

## Bovine colostrum is superior to enriched formulas in stimulating intestinal function and necrotising enterocolitis resistance in preterm pigs

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### Abstract

Milk contains immunomodulatory compounds that may be important to protect the immature intestine in preterm neonates from harmful inflammatory reactions involved in disorders like necrotising enterocolitis (NEC). We hypothesised that bovine colostrum and milk formulas enriched with sialic acids (SL), gangliosides (Gang) or osteopontin (OPN) would improve gastrointestinal function and NEC resistance in preterm neonates. Forty-seven caesarean-delivered preterm pigs were given total parenteral nutrition for 2 d followed by 1.5 d of enteral feeding. In Expt 1, a control formula was compared with an OPN-enriched formula ( $n$  13), while Expt 2 compared a control formula with bovine colostrum or formulas enriched with Gang or SL ( $n$  4–6). OPN enrichment decreased NEC severity relative to control formula ( $P < 0.01$ ), without any significant effects on NEC incidence, digestive enzyme activities and hexose absorption. Neither SL- nor Gang-enriched formulas improved NEC resistance or digestive functions, while all the intestinal functional parameters were significantly improved in pigs fed bovine colostrum, relative to formula. The effects *in vivo* were supported *in vitro* by bacteria- and dose-dependent modulation by colostrum whey of the cytokine response from bacteria-stimulated murine bone marrow-derived dendritic cells (DC). In conclusion, OPN had only moderate NEC-protective effects, while formulas enriched with Gang or SL were ineffective. The observed modulation of DC cytokine response by bovine colostrum whey *in vitro* may be due to a synergistic action of various milk bioactives, and it may explain its beneficial effects on NEC development and intestinal function in a piglet model of preterm infants.

**Key words:** Colostrum: Milk bioactives: Immunomodulation: Necrotising enterocolitis

Milk contains numerous bioactive substances including immunoglobulins, cytokines, growth factors and components possessing antibiotic and prebiotic activity<sup>(1,2)</sup>. Little is known about the biological effects of individual milk bioactives, despite the fact that natural milk improves intestinal function and development of a stable and beneficial microbiota in the gastrointestinal tract<sup>(3)</sup> relative to milk formula. The anti-microbial and anti-inflammatory properties of milk are most pronounced for the first milk (colostrum), although most known milk bioactives can be found throughout lactation. Milk components with preliminary evidence of positive effects on the intestinal immune system and microbiota include osteopontin (OPN), gangliosides (Gang) and sialic acids (SL)<sup>(1,4–10)</sup>. Characterisation of the biological effect of such components is important for optimal production of infant milk formulas to be used when mother's milk is not available. Furthermore, these

compounds could be particularly important for nutrition of sensitive newborns, such as preterm infants.

OPN is a phosphorylated acidic glycoprotein expressed by a number of different immune and non-immune cells and tissues, and it is also known as early T cell activation 1 protein<sup>(4,11,12)</sup>. Secreted OPN has cytokine-like properties. It enhances interferon- $\gamma$ -producing T helper 1 cell responses by inducing production of IL-12 and suppressing production of IL-10<sup>(4,13,14)</sup>. Hence, expression of OPN has been shown to be important in the efficient development of the T helper 1 immune response, and OPN is recognised as a key cytokine involved in T helper 1 cytokine expression at sites of inflammation. Many studies have reported increased production of OPN during various inflammatory processes<sup>(4,13–16)</sup>. Overall, OPN can be categorised as an important regulator of processes involved in inflammation and tissue repair; in particular, those responses characterised by the presence

**Abbreviation:** DC, dendritic cells; Gang, gangliosides; NEC, necrotising enterocolitis; OPN, osteopontin; PN, parenteral nutrition; SI, small intestine; SL, sialic acids; TPN, total PN.

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of macrophages and T cells. Besides lymphocyte activation and involvement in a number of other steps of cell-mediated immunity, OPN is also involved in cell survival and wound repair<sup>(11,14)</sup>. Due to the multifunctional immunoregulatory cytokine properties of OPN, and since it is present in such high amounts especially during early lactation, it is very likely that OPN plays a significant role in the early immunological development of infants.

Gang are complex glycosphingolipids comprising a hydrophobic ceramide tail attached to a hydrophilic oligosaccharide with at least one SL molecule. They are widely distributed in vertebrate tissues, attached to cell surfaces or secreted to plasma and other body fluids<sup>(17–20)</sup>. Breast milk contains Gang, and milk from different species has specific Gang profiles with contents varying during lactation<sup>(18,19,21–23)</sup>. Certain glycosylated milk Gang may serve as false receptor analogues for bacterial adhesins. Thus, they may be able to modify the intestinal microbiota in the neonate<sup>(7,19)</sup>. *In vitro*, Gang exhibit immunosuppressive effects on a number of immunocompetent cells, and these effects are dependent on Gang structure, concentration and the type of immune cell affected<sup>(17,18,24)</sup>.

SL comprise a diverse group of acidic sugars with a nine-carbon backbone. Typically, these are found on the terminal ends of glycan chains attached to lipids and proteins covering surfaces of all cells and a variety of secreted proteins in vertebrates. Here, they serve as components of binding sites for pathogens and toxins that recognise the SL unit in combination with different underlying carbohydrate chains of the cell surface glycans<sup>(10,25)</sup>. SL moieties on, for example, Gang can therefore inhibit attachment of pathogenic bacteria species such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* by acting as decoy receptors<sup>(26)</sup>.

Dendritic cells (DC) reside in the Peyer's patches and lamina propria of the intestine and are important antigen-presenting cells of the gut-associated immune system. We have previously shown that murine bone marrow-derived DC respond strongly to bacterial stimuli<sup>(27)</sup>, and this response can be modulated with various milk components<sup>(18,28)</sup>. Hence, the DC assay can be used as a screening tool to study the immunomodulatory effects of various milk bioactives.

In preterm pigs, maternal colostrum protects against necrotising enterocolitis (NEC) in the small intestine (SI)<sup>(29–31)</sup>, while infant formula leads to digestive and immune dysfunction and NEC lesions. We hypothesised that these diet differences are explained partly by the lack of milk bioactives like OPN, Gang and SL in commercial milk formulas, relative to mother's milk, or a diet like bovine colostrum. We therefore tested the *in vivo* intestinal response to milk formulas enriched with OPN, Gang or SL in preterm pigs. Furthermore, the immunomodulatory activity of the enriched milk fractions was investigated *in vitro* using DC stimulated with gut-associated bacteria. Both the overall bacterial load and specific groups (such as *Clostridia*) are suspected to play a role in NEC aetiology<sup>(32)</sup>.

## Materials and methods

### Animals, surgery and nutrition protocols

Forty-seven piglets (Large White × Danish Landrace) from three litters were delivered by caesarean section at 105 d of gestation as previously described<sup>(30)</sup>. While still anaesthetised from delivery, each piglet was fitted with an orogastric feeding tube (infant feeding tube 6F; Portex, Kent, UK) and a vascular catheter (4F; Portex) in the dorsal aorta via the transected umbilical cord<sup>(29)</sup>. Within 6 h of delivery, all the piglets were administered total parenteral nutrition (TPN), continuously infused through the arterial catheter for 48 h. Initially, the rate was 4 ml/kg body weight per h advancing to 6 ml/kg body weight per h after 12 h. The TPN solution was prepared aseptically as previously described and adjusted to meet the nutritional requirements of the preterm pig<sup>(29,33,34)</sup>. All the pigs were immunised with three gradually increasing doses of porcine plasma obtained from their mother<sup>(35)</sup>.

Enteral milk formulas used as control diets were prepared from commercially available formula products (Pepdite, Maxipro, Liquigen; SHS International, Liverpool, UK) and adjusted to match the energy and protein concentrations in porcine milk during lactation. The three enriched milk formulas were prepared by the addition of pure bovine OPN or Gang- or SL-enriched bovine milk fractions (all from Arla Foods Ingredients, Viby J, Denmark) as described later. The protein, lipid and carbohydrate contents were adjusted according to the additional nutrients added to each formula to obtain similar compositions for all formulas within the same experiment. As the Gang- and SL-enriched milk products were not completely pure, like the OPN product, these were tested in a separate experiment where the control formula was adjusted to match the composition of the Gang and SL formulas. Hence, the formula control diets of the two experiments were slightly different. The final composition of the applied formulas is shown in Table 1. Bovine colostrum was collected and pooled from two to three Holstein cows from the first milking after calving and stored at  $-20^{\circ}\text{C}$  until use. The content of OPN, SL and Gang in bovine milk has been documented elsewhere<sup>(21,22,36)</sup>.

After the TPN period, the pigs were switched to enteral nutrition. Every 3 h, all the pigs were manually given oral boluses of 15 ml/kg of their respective diets, and the pigs were monitored every 3 h for potential clinical signs of NEC (feeding intolerance, abdominal distension, diarrhoea and respiratory stress). The pigs were euthanised for sample collection 1.5 d after initiation of enteral feeding unless serious clinical NEC symptoms developed beforehand. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

### Expt 1: osteopontin-enriched formula to preterm pigs

Twenty-six preterm pigs derived from three litters were randomly assigned to two treatment groups. During the

**Table 1.** Composition of enriched (osteopontin (OPN), gangliosides (Gang) and sialic acids (SL)) and control formulas (per litre formula)

	OPN	Control	Gang	SL	Control
Energy (kJ)	4099.89	4051.85	3927.32	4171.20	3982.91
Protein (g)	61.77	63.20	58.17	63.63	60.52
OPN (g)	2.22	0.00	0.00	0.00	0.00
Polycose (g)	44.88	44.25	42.64	47.20	42.64
Dextrose (g)	8.40	8.78	3.85	4.00	3.85
Lactose (g)	0.02	0.00	8.16	10.56	8.80
Sialyl lactose (g)	0.00	0.00	0.00	8.66	0.00
Gang (g)	0.00	0.00	0.06	0.00	0.00
Protein-bound carbohydrate (g)	0.24	0.00	0.00	0.00	0.00
Total fat (g)	62.29	60.15	56.45	57.45	56.64
Saturated fat (g)	46.06	43.94	38.79	41.36	41.15
Monounsaturated fat (g)	9.12	9.21	8.69	9.01	8.69
Polyunsaturated fat (g)	3.43	3.44	3.61	3.74	3.61

2 d PN period, one group was given minimal enteral doses of 5 ml/kg body weight per 3 h of pure OPN diluted in sterile deionised water to a concentration of 2 g/l (OPN, *n* 13), while the controls (*n* 13) were given the same volume of deionised water. After the PN period, both the groups were switched to total enteral nutrition and fed either the standard milk formula (controls) or the same formula enriched with 2.2 g/l of OPN (OPN group).

#### *Expt 2: colostrum or gangliosides- or sialic acids-enriched formulas to preterm pigs*

Twenty-one pigs were obtained from three separate litters and administered TPN as described earlier. Following TPN, the pigs were assigned to a bovine colostrum group (*n* 5) and three formula groups. The formula groups were given either standard milk formula (control, *n* 4), formula with an SL-enriched bovine milk fraction (60 g/l corresponding to 8.7 g/l of sialyl lactose in the final solution, SL, *n* 6) or formula with Gang-enriched bovine milk fraction (3 g/l, final concentration of Gang was 0.06 g/l formula, Gang, *n* 6). The number of animals allocated to the treatment groups in Expt 2 (*n* 4–6) was kept relatively small because the studies quickly documented high NEC severity scores in all three formula groups, with marked differences relative to bovine colostrum. The concentration of SL used in this experiment corresponded to the concentration in human colostrum<sup>(7,37)</sup>, and immunomodulatory effects of milk proteins containing SL moieties have been documented *in vitro* and *in vivo*<sup>(9,25,28,37,38)</sup>.

#### *In vivo carbohydrate absorption*

The test was performed twice by the end of the PN period and 1 d after initiation of enteral nutrition. Boluses of 15 ml/kg body weight of 2% mannitol and 5% galactose were administered orally, and arterial blood samples were collected at intervals (0, 20, 40 and 60 min) after the two boluses. Concentrations of mannitol and galactose in plasma were analysed spectrophotometrically as previously described<sup>(39,40)</sup>.

#### *Necrotising enterocolitis evaluation and tissue collection*

When clinical symptoms of NEC were observed in several pigs in a litter or no later than 1.5 d after initiation of enteral nutrition, the pigs were anaesthetised and subsequently killed with an intra-cardiac injection of sodium pentobarbitone (60 mg/kg). The entire gastrointestinal tract was removed, and the SI, colon and stomach were weighed without contents. SI length was measured in a relaxed state, and the intestine was divided into three equal segments designated proximal, middle and distal SI. The wet mass of lungs, liver, spleen, heart and kidneys was recorded, and the lung volume was determined by immersion in water. Samples from the three SI regions were snap frozen in liquid N<sub>2</sub> and stored at –80°C before analysis of enzymatic activity. Another set of samples was fixed in 4% neutral-buffered paraformaldehyde for villus morphology as described previously<sup>(29,33)</sup>.

Each SI segment, stomach and colon were photographed and given a macroscopic pathologic lesion score on a scale from 1 to 6. Here, 1 indicates the absence of any NEC-associated symptoms; 2 indicates local hyperaemia, inflammation and oedema; 3 indicates hyperaemia, extensive oedema and local haemorrhage; 4 indicates extensive haemorrhage; 5 indicates local necrosis and pneumatosis intestinalis; and 6 indicates extensive necrosis and pneumatosis intestinalis. A pig having a score of 3 or more in any region was considered a case of NEC. An average NEC severity score across all five regions was calculated for each pig, and an average score for each treatment group was calculated from these average scores. The proportion of dry weight of mucosa was determined as previously described<sup>(30)</sup>.

#### *Intestinal mucosal morphology and enzyme activities*

Fixed SI samples were embedded in paraffin, sectioned (3 µm), and mounted on glass slides and stained with haematoxylin and eosin. Villus heights of ten intact villi from one representative cross-section from each pig were measured on scanned images of the tissue sections using

the software SoftWoRx Explorer version 1.1 (Applied Precision, Issaquah, Washington, DC, USA).

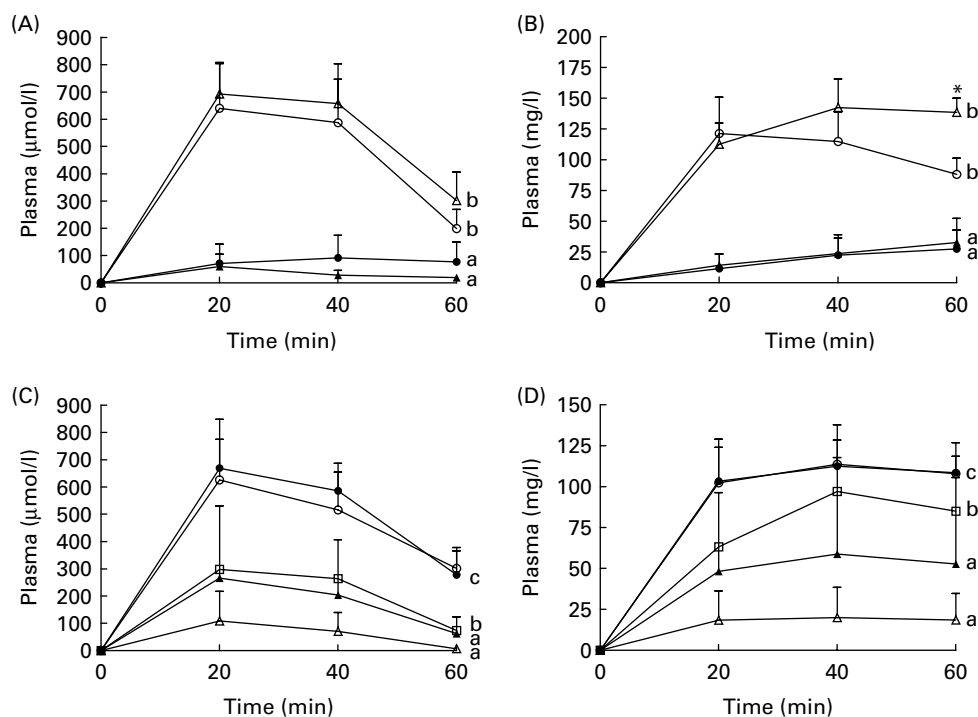
Homogenates of frozen samples from the three SI segments from each pig were assayed spectrophotometrically for activities of lactase, sucrase, maltase, dipeptidyl-peptidase IV, aminopeptidase N and aminopeptidase A. Lactose, sucrose, maltose, glycy-L-prolin-4-nitroanilide, L-alanine-4-nitroanilide and  $\alpha$ -L-glutamic acid 4-nitroanilide were used, respectively, as substrates, in accordance with a previously established protocol<sup>(41)</sup>. The hydrolytic release of 1  $\mu$ mol substrate per min at 37°C was considered to represent one unit (U) of enzyme activity, which was expressed per gram of wet intestine.

### Expt 3: in vitro immunomodulatory activity of milk bioactives

**Preparation of bacterial strains.** *Clostridium perfringens* NEC A20 (type A isolate from a premature piglet with NEC) was grown anaerobically overnight at 37°C in tryptone soya broth (Oxoid A/S, Greve, Denmark). *E. coli* Nissle 1917 (O6:K5:H1, isolate from human faeces) was cultured aerobically in de Man, Rogosa and Sharpe broth (Merck, Darmstadt, Germany) overnight at 37°C. Bacterial cultures were harvested by centrifugation at 2700g for 15 min, washed twice in sterile PBS (pH 7.2) and resuspended in PBS. Bacterial cells were killed by 1 h exposure to UV light and then stored at -80°C. Bacterial

concentrations were determined from dry mass of freeze-dried samples and corrected for buffer salt concentration.

**Preparation of dendritic cells and co-culturing with milk bioactives and bacteria.** Bone marrow cells were flushed with PBS from the dissected femur and tibia from C57BL/6 mice. The cells were washed twice in PBS and resuspended in Roswell Park Memorial Institute-1640 supplemented with 2 mM-L-glutamine, 10% (v/v) heat-inactivated foetal calf serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ M-2-mercaptoethanol (culture medium, all components from Lonza, Basel, Switzerland) and 15 ng/ml murine granulocyte-macrophage colony-stimulating factor (supernatant from a granulocyte-macrophage colony-stimulating factor transfected Ag8-653 myeloma cell line). The cells were incubated for 8 d in petri dishes at 37°C and 5% CO<sub>2</sub>. On days 3 and 6 of incubation, the cells were supplied with fresh culture medium containing 15 ng/ml granulocyte-macrophage colony-stimulating factor. On day 8, non-adherent cells were harvested and resuspended in culture medium with 10 ng/ml granulocyte-macrophage colony-stimulating factor. A total of  $2 \times 10^6$  cells/ml were seeded in forty-eight-well tissue culture plates, and 10  $\mu$ g/ml bacteria were added together with different concentrations of OPN, SL, Gang, bovine colostrum or colostrum whey diluted appropriately in culture medium. Colostrum whey was produced by coagulation with 3  $\mu$ l/ml bovine chymosin (Chr. Hansen, Horsholm, Denmark) for 30 min at 37°C and subsequent centrifugation for 10 min at 280g.



**Fig. 1.** Absorption of galactose ((A) and (C)) and mannitol ((B) and (D)) by the end of the 2 d total parenteral nutrition (TPN) period and 1 d after start of enteral feeding. Values represent increments in plasma galactose and mannitol at 20, 40 and 60 min (means with their standard errors). Enriched formulas are compared with colostrum and control formulas, respectively, and the entire response was analysed by repeated measures. <sup>a,b,c</sup> Mean values with unlike superscript letters assigned to the curves indicate galactose and mannitol responses that differ significantly ( $P < 0.05$ ). \* Mean values for the TPN-osteopontin (OPN) and OPN groups differ significantly specifically at 60 min in (B) ( $P < 0.05$ , by *t* test). ((A) and (C)) - $\Delta$ -, TPN-OPN; - $\circ$ -, TPN control; - $\blacktriangle$ -, OPN; - $\bullet$ -, control. ((B) and (D)) - $\circ$ -, TPN all pigs; - $\blacktriangle$ -, sialic acids; - $\Delta$ -, gangliosides; - $\bullet$ -, colostrums; - $\square$ -, control.



The final volume in each well was 600 µl. The cells were stimulated for 18–20 h at 37°C and 5% CO<sub>2</sub> before collection of cell culture supernatants, which were stored at –20°C until cytokine analysis. The concentration of cytokines IL-12p70, IL-10, IL-6 and TNF-α was determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**Statistical analysis**

ELISA data from the DC experiments were evaluated by one-way ANOVA with Dunnett's multiple comparison *post hoc* test using Graph Pad Prism (version 5.01; Graph Pad Software Inc., La Jolla, CA, USA). Villi heights were compared with one-way ANOVA and Tukey's *post hoc* test (Expt 2) or Student's *t* test (Expt 1) using Graph Pad Prism. Enzyme activity and NEC severity score data were analysed by two-way ANOVA for the two *in vivo* experiments separately using the mixed procedure of Statistical Analysis Systems (SAS/STAT version 9.1; SAS Institute, Cary, NC, USA). Treatment and intestinal region were fixed effects, while pig and litter were regarded as random variables. Sugar absorption data were analysed with repeated-measures analysis using Statistical Analysis Systems. NEC incidence was analysed using Fisher's exact procedure test with subsequent pairwise comparisons of treatment groups. Data are presented as means with their standard errors or means and standard deviations in all statistical analyses; *P* values <0.05 were considered significant.

**Results**

*Expt 1 and 2: in vivo studies on enriched bovine milk fractions and colostrum*

**In vivo carbohydrate absorption.** The *in vivo* response to oral boluses of galactose and mannitol by the end of the TPN period and 1 d after the initiation of enteral nutrition showed no significant difference between OPN and controls (repeated-measures analyses of graphs in Fig. 1). However, 60 min after the mannitol bolus, 2-d-old piglets supplemented with OPN during the TPN period had significantly elevated plasma mannitol levels, compared with controls (*P*<0.05, Fig. 1(B)). Both the groups had much lower plasma increments of galactose and mannitol during enteral feeding than during the TPN period.

In Expt 2 after initiation of enteral nutrition, Gang and SL lowered the galactose and mannitol absorption relative to controls, while the absorption capacity was higher in colostrum pigs compared with all formula-fed pigs (Fig. 1, *P*<0.01).

**Necrotising enterocolitis incidence and severity.** OPN supplementation (Expt 1) did not reduce the incidence of NEC (54 and 77% in the OPN and control groups, respectively, Table 2). However, the mean NEC severity score

**Table 2.** Necrotising enterocolitis (NEC) incidence and severity and small intestine structural and functional indices (Mean values with their standard errors)

Treatment	Expt 1						Expt 2					
	OPN		Control		Gang		SL		Control		Colostrum	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<i>n</i>	13	13	13	6	6	6	6	4	4	4	5	5
Daily intake of bioactive (mg/kg)	80/264†	0	0	7.2	7.2	1044	1044	0	0	0	–	–
NEC incidence (%)	54	77	77	83 <sup>a</sup>	83 <sup>a</sup>	83 <sup>a</sup>	83 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	40 <sup>a</sup>	40 <sup>a</sup>
NEC severity	2.54*	0.56	3.68	3.53 <sup>a</sup>	3.53 <sup>a</sup>	0.69	3.90 <sup>a</sup>	4.50 <sup>a</sup>	4.50 <sup>a</sup>	1.28 <sup>b</sup>	1.28 <sup>b</sup>	0.14
Villus ht (µm)	557	63.7	490	405 <sup>a</sup>	405 <sup>a</sup>	40.9	416 <sup>a</sup>	411 <sup>a</sup>	411 <sup>a</sup>	0.84	472 <sup>a</sup>	46.5
Enzyme activity (U/g tissue)‡												
Lactase	13.4	1.33	13.2	7.57 <sup>a</sup>	7.57 <sup>a</sup>	1.20	7.64 <sup>a</sup>	10.8 <sup>a</sup>	10.8 <sup>a</sup>	2.12	21.9 <sup>b</sup>	2.44
Maltase	1.36	0.095	1.48	1.40 <sup>a,b</sup>	1.40 <sup>a,b</sup>	0.167	1.49 <sup>a,b</sup>	1.13 <sup>a</sup>	1.13 <sup>a</sup>	0.127	2.01 <sup>b</sup>	0.220
Sucrase	0.182	0.0181	0.199	0.189 <sup>a,b</sup>	0.189 <sup>a,b</sup>	0.0214	0.212 <sup>a,b</sup>	0.157 <sup>a</sup>	0.157 <sup>a</sup>	0.0214	0.217 <sup>b</sup>	0.0126
ApA	2.27	0.188	2.14	1.73 <sup>a</sup>	1.73 <sup>a</sup>	0.196	1.47 <sup>a</sup>	1.39 <sup>a</sup>	1.39 <sup>a</sup>	0.253	2.79 <sup>b</sup>	0.176
ApN	3.38	0.272	3.17	2.47 <sup>a</sup>	2.47 <sup>a</sup>	0.232	2.35 <sup>a</sup>	2.61 <sup>a</sup>	2.61 <sup>a</sup>	0.401	4.76 <sup>b</sup>	0.229
DPP IV	0.882	0.0727	0.798	0.716 <sup>a</sup>	0.716 <sup>a</sup>	0.0672	0.627 <sup>a</sup>	0.600 <sup>a</sup>	0.600 <sup>a</sup>	0.0852	1.389 <sup>b</sup>	0.1394

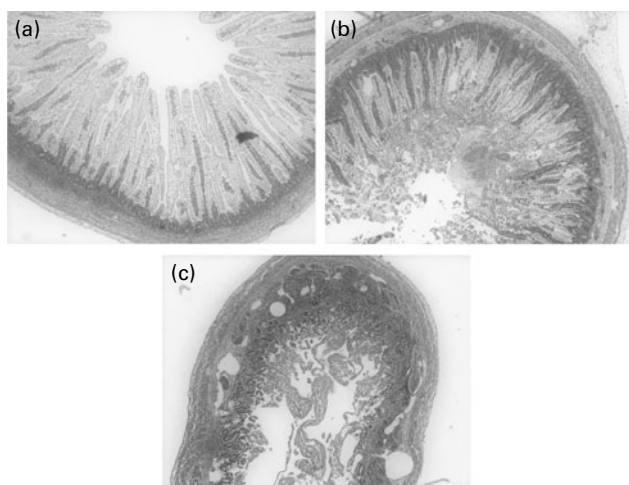
OPN, osteopontin; Gang, gangliosides; SL, sialic acids; ApA, aminopeptidase A; ApN, aminopeptidase N; DPP IV, dipeptidyl-peptidase IV.

<sup>a,b</sup> Means values within the same row with unlike superscripts letters were significantly different (*P*<0.05) for Expt 2.

\* Mean values were significantly different for OPN compared to controls in Expt 1 (*P*<0.05).

† OPN intake during total parenteral nutrition—minimum enteral nutrition and enteral feeding period, respectively.

‡ The hydrolytic release of 1 µmol substrate per min at 37°C represents one unit of enzyme activity, expressed per g wet intestine.



**Fig. 2.** Cross-sections of distal intestinal samples from sick and healthy pigs representing different degrees of necrotising enterocolitis (NEC) evaluated macroscopically at tissue collection and given a macroscopic pathologic lesion score on a scale from 1 to 6: 1 indicates the absence of any NEC-associated symptoms; 2 indicates local hyperaemia, inflammation and oedema; 3 indicates hyperaemia, extensive oedema and local haemorrhage; 4 indicates extensive haemorrhage; 5 indicates local necrosis and pneumatosis intestinalis; and 6 indicates extensive necrosis and pneumatosis intestinalis. (a) Control formula-fed pig, NEC score 1; (b) pig fed gangliosides-enriched formula, NEC score 4; (c) control formula-fed pig, NEC score 6.

was significantly reduced in the OPN group (2.5 (SEM 0.3) *v.* 3.7 (SEM 0.3),  $P < 0.01$ , Table 2). In Expt 2, all the three formula groups showed high NEC incidence (83–100%), while only 40% (2/5) of the pigs in the colostrum group developed NEC (Table 2). Only mild signs of NEC (grades 2 and 3) were observed in the colon and distal SI of two pigs fed colostrum. Hence, the NEC severity score of the colostrum group was significantly lower ( $P < 0.0001$ ) than all three formula-fed groups, which did not differ. In both the experiments, there were no significant differences in organ weights, intestinal dimensions and mucosal proportions among treated groups and their respective control groups (data not shown).

**Intestinal mucosal morphology and enzyme activities.** Microscopic examination of haematoxylin and eosin-stained sections of the distal SI revealed obvious pathological differences between pigs macroscopically diagnosed with NEC compared to those pigs diagnosed as healthy. The NEC pigs had extensive bleeding and mucosal atrophy, and in very severe cases (NEC severity score of 5–6), complete loss and disintegration of the mucosa, submucosa and muscularis layers were observed (Fig. 2). Measurement of villus height showed no significant differences between the OPN and control groups in Expt 1 or among the four groups in Expt 2 (Table 2).

There was no statistical difference in the activity of any of the measured enzymes between OPN and control pigs (Expt 1) and among the Gang, SL and control formula groups in Expt 2 (Table 2). Pigs fed bovine colostrum had significantly higher activities of all measured enzymes relative to the control formula group. Compared with the

Gang and SL groups, all the enzyme activities except for sucrase and maltase were also significantly improved with bovine colostrum, most pronounced for lactase (approximately a twofold activity increase, Expt 2, Table 2).

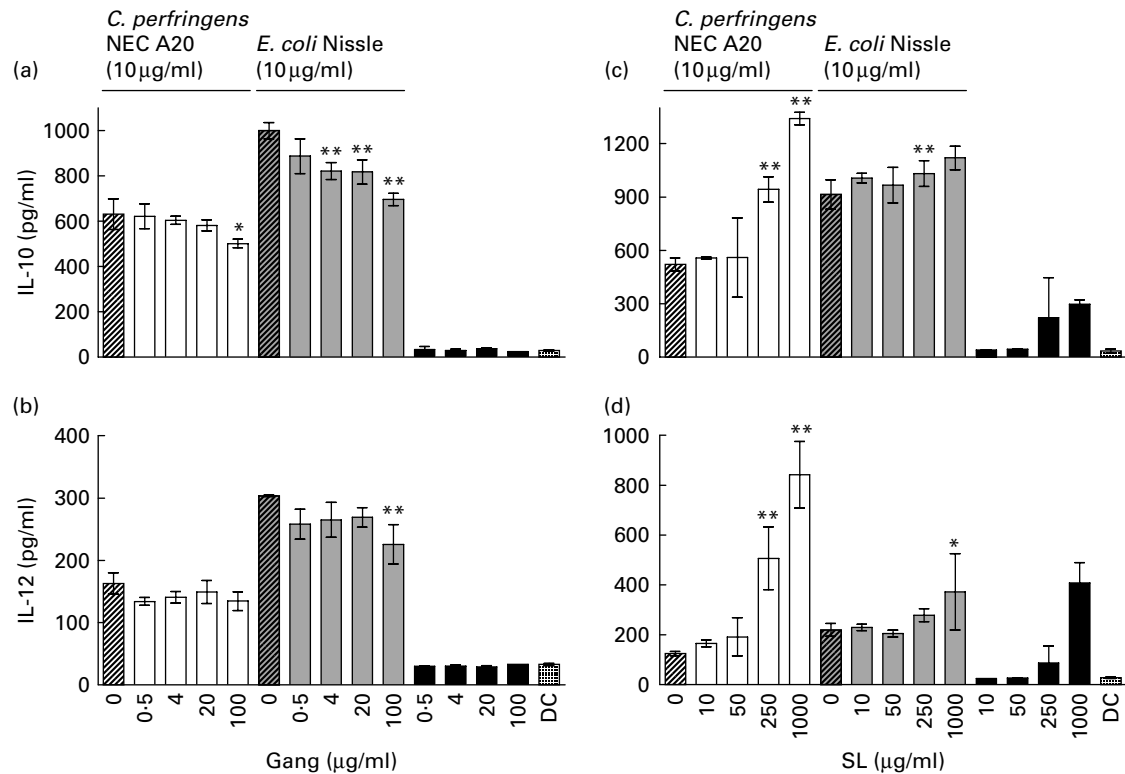
### Expt 3: *in vitro* immunomodulatory activity of milk fractions

For all four measured cytokines (IL-10, IL-12, IL-6 and TNF- $\alpha$ ), there were no significant differences between the bacteria-stimulated DC and the DC co-stimulated with bacteria and 0.1, 0.5, 1 and 5  $\mu\text{g/ml}$  of OPN (data not shown). The Gang-enriched milk product added to DC alone did not induce a cytokine response from the cells (Fig. 3(a) and (b)). High doses of the Gang-enriched milk product co-incubated with DC and bacteria resulted in reduced production of IL-10 and IL-12, with strongest inhibition of *E. coli* Nissle-induced cytokine production (Fig. 3(a) and (b)). There were no effects of Gang on the TNF- $\alpha$  and IL-6 responses (data not shown). When the SL-enriched milk product was added to the DC alone, it induced production of all four cytokines (Fig. 3(c) and (d)). Upon co-stimulation with bacteria, no effect on cytokine production of the SL-enriched milk product was observed for doses lower than 250  $\mu\text{g/ml}$ . Conversely, higher concentrations resulted in a general increase in cytokine production independently of bacterial stimulation (Fig. 3(c) and (d), IL-6 and TNF- $\alpha$  data are not shown).

Defatted colostrum was associated with high mortality of DC, and only 0.9% colostrum was tolerated by the cells resulting in similar effects as for low concentrations of whey (data are not shown). Therefore, cytokine production was measured after co-incubation with 10  $\mu\text{g/ml}$  *C. perfringens* NEC A20 or *E. coli* Nissle 1917 and 8.3, 2.8 and 0.9% (v/v) of bovine colostrum whey, which was not lethal to the DC. Colostrum whey alone induced some production of all four cytokines except IL-12 (Fig. 4). The whey fraction caused a bacteria-dependent modulation of the cytokine response from the stimulated DC. For *E. coli*-stimulated cells, whey caused a dose-dependent decrease in production of all cytokines (Fig. 4(a)–(d),  $P < 0.01$  and 0.001), while inhibiting effects on cytokine production were modest after *C. perfringens* stimulation. Production of IL-10 was induced with whey only after *C. perfringens* stimulation (Fig. 4(a),  $P < 0.05$  and 0.01).

### Discussion

The results show that enteral feeding with bovine colostrum reduces NEC lesions in preterm pigs, relative to formula, as shown previously for porcine colostrum<sup>(29,33–35,40,42,43)</sup>. It now remains to be investigated whether bovine colostrum, or fractions thereof, might also prevent gut inflammation and NEC in preterm infants lacking access to mother's milk. Formulas enriched with

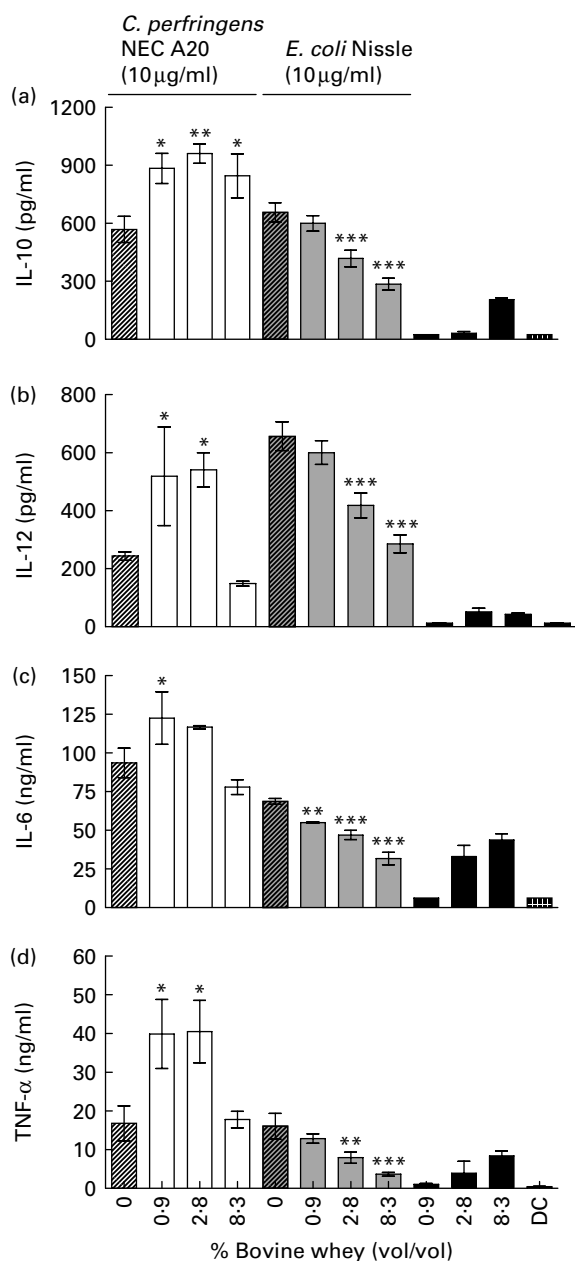


**Fig. 3.** IL-10 ((a) and (c)) and IL-12 ((b) and (d)) expression in dendritic cells (DC) co-incubated with 10 µg/ml *Clostridium perfringens* NEC A20 or *Escherichia coli* Nissle 1917 and different concentrations of sialic acids (SL) and gangliosides (Gang). The results are means and standard deviations of three replicates from one experiment, representative of two separate experiments. Values are compared to their respective controls without SL and Gang (striped bars). Mean values were significantly different: \* $P < 0.05$ , \*\* $P < 0.01$ .

Gang or SL failed to improve resistance in pigs, while OPN supplementation was associated with a modest decrease in NEC without any consistent effects on gut structural and functional indices. The potential immune-modulating activity of bovine colostrum was supported by the effects of colostrum whey on DC stimulated *in vitro* with either *E. coli* Nissle 1917 (a commensal probiotic bacteria) or *C. perfringens* NEC A20 (an opportunistic NEC pathogen). In contrast, the enriched milk fractions showed no consistent effects, except that high concentration of Gang reduced the IL-10 and IL-12 production in bacteria-stimulated DC.

*In vivo*, bovine colostrum improved several gut functional indices (galactose and mannitol absorption, dipeptidyl-peptidase IV, aminopeptidase A, aminopeptidase N and lactase activities), while the effects of enriched formulas were limited or absent, at least for the doses chosen in the present study. The activities of six intestinal brush-border enzymes were elevated in pigs fed bovine colostrum, relative to formula, confirming previous findings with porcine colostrum<sup>(29,33,34,40)</sup>. Digestive enzyme activities and nutrient absorptive capacity are sensitive parameters to detect digestive dysfunction, and they often correlate well with gut integrity and degree of inflammation<sup>(44)</sup>. In the present study, both the galactose and mannitol absorptions were reduced 1 d after initiation of enteral formula feeding. Other recent studies have indicated that this formula-induced decrease in nutrient

absorptive capacity may take place a few hours after the start of feeding<sup>(45)</sup>, coinciding with a very rapid formula-induced increase in tissue inflammatory markers such as IL-1 $\beta$ <sup>(31)</sup>, but before structural changes. In the present study, colostrum did not improve mucosal function by stimulating structural integrity, since we found no significant treatment differences for villus height, mucosa proportion or intestinal weight in the two experiments. Mean villus height in the bovine colostrum group was relatively low (472 µm) compared with preterm pigs fed porcine or bovine colostrum starting from the time of birth, without a TPN period (approximately 600 µm)<sup>(30,34)</sup>. Colostrum was not fully protective, as two of the five colostrum pigs showed mild NEC lesions in the distal intestine and colon. In agreement with our earlier observations<sup>(29)</sup>, a few days of TPN after birth may predispose preterm pigs to moderate inflammatory lesions, even when a near-optimal diet (porcine or bovine colostrum and OPN-enriched formula) is used as an enteral diet after TPN. Furthermore, we have also previously observed that minimal enteral doses of bovine colostrum during TPN followed by full enteral nutrition with bovine colostrum fully protect against NEC<sup>(46)</sup>. These previous findings with bovine colostrum indicate that it is only fully protective against NEC in the preterm pig model if given immediately after birth as it seems to counteract the detrimental effects of initial TPN.



**Fig. 4.** IL-10 (a), IL-12 (b), IL-6 (c) and TNF- $\alpha$  (d) expression in dendritic cells (DC) co-incubated with 10  $\mu$ g/ml *Clostridium perfringens* NEC A20 or *Escherichia coli* Nissle 1917 and different concentrations of a whey fraction from bovine colostrum. The results are means and standard deviations of three replicates from one experiment, representative of two separate experiments. Values are compared to their respective controls without whey (striped bars). Mean values were significantly different: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

*In vitro*, bovine colostrum whey caused a bacteria-dependent modulation of cytokine responses from stimulated DC. In recent studies with the DC model, we have found similar modulatory effects of porcine and human whey, indicating conserved immunomodulatory effects between different species (HK Møller, unpublished results). In contrast, the enriched milk fractions showed no consistent effects, except high concentration of Gang,

which reduced the IL-10 and IL-12 production in bacteria-stimulated DC.

*In vitro*, it has recently been shown that a bovine colostrum protein concentrate is able to modulate cytokine production from lipopolysaccharide-stimulated human peripheral blood mononuclear cells by increasing release of IL-2 while suppressing early production of TNF, IL-6 and IL-4<sup>(47)</sup>. The same study found an increase in IL-10 and IL-2 production and inhibition of interferon- $\gamma$  and TNF, when cells were stimulated with phytohaemagglutinin in the presence of colostrum<sup>(47)</sup>. Another study found that bovine colostrum was able to modulate interferon- $\gamma$  production in stimulated human peripheral blood mononuclear cells depending on the strength and type of antigenic stimulation<sup>(48)</sup>. Our findings using murine DC extend these properties to cell types other than peripheral blood mononuclear cells. As the present results were obtained using the whey fraction of colostrum, they, together with the Shing *et al.* study<sup>(47)</sup>, indicate that the immunomodulatory properties may be due to bioactive whey proteins. Previously, we have found immunosuppressive activity of sialylated milk proteins on the DC cytokine response to lipopolysaccharide, which again supports the notion that the proteins of colostrum play an important role in preventing inflammation<sup>(28)</sup>. It must also be noted that the *in vitro* data indicate a stimulus dependency of the effects of colostrum, which may be an important regulatory mechanism behind the protective effects of colostrum against inflammation *in vivo*.

The present study is the first to test luminal effects of OPN in a porcine model of gut immaturity and inflammation. Based on the immune-modulating effects of OPN documented by others under other conditions<sup>(4,5,8,11,13,14,49–51)</sup>, we hypothesised that milk-derived OPN played a role in the control of inflammatory responses in neonates. OPN exhibits pro-survival and anti-apoptotic properties, and thus aids cell survival<sup>(50)</sup>, wound healing and matrix formation<sup>(11,12,52)</sup>, supporting the observed tendency to positive clinical effects in the present study, where OPN may positively affect rebuilding of necrotic mucosa and tissue or through anti-apoptotic signals promoting epithelial cell survival. Further studies on OPN concentrations and mode of administration (enteral *v.* parenteral) are warranted. Although OPN significantly reduced NEC severity, the clinical effects of OPN on gut function parameters using the present treatment regimen were minimal. It is possible that OPN effects are developmentally dependent and that the gut-associated lymphoid tissue is too immature in preterm pigs to be able to fully respond to OPN.

Developmental immaturity may also contribute to the lacking effects of SL- and Gang-enriched formulas. Furthermore, the concentrations of these bioactives in the formulas may have been too low to provide beneficial effects *in vivo*. The finding that SL only affected DC



function at very high concentrations could indicate that a DC-stimulating contaminant, such as lipopolysaccharide, may have been present in the enriched formula. Different structures of Gang have been shown to exhibit immunosuppressive effects on various immunocompetent cell types *in vitro*, and these effects seem to be dependent on the Gang structure and type of immune cell affected<sup>(17,18,24)</sup>. Clinically, a Gang-enriched formula has been found to stimulate the development of a beneficial gut microbiota in preterm infants, relative to control formula<sup>(19)</sup>. Milk Gang and conjugated sialylated carbohydrates (glycoproteins and glycolipids) may also serve as false receptor analogues for bacterial adhesins, and thus be able to modify the intestinal microbiota in the neonate serving as a beneficial probiotic.

As in human milk, the predominant Gang of bovine milk are disialganglioside 3 (GD3) and monosialganglioside 3 (GM3), but in general, human milk contains higher amounts of Gang than bovine milk<sup>(7,19)</sup>. Hence, the Gang-rich milk product employed in the present study most likely contains GD3 and GM3, and concentrations approached those in human milk. We observed a dose-dependent suppression of *E. coli* Nissle 1917-induced IL-10 and IL-12 cytokine production, probably due to the effects of GD3 and GM3. This slight immunosuppressive effect of the Gang milk product on the DC cytokine response confirms our previous findings with pure Gang<sup>(18)</sup>. In conclusion, bovine colostrum was superior to milk formulas enriched with milk OPN, Gang or SL in protecting against NEC in preterm piglets. OPN tended to reduce NEC as indicated by a clearly reduced severity score but without any consistent improvement of structural and functional indices. The modest effect of milk bioactives may be explained by the immature state of the gut immune system in preterm pigs. Equally well, the effects of the enriched formulas may just be minimal compared with that of the synergistic actions of many bioactives present in intact bovine colostrum. These synergistic actions of colostrum bioactives appear to include inhibitory effects on bacteria-stimulated DC cytokine release.

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and P. T. S. wrote the paper. H. K. M. had primary responsibility for the final content. All authors read and approved the final manuscript.

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