MODEL OF INFECTION

Oligofructose contributes to the protective role of bifidobacteria in experimental necrotising enterocolitis in quails

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Bifidobacteria are dominant in the gut of full-term infants, although colonisation by them is often delayed in preterm neonates. Bifidobacteria are recognised to have beneficial effects on digestive disorders and they might prevent neonatal necrotising enterocolitis (NEC), a gastrointestinal disease that predominantly affects premature infants. They have been shown to protect gnotobiotic quails against NEC-like lesions when the birds were inoculated with faecal flora from preterm infants, decreasing the clostridial population. The present study was designed to investigate whether oligofructose, which stimulates the activity of bifidobacteria, may enhance their protective role. Experiments were done in eight groups of germ-free quails for 28 days. The groups differed as to their bacterial status, diet and environment. Quails were inoculated with one of two flora from premature twins. The first flora included *Bifidobacterium pseudo-catenulatum*, *Escherichia coli* and no clostridia. The second flora included clostridial species and was associated with *B. infantis-longum*. Caecal bacterial population and metabolism changes were investigated with a lactose (6%) diet *versus* a lactose-oligofructose (3%-3%) diet, either in a gnotobiotic environment or in an ordinary environment permitting post-colonisation by exogenous bacteria. In both environments and with both flora, oligofructose significantly increased the level of bifidobacteria and this was associated with a decrease of *E. coli* or *C. perfringens* and *C. ramosum*. The bacterial changes in the ordinary environment depended on the initial composition of the microflora and the colonisation resistance against exogenous bacteria was more efficient with the flora that included *B. pseudo-catenulatum*. The changes in caecal pH and short-chain fatty acids were minimal. It was demonstrated that, irrespective of the environmental conditions, the use of oligofructose helped to prevent the overgrowth of bacteria implicated in necrotising enterocolitis in preterm neonates.

Introduction

Necrotising enterocolitis (NEC) is one of the most common serious gastrointestinal diseases in neonatal intensive care units; it causes significant mortality and severe chronic morbidity after resection of necrotic bowel [1]. To date, none of the preventive treatments, i.e., antibiotic therapy, prophylactic treatment and parenteral feeding, is considered fully satisfactory.

In adults, prophylactic treatments that manipulate the autochthonous microflora with non-digestible oligosaccharides as a means of preventing digestive disorders have aroused interest for many years. Among these oligosaccharides, oligofructose (OF) has been demonstrated to be capable of increasing the numbers of *Bifidobacterium* spp. [2, 3] which are considered to be the best candidates for exerting biological activity promoting health [3, 4]. Clinical attempts to establish bowel colonisation with bifidobacteria showed that exogenous bifidobacteria were maintained only when fermented dairy products were consumed [5]. In healthy full-term babies fed breast milk, bifidobacterial colonisation of the gut appears in

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the first days of life and predominates at 2 weeks of age [6–9]. In contrast, this colonisation is often delayed for 2–3 weeks in preterm infants [6–8], thus favouring high levels of *Clostridium* spp., e.g., *C. butyricum*, *C. perfringens* and *C. paraputrificum*, implicated in the aetiology of NEC [10–14]. In a pair of premature twins, it was observed that at 3 days old the stools of one twin contained mainly bifidobacteria, whereas no bifidobacteria but high levels of clostridia, including *C. butyricum* and *C. perfringens*, were found in the second. The neonate harbouring bifidobacteria remained healthy while the other developed NEC [15].

Human breast milk has many specific and non-specific components thought to protect against potentially pathogenic bacteria. Among them, the oligosaccharide fraction is considered to be beneficial in full-term infants [16, 17], but has never been investigated in preterm neonates.

In order to study the pathogenesis and the prevention of NEC, gnotobiotic alactasic models, i.e., chicken and quail species which do not possess intestinal lactase, were developed in which caecitis associated with *Clostridium* spp. occurred [18–20]. The association of germ-free quails with the whole faecal flora from the premature twins demonstrated that bifidobacteria when initially present in the faecal flora or administered early prevented caecitis through the inhibition of clostridial species [15].

The aim of the present study was to investigate, with the gnotobiotic model, whether OF plays a protective role additional to that of bifidobacteria in a gnotobiotic environment and in an ordinary environment permitting post-colonisation by exogenous bacteria, as gut colonisation varies in preterm babies in neonatal intensive care units.

Materials and methods

Faecal flora from premature twin neonates

The two faecal flora came from premature dizygotic twins born at a gestational age of 33 weeks at the Centre de Pédiatrie Gatien de Clocheville, Tours. No antibiotic treatment was given at birth and enteral feeding started on day 2 with breast milk. One twin remained healthy, the other twin developed NEC; stool samples were collected from both twins a few hours before the onset of the disease. Both flora contained enterococci ($\log_{10}$ 3.7 and 8.6/g of faeces, respectively) as described previously [15]. In the flora of the healthy twin, *E. coli* was present ($\log_{10}$ 10.2/g), clostridia were not detected and a high level of bifidobacteria was found ($\log_{10}$ 8.9/g). The bifidobacteria were identified as either *B. catenulatum* or *B. pseudo-catenulatum* on the basis of phenotypic characters (biochemical and enzymic characters;

bioMérieux API, F-69280 Marcy l'Etoile, France). The numerical identification system developed by F. Gavini (personal communication) identified the isolates as *B. pseudo-catenulatum*. In the flora of the sick twin, the major differences observed with the healthy flora were the presence of two clostridial species – *C. difficile* ($\log_{10}$ 5.4/g) and *C. perfringens* ($\log_{10}$ 3.8/g). *E. coli* was not present, although *Proteus mirabilis* was detected ($\log_{10}$ 8.0/g). For the purpose of the study, this flora was associated with a *B. infantis-longum* strain isolated from the stool of a healthy premature infant (strain CUETM 89-215, collection of the Unité d'Ecotoxicologie Médicale, INRA, Ville-neuve d'Ascq).

The two flora were referred to as healthy flora and NEC-*bif* flora, respectively.

Diets

Two experimental semi-synthetic diets were compared in quails. One diet (already described [19]) contained lactose 6% w/w to mimic the proportion of lactose in milk. The other was a similar diet where half the lactose (3% w/w) was replaced by OF 3% w/w. OF was the oligosaccharide fraction of Raftilose (Orafti, Tienen, Belgium). It is composed of molecules of GF_n and Fn type (G, glucose; F, fructose; n, number of fructose moieties linked by β [2, 1] linkages in a ratio of c. 2:1), with n being 2–6, with an average polymerisation degree of 4. The diet was sterilised by gamma irradiation at 40 kGy. Diets were referred to as lactose diet and lactose/OF diet, respectively.

Experimental design

Maintenance and inoculation of quails. Germ-free quails (*Coturnix coturnix* subsp. *japonica*) were obtained by the method of Reynier and Sackteder [21] with the modifications described by Bousseboua *et al.* [19]. Two-week-old germ-free quails fed a non-lactose diet were transferred to various experimental germ-free isolators. The germ-free status of quails was checked before bacterial inoculation. Quails were inoculated orally with 100 μ l of cultures containing 10^8 viable cells of the bifidobacterial strain or a 10-fold dilution of the faecal flora, or both. Bacterial establishment was checked by examining fresh droppings at weekly intervals.

Experiment 1. Germ-free quails were separated into four isolators that provide absolute control of air-borne infection and prevent contact from exogenous organisms. The experimental groups consisted of gnotobiotic quails inoculated with either the healthy flora (group I) or the NEC-*Bif* flora (group II). Quails in each group were divided into two subgroups and fed with either the lactose diet (group I_L, 11 quails and group II_L, 8 quails) or the lactose/OF diet (groups I_{OF}, 11 quails and II_{OF}, 9 quails) (Table 1).

Table 1. Characteristics of groups of germ-free quails inoculated with healthy flora or NEC-*Bif* flora

Groups of quails	Number of quails	Diet	Inoculum	Environmental status
I _L	11	Lactose	Healthy flora*	Gnotobiotic
I _{OF}	11	Lactose/OF	Healthy flora*	Gnotobiotic
II _L	8	Lactose	NEC- <i>Bif</i> flora†	Gnotobiotic
II _{OF}	9	Lactose/OF	NEC- <i>Bif</i> flora	Gnotobiotic
III _L	12	Lactose	Healthy flora*	Ordinary‡
III _{OF}	12	Lactose/OF	Healthy flora*	Ordinary‡
IV _L	9	Lactose	NEC- <i>Bif</i> flora	Ordinary‡
IV _{OF}	8	Lactose/OF	NEC- <i>Bif</i> flora†	Ordinary‡

**B. pseudo-catenulatum* (bifidobacterium resident).

†*B. infantis-longum* CUETM 89-215 (bifidobacterium added).

‡Air filters of the isolators were removed to enable further infection by bacteria from the external environment.

Experiment 2. As in experiment 1, quails were separated into four isolators and inoculated with the healthy flora (group III) and the NEC-*Bif* flora (group IV). One week after inoculation the establishment of bacteria in their gut was checked. The air filters of the isolators were then removed to enable further infection by bacteria from the external environment. This was defined as ordinary environment. Then, quails of each group were divided into two subgroups and fed either the lactose diet (group III_L, 12 quails and group IV_L, 9 quails) or the lactose/OF diet (group III_{OF}, 12 quails and group IV_{OF}, 8 quails) (Table 1).

Sampling, bacterial counts and biochemical determinations

The quails were killed 28 days after inoculation. Caeca were collected immediately after death and the contents were removed for pH measurement, bacteriological and biochemical determinations. The weight of the caecal wall was expressed as the caecal wall weight to body weight ratio. Caeca were characterised macroscopically as normal (N), thickened (T), showing pneumatosis (P) and having haemorrhagic contents (H). Quails were considered as healthy when none of the three last criteria was observed.

Aerobic and anaerobic bacterial species were counted as described previously. Briefly, serial dilutions of the caecal contents were spread on various media allowing the enumeration of bifidobacteria, clostridia, *Bacteroides* spp., Enterobacteriaceae, enterococci and staphylococci [15]. Isolates were identified by standard laboratory methods. Bacterial counts were expressed as log₁₀ numbers (cfu/g of caecal content). The count threshold was 10³ cfu/g caecal content. Short-chain fatty acid (SCFA) concentrations were determined by gas chromatography as described previously [22].

Statistical analysis

Data are presented as the mean and SEM. Statistical significance was considered at $p < 0.05$. Analysis of variance followed by Fisher's test was used when comparing the two diets (lactose *versus* lactose/OF).

Results

None of the quails was sick in the presence of bifidobacteria irrespective of the presence or absence of OF and the two different environmental conditions.

Experiment 1: influence of diet in the gnotobiotic environment

Compared with the lactose diet, the lactose/OF diet significantly increased the bifidobacterial numbers whatever the origin of the strain (resident in the healthy flora or added in the NEC-*Bif* flora) (Table 2). In the healthy flora (groups I_L and I_{OF}) there was a significant decrease in the numbers of *E. coli*. In the NEC-*Bif* flora (groups II_L and II_{OF}) *C. perfringens* was no longer detected, the *C. difficile* count did not alter and the number of *P. mirabilis* decreased slightly. The numbers of enterococci were not modified in either flora.

The mean caecal wall weight to body weight ratio ranged from 3.7 to 4.2 whatever the flora and the diet (Table 3). In the healthy flora the introduction of OF in the diet led to a slight pH increase, but the SCFA concentrations were unaltered. In the NEC flora, pH was unchanged; SCFA concentrations decreased, but not significantly. For both flora, SCFA profiles mainly comprised acetate and the introduction of OF increased the proportion of *isobutyrate*, *butyrate*, *isovalerate* and *valerate*.

Experiment 2: influence of the diet in the ordinary environment

The lactose/OF diet significantly increased the bifidobacterial numbers in both groups. With the healthy flora the other bacterial numbers were not modified. In the NEC-*bif* flora OF significantly decreased the number of *E. coli*, *C. perfringens* and *C. ramosum* (Table 2). *C. difficile* decreased, but not significantly.

OF did not affect the caecal wall weight to body weight ratio, pH or SCFA concentrations, although slight modifications of butyrate ratio occurred with both flora (Table 3).

Table 2. Influence of OF on bacterial counts in the caecal contents of germ-free quails inoculated with healthy flora or with NEC-*Bif* flora

Groups of quails	Environmental status	Mean (SEM) log ₁₀ bacterial counts* (n = 6)							
		<i>B. cat</i>	<i>B. inf.</i>	<i>C. perf.</i>	<i>C. diff.</i>	<i>C. ram.</i>	<i>Entero.</i>	<i>E. coli</i>	<i>P. mir.</i>
I _L	Gnotobiotic	6.9 (0.2)	...	...	...	...	3.7 (0.2)	10.2 (0.2)	...
I _{OF}	Gnotobiotic	7.9 (0.3) [†]	...	...	...	...	3.3 (0.1)	8.4 (0.4) [†]	...
II _L	Gnotobiotic	...	8.6 (0.2)	3.8 (0.3)	5.4 (0.5)	...	8.6 (0.3)	...	8.0 (0.5)
II _{OF}	Gnotobiotic	...	9.2 (0.1) [†]	<3.0 [†]	5.2 (0.4)	...	9.0 (0.1)	...	7.1 (0.5)
III _L	Ordinary	7.8 (0.2)	...	...	...	...	8.5 (0.3) [‡]	8.3 (0.2)	8.0 (0.3)
III _{OF}	Ordinary	10.4 (0.2) ^{†‡}	...	...	...	...	8.5 (0.1) [‡]	8.1 (0.2)	8.3 (0.2)
IV _L	Ordinary	...	8.7 (0.3)	5.1 (0.5) [‡]	4.7 (0.4)	6.0 (0.3)	8.0 (0.2)	8.7 (0.0)	...
IV _{OF}	Ordinary	...	9.3 (0.1) [†]	3.7 (0.4) [‡]	3.9 (0.6) [†]	3.6 (0.4) [†]	8.3 (0.1)	7.8 (0.1) [†]	...

B. cat., *B. pseudo-catenulatum*; *B. inf.*, *B. infantis-longum* CUEM 89-215; *C. perf.*, *C. perfringens*; *C. diff.*, *C. difficile*; *C. ram.*, *C. ramosum*; *Entero.*, *Enterococcus*; *E. coli*, *E. coli*; *P. mir.*, *P. mirabilis*.

*cfu/g.

[†]p < 0.05 versus lactose diet for the same bacterial status and environment.

[‡]p < 0.05 versus gnotobiotic environment for the same bacterial status and diet.

Table 3. Influence of OF on caecal changes in germ-free quails inoculated with healthy flora or with NEC-*Bif* flora

Group of quails	Environmental status	pH value mean (SEM)	wt/body wt (10 ³) mean (SEM)	SCFA concentration: mean (SEM)		SCFA molar ratio (%)							
				caecal wall	μmol/g (wet wt)	ac.	prop.	isobut.	but.	isoval.	val.	isocap.	cap.
I _L	Gnotobiotic	5.5 (0.1)	4.20 (0.20)	19.7 (2.4)	93.7	1.0	1.0	2.7	0.8	1.0	0.4	ND	0.4
I _{OF}	Gnotobiotic	6.0 (0.1)	3.73 (0.46)	21.2 (3.6)	79.0	2.0	2.0	11.0	2.0	3.0	2.6	ND	0.4
II _L	Gnotobiotic	5.7 (0.2)	3.65 (0.28)	16.4 (6.3)	87.3	1.4	1.4	6.4	1.6	2.6	0.5	ND	0.2
II _{OF}	Gnotobiotic	5.6 (0.2)	4.07 (0.30)	9.1 (1.7)	77.0	2.0	2.0	11.0	4.0	3.0	2.6	ND	0.4
III _L	Ordinary	6.1 (0.2)	3.44 (0.37)	35.5 (4.8)	78.0	2.0	2.0	2.0	15.0	1.0	1.0	ND	1.0
III _{OF}	Ordinary	5.8 (0.2)	4.00 (0.32)	33.7 (7.9)	87.6	1.7	1.7	1.3	7.8	0.3	0.3	ND	1.0
IV _L	Ordinary	5.8 (0.3)	4.07 (0.32)	33.6 (1.0)	88.5	1.5	1.5	1.5	5.0	1.0	1.0	0.5	1.0
IV _{OF}	Ordinary	5.8 (0.1)	4.00 (0.36)	31.9 (2.2)	86.0	1.0	1.0	2.0	10.0	ND	ND	ND	1.0

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

Comparison of the gnotobiotic and ordinary environments

The intestinal microflora was altered qualitatively and quantitatively by the external environment. In the healthy flora, *P. mirabilis* appeared and the number of enterococci increased ($\log_{10}$ 8.5 in group III versus *c.* 3.5 in group I). In the NEC-*Bif* flora, *C. ramosum* and *E. coli* were acquired and *P. mirabilis* disappeared. The increase in the level of *B. pseudo-catenulatum* caused by the addition of OF was significantly greater in the ordinary environment than in the gnotobiotic environment ($+2.6 \log_{10}$ in group III versus $+1 \log_{10}$ in group I). In contrast, with NEC-*Bif* flora no difference was observed in *B. infantis-longum* numbers and *C. perfringens* counts were higher in the ordinary environment.

There were no significant differences in pH and caecal wall weight to body weight ratios. Whatever the diet, total SCFA concentrations were higher in quails kept in the ordinary environment (*c.* 33 $\mu\text{mol/g}$ in groups III and IV versus 9.1–21.2 $\mu\text{mol/g}$ in groups I and II). The proportion of butyrate was increased at the expense of isobutyrate.

Discussion

Clostridium spp. and *E. coli* are known to be responsible for NEC in preterm infants [23–27]. The present study showed how OF may participate in the health-promoting effect of bifidobacteria against NEC-like lesions in quails. In both environments studied, OF increased the numbers of bifidobacteria. With the healthy flora (*B. pseudo-catenulatum*, resident) the increase in bifidobacterial count was associated with a decrease of *E. coli*. In the NEC-*B.inf* flora groups (*B. infantis-longum*, added) the increase in the count of bifidobacteria was associated with strongly decreased numbers of *C. perfringens* and *C. ramosum*. These results were in agreement with both in-vitro [28, 29] and in-vivo [2] studies showing that OF favours the growth of bifidobacteria and inhibits the growth of clostridia and *E. coli*. However, *C. difficile* was unaffected by the increase of bifidobacteria. Its role in NEC is controversial. In rare cases, *C. difficile* toxins have been found in the faeces of infants with NEC [24]; no relationship has been found between the acquisition or the carriage of toxigenic *C. difficile* and intestinal symptoms [30].

The normal acquisition of flora is altered in preterm neonates by the prolonged stay in intensive care units associated with an increased risk of nosocomial infection. The ordinary environment investigated in this study simulated the conditions where infants are exposed to potentially pathogenic bacteria. Qualitative and quantitative changes were observed in the gut colonisation of the quails. With the healthy flora, *P. mirabilis*, usually found in preterm infants, was

acquired from the external environment, but no clostridia were detected. In the NEC-*bif* flora the number of *C. perfringens* was higher than in the gnotobiotic environment and *C. ramosum* was acquired. *C. ramosum* has been linked to various infections in children [31], but was not usually observed in the faecal bacterial population of neonates. This new bacterial composition is close to those found in neonatal intensive care units where Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Proteus* spp.) and enterococci are detected at a high level up to $\log_{10}$ 10/g of stool, clostridia are detected at $\log_{10}$ 5–8/g of stool depending on the species and bifidobacteria at $\log_{10}$ 0–10/g of stool in faecal specimens from healthy preterm neonates [6–9, 25].

The changes in the microflora observed depended on the composition of the initial microflora. The colonisation resistance against clostridial species appeared more efficient with flora containing *B. pseudo-catenulatum* than with flora containing *B. infantis-longum*. The numbers of the two different *Bifidobacterium* spp. did not explain this difference. This effect might be related to the bifidobacterial species; *B. pseudo-catenulatum* occurs at higher level than other bifidobacterial species but at a low frequency in breast-fed infants [9]. On the other hand, in-vitro fermentation experiments suggested that *Bifidobacterium* spp. differ in their responsiveness to protein and oligosaccharide growth promoters [32, 33].

Bifidogenic properties of OF were demonstrated whatever the flora, but their differences depended on the initial composition and were, therefore, more marked with the healthy flora. OF participate in the health promoting effect of bifidobacteria by exerting a beneficial effect on microflora balance, but not on the bacterial metabolites tested. The mechanism by which OF played a health-promoting role is still under discussion and has often been associated with fermentation of OF by bifidobacteria which subsequently led to a pH decrease [28, 29]. In the present study pH values did not differ statistically whatever the microflora and total SCFA concentrations were not linked to the presence of OF in the diet. Only slight changes in caecal SCFA profiles were observed.

Clinical trials of colonisation of the immature bowel of preterm infants with probiotics (*Lactobacillus* GG, *B. breve*) have shown that they need to be administered regularly (once or twice a day) [34–36]. In the present study, the daily intake of OF contributed to long-term colonisation with bifidobacteria at a high level after a single early administration of the probiotic and reduced colonisation by potentially pathogenic bacteria. These findings illustrated the potential application of the concept of synbiotics proposed by Gibson and Roberfroid as the combination of probiotics and prebiotics [3]. As an alternative to antibiotic therapy or to prophylactic treatments, an

early administration of probiotics associated with a nutritional programme including the use of OF could be a promising approach to the prevention of NEC.

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