mol/g}$ (wet wt)	ac	prop	isobut.	but.	isoval.	val.	cap.
A (11)	-	healthy	3	6.2 (0.2)	3	5.8 (0.0)	72.2	ND	6.9	20.9	ND	ND	ND
		sick	8	6.8 (0.1)	6	4.6 (0.9)	79.9	ND	4.5	15.6	ND	ND	ND
B (9)	-	healthy	3	6.2 (0.1)	2	28.3–33.1*	74.3	2.9	10.9	5.2	3.2	2.7	0.8
		sick	6	6.0 (0.2)	4	30.1 (5.3)	81.3	2.3	7.7	4.4	1.9	1.9	0.5
C (8)	+	healthy	8	6.2 (0.2)	6	19.7 (2.4)	93.7	1.0	2.7	0.8	1.0	0.4	0.4
		healthy	11	6.0 (0.2)	11	9.6 (1.2)	100	ND	ND	ND	ND	ND	ND
A + B.inf. (11)	+	healthy	8	5.7 (0.2)	5	16.4 (6.3)	87.3	1.4	6.4	1.6	2.6	0.5	0.2
B + B.inf. (8)	+	healthy											

ac, acetate, prop., propionate; isobut., isobutyrate; but., butyrate; isoval., isovalerate; cap., caproate. ND, not detected.

*Individual values.

†, ‡ See footnote to Table 2.

both cases, *B. infantis-longum* had a more important role in bacterial metabolism than in acidification.

With group C, the caecal pH and the SCFA concentration were close to the data obtained with group B + *B. inf.* The main differences concerned the SCFA profile, with a higher proportion of acetic acid (93.7%) and very low proportions of SCFAs originating from proteolytic or aminoacidolytic activities, or both.

Influence of bifidobacteria on H₂ excretion and butyric acid formation

Hydrogen excretion was seven times higher in group A than in group B (Table 5). No H₂ was detected in group A + *B. inf.*, whereas it was unchanged in group B + *B. inf.* Concerning the SCFA profiles, the relative proportion of butyric acid was four times higher in group A than in group B, but the concentration of this acid was not different between these two groups. In the presence of bifidobacteria, butyric acid disappeared in group A + *B. inf.* and decreased at least by a factor 8 in group B + *B. inf.* in both percentage and concentration. In group C, butyric acid concentration was similar to that of group B + *B. inf.*

Discussion

These results confirm and extend the validity of the experimental model previously developed for *C. butyricum* with monobiotic quails [24]. A whole flora including three *Clostridium* spp. isolated from an NEC patient led to NEC-like caecal lesions in gnotobiotic quails similar to those observed in human infants. Conversely, a whole flora including bifidobacteria isolated from a healthy premature neonate was not able to produce NEC-like lesions in this animal model. Such a gnotobiotic model is very useful for a better understanding of the disease, whose exact pathogenesis remains unknown. It allows the preservation of the initial flora composition for long-term studies. It is reproducible, easy to perform and it rapidly produces NEC lesions without invasive methods. The development of lesions in quails requires a combination of several factors present in premature human infants, i.e., (i) a low endogenous intestinal lactase activity, (ii) the presence of lactose in the diet, (iii) the presence of lactose-fermenting anaerobes, and (iv) physiological stasis due to the specificity of the quail digestive tract anatomy. The lesions were quite similar to those observed in human infants suffering from NEC, with different states ranging from haemorrhagic caecitis to peritoneal pneumatosis. Quails display a host-mediated response, as in both the *C. butyricum* group and the complex flora group 28% and 44% of quails, respectively, were free of any caecal lesions with the same high bacterial level. These findings are consistent with those observed in premature infants, as *Clostridium* spp.

Table 5. Influence of bifidobacteria on butyrate concentration and pattern in the caecal content and on in-vivo excretion of hydrogen in germ-free quails inoculated with various flora

Group of quails (n)	<i>Bifid.</i> status	Outcome	Number of quails	Butyrate		Hydrogen mean (SEM) ppm*
				molar ratio (%)	mean (SEM) concentration (μ mol/g)	
A (11)	—	healthy	3	21	1.2 (0.4)	35 (11)
		sick	8	15	0.7 (0.2)	
B (9)	—	healthy	3	5	1.6 (0.1)	5.2 (1.3)
		sick	6	4	1.25 (0.1)	
C (8)	+†	healthy	8	0.8	0.16 (0.03)	...
A + <i>B. inf.</i> (11)	+‡	healthy	11	ND	ND	ND
B + <i>B. inf.</i> (8)	+‡	healthy	8	1.6	0.21 (0.05) [§]	6.8 (1.7)

ND, not detected.

*Six quails from each group examined during a 4-h period in the respiratory chamber.

†,‡See footnote to Table 2.

§*p* < 0.01 versus quails in group B without bifidobacteria.

involved in NEC were found at high levels in stools from either sick or healthy premature infants [19].

Variations in intestinal bacteriology have been associated with a changing incidence of NEC [37]. The analysis of the two stool specimens did not reveal the presence of a great variety of micro-organisms, but their compositions were very different. This agrees with several previous observations that even 2 weeks after birth, the flora of premature infants do not always have the same composition [19] and the colonisation patterns differ from those of normal full-term infants [8, 10], in which a delayed bifidobacterial colonisation was noted [9, 10].

In the newborn infant who developed NEC, three *Clostridium* spp., *C. butyricum*, *C. perfringens* and *C. difficile*, were simultaneously isolated. In pre-term infants, colonisation by clostridia is observed in c. 10% of cases according to Blakey *et al.* [8] and a mixture of clostridia is quite unusual [8, 20, 21, 38]. In a study dealing with isolation of clostridia from 77 faecal samples, Westra-Meijer *et al.* [12] isolated four species from one faecal sample and two species from three samples from another infant.

Modifications were observed in the levels of the different *Clostridium* strains in quail droppings as compared with the original human neonate faecal inoculum. In quails, the counts for *C. difficile* were quite similar and *C. perfringens* colonised quails well, unlike in the neonate, whereas *C. butyricum* failed to establish. Hoy *et al.* [39] demonstrated that the quantitative composition of the premature faecal microflora may vary from day to day and that quantitative changes occurred immediately before the clinical onset of NEC. Therefore, it cannot be concluded that bacterial changes observed in quails would not have appeared in the premature infant.

In this study, a marked protective role *in vivo* by bifidobacteria was demonstrated against the onset of NEC-like lesions. This may be related to the decrease

of the *C. butyricum* population in group A + *B. inf.* and to the disappearance of *C. perfringens* in group B + *B. inf.* without unmodified *C. difficile* counts. Furthermore, *C. butyricum* was unable to colonise quails previously inoculated with the group C flora containing bifidobacteria. An association has been demonstrated previously between a high rate of colonisation by *Clostridium* spp., especially *C. perfringens* and an NEC outbreak [14, 15, 18]. The key role of *C. butyricum* in NEC development has been demonstrated previously with the two alactasic gnotobiotic models [23, 24, 40]. Neonatal colonisation by *C. difficile* is common even in pre-term infants and almost invariably asymptomatic, despite the detection in some cases of cytotoxin in stools [41–43]. Only in rare cases might *C. difficile* be involved in severe enterocolitis in neonates [44] and its possible role in NEC is controversial [45]. Despite the detection of *C. difficile* toxins in five infants with NEC, suggesting a role for this species [15], neither Chang and Areson [46] nor Bartlett *et al.* [47] found *C. difficile* cytotoxin in relation to NEC. From the results of the present study, *C. perfringens* and not *C. difficile* was shown to be responsible for the aetiology of the caecal lesions. The macroscopic examination of the caeca showed differences between sick animals in groups A and B. Haemorrhages, which predominated in group B, may be produced in relation to the action of the haemolytic *C. perfringens* toxins [18].

Bifidobacteria are thought to exert various beneficial effects on the host health including interactions with the colonic microflora. Different hypotheses have been proposed, among them the production of bacterial metabolites that have a direct inhibitory activity [48] or consequently lower the pH, or both [2, 6, 49], and the production of unknown antibacterial substances [5, 50].

Some authors observed an in-vitro inhibition by bifidobacteria of the growth of potential enteropathogens which was related to the production of lactic and acetic acids, thus lowering pH [6, 51]. Nevertheless, the inhibition can be observed even with pH-

neutralised supernatant fluids from *B. infantis* cultures and the most active fractions (obtained after extraction) contained neither acetic acid nor lactic acid [5]. Similarly, in the present study, direct protection by bifidobacteria through lowering pH did not seem to be the mechanism of action *in vivo* as the caecal pH was not significantly modified.

Depending on the bacteriological status of the group, total SCFA amounts ranged from 4 to 30 $\mu\text{mol/g}$ of content and were modified in response to bifidobacterial inoculation, in comparison with results reported by Bartram *et al.* [52], Hove *et al.* [53] and Okhusa *et al.* [54], but in their experiments increases in bifidobacteria were smaller than in this study.

The main effect in the present study was a dramatic decrease or a disappearance of butyric acid. The accumulation of large amounts of butyric acid produced from unabsorbed lactose was thought to be responsible for the increased caecal wall thickening in quails [24]. Then, a new step between colonic fermentation and its relevance to NEC is to involve butyric acid as a main factor in the onset of the disease. Butyric acid is considered to be essential for the integrity of the colonic epithelium [55]. It stimulates the proliferation of the epithelial colonic cells and is the major source of energy for the enterocytes. Nevertheless, it has been reported to be a cytotoxic agent in several isolated cell lines [56]. High concentrations of butyric acid were shown to exert toxic effects in animals leading to atony [57] and necrosis [58–60] of the intestinal epithelium. Butyric acid was related to the cytotoxic factor of *C. butyricum* strains involved in NEC [61]. In full-term babies, the butyric acid concentrations remain very low compared to premature infants [2, 62–65] and the SCFA pattern in breast-fed babies consists mainly of acetic acid. Besides *C. butyricum* and *C. perfringens* which are well known to form butyric acid from lactose, Carbonaro *et al.* [66] suggested that an increased ability for lactose fermentation for a *Klebsiella* strain and the ensuing production of organic acids may be a factor in the onset of NEC.

H₂ has been incriminated in the development of mucosal pneumatosis [67, 68]. In dioxenic animals (group A + *B. inf.*), H₂ was not excreted. In heteroxenic quails in group B, bifidobacteria had no inhibiting effect on H₂ production, which was low compared to production in monoxenic quails. In this case, H₂ production should be attributed to *C. difficile*, as *C. perfringens* counts decreased markedly. Therefore, in the absence of butyric acid, H₂ production has no deleterious effect.

This study is the first demonstration of a clear protective role for bifidobacteria against NEC and it is hypothesised that the inoculation of premature babies with selected *Bifidobacterium* strains could

prevent the development of NEC *via* inhibition of the growth of potentially pathogenic bacteria such as *C. butyricum*, *C. perfringens* and *C. difficile*.

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