Oligofructose and experimental model of neonatal necrotising enterocolitis

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The gut of preterm neonates is colonised with a paucity of bacterial species originating more from the environment than from the mother. Furthermore, a delayed colonisation by bifidobacteria promotes colonisation by potentially pathogenic bacteria. This may contribute towards the development of neonatal necrotising enterocolitis (NEC). The physiopathology of NEC is still unclear but immaturity of the gut, enteral feeding and bacterial colonisation are all thought to be involved. None of the current preventive treatments are considered satisfactory. Modulating the autochthonous microflora by probiotics or prebiotics could be a more reliable approach to prevention. Using gnotobiotic quails as an experimental model of NEC we have shown that onset of intestinal lesions requires a combination of low endogenous lactase activity, lactose in diet, and colonisation by lactose-fermenting bacteria such as the clostridia. The protective role of bifidobacteria was demonstrated in this model through a decrease in clostridial populations and in butyric acid. Oligofructose dietary supplementation was shown to enhance this effect with an increase in the bifidobacterial level and consequently a greater decrease in clostridia. However, oligofructose was unable to promote a bifidobacterial acquisition when the microflora was initially deprived of this group. Nevertheless, oligofructose can act as an anti-infective agent and decrease the occurrence or severity of the lesions depending on the bacteria involved. According to these results and to the fact that oligosaccharides are a major component of breast milk, the addition of oligofructose in formula milks may be a nutritional approach to favouring colonisation by a beneficial flora.

Premature infants: Necrotising enterocolitis: Oligosaccharides: Gut flora

Introduction

In recent years, research on infant nutrition has focused on a number of micro-components present in human milk and which appeared to have major physiological functions (Hilbrands & Streekstra, 1996). Among these, free oligosaccharides, the third largest solute in human milk, up to 18·5 g/l (Brand Miller *et al.* 1994), are considered to have functions ranging from modulation of morphogenesis and cell adhesion to anti-infective properties and promotion of beneficial gut bacteria (Kuntz & Rudloff, 1993). Their chemical structures suggest that they are unlikely to be hydrolysed by intestinal enzymes and may represent the 'dietary fibre' content of human milk (Brand Miller *et al.* 1994).

Oligofructose has been used as a bifidogenic prebiotic in order to prevent digestive disorders in adults and has been demonstrated as capable of specifically stimulating populations of bifidobacteria, and to lower *Clostridium* sp. and *Escherichia coli*, *in vitro* (Wang & Gibson, 1993), in humans (Gibson *et al.* 1995) and animal models (Campbell *et al.* 1997). Furthermore, there is some evidence that free oligosaccharides are potent inhibitors of bacterial adhesion, an initial stage of infection, by acting as cell receptor analogues (Kuntz & Rudloff, 1993; Newburg, 1997).

Taking this into account, we have focused on the effects of oligofructose on neonatal necrotising enterocolitis (NEC), a gastrointestinal disease in premature infants. These effects were investigated through the changes in the incidence of intestinal lesions, the microflora balance

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Abbreviations: NEC, necrotising enterocolitis; SCFA, short-chain fatty acid.
Note: For the definition of the terms inulin and oligofructose please refer to the introductory paper (p. S139) and its footnote.
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and the production of bacterial metabolites in an experimental model: the gnotobiotic quails associated with faecal flora from healthy or sick premature neonates.

Gut colonisation in premature neonates and the risk of necrotising enterocolitis

Despite significant advances in neonatal practice, NEC remains a major cause of gastrointestinal emergency in neonatal intensive care units (NICU) and the first cause of death in extremely premature infants. It is a very severe illness characterised by abdominal distension, gastrointestinal bleeding, mucosal ulcerations and intestinal pneumatosis (Neu, 1996). The physiopathology is still unclear and several factors including immaturity of the gut, enteral feeding and bacterial colonisation are involved (Neu, 1996). To date, none of the preventive treatments, i.e. antibiotic therapy, prophylactic aspects and parenteral feeding, is considered fully satisfactory. Thus, modulating the autochthonous microflora by probiotics or prebiotics could be a rational approach towards prevention.

Normally, the gastrointestinal tract, sterile during foetal life, is colonised at birth with bacteria acquired from the mother during delivery and/or from the environment, mainly *E. coli* and streptococci (Ducluzeau, 1990; Langhendries *et al.* 1998). Many factors affect this colonisation, especially the mode of feeding and gestational age. The gut of premature neonates is immature and colonised with few bacteria (Butel *et al.* 1999) (Table 1). This colonisation differs from that of full-term neonates because of the frequent use of antibiotics, isolation, an absence of feeding directly from the breast and local ecology in the NICU. Bifidobacterial colonisation, associated with a protection against infant digestive infection, is delayed for several weeks in favour of high levels of enterobacteria and clostridia in premature infants (Gewolb *et al.* 1999; Sakata *et al.* 1985). It has been postulated that this unbalanced intestinal microflora may contribute to a predisposition of NEC (Dai, 1998).

NEC is often associated with enterobacteria such as *Klebsiella sp* (Westra-Meijer *et al.* 1983), *E. coli* (Speer *et al.* 1976) or several clostridia (Blakey *et al.* 1985; Cashore *et al.* 1981; Kliegman *et al.* 1979; Kosloske, 1994; Loc *et al.* 1980). Among clostridia, the most commonly isolated species are *C. butyricum* (Gothefors & Blenkharn, 1978; Howard *et al.* 1977; Lawrence *et al.* 1982; Laverdière *et al.* 1978) and *C. perfringens* (Blakey *et al.* 1985; Kliegman *et al.* 1979; Kosloske *et al.* 1978; Kosloske, 1994).

Conversely, no relationship has been found between the acquisition or carriage of toxigenic *C. difficile* and intestinal symptoms (Delmée *et al.* 1988). Lawrence *et al.* (1982) postulated that in such infants a non pathogen, with few or no competitors, is able to multiply without interference. Thus bacterial metabolites, over produced in premature infants who are deficient in endogenous lactase, may be absorbed and cause mucosal damage, possibly initiating NEC. The anaerobic intestinal microflora converts carbohydrates to short-chain fatty acids (SCFA). Among them, butyric acid was first related to the cytotoxic factor of *C. butyricum* strains involved in NEC (Popoff *et al.* 1987). Its over production appear to be a critical factor in regulating the onset of the disease (Bousseboua *et al.* 1989; Butel *et al.* 1998b).

An experimental model of necrotising enterocolitis: gnotobiotic quails

Quails present some interesting similarities with premature infants in terms of gastrointestinal histology and physiology. They are natural alactasic species, their caeca are a pair of blind ending ducts which favour bacterial stasis and the structure of the caecal wall is comparable to that of the intestine with only slight variations (Hodges, 1979). We have developed an experimental model of NEC using gnotobiotic quails, i.e. germ-free quails reared in a sterile isolator, associated with faecal flora specimen belonging to premature infants (Fig. 1). They were fed a lactose diet sterilised by gamma irradiation and containing lactose 6 % (w/w) to mimic the proportion in human milk (Bousseboua *et al.* 1989). A combination of low endogenous lactase activity, lactose in diet, intestinal stasis and colonisation by lactose-fermenting bacteria such as *C. butyricum* (Szylit *et al.* 1997; Bousseboua *et al.* 1989) or faecal flora specimens from premature infants suffering from NEC (Butel *et al.* 1998b) have been shown essential to the onset of NEC-like caecal lesions. Gross necrosis was evaluated as follows: normal, thickened, showing pneumatosis and/or having haemorrhagic contents (Fig. 2). Histological findings were also carried out to complete the macroscopic observations (Fig. 3).

Oligofructose participates in the health promoting effects of bifidobacteria

The health promoting effect of bifidobacteria has been demonstrated in animal models. It appears that bifidobacterial

Table 1. Analysis of gut implantation in twelve premature infants born before 33 weeks of gestation (Butel *et al.* 1999)

	Median level (D21)	Range	
Staphylococci	$6 \cdot 10^{6}$*	$10^{3} - 9 \cdot 10^{9}$	
Enterococci	10^{8}	$4 \cdot 10^{3} - 3 \cdot 10^{9}$	3/12 non colonised at D26
Enterobacteria	10^{8}	$2 \cdot 10^{3} - 3 \cdot 10^{10}$	delayed colonisation
Clostridium	$2 \cdot 10^{6}$	$10^{4} - 2 \cdot 10^{7}$	8/12 colonised
Bacteroides		10^{4} and $2 \cdot 10^{7}$	2/12 colonised at D15 and D58
Bifidobacteria		$6 \cdot 10^{4}$ and $4 \cdot 10^{8}$	2/12 colonised at D15 and D64

* Bacterial counts expressed as CFU/g of feces.

➢ Germ-free quails, with no endogenous intestinal lactase and a physiological intestinal stasis

(caeca)

➢ Associated with either a single bacterial strain or various mixtures of bacteria (Butel *et al.*

1998)

➢ Fed a diet supplemented with lactose (6%)

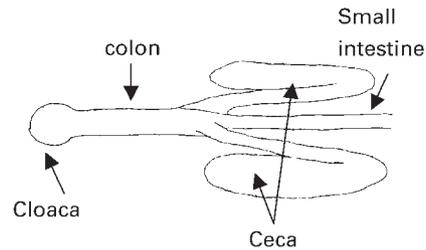| Strains | NEC-like lesions in quails fed a diet | |
	with lactose	without lactose
C. butyricum	YES	NO
C. perfringens	YES	NO
C. paraputrificum	YES	NO
C. difficile	NO	NO
Klebsiella	NO	NO

Fig. 1. Experimental model of NEC. Germ-free quails, with no endogenous intestinal lactase and a physiological intestinal stasis (caeca) Associated with either a single bacterial strain or various mixtures of bacteria (Butel *et al.* 1998*a*). Fed a diet supplemented with lactose (6%).

colonisation in the rat model results in a lesser risk for NEC through modulation of the inflammatory cascade (Caplan *et al.* 1999). In our experimental model fed the lactose diet, bifidobacteria were demonstrated to totally inhibit the development of the caecal lesions (Butel *et al.* 1998*b*; Butel & Szylit, 2000) (Table 2). First, a whole faecal flora including bifidobacteria and no clostridia, isolated from a healthy premature infant, was unable to produce NEC-like caecal lesions. Second, early

bifidobacterial supplementation of gnotobiotic quails associated with a NEC-flora resulted in a prevention of cecitis through a modulation of the intestinal microflora. This effect was related to a sharp decrease in clostridial populations and in SCFA concentrations, i.e. butyric acid and SCFA of protein origin.

Oligofructose effects on the intestinal microflora and the incidence of the digestive lesions were investigated in our experimental model in various situations. We compared the

Normal aspect

Thickening and first step of pneumatosis

Thickening, pneumatosis and haemorrhagic contents

Fig. 2. Varying macroscopic aspects of the caeca of gnotobiotic quails.

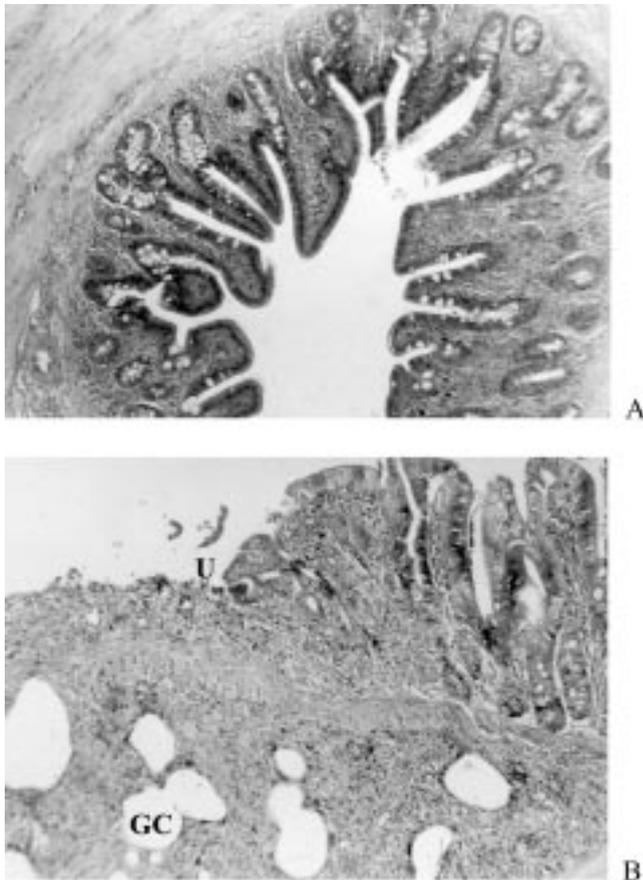**Fig. 3.** Varying histological examination of the caeca of gnotobiotic quails (hematoxylin−eosin stain × 100) A: Normal pattern; B: Large ulceration of the mucosa (U) and numerous gas cysts (GC).
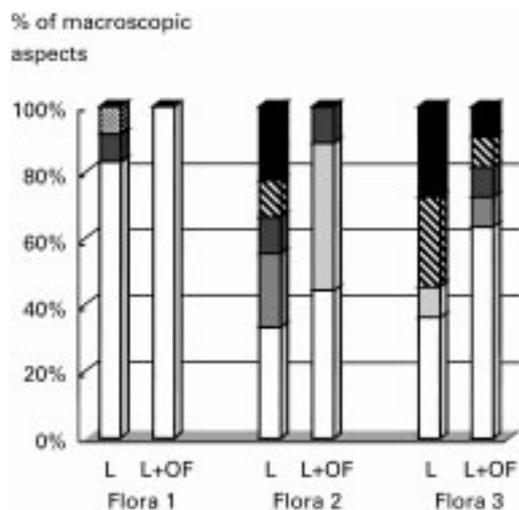

Fig. 4. Influence of oligofructose (OF) on caecal NEC-like lesions in gnotobiotic quails associated with fecal specimens from a healthy (flora 1) and two-NEC suffering (flora 2 and 3) premature infants. ■ = P + H + T, ◣ = P + H, ▧ = P + T, ■ = Pneumatosis (P), ▤ = Haemorrhagic (H), ▢ = Thickening (T), □ = normal.

occurrence of cecitis, bacterial population and metabolism changes between quails fed either the lactose diet (6 % w/w) or a lactose−oligofructose diet (3 %−3 % w/w).

Oligofructose is known to stimulate the activity of bifidobacteria in adults and similar results have been observed with gnotobiotic quails fed the oligofructose-supplemented diet (Table 2). This increased bifidobacterial level was associated with a decrease in *E. coli* and clostridial colonisation depending on the initial composition of the microflora (Catala *et al.* 1999). Therefore, oligofructose may participate in the health-promoting effect of bifidobacteria by exerting a beneficial effect on microflora balance of the premature infants. The mechanism by which oligofructose plays this role is still under discussion and has often been associated with fermentation of oligofructose by bifidobacteria which subsequently led to a pH decrease (Wang & Gibson, 1993; Gibson & Wang, 1994). In this study the change in pH and SCFA between the two bifidobacteria groups (fed with or without oligofructose) were minimal.

Protective effect of oligofructose in the absence of bifidobacteria

Because of the delayed bifidobacterial colonisation in premature neonates, the effect of oligofructose was investigated in absence of bifidobacteria and using clostridial species as a trigger of caecal injury in two specific cases.

In the first study, with gnotobiotic quails kept in the sterilised isolator, health promoting effects of oligofructose against NEC-like lesions caused by a polymicrobial infection were demonstrated, but these effects varied according to clostridial species, implantation level and bacterial association (Table 3). Three groups of germ-free quails were associated with three fecal flora from premature infants, yielding various mixed bacterial species implicated in the onset of NEC and involving *C. perfringens* known to produce several necrotising extracellular toxins (Finegold, 1977). When fed the lactose diet the occurrence of NEC-like lesions varied from 17 % with flora 1 (*C. perfringens* $< 10^6$ CFU/g caecal content) to 67 % with flora 2 (*C. perfringens* ca 10^7 CFU/g, *C. difficile*) and flora 3 (*C. perfringens* ca 10^8 CFU/g, *C. difficile, C. paraputrificum*), with different states of severity (Figs 3 and 4). Supplementation with oligofructose led to the inhibition of the lesions with flora 1, and to less extensive tissue necrosis and a sharp decrease in haemorrhages with floras 2 and 3. With flora 3, the main histological observation was an insignificant and simple caecal inflammation without any severe lesions (Fig. 3 A) when quails were fed the lactose+oligofructose diet. Furthermore, the oligofructose beneficial effect was associated with a decrease in bacterial species, i.e. *C. perfringens* (ca 10^6/g, $P < 0.05$) and *C. paraputrificum* only with flora 3. Whatever the flora, oligofructose did not alter caecal pH or short-chain fatty acid concentrations.

Capacity of oligofructose to allow a bifidobacterial colonisation

The study simulated the conditions in which infants are exposed to various bacteria in neonatal intensive care units. The ability of oligofructose to allow intestinal

Table 2. Influence of OF on bacterial counts in gnotobiotic quails associated with healthy or NEC-flora

Flora	NEC-flora		Nec-flora+Bifid		Healthy flora	
Diet*	Lactose	Lac+OF	Lactose	Lac+OF	Lactose	Lac+OF
Outcome of infection sick quails/total quails	6/9	5/9	0/8	0/9	0/11	0/11
Bacterial counts						
Bifidobacterium	...	...	8·6 (0·0)†	9·2 (0·1)‡	6·9 (0·2)	7·9 (0·3)‡
C perfringens	7·2 (0·4)	7·5 (0·3)	3·8 (0·3)	< 3‡	...	...
C difficile	5·4 (0·3)	5·8 (0·2)	5·4 (0·6)	5·2 (0·4)	...	...
Enterococci	9·9 (0·1)	9·5 (0·3)	8·6 (0·4)	9·0 (0·1)	3·7 (0·2)	3·3 (0·1)
Enterobacteria	8·6 (0·2)	8·0 (0·3)	8·1 (0·5)	7·1 (0·5)	10·2 (0·2)	8·4 (0·4)

Gnotobiotic quails were killed 28 days after inoculation.
* Quails were fed either a lactose (6%) or a lactose-OF (3%−3%) diet.
† Bacterial counts are expressed as mean (SEM) $\log_{10}$ CFU/g wet content, the threshold count was 3 $\log_{10}$ CFU/g.
‡ Significantly different from control diet within the same bacterial status ($P < 0.05$).

colonisation by exogenous bifidobacteria was determined in gnotobiotic quails associated with a flora deprived of bifidobacteria (Danan *et al.* 2000) (Table 4). To enable further infection by bacteria from the external environment, air filters were removed from all isolators one week after inoculation. Although quails were housed in an environment containing bifidobacteria, daily oligofructose consumption did not promote intestinal post colonisation by exogenous bifidobacteria in quails initially deprived of this bacterial genus. Nevertheless, the level of *C. perfringens* decreased (1·6 log lower). These results are in agreement with our previous study that demonstrated the specific inhibition of oligofructose against clostridia in gnotobiotic quails.

If several studies in adults (Garleb *et al.* 1996; Gibson *et al.* 1995) and in experimental models (Catala *et al.* 1999; Howard *et al.* 1995) showed that the bifidogenic effect of oligofructose is dependent on the initial level of bifidobacteria in the intestinal microflora, the regular ingestion of oligofructose did not entail bifidobacterial colonisation when bifidobacteria were initially absent.

Conclusion

Comparing the different studies in gnotobiotic quails, the protective effect of oligofructose observed appeared less effective than the probiotic effect of bifidobacteria or the symbiotic effect of oligofructose and bifidobacteria. This led to a complete disappearance of the caecal lesions due to a sharp decrease or the disappearance of clostridial species associated with the disappearance of caecal butyrate concentration. Oligofructose was unable to promote a bifidobacterial acquisition when the microflora was initially deprived of this species. Nevertheless, oligofructose can act as an anti-infective agent and was demonstrated to decrease the occurrence or severity of the intestinal lesions depending on the bacteria involved in the onset of the lesions.

Addition of free oligosaccharides has never been taken into account in premature formulae despite their high levels in human milk, particularly in milk from mothers delivering premature infants and despite the fact that they have major physiological functions. A supplementation with these prebiotics could offer — even in the absence of bifidobacteria — a new approach to improve pre-term formula milk and permit when bifidobacteria are present to maintain the beneficial flora for a long period. These positive results support the need for further clinical investigations on the role of oligofructose in bacterial colonisation and prevention of NEC.

Table 3. Influence of OF on caecal bacterial status of gnotobiotic quails associated with fecal specimens from a healthy (group 1) ot two NEC-suffering (groups 2 and 3) pre-term infants

Group	Flora 1		Flora 2		Flora 3	
Diet*	Lactose	Lac+OF	Lactose	Lac+OF	Lactose	Lac+OF
Outcome of infection sick quails/total quails	2/12	0/11	6/9	5/9	7/11	4/14
Bacterial counts						
C perfringens	5·1 (0·7)†	6·0 (0·5)	7·2 (0·4)	7·5 (0·3)	7·9 (0·2)	6·4 (0·1)‡
C paraputrificum	...	...	...	...	8·4 (0·1)	5·8 (0·3)‡
C difficile	...	...	5·4 (0·3)	5·8 (0·2)	5·6 (0·4)	5·1 (0·3)
K pneumoniae	7·8 (0·6)	8·1 (0·4)	...	...	...	...
Proteus mirabilis	...	...	8·6 (0·2)	8·0 (0·3)	...	...
Enterococci	9·3 (0·3)	8·5 (0·4)	9·9 (0·1)	9·5 (0·3)	8·4 (0·2)	9·0 (0·2)

Gnotobiotic quails were killed 28 days after inoculation.
* Quails were fed either a lactose (6%) or a lactose-OF (3%−3%) diet.
† Bacterial counts were expressed as mean (SEM) $\log_{10}$ CFU/g wet content, the threshold count was 3 $\log_{10}$ CFU/g; $n = 7$ or eight analyses per group and diet.
‡ Significantly different from control diet within the same bacterial status ($P < 0.05$).

Table 4. Cecal bacterial status in gnotobiotic quails in an ordinary environment and associated with a flora deprived of bifidobacteria

	Diet	
Bacteria	Lactose	Lactose+OF
C difficile	4·5 ± 0·5 * (5)†	5·2 ± 2·0 (3)
C perfringens	6·5 ± 0·9 (5)	4·9 ± 1·3 (4)‡
C malenominatum	5·7 ± 1·3 (4)	5·1 ± 1·1 (2)
Bacteroides	< 3	< 3
Bifidobacterium	< 3	< 3
Enterobacteria	< 3	< 3
Enterococci	9·0 ± 1·0 (5)	8·0 ± 1·4 (4)

Quails were housed in an environment containing bifidobacteria.
* Bacterial counts ± SD.
† Number of quails colonized.
‡ Significantly different from the lactose diet.

Acknowledgements

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