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Støy, Ann Cathrine Findal; Heegaard, Peter Mikael Helweg; Sangild, Per Torp

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The effect of bovine colostrum products on intestinal dysfunction and inflammation in a preterm pig model of necrotizing enterocolitis



PhD thesis
Ann Cathrine Findal Støy
December 2012

**The effect of bovine colostrum products on
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Ann Cathrine Findal Støy
2012

Innate Immunology Group
Section for Immunology and Vaccinology
National Veterinary Institute
Technical University of Denmark

Supervisors

Professor Peter M. H. Heegaard
Innate Immunology Group
Section for Immunology and Vaccinology
National Veterinary Institute
Technical University of Denmark

Professor Per T. Sangild
Clinical and Experimental Nutrition Group
Department of Human Nutrition
University of Copenhagen

Assesment committee

Senior Scientist Kirstine Klitgaard Schou (chairperson)
Section for Bacteriology, Pathology and Parasitology
National Veterinary Institute
Technical University of Denmark

Professor Hanne Frøkiær
Department of Veterinary Disease Biology
Faculty of Health and Medical Sciences
University of Copenhagen

Professor Björn Weström
Department of Biology
Lund University

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Front cover: Premature pig (Støy 2008)

Back cover: IPEC-J2 cells infected with *C. perfringens* (Støy 2011)

The effect of bovine colostrum products on intestinal dysfunction and inflammation in a preterm pig model of necrotizing enterocolitis

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Preface and acknowledgements

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Summary

Necrotizing enterocolitis (NEC), primarily seen in preterm infants, is associated with high morbidity and mortality. The pathogenesis is not fully understood but risk factors include prematurity, enteral feeding (especially with milk formula), and the intestinal microbiota. Mother's milk, rich in bioactive factors, has a protective effect against NEC, but not all preterm infants are able to receive mother's milk. The overall aim of this thesis was to investigate if bovine colostrum (BC), also rich in bioactive factors, could serve as an alternative to mother's milk. A preterm pig model of NEC was used to investigate the effect of BC products on intestinal structure, digestive and absorptive functions, microbiota, plasma and tissue proteins and tissue gene expression levels of inflammatory markers.

In **Study I**, the aim was to investigate if BC could correct intestinal dysfunction and reduce inflammation induced by total parenteral nutrition (TPN) combined with an abrupt transition to milk formula, which has shown to initiate detrimental intestinal responses. A group of preterm pigs fed milk formula followed by BC was compared with groups of preterm pigs fed either milk formula or BC alone after the TPN period. This study showed that BC feeding restores intestinal dysfunction and reduces a proinflammatory response induced by short term (6 hours) formula feeding to preterm TPN-fed pigs.

A prerequisite for the use of BC in clinical settings is that a standardized product is readily available, and in **Study II** it was investigated if pasteurized and/or spray dried BC had similar beneficial effects on intestinal dysfunction and inflammation as fresh BC. Preterm pigs were given TPN for two days followed by 6 hours of formula feeding before given either formula, BC, spray dried BC, or pasteurized and spray dried BC. The study showed that even though spray drying and pasteurization affected BC proteins, pasteurized and/or spray dried BC decreased the severity of NEC in pigs compared with milk formula, while a tendency towards lower NEC severity was observed in pig fed raw BC compared with milk formula. All three BC products maintained trophic and anti-inflammatory effects on the immature pig intestine.

A simple and standardized system was required to investigate the effects of milk formula versus BC on intestinal epithelial cells. In **Study III**, the IPEC-J2 cell line was evaluated as an *in vitro* model for the premature pig intestine. It was investigated if diet-induced effects could be observed on the expression of 48 epithelial- and immune response-related genes in IPEC-J2 cells stimulated with milk formula, BC or growth medium. Distal small intestinal samples from preterm pigs fed milk formula or colostrum were included for comparison. This study showed that careful considerations must be made prior to gene expression analysis of diet-induced responses in IPEC-J2 cells as a system for the premature intestine, since no diet-induced effects in the IPEC-J2 cells were detected.

C. perfringens is a pathogen associated with NEC in preterm infants and pigs, and in **Study IV**, the association between NEC and the abundance of *C. perfringens* and total bacteria in intestinal samples from preterm pigs was investigated using quantitative polymerase chain reaction. Furthermore, host-pathogen interactions were investigated in IPEC-J2 cells by analyzing the expression of 48 epithelial- and immune response-related genes after stimula-

tion with *C. perfringens*. An association between the abundance of total bacteria and *C. perfringens* and NEC was observed, and an *in vitro* study in IPEC-J2 cells showed that increased numbers of *C. perfringens* were associated with changes in gene expression response.

To increase the effect of BC against *C. perfringens*, **Study V** aimed to investigate if BC with increased activity toward *C. perfringens* could be produced. Western blot confirmed that anti-*C. perfringens* hyperimmune BC could be produced by immunization of pregnant cows with a clostridial specific vaccine.

In conclusion, BC products have a beneficial effect on the intestinal environment in preterm pigs based on improvements in intestinal structure, digestive and absorptive functions, the microbiota, and by reduced level of inflammatory markers. BC products, modified to meet the nutritional requirements of preterm infants, may serve as valuable alternatives to mother's milk during the first critical days after birth. However, well-designed studies in preterm infants are required to investigate if BC is a safe and effective alternative for these vulnerable infants when mother's milk is not available.

Sammendrag

Nekrotiserende enterocolitis (NEC), som primært ses hos præmature spædbørn, er forbundet med høj morbiditet og mortalitet. Patogenesen er ikke kendt fuldt ud, men risikofaktorer inkluderer præmaturitet, enteral ernæring (især moderermælksersætning) og tarmmicrobiotaen. Moderermælk, rig på bioaktive faktorer, har en beskyttende effekt imod NEC, men ikke alle for tidligt fødte spædbørn har mulighed for at få moderermælk. Formålet med denne afhandling var at undersøge om kocolostrum (BC), som også er rig på bioaktive faktorer, kunne tjene som et alternativ til moderermælk. En præmatur grisemodel for NEC blev anvendt i studierne til at undersøge effekten af BC produkter på tarmens struktur, fordøjelses- og absorptionskapacitet, microbiota, og inflammatoriske markører målt på både vævs- og plasma- proteinniveauer og på vævs-geneekspression niveau.

Formålet med **Studie I** var at undersøge, om BC kunne korrigere tarm dysfunktion og inflammation induceret af total parenteral ernæring (TPN) kombineret med en brat overgang til moderermælksersætning, hvilket har vist at igangsætte skadelige reaktioner i tarmen. En gruppe præmature grise fik moderermælksersætning efterfulgt af BC og blev sammenlignet med grupper af præmature grise, som fik enten moderermælksersætning eller BC efter TPN. Dette studie viste, at BC ernæring kunne reversere tarmdysfunktion og reducere inflammation forårsaget af kortvarig (6 timer) ernæring med moderate mængder af moderermælksersætning til præmature TPN-ernærede grise.

En forudsætning for brug af BC i klinikken er, at et standardiseret produkt er let tilgængeligt, og **Studie II** undersøgte om pasteuriseret og/eller spraytørret BC var lige så gavnligt som frisk BC. Præmature grise fik TPN i to dage efterfulgt af 6 timers moderermælksersætning for de fik enten moderermælksersætning, BC, spraytørret BC, eller pasteuriseret og spraytørret BC. Studiet viste, at selvom spraytørring og pasteurisering påvirker BC proteiner, så blev en lavere grad af NEC observeret i grise, som fik spraytørret BC, eller pasteuriseret og spraytørret BC, mens grise, som fik BC, havde en tendens til lavere grad af NEC en moderermælksernærede grise. All BC produkterne bevarede trofiske og antiinflammatoriske virkninger på den umodne tarm.

Et simpelt og standardiseret system var nødvendigt til at undersøge effekterne af moderermælksersætning versus BC på tarmepitelceller. I **Studie III** blev IPEC-J2 cellelinien vurderet som en mulig *in vitro* model for den præmature grisetarm. Diæt-inducerede forskelle på ekspressionen af 48 epitel- og immunrespons-relaterede gener blev undersøgt i IPEC-J2 celler stimuleret med moderermælksersætning, BC eller vækstmedium. Vævsprøver fra den distale tyndtarm fra præmature grise ernæret med moderermælksersætning eller BC blev inkluderet til sammenligning. Studiet viste, at grundige overvejelser skal foretages inden geneekspressionsanalyse af diæt-inducerede reaktioner i IPEC-J2 celler som et system for den præmature tarm, da diæt-inducerede effekter i IPEC-J2 cellerne ikke kunne observeres.

C. perfringens er en patogen bakterie associeret med NEC hos præmature spædbørn og grise, og i **Studie IV** blev mængden af *C. perfringens* samt total bakterier i tarmprøver fra præmature grise kvantificeret med qPCR. Endvidere blev vært-patogen interaktioner undersøgt i

IPEC-J2 celler ved at analysere ekspressionen af 48 epitel- og immunrespons-relaterede gener i IPEC-J2 celler stimuleret med *C. perfringens*. En association mellem *C. perfringens* eller total bakterier og NEC blev observeret, og *in vitro* studiet viste, at et øget antal *C. perfringens* er forbundet et ændret immunrespons. For at øge BCs aktivitet imod *C. perfringens*, blev det i **Studie V** undersøgt om hyperimmunt colostrum med øget aktivitet mod *C. perfringens* kunne produceres. Western blot blev anvendt til at bekræfte, at anti-*C. perfringens* hyperimmunt BC blev produceret ved immunisering af drægtige køer med en clostridia specifik vaccine.

Sammenfattende kan det konkluderes, at BC produkter har en gavnlig effekt på tarmmiljøet hos præmature grise baseret på en forbedring i tarmens struktur, fordøjelses- og absorptionsfunktioner, mikrobiotaen og et reduceret niveau af inflammatoriske markører. BC produkter, modificeret så de opfylder de ernæringsmæssige krav fra det præmature spædbarn, kan være værdifulde alternativer til modermælk i de første kritiske dage efter fødslen. Imidlertid er vel-designede studier i præmature spædbørn nødvendige for at undersøge om BC er sikkert og effektivt at bruge som ernæring for disse sårbare spædbørn, når modermælk ikke er tilgængelig.

Abbreviations

ApA	Aminopeptidase A	NEC	Necrotizing enterocolitis
ApN	Aminopeptidase N	NTC	No-template control
BC	Bovine colostrum	OA	Organic acids
BHI	Brain hearth infusion	PAF	Platelet-activating factor
BW	Body weight	PBS	Phosphate buffered saline
CD	Cluster of differentiation	PCA	Principal component analysis
cDNA	Complementary deoxyribonucleic acid	PCR	Polymerase chain reaction
CFU	Colony forming units	POW	Gamma-irradiated spray dried bovine colostrum
COLOS	Gamma-irradiated bovine colostrum	POWPAS	Pasteurized, gamma-irradiated spray dried pasteurized bovine colostrum
Cq	Quantification cycle	PUFA	Polyunsaturated fatty acids
DNA	Deoxyribonucleic acid	qPCR	Quantitative PCR
DPPIV	Dipeptidyl peptidase IV	RIN	RNA integrity number
ELISA	Enzyme-linked immunosorbent assay	RNA	Ribonucleic acid
FISH	Fluorescence <i>in situ</i> hybridisation	rRNA	Ribosomal RNA
IECs	Intestinal epithelial cells	RT-qPCR	Quantitative real-time reverse transcription PCR
Ig	Immunoglobulin	SDS-page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
IGF	Insulin-like growth factor	SEM	Standard error of the mean
IL	Interleukin	sIgA	Secretory IgA
Lf	Lactoferrin	SAA	Serum amyloid A
M	Molecular weight marker	TGF	Transforming growth factor
MEN	Minimal enteral nutrition	TLR	Toll-like receptor
MOI	Multiplicity of infection	TNF	Tumor necrosis factor
mRNA	Messenger RNA	TPN	Total parenteral nutrition
MSR	deMan, Rogosa and Sharpe		

Chapter 1 Introduction

When an infant is born prematurely, major concerns rise for the parents regarding the short- and long-term perspectives for their child due to the high risk of complications. One of the most common critical and potentially lethal diseases in the preterm gastrointestinal tract is necrotizing enterocolitis (NEC), which contributes significantly to infant morbidity and mortality in neonatal intensive care units. The primary predisposing factor for NEC is prematurity, but enteral feeding (especially with milk formula) and abnormal bacterial colonization are considered prerequisites for NEC (1-3).

Mother's milk is rich in bioactive factors that support, protect, and mature intestinal function and structure, immune system, and microbiota (4). Bovine colostrum (BC) also rich in bioactive factors could be an alternative for the preterm infant when mother's milk is not available.

The focus of this PhD project was to investigate the efficacy of BC and BC products on intestinal dysfunction and inflammation in a preterm pig model of NEC. The specific objectives that were addressed are

1. To investigate the ability of BC to restore intestinal dysfunction and reduce inflammation induced by total parenteral nutrition (TPN) combined with an abrupt transition to enteral feeding with milk formula.
2. To compare the efficacy of pasteurized and/or spray dried BC to raw BC in the treatment of intestinal dysfunction and inflammation.
3. To characterize the IPEC-J2 cell line as an *in vitro* model for the premature pig intestine based on the expression of multiple intestinal genes.
4. To determine the association between *C. perfringens* and total bacterial abundance in the distal small intestine with NEC.
5. To determine if hyperimmune BC against *C. perfringens* could be produced by immunization of cows with clostridial antigens.

Following this Introduction (Chapter 1), three chapters (Chapter 2, The preterm gut; Chapter 3, Colostrum; and Chapter 4, Models and Methods) will provide background knowledge for a discussion (Chapter 5) and conclusion (Chapter 6) of the objectives. The objectives were investigated through two papers submitted to peer-reviewed journals, one paper ready for submission, and two works still in progress.

Paper I (Støy *et al.* (I)): Ann Cathrine F. Støy, Peter M. H. Heegaard, Thomas Thymann, Mette Bjerre, Kerstin Skovgaard, Mette Boye, Barbara Stoll, Mette Schmidt, Bent B. Jensen, Per T. Sangild. Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs. Submitted to *Clinical Nutrition*.

Paper II (Støy *et al.* (II)): Ann Cathrine F. Støy, Per T. Sangild, Kerstin Skovgaard, Thomas Thymann, Mette Bjerre, Dereck E. W. Chatterton, Stig Purup, Mette Boye, Peter M. H. Heegaard. Mild heat treatment does not reduce the colitis-protective effects of bovine colostrum in preterm pigs. Ready for submission.

Paper III (Støy *et al.* (III)): Ann Cathrine F. Støy, Peter M. H. Heegaard, Per T. Sangild, Mette V. Østergaard, Kerstin Skovgaard. Gene expression analysis of the IPEC-J2 cell line as a simple system for the inflammation-sensitive preterm intestine. Submitted to *Comparative and Functional Genomics*.

Paper IV (Støy *et al.* (IV)): Ann Cathrine F. Støy, Camilla L. Delègue, Thomas Thymann, Per T. Sangild, Peter M. H. Heegaard, Kerstin Skovgaard, Sarmauli Manurung, Lars Mølbak. Total bacterial and *C. perfringens* abundance is associated with necrotizing enterocolitis in preterm pigs, and *C. perfringens* induces gene expression changes in IPEC-J2 cells. Work in progress.

Conference presentation (Støy *et al.* (V)): Ann Cathrine F. Støy, Per T. Sangild, Peter M. H. Heegaard. Bovine colostrum with hyperimmunity against Clostridia. Poster presentation at the “Alternatives to Antibiotics” Symposium in Paris, 25–28 September 2012. Work in progress.

Chapter 2 The preterm gut

A preterm infant is born before the 37th week's gestation with varying degree of immaturity of the organs including the gastrointestinal tract, which is a key risk factor for the development of NEC. In this section, a short description of NEC will be given, followed by a discussion of how immaturity of the intestine and the gut immune system, and an abnormal microbiota contributes to the pathogenesis of NEC. Throughout this chapter reference will be made to humans unless otherwise stated.

Necrotizing enterocolitis

NEC is the most common gastrointestinal disease in premature infants, affecting up to 12% of infants in neonatal intensive care units. Preterm infants account for 90% of the cases, and NEC is inversely related to gestational age at birth and birth weight, thereby making prematurity a key risk factor (1;5;6). Other contributing factors are bacterial colonization and enteral feeding, especially with milk formula. Altered blood flow leading to ischemia and mucosal injury may also contribute to the pathogenesis of NEC (1-3;7).

The most frequently affected part of the gastrointestinal tract is the ileo-colic region where a mucosal or transmural necrotic segment develops, but gangrene may extend from the stomach to the rectum (3;8-10). The affected part of the intestine becomes dull, dilated, and grey, and extensive hemorrhage and pneumatosis intestinalis may be observed (9-12). The staging system proposed by Bell *et al.* (13) can be employed to diagnose and classify the severity of NEC (Table 1). The onset can be insidious or fulminant and is characterized by inflammation and necrosis. The disease ranges from mild cases with diarrhea and occult blood in the stool to severe cases with pneumatosis intestinalis and transmural necrosis that rapidly can progress into peritonitis, sepsis, shock, or multisystem organ failure. NEC is fatal in up to 40% of the cases (1;5;6;9;14).

Due to the incomplete understanding of the mechanisms leading to NEC, the management strategies are only partly effective in many patients (15). Most cases of NEC are medically managed, and supportive care and medical stabilization including termination of enteral nutrition and provision of antibiotics is initiated when NEC is suspected (9;11;16). However, in approximately half of the cases, intestinal resection is necessary, which contributes to a considerable subsequent morbidity and a mortality rate of up to 60% (1;5;9).

The most serious long-term postoperative complication is short bowel syndrome that may result in growth failure and malnutrition, and moreover patients surviving NEC are at risk of impairments including increased risk of functional and developmental delay that affect family life (5;9;14;17). As presented here, NEC is a severe gastrointestinal disease with short- and long-term consequences for both the child and its parents, and being a major societal cost (18), and a disease with no exact prevention- or treatment strategy, this justifies the significant research done within this field.

Table 1. Modified Bell's criteria for staging of NEC (*adapted from (13;19)*).

Stage	Abdominal	Systemic	Radiographic
Stage 1a suspected NEC	elevated pregavage residuals mild abdominal distension occult blood in stool	temperature instability, apnea, bradycardia	normal or intestinal dilatation, mild ileus
Stage 1b suspected NEC	grossly bloody stool	same as above	same as above
Stage 2a definite NEC mildly ill	same as stage 1 plus lack of bowel sounds, possible abdominal tenderness	same as above	ileus, pneumatosis intestinalis
Stage 2b definite NEC moderately ill	same as above plus peritonitis, definite abdominal tenderness, possible abdominal cellulitis, right lower quadrant mass	same as stage 1 plus mild metabolic acidosis, mild thrombocy- topenia	same as above plus possible portal venous gas
Stage 3a advanced NEC severely ill; intact bowel	same as above, with marked tenderness and abdominal distention	same as stage 2b plus hy- potension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, dissemi- nated intravascular coagula- tion, and neutropenia	same as above plus ascites
Stage 3b advanced NEC severely ill, perfo- rated bowel	same as stage 3a	same as stage 3a	pneumoperitoneum

The preterm intestine

Intestinal maturation starts during the first trimester, and from mid-gestation the fetus begins to swallow amniotic fluid stimulating intestinal maturation due to its content of several of the same bioactive factor as found in human milk including transforming growth factors (TGF)- β , lysozyme, lactoferrin (Lf) and hormones (2;20-22). At birth, the initiation of enteral feeding and stimuli from bacteria colonizing the intestinal tract will further stimulate development and maturation of the gastrointestinal tract and the gut associated immune system (23;24). Thus, dependent on the gestational age at birth, the preterm infant may be born with a compromised intestinal function compared with mature newborns.

The gastrointestinal tract is a site for nutrient digestion and absorption, but it is also a major immunological defense barrier, and its ability to optimal conduct these tasks in parallel is due to the interplay and synergistic effects of a number of different cell types. Along the small intestine the overall structure is similar (Figure 1); furthest away from the intestinal lumen is the serosa, followed by the muscularis externa, and submucosa. The muscularis mucosae lies between the submucosa and the lamina propria, and is responsible for peristalsis, which is only mature at 8 months of gestation (25-28). The mucosa comprises the lamina propria with Peyer's patches, found only in the ileum, and the apical layer facing the intestinal lumen consist of intestinal epithelial cells (IECs) (27). IECs constitute the major cell population of the mucosal epithelium and consist of four cell types: enteroendocrine-, goblet-, Paneth-, and ab-

sorptive cells (29). The mucosal epithelium has a very large surface area (~200 m²) due to villi, and microvilli on the IECs, and begins to develop between 10 and 12 weeks of gestation (27;29). The small intestinal mucosa of newborn infants has mature crypt-villus architecture with continuous stem cell proliferation, differentiation and migration of cells to the top the villi where they are extruded (30). The IECs are joined by tight junctions responsible for the integrity and selective impermeability of the epithelial barrier (25;29). Tight junctions determine the guts permeability and regulate the fluctuation of fluids, selective permeability to small ions, absorption of nutrients, and functions as one of the first lines of defense towards luminal antigens. However, in preterm infants born before 34–35 weeks of gestation an increased permeability of the intestine is seen, increasing the risk of bacterial and toxin translocation (31).

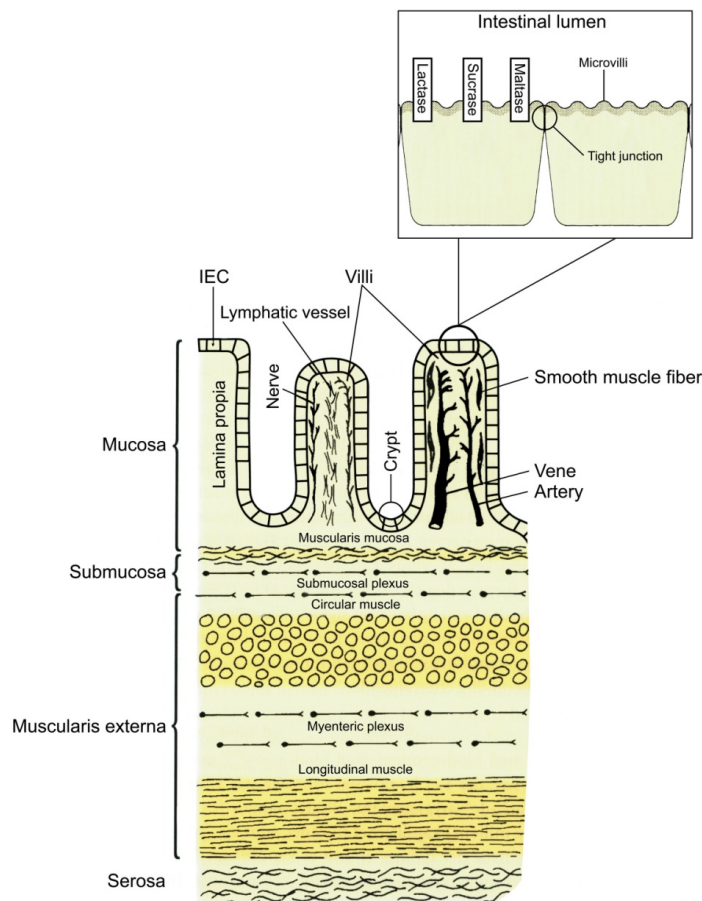


Figure 1. Simplified illustration of the general structure of the small intestinal wall (modified after (27) and reproduced with permission from Polyteknisk Forlag). IEC (Intestinal epithelial cell).

Brush-border enzymes

Mucosal injury as seen in NEC disrupts the crypt-villus architecture leading to reduced enterocyte renewal and villus heights. This has consequences for the intestinal digestive and absorptive functions and for the ability of the preterm infant to thrive. Brush-border enzymes are involved in digestion of carbohydrates and proteins and embedded in the microvilli (Figure 1). Lactase is located at the top of the villi, while sucrase and maltase are located at mid-villus level (32-34). The brush-border enzymes may therefore be lost during mucosal damage. Prematurity disturbs the normal developmental pattern of these enzymes. Although brush-border enzymes appear at 10–11 weeks of gestation (8), they first start to become active at 24 weeks of gestation and as the fetus approaches term a significant rise in activities takes place (29;35). As an example, the last of the disaccharidases to develop is lactase, and at 34 weeks of gestation the activity of lactase is only 30% of that seen in term infants (33;34). After initiation of enteral nutrition, especially mother's milk, the activity of lactase increases (36). This makes the investigation of diet-induced effects on brush-border enzyme function important, so the infant's ability to digest nutrients can be optimized.

The mucosal layer

The mucosal layer is formed by trefoil peptides and mucin glycoproteins secreted from goblet cells, and surfactant lipids secreted by epithelial cells and covers the apical surface of the mucosal epithelium (37;38). Besides being a complex physicochemical barrier between the underlying tissues and the luminal environment, it is also a site for host-bacterial interactions (2;37-39). The expression and structure of glycoconjugates in the microvillus membrane is controlled by the host's genome, and thus genetic susceptibility is a key factor in determining which bacteria have the best affinity to interact with the glycoconjugates (39-41). When indigenous bacteria colonize the intestine, they affect biochemical, physiological, and immunological functions of the host (42;43), and ferment nutrients and produce metabolites like organic acids (OA) and bacteriocins (39-41;44-47). This may affect the interaction of pathogenic bacteria with the mucosal layer and thus may prevent bacteria-induced diseases and intestinal damage. In the preterm gut, the composition of the glycoconjugates differ from that of the mature epithelium, and this immaturity combined with a reduced number of glycoconjugates in the immature epithelium may contribute to the development of an abnormal intestinal microbiota (2). Furthermore, a lack of trefoil factor peptides in infants with NEC is seen compared with healthy infants and adults, and this may impair the function and restoration of the mucosal layer (48).

The preterm gut immune system

The gut immune system is a very important part of the immune system due to the extensive contact and interaction with the external environment (29;39), and it has to respond in a proper way against environmental factors like microbes and nutrients. In addition to specialized immune cells, IECs are also involved in immune responses. Besides their function as a physical barrier, IECs initiate and coordinate the immune response through secretion of cytokines, chemokines and antibacterial peptides (39;49;50). Due to this central role in the immune response, it is relevant to study the IECs response towards diet and *C. perfringens* as performed with the IPEC-J2 cell line in Støy *et al.* (III;IV). Dendritic cell extensions sampling antigens in the intestinal lumen and intraepithelial lymphocytes are distributed between the IECs (29). If an antigen crosses the first line of defense, the innate immune system will be activated followed by the adaptive immune system. The innate immune system provides non-specific responses toward antigens, and is important in the immediate period after birth until activation of the adaptive immune system, which results in the establishment of a long lasting specific memory (28;29;51).

In addition to the impaired intestinal epithelial barrier, the immune system is immature, as seen by deficiencies in complement system proteins (52), and a lower number and functional deficiencies, including decreased cytokine release, of total lymphocytes, B- and T cells (12;53). Furthermore, altered cytokine production in fetal monocytes with impaired interleukin (IL-6) and tumor necrosis factor (TNF)- α synthesis, and reduced phagocytic capacity and expression of several adhesion molecules, but increased level of respiratory burst products in phagocytes (54) are seen in preterm infants compared with term infants and adults. This immaturity increases the risk of infections in the postnatal period, but the encounter with antigens and microbes and processing of these under controlled conditions within the lymphatic system is essential for the maturation of the immune system (12). The interaction between dendritic cells and external antigens like commensal bacteria is of importance for the achievement of a balanced immune system responding properly towards antigens, and immune responses are induced, resulting in either tolerance or activation of an immune response. Upon microbial priming, dendritic cells drive the differentiation of naive T cells into T helper 1 (proinflammatory), T helper 2 (anti-inflammatory/antibody stimulating) or T regulatory cells (maintaining immune cell homeostasis) through cytokine production (28;55-57). This stimulation will also lead to B cell differentiation and the production of antibodies (28).

The immature immune system and necrotizing enterocolitis

Several proinflammatory mediators and markers of inflammation including IL-8, IL-6, platelet-activating factor (PAF), C-reactive protein, serum amyloid A (SAA), and TNF have been found to be implicated in the pathogenesis of NEC due to increased levels in plasma during NEC (58-63). The chemokine IL-8 may play a key role in providing an increased cellular activity at the inflammation site, due to its stimulation of neutrophil migration. The concentra-

tion of IL-8 in plasma has been found to correlate positively with the degree of, severity and onset of symptoms of NEC (58;59). After removal of the necrotic intestinal segment, a decrease in circulating IL-8 was observed (58). In contrast, the level of anti-inflammatory factors varies. The level of plasma acetylhydrolase, which degrades PAF, has been found to be lower in preterm infant (60). The expression of fecal *HBD2* encoding the antimicrobial peptide β -defensin2 has been found to be lower in severe NEC cases compared with cases with a moderate NEC disease course (64). In contrast, the plasma concentration of IL-1 receptor antagonist and IL-10 is elevated in NEC infants compared with preterm infants without NEC (59;61).

A high expression of toll-like receptor (TLR)-4 in intestinal mucosa that recognizes lipopolysaccharides from Gram-negative bacteria has been associated with NEC in both human, and experimental models of NEC, inducing NEC with hypoxia (65;66). This may lead to an increased level of TLR-4, and upon activation with lipopolysaccharides, an increased activity of NF- κ B and consequently an increased proinflammatory response (8;28;67). In contrast, absence of TLR-4 has shown to be protective against NEC in mice and rat models of NEC (65;66).

Immunoglobulin (Ig) A is one of the main effectors of the mucosal immune system. It is released as secretory IgA (sIgA) into the intestinal lumen where it is responsible for immune exclusion by agglutination of microbes, and neutralization of pathogens and bacterial enterotoxins, that otherwise may contribute to bacterial invasion of the intestinal mucosa and translocation that would lead to a systemic immune response (68-70). In relation to *C. perfringens* type A associated NEC (see later section), the prevention of a close contact between this opportunistic pathogenic bacterium and the epithelial barrier is of importance, since this bacterium, as with *C. perfringens* type C, may up regulate toxin production when in close contact with the epithelium (71). The role of the commensal bacteria is of importance for the sIgA production. Bifidobacterial diversity may enhance mucosal sIgA maturation, while lack of bacterial exposure in germ-free animals may result in an underdevelopment of Peyer's patches, which are lymphoid follicles containing a large number of B- and T cells, and an deficiency of sIgA (28;29;72). It may take several weeks before a proper antibody response is provided (51), and not until two weeks after birth, sIgA producing plasma cells begin to appear in the lamina propria. The delay of sIgA may therefore be explained by the decreased number of sIgA producing plasma cells in the intestinal mucosa of newborn infants compared with older infant (73;74).

The microbiota

Colonization of the sterile neonatal gut is a complex process with great variations, and the colonization pattern in preterm infants is different from that of term infants, and these differences in colonization may be important for the development NEC.

In full-term vaginally delivered infants, the initial colonizing bacteria resembles those of the maternal gut- and/or vaginal microbiota and include facultative anaerobic or aerobic bac-

teria like enterobacteria, enterococci, streptococci, staphylococci, clostridia, and lactobacilli (75;76). In contrast, Caesarean section results in colonization of a larger number of bacteria from the environment (76-78), and delays the colonization process in both pigs and humans, but these differences are not permanent (76;79). Hereafter, the type of diet is a major determinant for the composition of the microbiota, but data on the nutritional impact, breast milk versus milk formula, on the microbiota is inconsistent. During the first month of life, which is of importance in relation to NEC, the predominant bacteria are *Bifidobacterium* and *E. coli* followed by *Lactobacillus*, *Bacteroides*, with *Bifidobacterium* being predominating in breast-fed infants while formula fed infants have a more complex microbiota (76;80-85).

The colonization pattern in preterm infants differs from that of full-term vaginally delivered infants. Preterm infants seem to have a lower bacterial density and a delayed colonization. Colonization with the natural inhabitants of the intestine, *Lactobacillus* and *Bifidobacterium*, may be deficient and delayed, which increases the risk of colonization with potentially pathogenic bacteria. This may in part be caused by the relatively aseptic environment in the neonatal intensive care units with isolation in incubators, hygiene procedures, and treatment with antibiotics (85-89).

The microbiota and necrotizing enterocolitis

An association between NEC and several enteric bacteria has been observed including *E. coli* (90;91), *Klebsiella* (90;91), *Enterobacter* (90), *Enterococcus* species and *Candida albicans* (92), *S. fecalis*, *S. aureus*, *B. fragilis* (91), and more strongly *Clostridium* species (90;91;93-95). In contrast, other studies have not observed an association between NEC and a specific bacterial species (89;96-99). The mucosal layer normally prevents close contact between host cells and bacteria however, during intestinal frailty due to immaturity of the gastrointestinal tract as previously discussed, bacteria may invade the intestinal epithelium or translocate if a total breach of the epithelial lining occurs and thereby contribute to further intestinal injury. Since NEC has not been seen in stillborn infants or before the intestine is colonized (3;7;100), the microbiota seems to play an important role in NEC development. Still, it may first be in the late pathogenesis; since the immaturity of the gastrointestinal tract affects the microbiota both in relation to its composition, but it may also affect the behavior of the bacteria, as will be described next for *C. perfringens*.

Gram-positive *C. perfringens* species are opportunistic pathogens associated with NEC in preterm infants, including *C. perfringens* type A (93), which also has been found in preterm pigs suffering from NEC (22;101). In Støy *et al.* (IV) the role of *C. perfringens* in NEC development was investigated, and therefore is its pathogenic properties elucidated in the following.

In preterm pigs with NEC, *C. perfringens* invaded the intestinal tissue, while it was located at the surface of the tissue in healthy pigs (102). This close contact between host cells and bacteria may lead to up regulation of toxins as showed by Vidal *et al.* (71), in which *C. perfringens* type C up regulated toxin production when in close contact with Caco-2 cell. This

may also be seen with other *C. perfringens* subspecies, for example type A, and thus contribute to NEC, since the immature and frail intestine may allow a closer contact between bacteria and host cells. The production of toxins by clostridia may lead to tissue necrosis, and thus may account for a faster development of and more extensive gangrene as seen in clostridia-associated NEC compared with NEC associated with *Klebsiella*, *E. coli*, or *Bacteroides fragilis* (91). However, the concentration of α -toxin from *C. perfringens* type A does not seem to correlate with disease severity or mortality in preterm infants (93). Furthermore, metabolites from carbohydrate fermentation including butyrate, acetate and lactate may be toxic to the intestine (100;103). The inoculation with *C. paraputrificum*, *C. butyricum*, and α -toxin producing *C. perfringens* type A in gnotobiotic quails are strongly implicated in the formation of cecal NEC lesions, possible through a short chain fatty acid profile with a high level of butyrate (100). This may result in more extensive pneumatosis intestinalis and increased incidence of portal venous gas as seen in infants with clostridia-associated NEC compared with NEC associated with *Klebsiella*, *E. coli*, or *Bacteroides fragilis* (91). In a study by Smith *et al.* (99) the presence of *C. butyricum* and *C. paraputrificum* correlated with histological pneumatosis intestinalis. Thus, when the opportunity is given, *C. perfringens* may be strongly implicated in the late events of disease progression and contribute to a rapid and more severe NEC progression than other bacteria.

Summary - necrotizing enterocolitis and the preterm gut

Deficiencies in peristalsis, barrier function and digestive capacities, concomitant with an inappropriate intestinal immune response increase the susceptibility to inflammatory diseases, such as NEC, in preterm infants. Immaturity of the intestine leads to accumulation of undigested nutrients in the intestinal lumen serving as bacterial substrate that can lead to bacterial overgrowth. Together with undigested nutrients, bacteria and toxins may not be flushed from the intestinal lumen due to increased transit time thereby stressing the mucosal barrier. This increases the risk of adherence of potential pathogenic bacteria, and combined with concomitant intestinal frailty due to hyper-permeability and impaired intestinal restitution of an already weakened intestinal barrier the risk of a breach of the intestinal epithelium is significant. A breach of the intestinal barrier increases bacterial and toxin translocation, which may result in pneumatosis intestinalis, and further activation of a systemic immune response. The immature immune response leads to an inflammatory cascade with increased tissue and serum levels of inflammatory mediators. This vicious cycle of events and ongoing inflammatory response may lead to NEC. These events might happen rapidly leading to shock, multisystem organ failure or be fatal. Dietary intervention with colostrum containing a high level of bioactive factors may support the intestine during this immature period by improvement of the intestinal structure and function, dampen an increased inflammatory response and contribute to the establishment of a beneficial microbiota.

Chapter 3 Colostrum

The type of nutrition and the feeding route play a significant role in the pathogenesis of NEC and several different nutritional strategies can be employed for preterm infants. Currently, research is performed in human infants and in several *in vivo* and *in vitro* models of NEC to determine the optimal nutritional strategy in relation to the type of diet, nutritional supplements, and feeding route and volume. Mother's milk is the natural and optimal diet for infants to support their nutritional demands. Nevertheless, alternatives need to be considered, because in several cases, mother's milk cannot be provided.

In the following sections, the focus will be on BC as one alternative to mother's milk. After an introducing section on colostrum and its nutritional composition, the focus will be on bioactive factors in colostrum and milk. Finally, the optimal nutritional strategy for the preterm infant and the effect of processing procedures on bioactive factors in colostrum will be discussed. The primary focus will be on the comparison of bovine and human colostrum, but comparison of bioactive factors in milk will be made if no reference is available on colostrum.

Definition and composition of colostrum

Colostrum not only serves as a basis for the growth of the newborn, it also has a high content of bioactive factors defined by Schrezenmeir *et al.* (104) as "*food components that can affect biological processes or substrates and hence have an impact on body function or condition and ultimately health*". The bioactive factors assist the newborn in the maturational process and provide passive immunity against infections seen in the newborns immediate environment (70;105). Colostrum contains a large number of interacting factors, including proteins, carbohydrates, lipids, vitamins, minerals, electrolytes, and trace minerals, in addition to bacteria and cells (Table 2 (106;107)).

Table 2. Classes of human milk constituents (adapted from (106)).

Proteins	Carbohydrates	Lipids	Vitamins	Minerals	Cells
Caseins	Lactose	Triglycerides	<i>Fat-soluble</i>	Calcium	Leukocytes
α-Lactalbumin	Oligosaccharides	Fatty acids	A and carotene	Phosphorus	Epithelial cells
Lactoferrin	Bifidus factors	Phospholipids	D	Magnesium	
Immunoglobulins	Glycopeptides	Sterols	E	Potassium	Bacteria ^a
β-Lactoglobulin		Hydrocarbons	K	Sodium	
Lysozyme			<i>Water-soluble</i>	Chlorine	
Enzymes			Thiamin	Sulfur	
Hormones			Riboflavin	<i>Trace elements</i>	
Growth factors			Niacin	Iodine	
Non-protein nitrogen compounds			Pantothenic acid	Iron	
Urea			Biotin	Copper	
Creatine			Folate	Zinc	
Creatinine			Vitamin B ₆	Manganese	
Uric acid			Vitamin B ₁₂	Selenium	
Glucosamine			Vitamin C	Chromium	
α-Amino nitrogen			Inositol	Cobalt	
Nucleic acids			Choline		
Nucleotides					
Polyamines					

^aReferences (107;113)

In humans, colostrum is produced during the first five days after birth, and gradually changes to mature milk during the first two weeks after birth (108;109). In this thesis, the following definitions are used regarding human milk. Preterm human colostrum and milk were collected from mothers giving birth preterm (~29 week of gestation), while full term colostrum and milk were collected from mothers giving birth at full term (> 37 week of gestation). Preterm and full term colostrum were collected at day 3–5 after birth, while preterm and full term milk were collected between day 8–29 after birth (110;111).

BC is referred to as milk produced during the first 48 hours after parturition, and the highest amount of bioactive factors is found in colostrum from the first milking after birth (4;112). Therefore, colostrum from the first milking was used in the studies included in this thesis.

In addition to a fluctuation of the amount of nutrients over time, the composition of colostrum is species dependent, it varies between individuals within a species, within each nursing, and is influenced by the health status of the mother (106;109). Fluctuations of macronutrients: proteins, carbohydrates and lipids over time and within species are evident (Table 3) and underline that the neonate has specific needs at a certain time point and between species. This should be considered when formulating nutritional products to human infants based on BC and bovine milk. This is also an issue in the translational studies included in this thesis, in which bovine products are tested in preterm pigs, for which sow's colostrum and milk are the optimal nutrient (Table 3). However, BC may be equal to sow's colostrum in decreasing the risk of NEC development in preterm pigs (102). Besides, sow's colostrum will probably not be considered as an alternative nutritional source in the clinics due to cultural considerations and production difficulties. This justifies the investigation of the possible use of BC products as possible nutritional alternatives for the human infant, and the use of a preterm pig model of NEC to study its effects.

Proteins

Milk proteins are broadly classified as caseins and whey proteins, and dependent on the species, caseins constitute up to 80% of the total protein fraction (116). In human milk the whey-to-casein ratio is 70:30 while it is 20:80 in bovine milk (117;118). Caseins: α -casein, β -casein and κ -casein, are phosphoproteins present in colostrum and milk only, and form micelles with calcium, phosphate, and magnesium ions (106). In the stomach, caseins are precipitated by gastric acids and enzymes including chymosin, and form a curd that retains the caseins in the stomach for a longer period of time than whey proteins, which remain soluble in the milk serum (109;116;119). In the polypeptide chain of caseins, bioactive factors with possible immune-modulating and antimicrobial capacities are hidden and can be released during enzymatic digestion of caseins (116;120). Whey proteins such as Ig, Lf, lactalbumin, lactoglobulin, and serum albumin passes through the stomach to the small intestine after clotting of casein (45;116;118).

The protein content is higher in preterm human milk than full term human milk (110). Furthermore, the content of several free amino acids, especially the essential amino acids, is significantly higher in preterm human milk than full term human milk (121).

Table 3. Macronutrient composition in human, bovine and porcine colostrum and milk^a. Additionally, the composition of the products used in the studies in this thesis is shown.

	Total energy (Kcal/L)	Protein (g/L)	Lactose (g/L)	Lipids (g/L)
Human				
Preterm colostrum	580 ± 20 ^b	~23 ^{b, c}	50.4 ± 1.2 ^b	30.0 ± 2.3 ^b
Preterm milk	710 ± 20 ^b	~15 ^{b, c}	59.7 ± 1.0 ^b	43.3 ± 2.4 ^b
Full term colostrum	480 ± 30 ^b	~20 ^{b, c}	51.4 ± 2.2 ^b	18.5 ± 3.5 ^b
Full term milk	590 ± 20 ^b	~15 ^{b, c, d}	~70 ^{b, d}	~35 ^{b, d}
Bovine				
Colostrum ^e	1430 ^f	~150 ^{f, g}	~26 ^g	~60 ^{f, g}
Milk	660 ^f	32 ^{f, g}	~50 ^g	~45 ^{f, g}
Porcine^h				
Colostrum	~1500	~138	~32	~60
Milk	~1070	~53	~55	~66
Products used in the studies included in this thesisⁱ				
Colostrum				
COLOS	1000	110	35	50
POW	900	100	35	40
POWPAS	900	95	45	35
Milk formula				
	1100	60	60	70

^a Data are presented as mean ± standard error of mean, or as an approximate value (~) when estimated from several studies or if SEM was not given.

^b (110).

^c (111).

^d (106).

^e First milking.

^f (112).

^g (114).

^h All data on sow's colostrum and milk are obtained from (115).

ⁱ Gamma-irradiated BC (COLOS), gamma-irradiated, spray dried BC (POW), pasteurized, gamma-irradiated spray dried BC (POWPAS).

Carbohydrates

Lactose is the principal carbohydrate in human milk and consists of D-galactose and D-glucose bonded through a β(1-4) glycosidic linkage, and is hydrolyzed by lactase (β-galactosidase) to galactose and glucose, which are absorbed in the small intestine (33;106;122). In contrast to protein and fat, the concentration of lactose is lower in preterm human milk than in full term human milk (110).

Human milk contains a range of moderate-chain-length carbohydrates, oligosaccharides and glycoconjugates with bioactive abilities (106). In contrast, the level of oligosaccharides in bovine milk is very low (123;124). Oligosaccharides function as prebiotics, which are non-digestible food compounds acting as selective substrates for commensal bacteria in the colon by stimulating their growth and activity. This induces beneficial effects on the host's health

(125). They thereby exert an indirect defense function by stimulating the succession of a beneficial microbiota, and inhibition of pathogen attachment to intestinal glycoconjugates by acting as analogs for these (105;126).

Lipids

Milk triglycerides account for 98% of the lipid content with the remaining content consisting of phospholipids, cholesterol, and free fatty acids (106;127;128). Lipids are the major source of energy in milk and are contained within membrane-enclosed milk fat globules that are dispersed in the milk serum. The globule membrane consists of phospholipids, cholesterol, and proteins while triglycerides constitute the core (106). Preterm human milk has a higher content of lipids than full term human milk (110). Long-chain polyunsaturated fatty acids (PUFA) have shown to reduce the incidence of NEC and intestinal inflammation in a neonatal rat model, and *TLR4* and *PAFR* expression in IEC-6 cells has been blocked by PUFA supplementation (129;130).

Bioactive factors in colostrum

Colostrum contains a huge number of bioactive factors (Table 4) that may aid in gastrointestinal maturation, priming of the immune system, and the succession of a beneficial microbiota (4;12;22;105;131). These factors are relatively easy to isolate from colostrum or milk, but to provide evidence for their relevance is more difficult. The mere presence of a factor with a known effect in colostrum is not adequate to assume an effect in the gastrointestinal tract (12). To have physiological relevance, the bioactive factor has to pass through the gastrointestinal tract to the site of action without being degraded. To facilitate this, colostrum contains glycoproteins and protease inhibitors (inhibiting trypsin, chymotrypsin, and elastase) and furthermore, some factors are hard to digest by being acid resistant (132;133). On the contrary, some factors are activated by the acidic environment in the stomach, and furthermore, bioactive peptides may be released from proteins during enzymatic digestion in the gastrointestinal tract (116;120;134). Thus, bioactive factors have to be present in sufficient quantity, in an active state, and not being inhibited by other factors (12;131).

The antimicrobial effect of bioactive factors can be due to a direct effect on the microbiota by agglutination of microbes and neutralization of pathogens by for example Igs, and degradation of the bacterial cell walls by for example lysozyme and defensins (68-70;126). The antimicrobial effect may also be through modulation of the immune system. As an example, cluster of differentiation (CD) 14 is involved in the binding of lipopolysaccharides to TLR-4. Soluble CD14 is found in human colostrum and BC, but not milk formula, and may mediate this binding, and thereby play a role in the defense against Gram-negative pathogens (135).

Both pro- (including IL-1 β , IL-6, IL-8, and TNF- α) and anti-inflammatory (including IL-10, TGF- β) cytokines are present in colostrum and milk (reviewed in (4;131)) and they have the potential to affect the immune response in the intestine. The concentration of cytokines (IL-1 β , IL-6, TNF- α , INF- γ and IL-1 receptor antagonist) has been found to be higher in bo-

vine colostrum whey than milk whey (136). At concentrations found in human milk, TGF- β and the hormone erythropoietin have shown to mediate a suppressive effect on TNF- α and IL-1 β induced IL-8 secretion in a fetal human enterocyte cell line (137). The presence of PAF-acetylhydrolase in preterm human milk and full term human milk may compensate for the low plasma level of PAF-acetylhydrolase in preterm infant with NEC compared with age-matched control infants (60;138).

Table 4. Overview of some immunological and antimicrobial factors found in colostrum and milk (adapted from (131)).

Antimicrobial	Immune development	Anti-inflammatory	Tolerance/priming
Immunoglobulins: sIgA, SIgG, SIgM	Macrophages	Cytokines: IL-10, TGF- β	Cytokines: IL-10 ; TGF- β
Lactoferrin, lactoferricin B and H	Neutrophils	IL-1 receptor antagonist	Anti-idiotypic antibodies
Lysozyme	Lymphocytes	TNF- α and IL-6 receptors	
Lactoperoxidase	Cytokines	sCD14	
Nucleotide-hydrolyzing antibodies	Growth factors	Adhesion molecules	
κ -Casein and α -lactalbumin	Hormones	Long-chain PUFA	
Haptocorrin	Milk peptides	Osteoprotegerin	
Mucins	Long-chain PUFA	Lactoferrin	
Lactadherin	Nucleotides	<i>Hormones and growth factors</i>	
Free secretatory component	Adhesion molecules	cortisol, estrogen, pregnadiol, progesterone, thyroid hormones, erythropoietin, gonadotropin, insulin, leptin, prolactin, procalcitonin	
Oligosaccharides and prebiotics			
Fatty acids			
Maternal leukocytes and cytokines			
sCD14			
Complement and complement receptors			
β -Defensin-1			
Toll-like receptors			
Bifidus factor			

The achievement of tolerance may be affected by colostrum factors. For example, TGF- β , IL-6, and IL-10 coupled with commensal bacteria and dietary antigens found in colostrum may aid in development of tolerance toward the commensal microbiota and dietary antigens. This is done through modulation of lymphocyte, macrophage and dendritic cell differentiation, proliferation and activation (12;56;131 ;139;140). Finally, it is speculated that breast milk lymphocytes with immunological memory may have some modulating effects on the infant's immune response (56). The content of hormones and growth factors in colostrum may enhance intestinal barrier function and recovery after injury and modulate the intestinal response as described for erythropoietin (12;126).

This short review of some of the effects of the bioactive factors presented in Table 4 clearly shows that a huge number of factors may be of importance in achieving a beneficial gut environment in the preterm infant. All are probably of importance to some extent, but the

exact function of many of the factors is difficult to determine. It would be a very comprehensive task to discuss them all in detail therefore, only the bioactive factor analyzed in Støy *et al.* (II): Ig, Lf, lactoperoxidase, insulin-like growth factor (IGF)-I and TGF- β will be further discussed.

Immunoglobulins

In mammals, five major classes of Igs have been classified: IgG, IgA, IgE, IgM, and IgD, which share the same basic chemical structure, but exert different biological functions in relation to the host defense against microbes (28). sIgA is the major Ig in human colostrum and decreases in mature milk. IgM and IgG are present in small amounts in human colostrum and milk (106). Igs remain stable on digestion with pepsin, trypsin, and chymotrypsin, thereby staying active *in vivo* to exert effects in the intestine (134). In humans, Igs are transferred from mother to fetus and provide passive immunity in the infants during the first period of life. However, this transfer occurs after 32 weeks of gestation thereby leaving preterm infants born earlier without this passive protection (12). In human colostrum and milk, the concentration of IgA and sIgA ranges between 2–9 g/L (106;141;142). The supply of antibodies is essential for the newborn infant due to the decreased number of B cells and sIgA in the intestinal mucosa during the first period after birth, as described previously (73;74). One study found that sIgA in preterm human milk seemed to be constant for a longer period of time, while it decreased in full term human milk during the first month (142). This gives a higher and much needed protection from sIgA in preterm infants during a longer period of time. However, mothers of preterm infants may not be able to provide colostrum and in these cases an alternative source of Igs could be BC.

A high level of Igs in BC is important for the newborn calf, due to a lack of transfer of Igs over the placenta (143). In BC and bovine milk the primary Ig is IgG, and in BC the concentration is approximately 80 g/L (total Ig: range 20–150 g/L) and drops to less than 2 g/L in mature milk (112;118;144). Hence, preterm infants not so fortunate to receive mother's colostrum could get a passive protection from the IgG fraction in BC. To increase its effect on specific pathogens, hyper-immune colostrum with increased amount of Igs targeting a specific pathogen could be produced. Hyper-immune colostrum is produced when cows are immunized during gestation with specific antigens, for example attenuated pathogens and their toxins. After calving, a higher concentration of antibodies against these antigens will be secreted into colostrum. Hyperimmune colostrum products have been beneficial in the prevention and treatment of infections caused by *E. coli* (145) and rotavirus (146). One of the opportunistic pathogens associated with NEC is *C. perfringens* as previously described, and hyper-immune colostrum towards this bacterium could be used in cases with *Clostridium* associated NEC. Støy *et al.* (V) confirmed that hyper-immune BC against *C. perfringens* could be produced by immunizing cows with a clostridial vaccine. The increased level of anti-*C. perfringens* antibodies could possibly neutralize *C. perfringens* in the intestinal lumen.

Lactoferrin and lactoperoxidase

Lf is the dominating whey protein in human colostrum, and the levels in preterm- and full term human colostrum and milk vary (approximately 5 g/L (111;147)). However, in preterm human milk the concentration of Lf seems to remain rather constant for a longer period of time than full term human milk (111) and thus maintain an increased protection of the preterm infants for a longer period of time. In bovine milk the concentration is approximately 2 g/L in the first milking after parturition (112).

Lf possesses many biological activities including antimicrobial- and immune modulatory effects, and thus plays an important role in the innate immune defense against infections (148). Lf has an antimicrobial effect due to iron-sequestration thereby competing for iron with iron-demanding bacteria for example *E. coli* (149). By reducing the availability of iron, Lf may reduce the numbers of possible pathogens, and thereby be indirectly involved in down regulation of immune cell recruitment and activation. Furthermore, Lf has a direct immune modulating effects, both anti- and proinflammatory (150;151). Lf remains stable on digestion with pepsin, trypsin, and chymotrypsin (134), but antimicrobial peptides, lactoferricin B and lactoferrampin with effects toward Gram-positive- and negative bacteria may be released if Lf is digested (126;152;153).

Lactoperoxidase is involved in the antimicrobial defense, and has a synergistic effect with Lf (154). Lactoperoxidase is found in BC and milk (approximately 0.03 g/L) (118), and in human colostrum (0.004% of the total protein content) (155).

IGF-I and TGF- β

The content of growth factors in colostrum is species dependent; in human milk, epidermal growth factor is dominating, while IGF-I and IGF-II dominates in BC (4). The concentration of IGF-I is much higher in bovine than human colostrum (300 versus 18 g/L) (112;156), and hereafter the level of IGF-I decreases rapidly (112). TGF- β and TGF- β -like molecules are present in high concentrations in BC (20–40 mg/L) and bovine milk (1–2 mg/L) (4), while in human colostrum the concentration of TGF- β 1 is 650 pg/mL (range 0–8000 pg/L) and in milk, 250 pg/mL (range 0–8000 pg/L) (157). IGF-I and TGF- β survives the acidic conditions in the stomach thereby retaining their biological activity. IGFs are involved in the transmembrane transport and metabolism of various nutrients, and in the regulation of cell proliferation and differentiation. TGF- β is involved in immune regulation for example by its anti-inflammatory properties and in intestinal recovery after injury (reviewed in (4;131)). In preterm infants, the level of epidermal growth factor is reduced making the nutritional supply of growth factors important to stimulate intestinal barrier maturation (158).

Nutritional strategy for the preterm infant

One of the central risk factors for NEC development is the feeding regimen and the majority of infants developing NEC have received enteral nutrition (159;160). Human colostrum and milk is the natural diet for infants and is looked upon as the golden standard for infant nutri-

tion. The World Health Organization recommends exclusive breastfeeding for all healthy women and infants from within an hour after birth and throughout the first 6 month of life (161). Mothers of preterm infants are often unable to provide milk, or enough milk, to their preterm infants (162). However, provision of the most optimal diet is of utmost importance for these vulnerable infants at high risk of complications including NEC. Recently the American Society for Parenteral and Enteral Nutrition put forward the clinical guidelines regarding nutritional support of neonatal patients at risk of NEC (18). These guidelines are based on a systematic review to provide evidence for different types of nutritional support to neonates at risk of NEC (Table 5). Question 1 (when and how should feeds be started in infants at high risk for NEC?) and question 2 (does the provision of mother’s milk reduce the risk of developing NEC relative to bovine-based products or formula?) are of special interest in relation to the objectives put forward in this thesis, and will be discussed next.

Table 5. Nutrition support guideline recommendations for neonatal patients at risk for NEC put forward by the American Society for Parenteral and Enteral Nutrition (18).

Question	Recommendation	Evidence
1 When and how should feeds be started in infants at high risk for NEC?	We suggest that minimal enteral nutrition should be initiated within the first 2 days of life and advanced by 30 mL/kg/d in infants ≥ 1000 g.	Weak
2 Does the provision of mother's milk reduce the risk of developing NEC relative to bovine-based products or formula?	We suggest the exclusive use of mother's milk rather than bovine based products or formula in infants at risk for NEC.	Weak
3 Do probiotics reduce the risk of developing NEC?	There are insufficient data to recommend the use of probiotics in infants at risk for NEC.	Further research needed
4 Do certain nutrients either prevent or predispose to the development of NEC?	We do not recommend glutamine supplementation for infants at risk for NEC. There is insufficient evidence at this time to recommend arginine and/or long-chain polyunsaturated fatty acid supplementation for infants at risk for NEC.	Strong Further research needed
5 When should feeds be reintroduced to infants with NEC?	There are insufficient data to make a recommendation regarding time to reintroduce feedings to infants after NEC.	Further research needed

TPN may be necessary to ensure adequate energy intake in cases with feeding intolerance. Unfortunately, negative consequences are observed with TPN, which have shown to result in mucosal atrophy and dysfunction and an increased incidence and degree of NEC in preterm pigs after the initiation of enteral feeding. Additionally, TPN may suppress intestinal function, and lactose digestion when enteral feeding is initiated (102;163). The optimal nutritional strategy may be minimal enteral nutrition (MEN) which is the combination of small doses of enteral nutrition and parenteral nutrition. This will provide the infant with adequate intake of nutrients and still provide the beneficial effects of enteral nutrition on intestinal and immune

system maturation, especially in preterm infants for who it is possible to give mother's own milk or donor breast milk. As stated in Table 5, the effect of MEN remains uncertain in preterm infants (18;164). In contrast, the protection against NEC in preterm pigs is completely when colostrum is provided in slowly increasing volumes from the time of birth (165). Therefore, further research is of importance to elucidate the optimal dose, volume initiation and advancement of MEN.

There is no doubt that human breast milk should be the preferred choice of feeding for neonates, but for mothers unable to breast feed or express milk, donor milk is the preferred alternative option, while the second alternative is bovine milk-based infant formula. However, modified BC-based products could be a third alternative. Fluctuations of nutrients in colostrum and milk within time and species indicate that the milk is designed to different developmental stages and species. The question is therefore, how important are these differences, and is it possible that colostrum or milk from different lactation periods and species can be good and safe alternatives to mother's milk. A prospective observational study comparing preterm infants exclusively on mother's own milk or mother's own milk supplemented with donor milk showed that the amount of maternal milk was inversely correlated to NEC. Furthermore, the weight gain was improved in preterm infants fed exclusively mother's own milk compared with preterm infants fed mother's own milk supplemented with donor milk (166). When comparing the two alternatives to human breast milk: donor milk and milk formula, a Cochrane review concluded that infant formula increased the risk of NEC in preterm infant compared with donor breast milk (167). Nutrient-fortified donor milk is often used for preterm infants, but the majority of studies are conducted with non-fortified donor milk. Therefore, additional studies are required to investigate the effect of nutrient-fortified donor milk on the preterm infant in relation to for example NEC (167). Until now, results from studies comparing fortified diets, either human-based or bovine/human-based, are inconsistent (162;168). In contrast, results from the preterm pig model of NEC are more clear, showing that preterm pigs fed bovine or porcine colostrum, or human banked milk have lower NEC incidence compared with formula fed pigs (79;102;169;170). This also shows that colostrum or milk from one mammalian species may be effective in another species. In contrast, formula feeding reduces brush-border enzyme activity and nutrient absorption, and induces mucosal atrophy compared with colostrum feeding in preterm pigs, and this could potentially lead to inflammatory responses and NEC (79;102).

As shown in Table 5, several other potential nutrition therapies have been considered, but none has yet proven a success (18). Based on Cochrane reviews, no evidence support administration of oral Ig for the prevention of NEC (171), and there is no evidence of the efficacy of oral Lf prophylaxis to prevent NEC when given together with antibiotic therapy (172;173). Thus, it is of relevance to investigate which nutritional strategy is the safest and most beneficial for the preterm infant when mother's milk is not available.

Processing procedures

The bacterial contamination standards are set at a very low level for infant formula or donor breast milk given to preterm infant (174). A prerequisite for the use of BC in a clinical setting is that a product with low bacterial contamination and which is easy to handle and store can be produced. Several processing methods can be employed to increase storage time, reduce bacterial load and facilitate handling of BC products. However, the bioactivity of BC also needs to be preserved, leaving the number of feasible methods limited. Pasteurized, gamma-irradiated, and/or spray dried BC products were used in the studies in this thesis, and these processing methods will be presented and discussed next.

Pasteurization does not lead to a sterile product; instead the number of viable organisms is reduced, thereby reducing the number of pathogens able to cause disease. Two common methods of pasteurization are used for milk and colostrum: high temperature, short time pasteurization (72 °C for 15 sec) as used in Støy *et al.* (II), and Holder (batch) pasteurization at approximately 63 °C for 30 min (143). Spray drying, performed by filter mat spray drying in Støy *et al.* (II) (confidence procedure, Biofiber-Damino), besides being a dry preservation method, increases handling and storage capacity of the colostrum. Spray drying is faster and less costly than other dehydration methods like freeze-drying and microwave vacuum evaporation (175).

Besides destruction of microorganisms, heat treatment may cause Maillard reactions (176), protein aggregation (177), inactivation of bioactive peptides and enzymes, and loss of certain vitamins, but the extent is temperature dependent (178;179). A review of the effects of pasteurization on several compounds in human milk can be found in (174), but the effects of pasteurization on the bioactive factors in focus in this thesis will be presented next.

Holder pasteurization is often used to pasteurize donor milk (180), and has been found to both have little effect on the levels of IgA and Lf, but also to reduce the levels of IgA, IgG and Lf in human milk (181-183). The level of TGF- α and TGF- β 2 (184), oligosaccharides (185) and PUFA (180) is preserved after Holder pasteurization. Pasteurization of colostrum at 75 °C for 5 min reduces IgG by ~20% while a 59% reduction is seen at 95 °C for 15 s. In contrast, isolated IgG in phosphate buffered saline is reduced by a 100% at 95 °C for 15 s. This difference in reduction may be explained by the content of sugars or glycerol in colostrum, which protect and stabilize IgG during heat treatment (186). Furthermore, heat treatment causes conformational changes in the IgG molecule (187) and reduces the antigen-binding activity of bovine IgG (177). Pasteurization does not seem to alter the levels of IGF-I in milk, but heat treatment at 121 °C for 5 min destroys IGF-I (188). Hence, the lower the temperature, the smaller is the negative effect on the concentration and bioactivity of several bioactive factors. Compared with other dehydration methods (freeze-drying and microwave vacuum evaporation), spray drying may preserve the quantity and function of Ig (175). Spray dried milk powder may have a poor solubility (176), which also was observed in Støy *et al.* (II). This may be caused by the formation of free milk lipids coating the powder particles thereby

reducing their solubility in water, but the solubility can be increased by coating the particles with surfactants like lecithin (176).

The BC products used in the studies in this thesis were also sterilized by gamma-irradiation to eliminate possible contaminating organisms causing disease. Studies have shown that both structural changes and the amount of milk proteins (casein, β -lactoglobulin, IgA, and Lf) can be affected in varying degree by gamma-irradiation (181;189). The conditions (liquid or powder) can also affect the proteins. No significant changes of gamma-irradiation were observed for β -lactoglobulin in a solid phase, while the protein aggregated when in a solution (190). A joint “Food and Agriculture Organization of the United Nations/International Atomic Energy Agency/World Health Organization” study group on high-dose irradiation has concluded that it is safe to consume gamma-irradiated food and that it does not cause toxicological hazard based on thorough scientific evidence (191).

Briefly, it should be mentioned that colostrum may maintain its immunological properties during storage at -20°C for more than 6 months (192). This is relevant for the studies in this thesis, since the products have been stored at -20°C between production and use.

Chapter 4 Models and methods

In vivo and *in vitro* models are important to investigate human diseases, and can be used to investigate physiological mechanism, invasive intervention, or experimental designs not ethical to perform or practical to conduct in humans. These are significant considerations in relation to NEC, due to the vulnerability of preterm infants and the period in which action can be taken, since they may rapidly become seriously ill or are dying. For both *in vivo* and *in vitro* models, one should be cautious to apply results directly into the clinic due to interspecies variations. Additionally, ethical approval and considerations should be considered when employing *in vivo* models.

In the following sections, the preterm pig model of NEC, the IPEC-J2 cell line, and the methods used to investigate the intestinal environment will be discussed, thereby justifying their usefulness to investigate the objectives put forward in this thesis.

The preterm pig model of necrotizing enterocolitis

Several animal models including flies, worms, mice, rats, rabbits, sheeps, quails, chickens and pigs can be used in NEC research (15;22;25;100;193-195). In this thesis, the preterm pig model of NEC has been chosen as *in vivo* model for NEC. Other models induce NEC with physical- (hypoxia) (3), chemical- (casein, endotoxin, PAF) (3;196), or microbiological (*Clostridium*) stress factors (100;193;195;197). In preterm pigs, NEC develops due to prematurity, bacterial colonization and feeding strategies (22), much like in preterm infants. Additionally, the clinical setting with incubators, oxygen supply, heat-regulation, and the nutritional strategy, including a TPN period is also used in the preterm model of NEC.

Enteral nutrition is a risk factor for NEC in both preterm infants (198) and preterm pigs (102). In preterm pigs, NEC only develops after the initiation of enteral nutrition (102) as in preterm infants, but the degree of NEC is dependent on the feeding regimen. If a TPN period precedes enteral nutrition, a higher incidence of NEC is seen in formula- (62% versus 39%) and sow's colostrum (26% versus 5%) fed preterm pigs (102). The best protection against NEC in preterm pigs is seen when colostrum is given as MEN (165), while the effect of MEN in preterm infants remains uncertain (18;164). This indicates that the transition from TPN to enteral nutrition is challenging for the preterm neonate. Consequently, it is relevant to investigate if it is possible to revert and restore any damage that happens during this challenging period as performed in Støy *et al.* (I).

The pathogenesis and clinical signs of NEC in preterm pigs also closely resembles those observed in preterm infants (22;24;194). As shown in Table 1, the staging system proposed by Bell *et al.* (13) can be used to diagnose and classify the severity of NEC in preterm infants. In preterm infants and pigs, abdominal distension, feeding intolerance, regurgitation, and lethargy are some of the first signs of NEC (1;11;13;22). In preterm infants and pigs, NEC is most often seen in the ileo-colonic region, but in severe cases, the entire gastrointestinal tract can be affected (3;8-10;22;102), which also was observed in a few pigs in Støy *et al.* (I;II).

Furthermore, in some preterm pigs, NEC-like lesions were observed only in the stomach. This may be caused by the continued bolus feeding in preterm pigs despite appearance of clear signs of distress, which in preterm infants would lead to termination of enteral nutrition and bowel rest (9;11;16). Thus, several conditions and similarities favor the use of the preterm pig model of NEC in NEC research, but differences between humans and pigs exist, which may complicate interpretations of the results. In humans, Igs are transferred *in utero* from the mother to the fetus and will protect the newborn during the first period of life. This transfer lacks in pigs and makes them more vulnerable toward infections after birth (199). Hence, the preterm pigs are given maternal plasma during the first 24 hours after birth. As reviewed in (49), some part of the immune system in pigs differs from that of humans: pigs have some unusual immune cells including CD4/CD8 double-positive $\alpha\beta$ T cells; their lymph nodes have an inverted structure that could affect T cell trafficking; they have up to eight IgG subclasses; IgA is found primarily as dimers and larger polymers while monomeric IgA dominates in human serum; and antibody diversification happens early in life in Peyer's patches and not in bone marrow throughout life as in humans. Furthermore, acute phase proteins may behave very differently during an acute phase reaction (200). However, the immune system appears to function in a similar fashion, and with the same result as in other species (49). Several of these differences are related to the adaptive immune response, and thus are of less relevance in relation to the preterm pig model of NEC, because the pigs are euthanized after 5 days, preventing a full initiation of the adaptive immune response.

The gestational length of the pig is shorter than of the human (114–116 days versus approximately 270 days) and the functional maturation of the gastrointestinal tract starts earlier in humans than pigs. This delayed maturation in pigs results in poor viability until 90% gestation is completed, whereas preterm infants can survive when 70% of the gestation is completed. However, the clusters of maturational changes of the GIT take place during rather similar periods (201). Therefore, it is of importance to choose a gestational time for delivery of the pigs by Caesarean section, where the maturational state of the preterm pigs, especially the gastrointestinal tract, most closely resembles that of preterm infants and where the pigs are able to survive. The optimal time for preterm delivery of pigs is therefore at 92% gestation (105–106 days) comparable to approximately 80% gestation (30 weeks) in humans (201). Figure 2 illustrates the overall experimental design, and more detailed protocols can be found in Støy *et al.* (I;II).

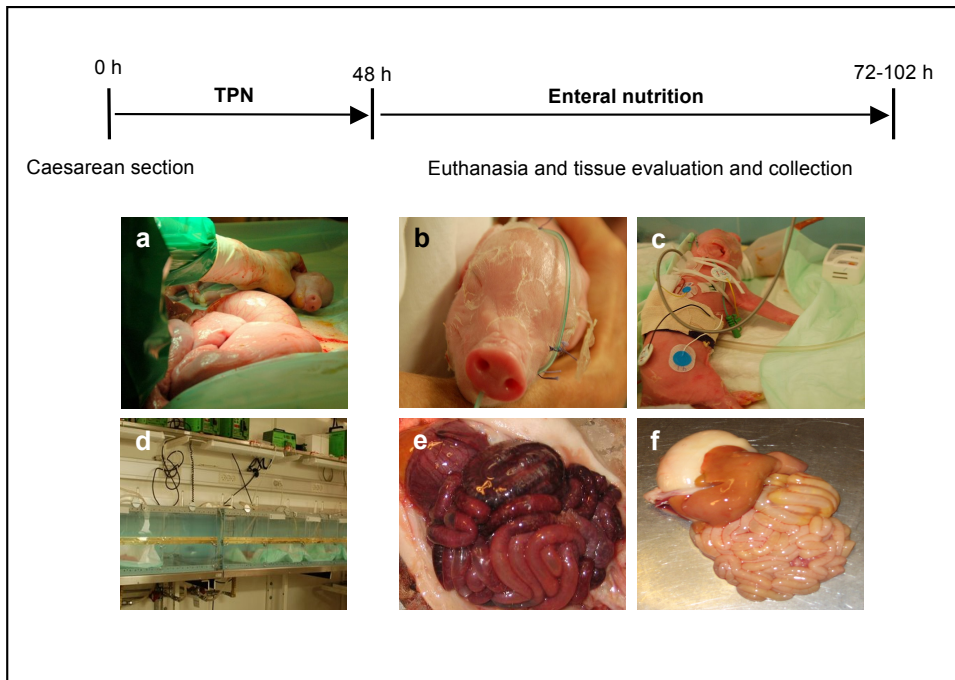


Figure 2. Experimental setup in the preterm pig model of NEC. Preterm pigs were delivered by Caesarean section at 92% gestation and placed in incubators. They were given maternal serum during the first 24 hours after birth, and fed TPN for 2 days through a vascular catheter. Hereafter, the pigs received enteral nutrition through an orogastric feeding tube and after ~2 days of enteral feeding pigs were euthanized, the tissue collected, and the intestine evaluated for NEC lesions. Caesarean section (**a**), preterm pig with an oral feeding tube (**b**), preterm pig in an incubator (**c**), incubators with preterm pigs (**d**), intestine with severe NEC (NEC severity score = 6, **e**), and a healthy intestine (NEC severity score = 1, **f**) (Støy 2007; Støy 2008).

IPEC-J2 cell line

Cell lines give the opportunity to simplify the biological system of interest, in this case the small intestine, and investigate a limited number of interacting factors and components, which is impossible in the complex and heterogeneous intestinal tissue. The IPEC-J2 cell line is a non-transformed, nontumorigenic small intestinal cell line that originates from jejunal epithelia isolated from an unsuckled neonatal pig less than 12 hours old (202;203) (Figure 3). The IPEC-J2 cell line has been a valuable tool in investigating host-pathogen interactions (202-209), and thus could be a possible cell line for studying the effects of pathogens and diet on the IECs in relation to NEC research.

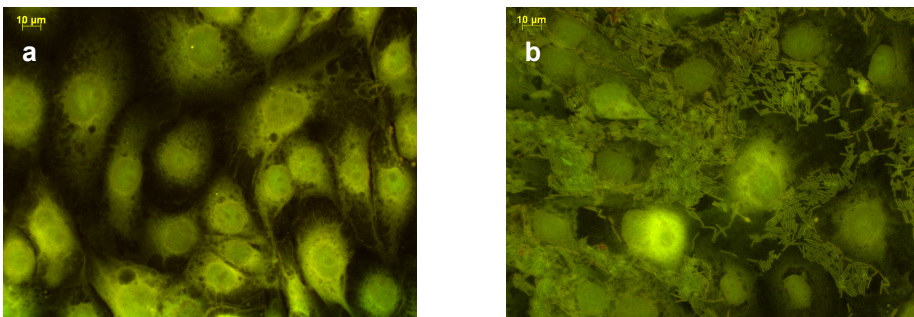


Figure 3. IPEC-J2 cells (a) and IPEC-J2 cells infected with *C. perfringens* (Multiplicity of infection (MOI) = 20, b). Pictures are obtained from an infection study in chamber slides with essentially the same experimental set up as used in Støy *et al.* (III), (Støy 2011).

The IPEC-J2 cell line shows great similarity with IECs with the presence of microvilli and tight junction, and in addition, they express porcine β -defensins, cytokines and TLRs, secrete mucin, and produce cytokines (202;203;209;210). Thus, the IPEC-J2 cell line is a convenient system to simulate innate immune functions of the intestinal epithelium and study the response against diet and pathogens (*C. perfringens*) as performed in Støy *et al.* (III;IV). Despite, that the IPEC-J2 cell line is capable of producing the same cytokines as IECs, cell lines will never be identical to the original tissue, and can have a highly variable gene expression pattern. As with the preterm pig model of NEC, the cellular response is species-specific and this can lead to misinterpretation of results when transferring the results to humans. Furthermore, due to the simplicity of the cell line and thus lack of the interacting response from other cell types and tissues in the body, one should be careful not to over-interpret the results.

To achieve the most optimal growth conditions for the IPEC-J2 cell line, Transwell-COL collagen-coated membranes were used in Støy *et al.* (III), which have shown to optimize the growth of IPEC-J2 cells so they differentiate into a single monolayer of polarized enterocyte-like cells with apical microvilli, tight junctions, expression of occludin, and intercellular spaces (211). Furthermore, trans-epithelial electric resistance, a measure of functional integ-

rity of the monolayer (confluence and viability), can be used as an indication of when to initiate experiments (211).

Laboratory analyses

The methods used to analyze the intestinal response are divided into 3 areas of investigation: intestinal digestive function and morphology, immune response, and microbiota, and will be presented in the following sections.

Intestinal digestive function and morphology

To measure intestinal loss of structure and integrity, the following morphological measurements can be used. Villi heights and crypt depths decrease during intestinal disease or injury, while intestinal circumference increases due to loss of muscular tone, and finally can mucosal mass decrease due to reduced activity of the mucosal surface (22). When the intestine loses its structure and integrity, the brush-border enzymes are lost, and thus the measure of brush-border enzyme activity is both a measure of the activity of the enzymes, but a decreased activity may also give indication of intestinal injury. Plasma galactose absorption can be used as an estimate of the function of the sodium-glucose linked transporter 1, and thus the active carrier-mediated transport, while mucosal permeability can be estimated by the urinary lactulose-to-mannitol ratio and thus give an idea of mucosal integrity and tight junction function (22;102;212). The use of sugars of different sizes has been used previously to determine intestinal permeability (213;214). Collectively, these methods to evaluate intestinal digestive function and morphology give a very good indication of the health status of the intestine, and how NEC progression and diet interventions affect the developing intestine.

Intestinal immune response

Gene expression was analyzed using quantitative reverse transcription polymerase chain reaction (RT-qPCR), while the protein levels of inflammatory factors were investigated using a Luminex multiplex assay and an enzyme-linked immunosorbent assay (ELISA).

Gene expression analysis

RT-qPCR allows quantification of complementary deoxyribonucleic acid (cDNA), reverse transcribed from messenger ribonucleic acid (mRNA), by measuring product formation upon amplification of cDNA at each polymerase chain reaction (PCR) cycle during the PCR reaction. During the first PCR cycles, no product is detected because the fluorescent signal of the amplicons is below the detection threshold. At one point, the fluorescence from the accumulating amplicons will be detected dependent on the amount of starting material. Hereafter, in theory, an exponential accumulation of amplicons occurs, resulting in a doubling of the PCR product at each cycle. At some point, a shortage of PCR reagents will lead to a level where the net synthesis approximates zero and the curve becomes horizontal. Quantification is done by placing a threshold line somewhere at the exponential

phase thereby assigning a quantification cycle (Cq) value to each sample, which is inversely proportional to the start amount of transcript for the gene of interest in question (215). Thus, a high Cq value is proportional to a low amount of template in the sample.

Two quantitative PCR (qPCR) platforms were used in the studies to investigate the effect of diet on the gene expression in distal small intestinal tissue from preterm pigs, and of diet and *C. perfringens* in IPEC-J2 cells. A Rotor gene Q platform (Qiagen, Ballerup, Denmark) was used to investigate a few genes related to inflammation in distal small intestinal tissue (Støy *et al.* (I;II)), while a high throughput platform (BioMark, Fluidigm Corporation, San Francisco, CA, USA) was used for analysis of multiple epithelial- and immune response-related genes in the distal small intestinal tissue from preterm pigs and in IPEC-J2 cells (Støy *et al.* (III;IV)).

To ensure an optimal experimental setup, the “Minimum information for publication of quantitative real-time PCR experiments” guidelines (216), which describe the minimum information necessary for evaluating qPCR experiments, were taken into account. Throughout the working procedures, from sample collection to data interpretation, the most optimal procedures possible were used to preserve the quality and integrity of the ribonucleic acid (RNA), which is the single most important factor to obtain a valid quantification (217). The scope of this section is not to give a thorough explanation of the methods or all considerations done; this can be found in Støy *et al.* (I;III). The focus will be on how to evaluate the quality and quantity of the RNA thereby assuring that the obtained results can be trusted.

The quality of the purified RNA is variable and depends on initial sample (healthy or necrotic tissue), storage, and extraction procedure. The RNA should be free of protein, genomic deoxyribonucleic acid (DNA), inhibitors (biological components from the sample or extraction reagents), and nucleases (217;218). Therefore, optical density measurement to quantify and qualify the RNA and DNase treatment to eliminate genomic DNA were included. Furthermore, to examine the integrity of the RNA, a RNA integrity number (RIN) was calculated based on an algorithm that involves the 18S:28S ratio, 5S and other regions of ribosomal RNA (rRNA) (217;219). RIN ranges from 1 (degraded RNA) to 10 (high quality non-degraded RNA). In the studies in this thesis, the mean RIN number was 6.5 (range 4.4–9.9) indicating that the RNA in general was of average quality and suitable for further analysis (217). The PCR efficiency, measured by the steepness of the standard curve, can assess the presence of inhibitors that can cause inaccurate or false-negative results (218;220). The optimal qPCR reaction is a doubling of the amount of cDNA in each cycle indicated by a PCR efficiency of 100% but this is seldom achievable. The fluorescence dyes, SYBR Green and EVA Green, are non-specific dyes binding to double stranded DNA, and could lead to formation of primer-dimers resulting in additional fluorescence. Therefore, melting curves were evaluated to make sure that the right amplicon was obtained. Finally, no-template controls were included and evaluated to exclude contamination and nonspecific amplification.

Normalization of the values to a number of reference genes is important to eliminate sample-to-sample differences in amount of mRNA and in the upstream procedures of qPCR. The

reference genes are stable genes adequately expressed in the tissue of interest, but with little expression variation between samples and thereby not affected by disease or experimental conditions. It is important to choose more than one reference gene as the expression of the gene can vary between tissues, cell lines and individuals (221-223). Several programs can be used to rank putative reference genes including GeNorm, which were used in the studies in this thesis. In GeNorm a stepwise exclusion of putative reference genes based on a gene-stability measure followed by geometric averaging of the reference genes selected to be most stable gives the normalization factor (224). After normalization, the relative values between experimental groups can be compared.

The gene expression does not always correlate with the protein level (225) however; gene expression analysis may provide knowledge of the immediate host response to a particular stimulus since mRNA is a measure of the immediate steady-state because it is short lived, while an accumulation of proteins is seen (225).

Protein analysis

For protein analysis of intestinal tissue and plasma, the Luminex multiplex assay (IL-1 β , IL-6, IL-8, and IL-10) and ELISA (SAA) were used in Støy *et al.* (I;II). The two assays built on essentially the same sandwich principle, but in a multiplex assay, the primary antibody is coupled to a bead, while it is coupled to the well in an ELISA.

The multiplex assay is based on color-coded beads (regions), where each region is coupled with monoclonal antibodies specific for one of the proteins of interest. Thus, several different regions each coupled with a specific monoclonal antibody can be added to the same well in a microtiter plate, and give the opportunity of simultaneous detection of multiple proteins of interest, for example inflammatory factors, within the same sample. The protein is then captured and detected by a biotinylated antibody specific for another region of the antigen than the primary antibody. When reading the sample in the Luminex reader, the beads pass by a dual laser, where the color code of the bead determines the protein type, while fluorescence of phycoerythrin-labeled streptavidin bound to the secondary antibody determines the amount of the protein (226-229). Several commercially multiplex kits are available for the simultaneous detection of proteins in humans and rodents, but only a limited number of assays are available for pigs (229;230).

For both types of assays, multiplex or ELISA, several considerations have to be taken in relation to sensitivity, specificity, simplicity, reliability, costs and hand-on time (228), but it is not the scope of this thesis to thoroughly examine these.

Intestinal microbiota

Up to 500 bacterial species may colonize the intestine and the number of bacteria reaches 10¹² colony forming units/g contents in the colon (23;231). To analyze this complex microbiota several methods can be used, and in this thesis, the density of the microbiota was analyzed

using conventional culture-dependent microbiology, fluorescence *in situ* hybridization (FISH) and qPCR.

Conventional culturing gives an estimate of the total amount of bacteria in a sample. In Støy *et al.* (I), the nutrition rich C-calves blood agar was used, which promotes the growth of a wide variety of microorganisms (232). However, approximately 50% of the intestinal microbiota consist of non-cultivable species, and moreover, bacteria are sensitive towards incubation conditions, and may require certain nutrients thereby affecting the growth of certain bacteria, which can distort the results (47;233;234). Furthermore, culture-dependent methods are time- and labor consuming (47;235), but they give a visual presentation of the bacterial colonies, and pure isolates can be identified through sequencing of the 16S rRNA gene (233;236).

FISH is a molecular method using bacterial oligonucleotide probes, either general or specific, that target the 16S- or 23S rRNA gene. FISH provides the opportunity to visualize bacterial cells *in situ*, gives information of the ecosystem under study, and is quantitative, thereby allowing enumeration in a culture-independent manner (47;237). However, cell impermeability can lead to insufficient access of probes to the target site and may result in low fluorescent signal and erroneous interpretations. Furthermore, only probes to bacterial groups with a known 16S- or 23S rRNA gene can be designed. FISH is unsuitable for analysis of the diversity of the total microbial community, since only a few species can be visualized at the same time (237;238).

qPCR on the Rotor gene Q platform (Qiagen) was used to determine the absolute abundance of total bacteria and *C. perfringens* in Støy *et al.* (IV). This analysis was based on essentially the same principle as the qPCR described for gene expression analysis, but using TaqMan probe-based chemistry instead of DNA binding fluorescent dyes.

Finally, organic acid (OA) measurements can provide information of abnormal bacterial fermentation of carbohydrates. Excess bacterial fermentation of carbohydrates leading to increased concentration of OA or a non-favorable OA profile may injure the intestine and contribute to NEC development (100;102;239).

Chapter 5 Summarizing discussion

Dietary intervention with colostrum may support the function of the gastrointestinal tract and gut associated immune system, and the establishment of a beneficial microbiota, due to the high level of bioactive factors in colostrum as reviewed in Chapter 3. Studies have shown that raw BC protects against NEC in preterm pigs (102;240). Støy *et al.* (I) now documented that BC also has a beneficial and restoring effect on intestinal function and reduce pro-inflammatory responses after milk formula-induced inflammation, which have shown to be present after a short period (8 hours) of milk formula feeding to preterm TPN-fed pigs (241). Furthermore, we showed that pasteurized and spray dried BC products reduced the severity of NEC lesions induced by formula feeding to TPN-fed preterm pigs (Støy *et al.* (II)), while feeding raw BC showed a tendency towards lower NEC severity than milk formula feeding to TPN-fed preterm pigs. Further studies are required to investigate what is responsible for this tendency towards an improved effect on NEC severity of pasteurized and/or spray dried BC products compared with raw BC and milk formula.

A lower intestinal circumference (indicating better muscular tone) and colonic lactic acid production, in addition to increased villus height, enzyme activities and hexose absorption were observed in preterm pigs fed BC products (raw colostrum, pasteurized and/or spray dried colostrum) compared with preterm pigs fed milk formula. This supports previous studies showing a reduction of brush-border enzyme activity and nutrient absorption, and an induction of mucosal atrophy in milk formula- compared with colostrum fed preterm pigs (79;102). Furthermore, BC products may affect the immune response as observed by decreased IL-1 β and IL-8 tissue concentrations in pigs fed BC products compared with milk formula (Støy *et al.* (I;II)). A reduced concentration of intestinal IL-1 β has also been observed in preterm pigs fed sow's colostrum compared with milk formula (169). Minekawa *et al.* (242) found that human breast milk suppresses IL-8 secretion induced by IL-1 β and LPS in Caco-2 cells, while BC may increase the production of IL-8 compared with skim milk or growth medium in HT-29 cells (243). Therefore, caution should be applied when considering BC products to preterm infants.

A positive correlation between IL-1 β tissue concentration, the expression of *IL1B*, *IL6*, *IL8*, and *SAA*, and mean NEC severity was observed, while no correlation was observed for IL-8 tissue concentration or serum concentration of IL-10 and *SAA* (Støy *et al.* (I;II)). Increased concentrations of plasma IL-6, IL-8 and *SAA* have been observed in preterm infants with NEC (58;59;61-63), while no association between plasma IL-1 β concentration and NEC has been observed (59). No correlation between the expression of *TLR4* in intestinal tissue and NEC progression was observed in Støy *et al.* ((I;II)), as seen in other experimental models of NEC and in preterm infants (65;66). These differences, observed in inflammatory responses between preterm infants and pigs may be due to a different behavior of these inflammatory markers in different species, as observed for acute phase proteins (200). The expression of the anti-inflammatory cytokine *IL10* in distal intestinal tissue was positively correlated with NEC in Støy *et al.* (I). In preterm infants, a higher plasma concentration of anti-

inflammatory factors, IL-10 and IL-1 receptor antagonist, has been observed compared with infants not suffering from NEC (59;61). Furthermore, the expression of *IL1RN*, *IκBA* and *TNPAIP3* was increased in IPEC-J2 cells concomitant with proinflammatory factors (Støy *et al.* (III;IV)). Up regulation of anti-inflammatory factors concomitantly with a proinflammatory response is thus a part of a general immune response during infection or mucosal injury (59;61;225).

No association between the type of diet and bacterial density was observed in Støy *et al.* (I;II). Thus, the bacterial density at the mucosal surface may be more correlated with disease progression than with the type and amount of the enteral diet (165). This is supported by a positive correlation between the severity of NEC and the total number of bacteria determined by FISH (Støy *et al.* (I;II)) and qPCR (Støy *et al.* (IV)). Also intestinal tissue from preterm infants suffering from NEC has been observed to be more heavily colonized by bacteria than tissue from infants not suffering from NEC, although no positive correlation was observed between bacterial density and NEC (99). Therefore, bacterial density more than specific bacteria may be important for the development of NEC as previously suggested (101). Intestinal immaturity may be responsible for bacterial overgrowth due to accumulation of undigested nutrients serving as substrate for bacterial fermentation and increased intestinal transit time (reviewed in Chapter 1). Furthermore, intestinal frailty during NEC may lead to increased access for bacteria to the intestinal epithelial, as observed by increased penetration into villi and translocation to muscularis mucosae (102;Støy *et al.* (I)). Collectively, this indicates that the involvement of bacteria in NEC development may not be part of the primary pathogenesis, but first contributes in the later events. One could speculate, that the association between a specific bacteria and NEC (90;91;93-95;Støy *et al.* (IV)) may be a cause of the general increase in total bacteria. However, the role of the specific pathogens like *C. perfringens* in intestinal injury may still be significant due to production of toxin and metabolites as reviewed in Chapter 1. Furthermore, pathogenic bacteria may contribute to changes in gene expression in host cells as observed for *C. perfringens* in Støy *et al.* (IV) that may lead to adverse effects in the intestine.

A preterm pig model of NEC and an IPEC-J2 cell line were used to investigate the effect of nutritional intervention on intestinal mucosal immunity and microbiota. As shown in Støy *et al.* (III), diet effects were seen on the intestinal gene expression in the preterm pig model of NEC, while no diet effects were seen in the IPEC-J2 cell line. In contrast, several genes were affected by increasing numbers of *C. perfringens* (Støy *et al.* (IV)), supporting other studies showing that the IPEC-J2 cell line is a valuable system for investigation of host-pathogen interactions (202-209). Cell lines provide important systems to investigate the interaction between a limited numbers of factors in a standardized setting, while animal models gives the opportunity to study dietary interventions on the organism as a whole. The cellular response is species-specific and this can lead to misinterpretation of results when transferring the results to humans. However, human research is dependent on studies in animal model and cell lines, since they provide the possibility to investigate physiological mechanism, invasive interven-

tion, or experimental designs that are not ethically possible or practical to conduct in humans. In relation to the diet-dependent research of NEC, further studies *in vitro*, *in vivo*, but also in human preterm infants are required to investigate which nutritional strategy is most beneficial and safe for preterm infants.

Clearly, mother's colostrum and milk is arguably the first choice of nutrient, but milk formula is a lifesaving alternative when mother's milk or human donor milk is not available. The BC products investigated in this thesis, showed great potential against NEC in a preterm pig model of NEC. The beneficial effects of colostrum on the intestinal environment are undoubtedly due to multiple factors acting in concert to exert immune modulation, promote intestinal growth and the succession of a beneficial microbiota (reviewed in Chapter 3), and it is very unlikely that only a single factor is responsible. However, caution should be applied when considering BC for preterm infants, especially unmodified, due to the marked species differences in nutritional content (Chapter 3, Table 3). Results from studies in preterm infants are inconsistent regarding the evidence for an association between increased risk of NEC when giving a human-based diet including bovine based products compared with an exclusively human-based diet (162;168). In contrast, results from the preterm pig model of NEC show that preterm pigs fed either bovine or porcine colostrum, or human banked milk have lower NEC incidence compared with pigs fed milk formula (79;102;169;170).

When considering alternatives to mother's own milk, processing procedures are required to achieve a low bacterial safety level. A temperature-dependent protein denaturation has been observed previously, showing that a higher temperature during heat treatment leads to increased protein denaturation of some milk proteins, including IgG (182;186). However, the pasteurized and/or spray dried BC products maintained the beneficial biological effects on the preterm pig intestine to the same degree as BC, thereby suggesting that some extent of protein denaturation may not necessarily reduce the biological effect of colostrum bioactive factors (174). Furthermore, heat treatment of colostrum at temperatures similar to those used during pasteurization and spray drying of colostrum in Støy *et al.* (II) has shown not to radically affect the important passive transfer of immunity from colostrum to newborn calves (reviewed in (69;143)). As reviewed in Chapter 3 (Table 4), several other non-protein bioactive factors for example PUFA (106) are present in colostrum, and could account for some of the beneficial effects observed by the pasteurized and/or spray dried BC products. PUFA are not affected by Holder pasteurization (180;185), and one could speculate that PUFA also are preserved during high temperature, short time pasteurization. Furthermore, IGF-I was not affected by heat treatment in Støy *et al.* (II), which also was observed in (188). Further studies in the preterm pig model of NEC could include groups of preterm pigs receiving BC products exposed to pasteurization and/or spray drying at lower or higher temperatures, to explore the effects of heat treatment on the beneficial effects of BC products seen in the preterm pig model of NEC. Gamma-irradiation did not appear to lead to significant denaturation of proteins compared with non-irradiated colostrum from the same pool of colostrum (unpublished data, Støy *et al.* (II)), while other studies have shown that gamma-irradiation may lead to

structural changes in milk proteins (181;189). While powdered colostrum may be easier to handle and store, a poorer solubility of such products can be observed (176;Støy *et al.* (II)). However, solubility increases by coating of milk particles with surfactants like lecithin (176) or by gentle heating (< 50 °C) (unpublished observations). To avoid processing, one alternative could be to feed raw donor breast milk as is currently practiced in some countries. However, the clinical outcome of this should be monitored, due to the presence of possible contaminating organisms in the raw milk that could cause diseases (174).

The research done in this thesis showing a beneficial effect of BC products on the immature gastrointestinal tract may also benefit other patient groups by providing knowledge about the effects of a bioactive product in a sensitive model for intestinal inflammatory disorders, as the preterm pig model of NEC. Even though one should keep in mind that species and maturational (term born infants and adult) specific responses are often present, these studies provide some general knowledge with regards to the nutritional impact on gut disease in general. Thus, BC products may prove to be beneficial for patients with other gastrointestinal diseases like short-bowel syndrome, inflammatory bowel disease, and mucosal injury caused by non-steroidal anti-inflammatory drugs or chemotherapy (4;139;214;244). Despite a questionable use of hyperimmune colostrum for preterm infants (Støy *et al.* (V)) due to the involvement of several bacteria in the pathogenesis of NEC, it still may prove to be useful in the prevention and treatment of diseases caused by specific known pathogens (145;146;245). In relation to investigation of hyperimmune colostrum towards *C. difficile*, the preterm pig model of NEC could be a sensitive model to investigate the efficacy of anti-*C. difficile* hyperimmune colostrum, while the IPEC-J2 cell line could be used to test the anti-*C. difficile* activity.

In relation to preterm infants, it is still relevant to investigate the efficacy and safety of BC products. However, some modifications of the BC products may be necessary to achieve a product that is safe and meets the nutritional requirements of preterm infants.

Chapter 6 Conclusion

Based on the work presented in this thesis, it can be concluded that

1. BC has a restoring effect on intestinal function and reduces intestinal inflammation after formula-induced intestinal inflammation in TPN-fed preterm pigs.
2. Pasteurized and/or spray dried BC decrease the severity of NEC, while a tendency towards lower NEC severity is observed when feeding raw BC compared with milk formula. All three BC products improve intestinal structure, function, and reduce the level of inflammatory markers relative to milk formula. Thus, pasteurized and/or spray dried BC are as effective as raw BC in the treatment of intestinal dysfunction and inflammation.
3. Differences in gene expression to nutritional factors are seen between intestinal tissue from preterm pigs and IPEC-J2 cells, and no diet effects are observed in the IPEC-J2 cells. Therefore careful considerations must be made prior to analysis and interpretation of diet-induced effects on gene expression in the IPEC-J2 cell line. In contrast, increasing numbers of *C. perfringens* lead to changes in gene expression proving that the IPEC-J2 cell line is valuable in investigation of host-pathogen interactions.
4. Increased abundance of total bacteria and *C. perfringens* is associated with NEC in a preterm pig model of NEC.
5. Anti-*C. perfringens* hyper-immune BC is produced after immunization of late-gestational cows with an Clostridia specific vaccine.

The results from this thesis underline the importance of an optimal diet composition during the first enteral feeding period in preterm neonates. BC products, modified to meet the nutritional requirements of preterm infants, may benefit preterm infants during the first critical and challenging period after birth. Further experimental studies *in vivo* and *in vitro*, in addition to well-designed studies in preterm infants are required to investigate if BC products could serve as safe and effective alternatives when mother's milk is not available.

Chapter 7 Reference list

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Chapter 8 Papers included in the PhD thesis

Paper I (Støy *et al.* (I)): Ann Cathrine F. Støy, Peter M. H. Heegaard, Thomas Thymann, Mette Bjerre, Kerstin Skovgaard, Mette Boye, Barbara Stoll, Mette Schmidt, Bent B. Jensen, Per T. Sangild. Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs. Submitted to *Clinical Nutrition*.

Paper II (Støy *et al.* (II)): Ann Cathrine F. Støy, Per T. Sangild, Kerstin Skovgaard, Thomas Thymann, Mette Bjerre, Dereck E. W. Chatterton, Stig Purup, Mette Boye, Peter M. H. Heegaard. Mild heat treatment does not reduce the colitis-protective effects of bovine colostrum in preterm pigs. Ready for submission.

Paper III (Støy *et al.* (III)): Ann Cathrine F. Støy, Peter M. H. Heegaard, Per T. Sangild, Mette V. Østergaard, Kerstin Skovgaard. Gene expression analysis of the IPEC-J2 cell line as a simple system for the inflammation-sensitive preterm intestine. Submitted to *Comparative and Functional Genomics*.

Paper IV (Støy *et al.* (IV)): Ann Cathrine F. Støy, Camilla L. Delègue, Thomas Thymann, Per T. Sangild, Kerstin Skovgaard, Peter M. H. Heegaard, Sarmauli Manurung, Lars Mølbak. Total bacterial and *C. perfringens* abundance is associated with necrotizing enterocolitis in preterm pigs, and *C. perfringens* induces gene expression changes in IPEC-J2 cells. Work in progress.

Conference presentation (Støy *et al.* (V)): Ann Cathrine F. Støy, Per T. Sangild, Peter M. H. Heegaard. Bovine colostrum with hyperimmunity against Clostridia. Poster presentation at the “Alternatives to Antibiotics” Symposium in Paris, 25–28 September 2012. Work in progress.

Paper I

Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs

Ann Cathrine F. Støy, Peter M. H. Heegaard, Thomas Thymann, Mette Bjerre, Kerstin Skovgaard, Mette Boye, Barbara Stoll, Mette Schmidt, Bent B. Jensen, Per T. Sangild

Submitted to Clinical Nutrition

TITLE PAGE

Bovine colostrum improves intestinal function following formula-induced gut inflammation in preterm pigs

Short title: Colostrum and necrotizing enterocolitis in preterm pigs

Ann Cathrine F. Støy^a, Peter M. H. Heegaard^a, Thomas Thymann^b, Mette Bjerre^c, Kerstin Skovgaard^a, Mette Boye^a, Barbara Stoll^d, Mette Schmidt^e, Bent B. Jensen^f, Per T. Sangild^{1b*}

^a National Veterinary Institute, Technical University of Denmark, DK-1870 Frederiksberg C, Denmark

^b Department of Human Nutrition, University of Copenhagen, DK-1958 Frederiksberg C, Denmark

^c The Medical Research Laboratories, Department of Clinical Medicine, Faculty of Health Sciences, Aarhus University, Aarhus C, Denmark

^d U. S. Department of Agriculture - Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, USA

^e Department of Large Animal Sciences/Veterinary Reproduction and Obstetrics, University of Copenhagen, DK-1958 Frederiksberg C, Denmark

^f Department of Animal Science, Aarhus University DK-8030 Tjele C, Denmark

*Corresponding author

Per Sangild, Department of Human Nutrition, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark; tel. +45 3533 2698; fax. + 45 3533 2469.

Conference presentation

An abstract, based on this study, was accepted and selected for oral presentation at the International Society for Research in Human Milk and Lactation in Trieste in September 2012.

Abstract

Background & Aims: In preterm infants, rapid transition from total parenteral nutrition to enteral nutrition, especially using infant formula, predisposes to necrotizing enterocolitis (NEC). A few hours of formula feeding induce proinflammatory effects in the preterm pig model of NEC. We hypothesized that bovine colostrum, rich in bioactive factors, may improve intestinal function following exposure to formula.

Methods: After receiving total parenteral nutrition for 2 days, preterm pigs were fed formula (FORM, n = 14), bovine colostrum (COLOS, n = 6), or formula (6 h) followed by bovine colostrum (FCOLOS, n = 14). Intestinal lesions, function, and structure, number and location of bacteria, and inflammation markers were investigated.

Results: NEC severity and ileal IL-1 β and IL-8 protein concentrations were lower in COLOS and FCOLOS pigs, relative to FORM pigs. Villus height, galactose absorption, and brush-border enzyme activities were increased in COLOS and FCOLOS pigs. Expression of *IL1B*, *IL6*, *IL8*, *IL10* and *SAA* and bacterial number and location score correlated positively with NEC severity.

Conclusions: Bovine colostrum restores intestinal function after initial formula-induced inflammation in preterm pigs. Further studies are required to test if bovine colostrum may also benefit preterm infants during the challenging transition from total parenteral nutrition to enteral nutrition, when human milk is unavailable.

Keywords: Bovine colostrum; necrotizing enterocolitis; inflammation; animal model.

Introduction

Necrotizing enterocolitis (NEC) is a serious gastrointestinal disease that mainly affects pre-term infants. Short gestational age, aggressive formula feeding, and abnormal bacterial colonization are factors that predispose to NEC¹⁻³. Before the initiation of enteral nutrition, pre-term neonates may require a period of total parenteral nutrition (TPN), and NEC occurs very seldom in the absence of enteral food in infants⁴ and pigs⁵. Although TPN may be a life-saving therapy for many infants, TPN may also reduce the digestive- and absorptive function⁶, increase intestinal permeability⁷, and cause a degree of mucosal atrophy in the developing intestine⁸. When milk formula feeding is initiated after a few days of TPN, this may be associated with a more difficult and less tolerant transition to enteral nutrition, as indicated from studies in preterm pigs⁵. An abrupt transition to milk formula reduces villus height, mucosa percentage, digestive enzyme activities and nutrient absorption, and increases bacterial adherence, mucosal atrophy and inflammatory cytokine levels within just 8 hours of feeding^{5,9,10}. In contrast, preterm pigs fed porcine or bovine colostrum are almost protected from NEC^{5,10,11}, most completely when colostrum is provided in slowly increasing volumes already from the time of birth¹².

Colostrum is rich in bioactive compounds such as growth factors, antioxidants, antimicrobial and immune-modulatory factors, which may support intestinal maturation, balance and priming of the immune system and establishment of a beneficial gut microbiota^{13,14}. Although it is known that colostrum may prevent the inflammatory cascade leading to NEC lesions¹², the ability of colostrum to regenerate an already compromised gut exposed to a short period of formula is unknown. This is important because lack of mother's milk following preterm birth may require formula feeding for some time after birth. Formula feeding may last for much longer if breast-feeding remains impossible and if banked human milk is not available. There is a need to know how mother's own milk, or a possible substitute bioactive product like bovine colostrum, may help to suppress the pro-inflammatory state of the immature intestine resulting from a few days of TPN followed by a period of formula feeding.

We hypothesized that the gut dysfunction induced by the combination of TPN with an abrupt transition to milk formula, is corrected if bovine colostrum is fed shortly after the formula feeding period. We used a preterm pig model of NEC^{5,10} to investigate the effects of bovine colostrum on intestinal structure, digestive and absorptive functions, microbiota, and plasma and tissue proteins and tissue mRNA levels of inflammatory markers. A group fed formula followed by colostrum was compared with groups of pigs fed either formula (negative control) or colostrum alone (positive control) after the TPN period.

Materials and methods

Animals and their treatment

The procedures for caesarean section and nursing of the preterm pigs followed a standard protocol^{5,15}. Thirty-four preterm pigs were delivered from four sows by caesarean section (Large White × Danish Landrace × Duroc, Askelygaard Farm, Roskilde, Denmark) at 105–107 d gestation (90–92% gestation). The pigs were fitted with an oro-gastric feeding tube (6F, Portex, Kent, UK) and a vascular catheter (4F, Portex) in an umbilical artery, and placed in temperature-regulated incubators with oxygen supply. To provide passive immunity, pigs were given maternal plasma three times (4, 5, and 7 ml/kg body weight) through the vascular catheter during the first 24 h after birth. The following 48 h, pigs were given TPN through the vascular catheter (4 ml/kg/h advancing to 6 ml/kg/h). The TPN solution was based on Nutriflex Lipid Plus (Braun, Melsungen, Germany) and adjusted in nutrient composition to meet the requirements of pigs⁵. After the TPN period, pigs were abruptly shifted to total enteral nutrition for an additional two days before euthanasia and tissue collection. At transition to enteral nutrition the pigs were randomly assigned according to birth weight into three enteral nutrition groups fed either a milk formula (FORM, n = 14), bovine colostrum (COLOS, n = 6), or 6 h of milk formula followed by bovine colostrum until euthanasia (FCOLOS, n = 14). The feeding dose for all groups was 15 ml/kg body weight/3 h. The milk formula contained 80 g Peptide, 70 g Maxipro, and 75 g Liquigen/L of water⁵, all products kindly donated by Nutricia, Allerød, Denmark. Bovine colostrum was obtained from the first milking after parturition (kindly donated by Biofiber-Damino, Gesten, Denmark). Before use, the colostrum was sterilized by gamma-irradiation (1 × 10 kGy; Sterigenics, Esbjerg, Denmark) and stored at -20 °C. The products were warmed to body temperature in a water bath and colostrum was dissolved in tap water to obtain the same dry matter content as in the milk formula before use. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation (Licence: 2004/561-910).

Clinical evaluation and tissue collection

Pigs were monitored closely and euthanized if clinical symptoms of NEC, such as abdominal distension, lethargy, cyanosis or bloody diarrhea were observed. Pigs not showing clinical signs of NEC were euthanized after 45–50 h of enteral feeding. Euthanasia and tissue collection were performed according to earlier protocols¹¹. Proximal, middle and distal small intestine, stomach and colon were given a NEC severity score: 1 = No or minimal focal hyperaemic gastroenterocolitis; 2 = Mild focal gastroenterocolitis; 3 = Moderate locally extensive gastroenterocolitis; 4 = Severe focal gastroenterocolitis; 5 = Severe locally extensive hemorrhagic and necrotic gastroenterocolitis; or 6 = Severe extensive hemorrhagic and necrotic gastroenterocolitis. Pigs with a severity score of 3 or more in any gastrointestinal region were considered suffering from NEC. To determine small intestinal enzyme activities, gene expression, and cytokine concentrations, full thickness tissue samples from the small intestinal re-

gions were immediately snap-frozen in liquid nitrogen and stored at -80 °C. A 10 cm segment of each small intestinal region was used to measure intestinal circumference and to determine the proportion represented by mucosa according to⁵. Samples from the distal small intestine were collected and fixed in 4% neutral buffered paraformaldehyde for 24 h and transferred to 70% ethanol before preparation for fluorescence *in situ* hybridization (FISH) and evaluation of gut morphology.

Blood collected at euthanasia was used for later determination of inflammatory factors (see below). All blood samples were collected in EDTA- or heparin coated tubes, placed on ice, centrifuged for 10 min at 4 °C and 2,500g and the plasma was stored at -20 °C until further analyses.

Gut morphology and intestinal function

Distal intestinal villus height and crypt depths were evaluated on scanning pictures obtained from FISH analysis using the morphometric software SoftWoRx Explorer version 1.2.0 (Applied Precision, Issaquah, WA, USA). One representative cross-section was selected from each pig and 10 representative villi and crypts were measured. Intestinal function was evaluated by measuring enzyme activities of dipeptidyl peptidase IV, aminopeptidase N, aminopeptidase A, lactase and maltase according to¹⁶. Finally, trehalase activity was measured as described for lactase and maltase in Sangild *et al.*¹⁶ using 0.6 M D-(+)-trehalose dihydrate (EC 202-739-6, Sigma-Aldrich, Brøndby, Denmark) as a substrate. The enzyme activities were expressed as U/g of wet intestine, where one unit of activity correspond to 1 µmol substrate released per min at 37 °C. *In vivo* galactose absorption was used as an index of the hexose uptake capacity via the sodium-glucose linked transporter 1. Plasma galactose was measured before enteral nutrition (0 h) and at 6 h and 30 h after enteral food introduction by collection of a blood sample 20 min after an oral bolus (15 ml/kg, 5% galactose) via the oro-gastric feeding tube. Plasma concentrations of galactose were measured according to Thyman *et al.*¹⁷ and the results from each treatment group at 6 h and 30 h were compared with the baseline level at 0 h. To get an estimate of gut permeability, an oral bolus of 5% lactulose and 2% mannitol (15ml/kg) was given 4 to 6 h prior to kill and urine was sampled from the bladder by cystocentese. The urinary ratio of lactulose to mannitol was determined as previously described⁵.

Gene expression analyses of inflammatory factors

The expression of *IL1B*, *IL6*, *IL8*, *IL10*, *PBD-2*, *TLR4*, *TNF* and *SAA* and reference genes *B2M*, *ACTB* and *HPRT1* in distal intestinal tissue was determined using quantitative real-time PCR. Total RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen, Ballerup, Denmark) and on-column DNase treated using RNase-free DNase set (Qiagen) according to manufacturer's protocol. The quantity of extracted total RNA was measured on a Nanodrop ND-1000 UV-spectrophotometer (Saveen Biotech, Aarhus, Denmark) and the quality of extracted total RNA was determined using on-chip electrophoresis on an Agilent 2100 Bioana-

lyzer (Agilent Technologies, Nærum, Denmark) yielding an RNA integrity number (RIN) assigned to each sample using the 2100 Expert software (Agilent Technologies, Hørsholm, Denmark). One µg of total extracted RNA was converted into first-strand cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions and stored at -20 °C. Quantitative PCR was performed using diluted cDNA (1:5 or more) in a total reaction volume of 25 µl containing gene-specific primers (300 nM; Table 1), SYBR Green PCR Master Mix (Sigma-Aldrich) and MgCl₂ (Table 1). PCR cycling was performed on a RotorGene 3000 Detection System (Corbett Research, Sydney, Australia) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles with 15 s at 95 °C and 1 min at 59–62 °C (Table 1). Sequences for primers were obtained from Genbank and NCBI, and to confirm total gene specificity of the primer sequences and absence of polymorphisms at the primer site, an *in silico* screen was performed in BLAST. Primers were designed using Primer3 ([5http://frodo.wi.mit.edu/4](http://frodo.wi.mit.edu/4)) and synthesized at TAG Copenhagen, Copenhagen, Denmark. To confirm a single PCR product having the predicted melting temperature, melting curves were generated after each run and selected amplicons were initially validated by sequencing. All reactions were performed in triplicate and outliers excluded using Grubbs test¹⁸. To exclude contamination and nonspecific amplification problems, no-template controls were included in each run. Standard curves of samples expressing the gene of interest at a high level were included to calibrate the assay in order to calculate relative expression levels, and to assign primer efficiency and dynamic range. The primer efficiency for the standard curves ranged between 0.85 and 1.10 with a correlation coefficient > 0.99. Interrun calibration was performed based on several samples. Rotor-Gene series Software 6000 1.7 (Corbett Research) was used to analyze the data and the quantification cycle was defined as the cycle number at which the reporter fluorescence reached a predetermined threshold. To normalize relative expression levels of samples, a normalization factor was calculated in geNorm¹⁹ based on the three most stable reference genes *B2M*, *ACTB* and *HPRT1* (Table 1) out of five potential reference genes, thereby excluding *RPL13A* (NM_001244068) and *GAPDH* (AF017079) as reference genes. To assess differential gene expression among the three treatment groups, the normalized mean for the COLOS group was set to one, and the normalized mean of the gene in question from the two other groups was displayed as fold change compared to this.

Levels of circulating and tissue inflammatory factors

A multiplex assay was used to determine the concentrations of interleukin (IL)-6, IL-8 and IL-10 in distal small intestinal samples homogenized in 1% Triton X-100 and in plasma samples, as previously described²⁰, with analysis of IL-1β added to the assay. Briefly, magnetic beads coupled with the specific antibodies were incubated with plasma samples or intestinal homogenates. For plasma analysis, standards, controls and samples were diluted in assay buffer, to which was added 10% filtered and heat inactivated (56 °C for 30 min) normal porcine plasma. Biotinylated antibodies were added followed by wash and incubation with phy-

coerythrin-labeled streptavidin and reading by the Luminex100 and the data was analyzed using BioPlex software Manager 4.1.1 (Bio-Rad Laboratories, Copenhagen, Denmark). The results were expressed relative to total protein concentration in each sample determined by spectrophotometry (E280).

Plasma concentrations of serum amyloid A (SAA) were determined using a sandwich- Enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Tridelata Developments Ltd., Maynooth, County Kildare, Ireland).

Microbiology and organic acids

The luminal contents of the distal small intestine were immediately stored on ice and plated on C-calves blood agar (SSI Diagnostica, Hillerød, Denmark), and incubated under anaerobic and aerobic conditions at 37 °C for 24 h to determine the number of colony forming units (CFU)/g luminal content. The abundance and location of bacteria in the distal small intestine were evaluated using FISH. Cross-sections of 3 µm formalin-fixed and paraffin-embedded distal small intestinal tissue samples were mounted on SuperFrost/plus slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized in xylene, and dehydrated in 99% ethanol. Hybridization was performed using an AlexFluor555-oligonucleotide probe targeting 16S rRNA in most bacteria (5'-GCT GCC TCC CGT AGG AGT-3' ((21), Eurofins MWG Operon AG, Ebersberg, Germany) according to (22) with minor modifications. The slides were scanned on an ArrayWoRx[®] microarray scanner (Applied Precision, Issaquah, WA, USA) and evaluated using Adobe Photoshop version C4 (Adobe Systems, San Jose, CA, USA). All slides were evaluated using the two following semi-quantitative scores: Bacterial abundance: 1 = No or very few microcolonies; 2 = Few microcolonies; 3 = Intermediate number of microcolonies; 4 = Abundant number of microcolonies; 5 = Extensive colonization. Bacterial location was scored as follows: 1 = Present only in the lumen; 2 = Present in the lumen and in association with the tip of the villus; 3 = Present in the lumen and between villi; 4 = Present in the lumen and between villi and translocation to muscularis mucosae. As a marker of bacterial metabolic activity, the concentrations of 15 organic acids (OA) were measured in stomach and colon contents as described by Canibe *et al.*²³, however only one gram gastrointestinal sample were used in the extraction procedure.

Statistical analysis

Group differences in NEC incidences were evaluated using Fisher's exact test. All other statistical analyses were based on parametric ANOVA using the MIXED model of SAS with treatment and region as fixed variables and litter as random variable, a non-parametric Kruskal-Wallis or Mann-Whitney test in SAS or GraphPad Prism 5 (Version 5.02, La Jolla, CA, USA). Correlation analyses were performed in GraphPad Prism 5 using Spearman correlation according to Zar²⁴. Results are presented as means with standard error of mean (SEM) and $p < 0.05$ was used as the critical level of significance.

Results

Clinical outcome, organ dimensions and intestinal function

The NEC severity score was lower in COLOS pigs and tended to be lower in FCOLOS pigs ($p = 0.07$, Figure 1a), relative to FORM pigs. The incidence of NEC did not differ significantly among groups (FORM: 71%, 10/14; FCOLOS: 64%, 9/14; COLOS: 50%, 3/6). Mean daily weight gain was higher in FCOLOS pigs (21 ± 6 g/d, $p < 0.01$) than in FORM pigs (6 ± 3 g/d), with COLOS pigs being intermediate (17 ± 2 g/d).

Intestinal villus height was lower in FORM relative to FCOLOS pigs, with intermediate values in COLOS pigs (Figure 1b), and small intestinal circumference was higher in FORM (10.3 ± 0.2 mm) relative to FCOLOS pigs (9.6 ± 0.13 mm, $p < 0.01$), with values in COLOS pigs being in-between (9.7 ± 0.2 mm). The two groups fed colostrum had a higher proportion of dry mucosa in the small intestine (0.70 ± 0.01 , pooled values), relative to the FORM group (0.65 ± 0.02 , $p < 0.05$). The galactose absorption capacity (Figure 2) decreased in the FCOLOS groups after the initiation of enteral feeding. With subsequent colostrum feeding in the FCOLOS group, the values increased to reach values at 0 h and the COLOS group at 30 h of enteral feeding, while the value in FORM decreased further. The digestive capacity, indicated by small intestinal enzyme activities across the three small intestinal regions, were increased for both colostrum groups (COLOS, FCOLOS) relative to the FORM for four of the six enzymes measured (Figure 1c-f), and FCOLOS had a higher maltase activity than COLOS and FORM (Figure 1g). Only trehalase activity did not show any difference among the groups (0.30 ± 0.02 U/g of wet tissue, pooled values). Intestinal permeability measured as the ratio of lactulose to mannitol did not differ among the three groups (0.12 ± 0.04). No differences were observed between the groups in the following parameters (pooled values): body weight at birth (919 ± 28 g) and at euthanasia (973 ± 28 g), total life span (89 ± 2 h), relative length of the small intestine (308 ± 9 cm/kg body weight), ileum crypt depth (93 ± 4 μ m), total small intestinal area (303 ± 11 cm²), or relative weights (to body weight, g/kg) of stomach (7.0 ± 0.4), small intestine (29.6 ± 1.1), large intestine (9.6 ± 0.5), heart (7.9 ± 0.2), lungs (20.3 ± 0.7), liver (26.0 ± 0.5), kidneys (10.1 ± 0.3), and spleen (2.1 ± 0.1).

Tissue and circulating cytokine mRNA and protein levels

The intestinal tissue expression of *IL6*, *IL8*, *IL10*, *PBD-2*, *TLR4*, *TNF* and *SAA* did not differ between the three groups, while *IL1B* was lower in FORM compared with FCOLOS (Table 2). Positive correlations were observed between the mean NEC severity and the expression of *IL1B* ($r = 0.594$, $p < 0.001$), *IL6* ($r = 0.576$, $p < 0.001$), *IL8* ($r = 0.669$, $p < 0.001$), *IL10* ($r = 0.415$, $p < 0.05$) and *SAA* ($r = 0.376$, $p < 0.05$), whereas no correlation was observed with *PBD-2*, *TLR4* and *TNF* and mean NEC severity (not shown).

The protein concentrations of IL-1 β in the distal small intestine were higher in FORM pigs than in COLOS and FCOLOS pigs (Figure 1h). Furthermore, the IL-1 β tissue concentration correlated positively with mean NEC severity ($r = 0.350$, $p < 0.05$) and with the expression of

IL1B in tissue ($r = 0.656$, $p < 0.001$). The protein concentration of IL-8 was higher in FORM than FCOLOS, while the difference between FORM and COLOS was $p = 0.062$ (Figure 1i). No significant correlations were found for the protein concentration of IL-8 and the total mean NEC severity or the expression of *IL8* (not shown). The distal small intestinal concentrations of IL-6 and IL-10 were generally below detection limit and hence no results are shown for these cytokines. Plasma levels of IL-10 (58.1 ± 6.2 pg/mL, pooled values) and SAA (81 ± 12 g/mL, pooled values) showed similar values across the three groups, and no correlation was observed between either IL-10 or SAA, and the mean NEC severity (not shown). The plasma levels of IL-1 β , IL-6, and IL-8 were generally below the detection limit (not shown).

Microbiology and organic acid concentrations

The luminal concentration of bacteria in the distal small intestine was in the range of 10^8 CFU per g contents and did not differ among the three groups neither for anaerobic bacteria ($6.3 \times 10^8 \pm 2.0 \times 10^8$ CFU/g luminal content, pooled values) nor aerobic bacteria ($4.4 \times 10^8 \pm 1.5 \times 10^9$ CFU/g luminal content, pooled values). Likewise, FISH analyses of the distal small intestinal mucosa-associated microbiota revealed no differences in the score for bacterial abundance (1.9 ± 0.2 , pooled values) or location (2.2 ± 0.2 , pooled values) among the three groups (Figure 3a, b). However, a positive correlation between mean NEC severity and the bacterial abundance score ($r = 0.630$, $p < 0.001$) and bacterial location score ($r = 0.381$, $p < 0.05$) was observed.

The total concentration of OA in stomach contents (Figure 4a) did not differ between groups. Of the individual organic acids, octanoic acid was lower in the COLOS group than in the FORM group, with FCOLOS group being intermediate. Furthermore, the concentration of butyric acid in the stomach was lower in FORM compared with FCOLOS and COLOS, which was similar.

The concentration of OA in the colon contents (Figure 4b) was higher in the FORM group compared with values of the two colostrum groups combined (pooled values, $p < 0.01$). Furthermore, a significantly higher concentration of lactic acid was observed in the FORM pigs compared with COLOS and FCOLOS pigs.

Discussion

We have previously shown that an abrupt transition from parenteral to moderate amounts of an enteral diet induces an intestinal dysfunction that may predispose to development of NEC, at least in preterm pigs^{5-8,10,11}. Changes to intestinal structure and function occur within just 8 hours of enteral feeding and are much more pronounced with formula, relative to sow's colostrum⁹, and we now document that bovine colostrum feeding reverses the immediate pro-inflammatory effects of a short term (6 hours) feeding with moderate amounts of formula to preterm TPN-fed pigs. This was seen by the observed lower NEC severity and intestinal circumference (indicating better muscular tone), increased villus height, enzyme activities and

hexose absorption and lower tissue IL-1 β and IL-8 levels and colonic lactic acid production in the FCOLOS group, relative to the FORM group. The intact colostrum diet had this effect even when it was obtained from another species (bovine) and after sterilization by gamma-irradiation. Further studies will show whether intact bovine colostrum could be considered as a supplemental diet also for preterm infants during the challenging parenteral-enteral nutritional transition.

As in previous studies, formula-fed pigs had an increased severity of NEC, compared with pigs fed bovine colostrum^{5,25}. Colostrum feeding did not fully protect against NEC, as a mild degree of NEC was observed in both colostrum groups. This was not surprising, as it has previously been documented that bovine colostrum only protects 100% against NEC when provided already from the time of birth, and in slowly increasing volumes¹². An abrupt transition to enteral colostrum feeding after TPN may also be associated with a higher incidence of NEC-like lesions in the stomach region^{5,26} and indeed, two of the three NEC-diagnosed COLOS pigs had high NEC scores only in the stomach region. The overall higher NEC severity in the FORM group was accompanied by a reduced activity relative to the colostrum groups of five brush border enzymes. While the direct diet effects have been demonstrated previously^{11,15,25}, the present study proves for the first time that colostrum feeding can reverse the diet-induced effects on enzyme activities known to take place already within the first hours of formula feeding⁹.

Results that clearly illustrate the restorative effect of colostrum feeding were the galactose absorption tests. As expected, the absorption capacity fell in the FCOLOS and FORM groups during the first 6 hours after initiation of enteral feeding, whereas the absorption capacity of galactose in COLOS pigs remained at the 0-hour level during this period. Thus, as demonstrated previously, the initial formula feeding appears to induce a very rapid decrease in the function of the sodium-glucose linked transporter 1^{12,25}. The decrease was reversed by colostrum feeding since the galactose level in FCOLOS pigs at 30 hours after start of feeding were similar to that in COLOS pigs, while the level very decreased further in FORM pigs at 30 hours. The improved digestive and absorptive capacity not only removes more nutrients from the intestinal lumen but also reduces the amount of substrate available for bacterial fermentation. This can explain that the concentration of colonic lactic acid was reduced in both colostrum groups, relative to formula.

Colostrum feeding resulted in a decrease of the formula-induced increases in both IL-1 β and IL-8 production in the distal small intestine. A previous study showed that feeding sow's colostrum to preterm pigs down-regulates IL-1 β and the initial feeding-induced intestinal lesions relative to formula¹⁰, and this study shows that bovine colostrum has a similar effect. The group differences seen at the protein level for IL-1 β and IL-8 were not observed at the mRNA level and for IL-1 β a higher tissue level in the FCOLOS group was observed compared with the FORM group. The lack of correlation between protein- and mRNA levels may be due to differences in onset of the expression of the mRNA and the following secretion of the protein²⁷. The positive correlation between the expression of *IL1B*, *IL6*, *IL8*, *IL10* and *SAA*

and the mean NEC severity suggests that the expression of these inflammatory factors is more correlated to disease progression than to the type and amount of nutrition. No correlation was observed between the IL-10 and SAA levels in plasma and the mean NEC severity. Plasma SAA levels have been found to correlate with NEC disease progression in infants^{28,29}. However, acute phase proteins like SAA may behave very differently in different species³⁰. Furthermore, protein levels of IL-1 β , IL-6 and IL-8 in plasma were below detection preventing their use as biomarkers for NEC in preterm pigs.

The density of the gut microbiota in the distal small intestine, as well as the bacterial attachment to the mucosa, was not significantly affected by diet in our study, however, mean NEC severity correlated positively with both the bacterial abundance and location scores. Bacterial density at the tissue surface is therefore correlated more with the progression of NEC than with the nature and amount of the enteral diet¹². The trend towards higher total amount of colonic OA in the FORM group suggests that FORM resulted in a higher microbial activity in the colon. Thus, colostrum does not suppress bacterial density and mucosal attachment, but rather support tissue responses towards feeding and bacteria. This study underlines the importance of diet composition in the first enteral feedings to preterm neonates to promote intestinal maturation and resistance against NEC. It remains to be investigated whether bovine colostrum could be used as a safe and effective substitute product to preterm infants when human mother's milk or colostrum is not available during the challenging parenteral to enteral nutrition transition. We do underline that preterm pigs may not accurately reflect the physiology of preterm infants born at different gestational ages and with different clinical complications, and studies in infants are clearly needed. Nevertheless, further studies in preterm pigs may help to better define the optimal diet, timing and amount of the first enteral food for the immature infant intestine.

Acknowledgements

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Conflict of interest

The study was partly supported by Biofiber-Damino, which also donated the colostrum used in the study. This commercial partner had no influence on any decisions regarding study design, results interpretation and conclusion of the study.

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FIGURE LEGENDS

FIGURE 1: Mean NEC severity scores across the five gut regions based on macroscopic tissue evaluation (a), distal small intestinal villus height (b), activity of brush-border enzymes: dipeptidylpeptidase IV (c), aminopeptidase A (d), aminopeptidase N (e), lactase (f), and maltase (g) across the three small intestinal regions and values for IL-1 β (h) and IL-8 (i) protein levels in distal small intestinal tissue in FORM pigs (white), COLOS pigs (gray), and FCOLOS pigs (black). All data are presented as means \pm SEM, and means not sharing the same superscript symbols are significantly different ($p < 0.05$).

FIGURE 2: Arterial plasma concentrations of galactose after an oral bolus of galactose given before (0 h, pooled values, horizontal lines) as well as 6 h and 30 h after initiation of the enteral feeding period (means \pm SEM) in FORM pigs (white), COLOS pigs (gray), and FCOLOS pigs (black). Means at 6 h and 30 h are compared with the mean for 0 h, and significant differences are shown by different superscript symbols ($p < 0.05$).

FIGURE 3: Examples of FISH analyses applied to cross-sections of the distal small intestine. Scanning photos showing bacteria (red signal) of representative pigs: bacterial abundance score = 1, no or very few microcolonies and distal NEC score = 1, no or minimal focal hyperaemic gastroenterocolitis (a), and bacterial abundance score = 5, extensive colonization and distal NEC score = 5 (b).

FIGURE 4: Total concentration (means \pm SEM) and the concentration of the dominating OAs in stomach contents (a) and colon contents (b) in FORM pigs (white), COLOS pigs (gray), and FCOLOS pigs (black). Different superscript symbols indicate significant differences ($p < 0.05$).

TABLES

Table 1. Primer sequences and reaction conditions for quantitative real-time PCR analysis.

Gene	Accession number	Sequence (5' - 3')	Amplicon length	Ta (°C) ^a	MgCl ₂ (mM) ^b
<i>B2M</i>	DQ845172.1	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	70	62	1.5
<i>ACTB</i>	DQ845171.1	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76	62	1.5
<i>HPRT1</i>	AF143818.1	F: AACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71	60	1.5
<i>IL1B</i>	NM_214055.1	F: CCAAAGAGGGACATGGAGAA R: GGGCTTTTGTCTGCTTGAG	123	59	3.0
<i>IL6</i>	AF518322.1	F: GCAGTCACAGAACGAGTGGA R: CAGGCTGAACTGCAGGAAAT	82	59	3.0
<i>IL8</i>	NM_213867.1	F: GAAGAGAAGTGAAGCAACA R: TTGTGTTGGCATCTTTACTGAGA	99	60	3.0
<i>IL10</i>	NM_214041.1	F: CTGCCTCCACTTCTCTTTG R: TCAAAGGGGCTCCCTAGTTT	95	60	1.5
<i>PBD-2</i>	NM_214442.1	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99	60	1.5
<i>TLR4</i>	GQ304754.1	F: TTTCCACAAAAGTCCGAAGG R: CAACTTCTGCAGGACGATGA	145	60	3.0
<i>TNF</i>	JF831365.1	F: CCCCAGAAAGGAAGATTTC R: CGGGCTTATCTGAGGTTTGA	92	62	1.5
<i>SAA</i>	EF362780.1	F: TAAAGTGATCAGCAATGCCAAA R: TCAACCCTTGAGTCCTCCAC	96	60	3.0

^a Annealing temperature.

^b MgCl₂ is the total concentration of MgCl₂ in the reaction mix.

Table 2. Relative expression of inflammatory markers in the distal small intestine.

Gene ^a	FORM	COLOS	FCOLOS
<i>IL1B</i>	8.6 ± 2.8 [*]	1.0 ± 0.1	25 ± 17 [§]
<i>IL6</i>	1.8 ± 0.7	1.0 ± 0.2	7.6 ± 5.2
<i>IL8</i>	3.0 ± 1.0	1.0 ± 0.4	2.7 ± 1.5
<i>IL10</i>	1.8 ± 0.5	1.0 ± 0.3	1.4 ± 0.5
<i>PBD-2</i>	1.5 ± 0.5	1.0 ± 0.3	0.9 ± 0.2
<i>TLR4</i>	1.5 ± 0.3	1.0 ± 0.1	1.0 ± 0.2
<i>TNF</i>	1.0 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
<i>SAA</i>	1.9 ± 0.5	1.0 ± 0.6	1.3 ± 0.6

^a Values are presented as mean ± SEM, and means not sharing the same superscript symbol within each gene are significantly different ($P < 0.05$).

FIGURE 1

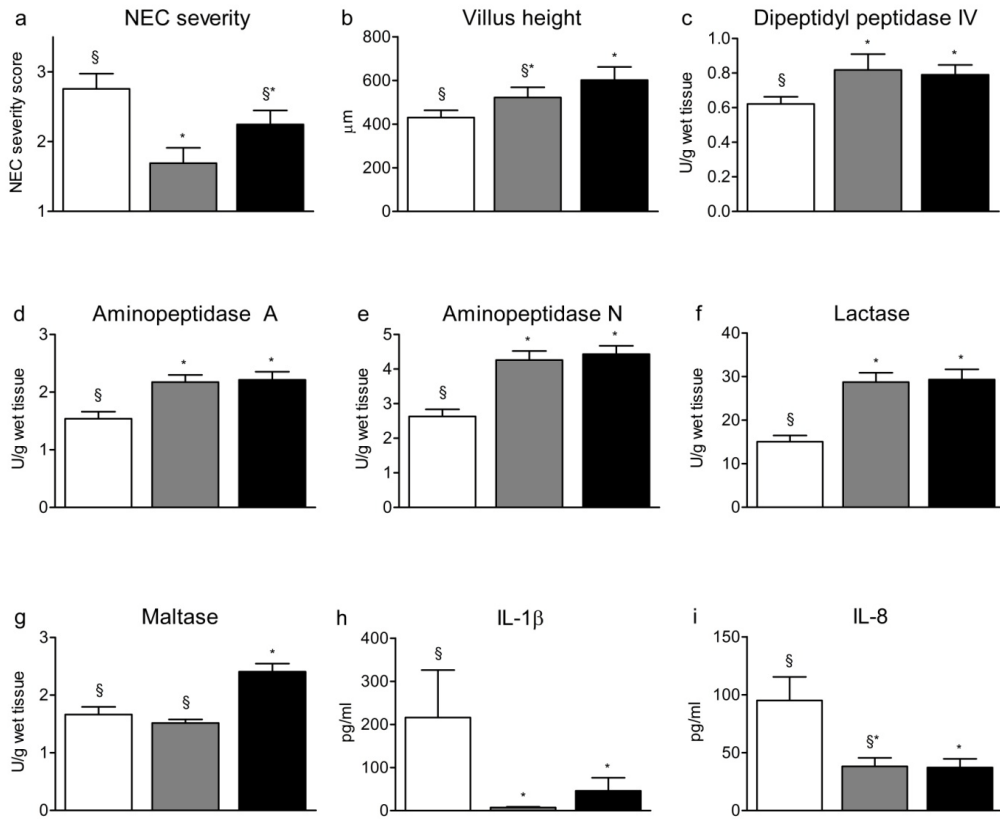


FIGURE 2

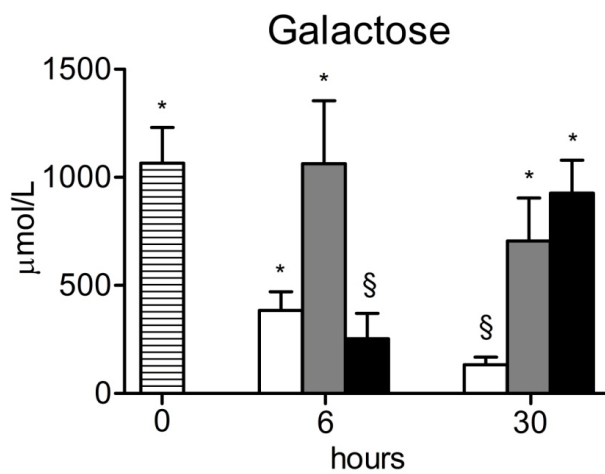


FIGURE 3

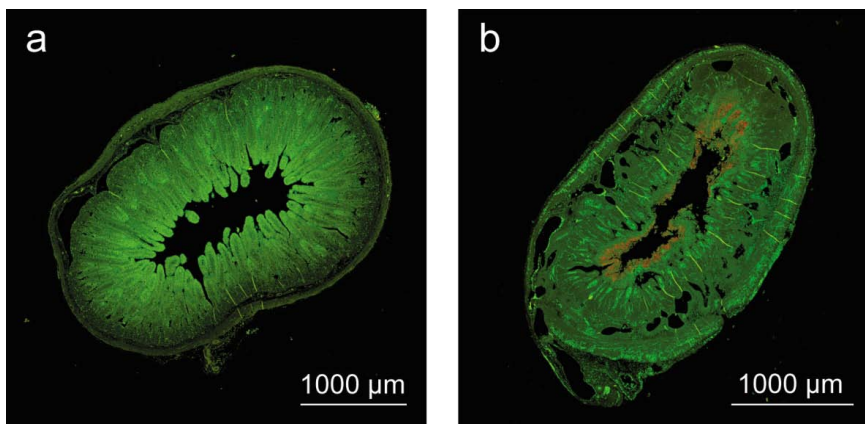
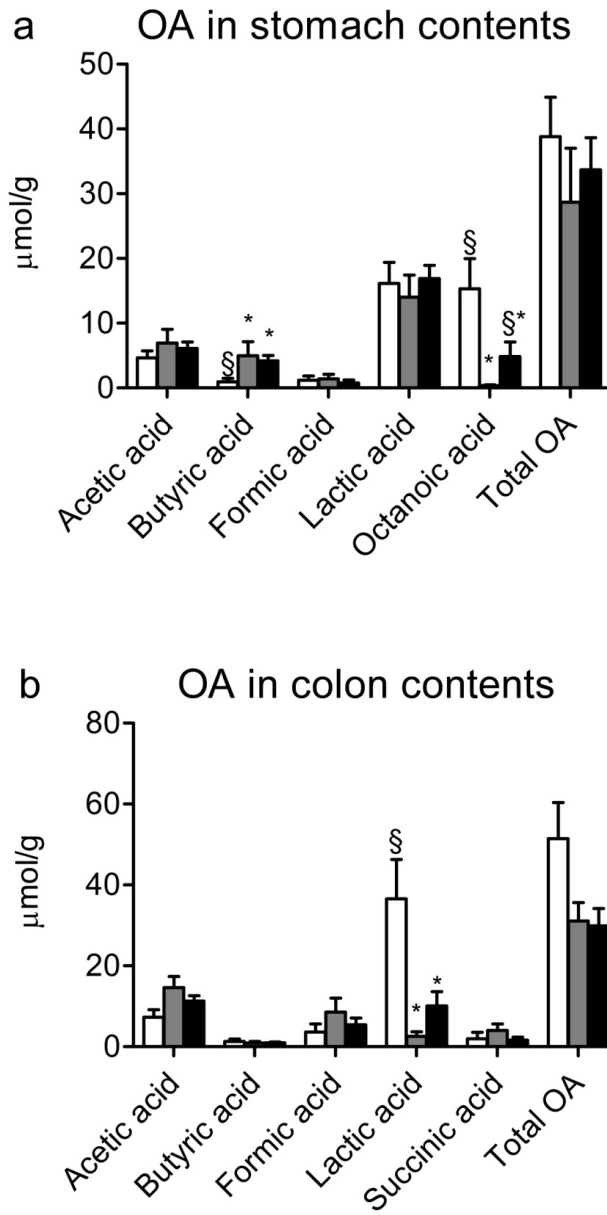


FIGURE 4



Paper II

Mild heat treatment does not reduce the colitis-protective effects of bovine colostrum in preterm pigs

Ann Cathrine F. Støy, Per T. Sangild, Kerstin Skovgaard, Thomas Thymann, Mette Bjerre, Dereck E. W. Chatterton, Stig Purup, Mette Boye, Peter M. H. Heegaard

Manuscript ready for submission

TITLE PAGE

Mild heat treatment does not reduce the colitis-protective effects of bovine colostrum in preterm pigs

Running title: Spray drying and pasteurization of colostrum

Ann Cathrine F. Støy¹, Per T. Sangild², Kerstin Skovgaard¹, Thomas Thymann², Mette Bjerre³, Dereck E. W. Chatterton⁴, Stig Purup⁵, Mette Boye¹, Peter M. H. Heegaard^{1*}

¹ National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark

² Department of Human Nutrition, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

³ The Medical Research Laboratories, Department of Clinical Medicine, Faculty of Health Sciences, Aarhus University, DK-8000 Aarhus C, Denmark

⁴ Department of Food Science, Dairy Technology, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

⁵ Department of Animal Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

*Corresponding author

Peter M. H. Heegaard, National Veterinary Institute, Technical University of Denmark, 27 Bülowsvej, DK-1870 Frederiksberg C, Denmark; tel. +45 35886241; fax. +45 35886001. e-mail: pmhh@vet.dtu.dk.

Abstract

Fresh bovine colostrum (BC) prevents development of necrotizing enterocolitis (NEC) in pre-term pigs. Spray drying and pasteurization are required to use BC in clinical settings but this may also reduce its bioactivity.

In studies on preterm pigs, we compared raw BC with spray dried and pasteurized BC. Pre-term pigs were fed a few hours of formula followed by two days of formula (FORM, n = 14), fresh BC (COLOS, n = 14), spray-dried BC (POW, n = 8), or spray dried, pasteurized BC (POWPAS, n = 9).

POW and POWPAS pigs showed lowered gut NEC severity, IL-1 β and -8 levels and lactic acid levels, and higher intestinal villus heights, hexose absorption, hydrolase activities (lactase, maltase, peptidases) than FORM pigs (all $P < 0.05$). These values in POW and POWPAS groups were similar to those in the COLOS group. Intestinal expression of *IL1B*, *IL6* and *IL8* and bacterial abundance score were positively correlated with NEC severity ($P < 0.05$). All three colostrum diets stimulated wound-healing capacity of intestinal IEC-6 cells *in vitro*, but spray drying, and especially pasteurization, increased the breakdown of growth factors (TGF- β 1 and - β 2) and the aggregation of milk proteins.

Spray drying and pasteurization affect BC proteins but such treatments do not necessarily decrease its trophic and anti-inflammatory effects on the immature intestine. It remains to be studied if such colostrum products also improve gut maturation in preterm infants.

Keywords: Pasteurized bovine colostrum, spray dried bovine colostrum, animal model, necrotizing enterocolitis.

Introduction

Colostrum, the first milk after birth, is particularly rich in bioactive compounds including antioxidants, growth factors, and antimicrobial- and immune-modulatory factors. These bioactives are essential in stimulating gastrointestinal- and immunological maturation, and establishment of a beneficial gut microbiota (1-3). Compared with mature milk, the concentration of many bioactive factors is several times higher in colostrum, and some are found to be elevated also in preterm milk (2, 4-9). Provision of colostrum is particularly important for preterm neonates, because the intestinal immaturity makes these individuals very sensitive to maldigestion, abnormal bacterial colonization and dys-regulated immune responses, which potentially can lead to necrotizing enterocolitis (NEC). Feeding infant formula, both alone and in combination with mother's milk, predispose to infant NEC (10-13). In a preterm pig model of NEC, formula-fed preterm pigs had decreased villus heights and mass of the intestinal mucosa, decreased digestive enzyme capacity and nutrient absorption and more NEC lesions compared with preterm pigs fed bovine colostrum (BC) or porcine colostrum (14, 15). These studies also indicate that the beneficial actions of colostrum or milk from one mammalian species may be effective in another species and that both BC and donor human milk decreased NEC in preterm pigs compared with infant formula (16). Thus, when human colostrum and milk are not available for preterm infants during the first critical days after birth, an alternative to infant formula could be BC, adjusted in macro- and micronutrients composition to meet the nutritional requirement of the preterm infant.

A prerequisite for use of BC in clinical settings is a readily available standardized and with a low bacterial contamination. It is well known that heat treatment is associated with loss of bioactivity to a varying degree dependent on the processing conditions (17-19). We hypothesized that gentle spray drying, combined with high-temperature, short-time pasteurization would not markedly affect the beneficial effects of BC in the preterm pig intestine. Using a preterm pig model of NEC (14), we investigated if spray dried and sterilized BC would be superior to infant formula, and similar to raw BC, with respect to effects on NEC lesions, intestinal structure, digestive and absorptive functions, microbiota, and tissue protein and mRNA levels of immune factors. Preterm pigs were given TPN for two days followed by 6 hours of formula feeding, which have been shown to initiate detrimental intestinal changes (20), before being given either formula or either one of the three different colostrum products. *In vitro* cell migration assay was used to determine wound healing in the presence of increasing doses of the three colostrum products. Finally, we determined the concentrations of some bioactive proteins in the colostrum products and studied the possible treatment-related aggregation of proteins.

Materials and methods

Animals and their treatment

The procedures for caesarean section and nursing of the preterm pigs followed a standard protocol (14, 21). Forty-five preterm pigs were delivered from four sows by caesarean section (Large White × Danish Landrace × Duroc, Askelygaard Farm, Roskilde, Denmark) at 105–107 d gestation (90–92% gestation). The pigs were fitted with an oro-gastric feeding tube (6F, Portex, Kent, UK) and a vascular catheter (4F, Portex) fitted into an umbilical artery and placed in temperature-regulated incubators with extra oxygen supply if necessary. To provide passive immunity, the pigs received isolated maternal plasma three times (4, 5, and 7 mL•kgbody weight (BW)⁻¹ at 3, 8 and 12 h after birth, respectively) through the vascular catheter. Pigs were given TPN for 48 h via the vascular catheter based on Nutriflex Lipid Plus (Braun, Melsungen, Germany; 4 mL•kgBW⁻¹ advancing to 6 mL•kg BW⁻¹ per h) and adjusted in nutrient composition to meet their nutritional requirements (14). Following the TPN period, pigs were shifted to total enteral nutrition for an additional 2 d before euthanasia and tissue collection. Over the first 6 h of enteral feeding, all the pigs received milk formula (containing 80 g Pepdite, 70 g Maxipro, and 75 g Liquigen per L of water, all products kindly donated by Nutricia, Allerød, Denmark (14)). Hereafter, the pigs were randomly assigned according to birth weight into four enteral nutrition groups fed either raw BC (COLOS, n = 14), spray dried BC (POW, n = 8), pasteurized spray dried BC (POWPAS, n = 9), or milk formula (FORM, n = 14). The feeding dose was 15 mL/kg BW/3 h for all groups. The colostrum products were obtained from the same batch of first milking after parturition and kindly donated by Biofiber-Damino, Gesten, Denmark. Pasteurization of liquid BC was performed using high temperature, short time pasteurization at 72 °C for 15 seconds, while the powdered BC was produced by filter mat spray drying (Biofiber-Damino). COLOS, POW and POWPAS were sterilized by gamma-irradiation (1 x 10 kGy, Sterigenics, Espergærde, Denmark). The products were tested free of bacterial contamination, and were stored at -20 °C. The formula and three BC products were all dissolved in tap water to obtain the same dry matter content (approximately 15 g•L⁻¹) and warmed to body temperature in a water bath before use. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

Clinical evaluation and tissue collection

Pigs were monitored closely and euthanized if clinical symptoms of NEC such as abdominal distension, lethargy, cyanosis or bloody diarrhea were observed. Pigs not showing clinical signs of NEC were euthanized after 45–50 h of enteral feeding. Euthanasia and tissue collection were performed according to earlier described protocols (15). In all pigs the proximal, middle and distal small intestine, stomach and colon were given a NEC severity score: 1, no or minimal focal hyperaemic gastroenterocolitis; 2, mild focal gastroenterocolitis; 3, moderate locally extensive gastroenterocolitis; 4, severe focal gastroenterocolitis; 5, severe locally ex-

tensive hemorrhagic and necrotic gastroenterocolitis; or 6, severe extensive hemorrhagic and necrotic gastroenterocolitis. Pigs with a severity score of minimum 3 in any gastrointestinal region was regarded as a case of NEC. Mean NEC severity was expressed as the mean of the NEC severity score from the five regions. Full thickness tissue samples from the small intestinal regions were immediately snap-frozen in liquid nitrogen and stored at -80 °C until analysis of small intestinal enzyme activities, gene expression, and cytokine concentrations. A 10 cm segment of each small intestinal region was used to measure intestinal circumference and to determine the proportion of dry mucosa as described in (22). Samples from the distal small intestine were collected and fixed in 4% neutral buffered paraformaldehyde for 24 h before being transferred to 70% ethanol and prepared for fluorescence *in situ* hybridization (FISH) and gut morphology investigations.

Gut morphology and intestinal digestive function

Villus heights and crypt depths in the distal small intestine were evaluated on scanning pictures obtained from FISH analysis using the morphometric software SoftWoRx Explorer version 1.2.0 (Applied Precision, Issaquah, WA, USA). For each pig, one representative cross section was selected and 10 random selected villi and crypts were measured. To determine the *in vivo* galactose absorption capacity, each pig was given an oral bolus (15 mL•kg BW⁻¹, 5% galactose) via the oro-gastric feeding tube before enteral nutrition, 6 h and 30 h after enteral food introduction. Arterial blood was obtained exactly 20 min after administration of each bolus for measurements of plasma galactose concentrations (23). All blood samples were collected in EDTA- or heparin coated tubes, placed on ice, centrifuged for 10 min at 4°C and 2,500 × g and the plasma stored at -20°C until further analyses. Blood collected at euthanasia was used for later determination of plasma cytokine levels. The ratio of lactulose to mannitol in urine was used as a surrogate marker of intestinal permeability. Pigs received an oral bolus containing 5% lactulose and 2% mannitol (15 mL•kg BW⁻¹) four to six hours prior to euthanasia, at which time urine was sampled from the bladder by cystocentesis and the urinary ratio of lactulose to mannitol in urine was determined as previously described (14). Finally, intestinal digestive capacity was measured on frozen intestinal sections as hydrolytic activities expressed as U•g⁻¹ of wet intestine of lactase, maltase, aminopeptidase N, aminopeptidase A and dipeptidyl-peptidase IV, as described previously (24). Trehalase activity was measured as described for lactase and maltase in (24) using 0.6 M D-(+)-trehalose dihydrate (EC 202-739-6, Sigma-Aldrich, Brøndby, Denmark) as a substrate.

Immune gene expression in intestinal tissue

Gene expression of *IL1B*, *IL6*, *IL8*, *IL10*, defensin β 4A (*DEFB4A*), toll-like receptor 4 (*TLR4*), TNF- α (*TNF*) and serum amyloid A (*SAA*) and reference genes β -2-microglobulin (*B2M*), β -actin (*ACTB*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) in distal intestinal tissue was determined using quantitative real-time PCR. Total RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen, Ballerup, Denmark) and on-column DNase

treated using RNase-free DNase set (Qiagen) according to manufacturer's protocol. Quantity and quality of extracted total RNA was measured as described previously (25). One μg of total extracted RNA was converted into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. Quantitative PCR was performed using diluted cDNA (1:5 or more) in a total reaction volume of 25 μl containing gene-specific primers (300 nM), SYBR Green PCR Master Mix (Sigma-Aldrich) and MgCl_2 (Table 1). PCR cycling was performed on a RotorGene 3000 Detection System (Corbett Research, Sydney, Australia) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles with 15 s at 95 °C and 1 min at 59–62 °C (Table 1). Primer design and validation were performed as described in (25), and synthesized at TAG Copenhagen, Copenhagen, Denmark. All PCR reactions were performed in triplicate and outliers excluded using Grubbs test (26). To exclude contamination and nonspecific amplification problems, no-template controls were included in each run. Standard curves of samples expressing the gene of interest at a high level were included to assign primer efficiency and dynamic range. The primer efficiency for the standard curves ranged between 0.85 and 1.10 with a correlation coefficient > 0.99 . Inter-run calibration was performed based on several samples. Expression levels of all samples were normalized to the normalization factor calculated in geNorm (27) based on the geometric mean of three out of five most stable reference genes: *B2M*, *ACTB*, and *HPRT1* (Table 1). To assess differential gene expression between the four treatment groups the mean for the COLOS group was set to one, and the mean of the gene in question from the three other groups was displayed as fold change compared to the COLOS group.

Intestinal and plasma levels of cytokines

A multiplex assay was used to determine the concentrations of IL-6, IL-8, and IL-10 in distal small intestinal samples homogenized in 1% Triton X-100 and in plasma samples, as previously described (28) with the addition of IL-1 β to the assay using the conditions described (28). Briefly, plasma samples or intestinal homogenates were incubated with magnetic color-coded beads coupled with specific antibodies. For the plasma analysis, standards, controls and samples were diluted in assay buffer, to which was added 10% filtered and heat inactivated (56 °C for 30 min) normal porcine plasma. Biotinylated antibodies were added followed by wash and incubation with phycoerythrin-labelled streptavidin. The beads were read by the Luminex100 followed by analysis using the BioPlex software Manager 4.1.1 (Bio-Rad Laboratories, Copenhagen, Denmark). The results were expressed relative to total protein concentration in each sample determined by spectrophotometry (E280).

SAA plasma concentrations were determined with ELISA, according to the manufacturer's instructions (Tridelta Developments Ltd. Maynooth, County Kildare, Ireland).

Bacterial abundance and location and organic acid concentrations

Bacterial abundance and location were evaluated using FISH analysis. Cross-sections of 3 μm formalin-fixed and paraffin-embedded distal small intestinal tissue samples were mounted on

SuperFrost/plus slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized in xylene, and dehydrated in 99% ethanol. Hybridization was performed using an AlexFluor555-oligonucleotide probe targeting 16S rRNA in most bacteria (5'-GCT GCC TCC CGT AGG AGT-3' ((29), Eurofins MWG Operon AG, Ebersberg, Germany) according to (30) with minor modifications. The slides were scanned on an ArrayWoRx^e microarray scanner (Applied Precision, Issaquah, WA, USA) and evaluated using Adobe Photoshop version C4 (Adobe Systems, San Jose, CA, USA). The following two scoring systems were used to evaluate bacterial abundance: 1, no or very few microcolonies; 2, few microcolonies; 3, intermediate number of microcolonies; 4, abundant number of microcolonies; 5, extensive colonization. Bacterial location was scored as: 1, present only in the lumen; 2, present in the lumen and in association with the tip of the villus; 3, present in the lumen and penetration into villus; 4, present throughout the section and translocation to muscularis mucosae.

The concentrations of organic acids (OA) in gastrointestinal content were measured as described by (31), however only one gram gastrointestinal sample were used in the extraction procedure.

Migration of intestinal epithelial cells *in vitro*

Rat intestinal epithelial cells obtained from DSMZ (IEC-6, Braunschweig, Germany; ACC 111) were cultured in 75 cm² flasks (Thermo Fisher Scientific, Roskilde, Denmark) in Dulbecco's minimal essential medium (11960-044; Invitrogen, Tåstrup, Denmark), supplemented with fetal calf serum (FCS, 10%; Cambrex Bio Science, Copenhagen, Denmark), sodium pyruvate (0.4 mM; Invitrogen), Glutamax (2 mM; Invitrogen), and penicillin/streptomycin solution (1 v/v%, P-0781; Sigma-Aldrich, Vallensbæk Strand, Denmark). A scratch wound assay was performed as an *in vitro* wound healing model. Cells were plated in 12-well plates at a density of 200,000 cells per well at the day before the experiments. Monolayers of cells were scratched with a pipette tip to create a standardized cell-free area. One scratch was established per well. Cells were then carefully washed with Dulbecco's Phosphate-Buffered Saline (14080-048, Invitrogen) to remove residual cell debris. Whey was prepared from raw colostrum (COLOS) diluted 1:5 in sterile water, and POW or POWPAS in solutions of 10% (v/v) in sterile water as previously described (7). Treatment medium containing concentrations of 0.01, 0.1, 1, 5 and 10% (v/v) of whey but without FCS was then added to cells. As positive and negative controls, serum-free medium alone or medium containing 10% FCS was included in each assay (positive control not shown). The distance migrated from the original wound line was determined by taking photomicrographs after wounding and 5–6 h after addition of treatment medium. An inverted microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany) and a Leica digital camera (DFC320) were used to obtain photomicrographs. Identical regions were examined at each time point by pre-marking the base of the plates to facilitate alignment. Six measurements were performed in each of two replicate wells giving 12 replicate measurements for each treatment.

Characterization of proteins in colostrum products

Whey fractions of COLOS, POW and POWPAS were prepared according to (7). Concentration of insulin-like growth factor (IGF-I), TGF- β 1 and TGF- β 2 in the whey fractions were determined in two replicate measurements by time-resolved immune-fluorescence assay (IGF-I) and ELISA (TGF- β 1, TGF- β 2) as previously described (7).

Colostrum protein denaturation and aggregation were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) analysis. One % (w/v) solutions of proteins were prepared and used without further treatment, or centrifuged at $13,000 \times g$ for 15 min. Following centrifugation, the supernatant was sampled without disturbing the lipid layer. Samples were heated to 95°C for 3 min in non-reducing Laemmli sample buffer and then $11.25 \mu\text{g}$ of protein from each sample was applied to 12% Tris/Glycine SDS PAGE gels and proteins separated using electrophoresis. Proteins were visualized by staining with Coomassie Brilliant Blue. The relative molecular weight of the proteins was determined by comparison to the migration of molecular weight marker (M).

Statistical analysis

Group differences in NEC incidences were evaluated using Fisher's exact test. All other statistical analyses were based on parametric ANOVA in GraphPad Prism 5 (Version 5.02, La Jolla, CA, USA) or using the MIXED model of SAS (SAS Institute, Cary, NC, USA) with treatment and region as fixed variables and litter as random variable, while non-parametric data were analyzed using a non-parametric Kruskal-Wallis test or Mann-Whitney test in SAS or GraphPad Prism 5. Correlation analyses were performed in GraphPad Prism 5 using Spearman correlation according to (32). Statistical analyses of migration data were performed using the general linear models procedure (PROC GLM) of SAS. Results are presented as mean with standard error of the mean (SEM) and $P < 0.05$ was used as the critical level of significance.

Results

Clinical outcome and organ dimensions

Twenty-five pigs were euthanized due clinical symptoms of NEC during the 50 h of enteral feeding, and afterwards evaluated as suffering from NEC based on the intestinal NEC severity score. This resulted in a higher mean NEC severity in FORM than POWPAS and POW pigs, while POWPAS had lower severity of NEC than COLOS pigs (Fig. 1A). Additionally, COLOS showed a tendency towards lower NEC severity than FORM pigs ($P = 0.06$). The incidence of NEC decreased in the order FORM (71%, 10/14), COLOS (64%, 9/14), POW (38%, 3/8), POWPAS (33%, 3/9), with a significant difference between FORM and POWPAS pigs ($P < 0.01$). FORM pigs had reduced life span (85 ± 4 h) compared with POW and POWPAS pigs (both 98 ± 1 h, $P < 0.01$), with COLOS pigs being intermediate (90 ± 3 h). Distal small intestinal villus heights were lower in the FORM group relative to the three colostrum groups

(Fig. 1B). In addition, FORM pigs (10.3 ± 0.22 mm) had an increased small intestinal circumference compared with POWPAS (9.5 ± 0.22 mm) pigs, while the small intestinal circumference in COLOS (9.6 ± 0.13 mm) and POW (9.7 ± 0.16 mm) pigs were intermediate. No significant group differences (pooled value) were observed in BW at birth (898 ± 28 g) or at euthanasia in BW (947 ± 29 g), relative length of the small intestine (322.4 ± 9.9 cm \cdot kg $^{-1}$), crypt depth (96 ± 3 μ m), total small intestinal area (291 ± 9 cm 2 , pooled values), or relative weights (to BW, g \cdot kg $^{-1}$) of stomach (6.7 ± 0.3), small intestine (30.0 ± 0.9), large intestine (9.5 ± 0.4), heart (8.0 ± 0.2), lungs (21.6 ± 0.8), liver (26.0 ± 0.4), kidneys (9.9 ± 0.3) and spleen (2.0 ± 0.1).

Intestinal digestive capacity, absorption and permeability

Across the small intestinal regions, the activity of five of six enzymes measured was higher in pigs from the colostrum groups compared with FORM pigs, and some differences were observed between the three colostrum groups (Fig. 1C–G). The activity of trehalase was similar between groups (0.32 ± 0.01 U \cdot g $^{-1}$ of wet intestine, pooled values). The three colostrum groups combined had a higher proportion of dry mucosa in the small intestine (0.69 ± 0.01 , pooled values), relative to the FORM group (0.65 ± 0.02 , $P < 0.05$). Intestinal permeability measured as the ratio of lactulose to mannitol did not differ between groups (0.08 ± 0.02 , pooled values). Across groups, galactose absorption decreased during the first 6 h in which all pigs received formula, and with subsequent colostrum feeding in the COLOS, POW and POWPAS groups the values increased to become significantly higher than for the FORM group at 30 h (Fig. 2).

Cytokine expression and concentration

IL1B, *IL8*, *IL6*, *IL10*, *TLR4*, *TNF* and *SAA* expression in distal intestinal tissue did not differ between the groups, whereas *DEFB4A* expression was significantly higher in POWPAS pigs compared with COLOS pigs (Table 2). Positive correlations were observed between the mean NEC severity and the expression of *IL1B* ($r = 0.55$, $P < 0.001$), *IL6* ($r = 0.40$, $P < 0.01$), and *IL8* ($r = 0.52$, $P < 0.001$), while no correlations were observed between the mean NEC severity and the expression of *IL10*, *PBD-2*, *TLR4*, *TNF* or *SAA* (not shown).

The protein concentration of IL-1 β in the distal small intestine was higher in FORM pigs than COLOS and POWPAS pigs with POW pigs being in-between (Fig. 1H). IL-1 β correlated with the mean NEC severity ($r = 0.50$, $P < 0.01$) and with the expression of *IL1B* ($r = 0.56$, $P < 0.001$). The protein concentration of IL-8 (Fig. 1I) was higher in the FORM group compared with the COLOS group, with intermediate values in the POW and POWPAS groups. No significant correlations were found for the protein concentration of IL-8 and the expression of *IL8* or the total mean NEC severity (not shown). IL-10 (53.0 ± 5.5 pg \cdot mL $^{-1}$, pooled values) and SAA (77.7 ± 12.4 g \cdot mL $^{-1}$, pooled values) levels were similar across groups, and no correlation was observed between either IL-10 or SAA, and the mean NEC severity (not

shown). The distal small intestinal tissue concentration of IL-6 and IL-10, and plasma levels of IL-1 β , IL-6, and IL-8 were generally below detection limit (not shown).

Bacterial abundance and location and organic acid concentrations

FISH analyses of the distal small intestinal mucosa-associated microbiota revealed no differences in the score for bacterial location score (2.4 ± 0.1 , pooled values) or abundance score (1.9 ± 0.2 , pooled values) between the four groups (Fig. 3A, B). A positive correlation between the mean NEC severity and the bacterial abundance score ($r = 0.38$, $P < 0.05$) was observed.

In the stomach, no differences in the total concentration of OAs were observed between groups (Fig. 4A), but of the major OAs in the stomach, the concentration of butyric acid was lower in FORM pigs than pigs from the three colostrum groups. The concentration of octanoic acid in the stomach was significantly higher in FORM and COLOS than POWPAS pigs, with POW being intermediate. The concentration OAs in the colon (Fig. 4B) was higher in the FORM group (51.4 ± 9.0 mmol \cdot kg wet weight $^{-1}$) compared with the three colostrum groups combined (30.0 ± 2.9 mmol \cdot kg wet weight $^{-1}$, $P < 0.01$). A higher concentration of lactic acid was observed in the FORM group compared with the three colostrum groups, while the concentration of acetic acid in the colon was significantly lower in FORM compared with POW and POWPAS pigs. No differences were found for the remaining OAs in the stomach or colon contents.

Migration of intestinal epithelial cells *in vitro*

Intestinal epithelial cell migration (Fig. 5) was increased in a dose-dependent way by whey produced from COLOS and POWPAS (both $P < 0.05$). Maximum increase in migration was obtained at the highest whey concentration (10%) where the distance migrated was increased 1.9 times compared with the control (no whey added). Overall, migration tended ($P < 0.06$) to be increased by whey produced from POW, and migration was significantly increased in a concentration of 1% relative to control ($P < 0.01$). In cells treated with POW whey, migration at a concentration of 5% was lower than at 1% of whey, and at 10%, cells were lysed indicating a too high concentration of whey added.

Characterization of proteins in colostrum products

The concentration of IGF-I was similar in whey produced from COLOS, POW and POWPAS (1212 ± 26 ng \cdot g dry matter $^{-1}$, pooled values). The concentration of TGF- β 1 was higher in COLOS (35.1 ± 0.5 ng \cdot g dry matter $^{-1}$) compared with POW (18.7 ± 0.1 ng \cdot g $^{-1}$) and POWPAS (15.1 ± 0.4 ng \cdot g $^{-1}$), while the concentration of TGF- β 2 was lower in POWPAS (166 ± 5 ng \cdot g $^{-1}$) compared with COLOS and POW (607 ± 5 and 558 ± 1 ng \cdot g $^{-1}$, respectively).

Non-centrifuged samples had a similar protein profile on the SDS page gels (Fig. 6). Caseins were observed at ~ 36 kDa, and centrifugation resulted in a removal of the majority of casein micelles (average diameter 120–180 nm (33)) in all colostrum products, while a smaller population of residual casein micelles (average diameter 60 nm (34)) remained. Quali-

tatively, protein aggregation was most pronounced in the POWPAS product, observed by a reduction in Ig (~250 kDa) and proteins in the region 64–98 kDa, including lactoferrin and lactoperoxidase observed at ~80 kDa. Still, all proteins of interest were detected also in the POWPAS group.

Discussion

The majority of mothers giving birth to preterm infants are unable to provide enough milk for their infants during the first critical weeks after birth (35). Parenteral nutrients may help, but a gradual transition to supplementary enteral feeding is required to obtain sufficient growth and development. When mother's milk or human donor milk is not available, the source of this enteral nutrition is artificial milk replacers. Natural milk contains not only nutrients but also a broad range of growth factors and bioactive components that mediate antimicrobial and immune-modulatory effects in the newborns of different species. Colostrum is particularly rich in these factors and fresh colostrum is known to be highly important for compromised newborns, such as those born prematurely. Fresh BC protects against NEC in preterm pigs (14, 36) and the potential exists that BC may be beneficial also for preterm infants. Surprisingly, in this study, POWPAS and POW pigs, but not COLOS pigs had a lower NEC severity than FORM pigs, and furthermore, of the three colostrum groups only POWPAS pigs had a lower NEC incidence than FORM pigs. Future studied is required to investigate what is responsible for this tendency towards an improved effect on NEC severity in POW and POWPAS pigs compared with COLOS and FORM pigs.

However, we documented that the majority of parameters reflecting intestinal morphology and gut functions were improved to a similar extent in all three colostrum groups relative to the formula group. This is encouraging for the possible use of BC products, or fractions thereof, in a clinical hospital setting. A smaller intestinal circumference in the POWPAS group, and increased villus heights and distal small intestinal enzyme activity in all three colostrum groups, relative to the formula group, indicate increased protection against loss of muscular tone, mucosal atrophy and brush-border enzyme dysfunction. With regard to hexose absorptive function, measured by plasma galactose levels, the capacity decreased dramatically during the 6 hours of formula feeding in all four groups. However, the mucosa regained its capacity to absorb hexose when pigs subsequently were fed either one of the three colostrum products, while the values remained low after continued formula feeding, as assessed by plasma galactose levels 30 hours after start of enteral feeding. Again, the results suggest that processed colostrum products (POW and POWPAS) have the same effects as raw colostrum on intestinal maturation and development.

We observed some inflammation in all three colostrum treated groups based on the NEC severity score. This is in agreement with earlier observations showing that a few days of TPN after birth followed by formula feeding, in contrast to colostrum feeding, predispose preterm pigs to inflammatory lesions, impaired intestinal function and NEC (14, 15, 22, 37). These changes are present after just 8 hours of formula feeding after the TPN period (20). This ex-

plains the relatively high incidence of NEC in the COLOS group compared with colostrum-fed preterm pigs from other studies in which colostrum was the only enteral diet introduced after the TPN period (14, 36). The present study now shows that colostrum acts to repair damage induced by a short initial period of formula feeding given after a two-day TPN period. Small amounts of colostrum given during parenteral nutrition may also prevent inflammatory lesions induced by later aggressive formula feeding, and full prevention of NEC is achieved when colostrum is given as a combination of minimal and full enteral feeding (38). These results are supported by the *in vitro* study in which COLOS, POW and POWPAS increased migration of intestinal epithelial cells as a measure of wound healing properties. The ability of colostrum to stimulate cell migration and healing of epithelial wounds might be particularly important for the sensitive epithelium of preterm newborns.

Colostrum feeding resulted in a decreased level of IL-1 β and IL-8 production in the distal small intestinal tissue and thereby reverted an initial pro-inflammatory state induced by TPN- and formula feeding. Thus, results from this study support previous observations showing that feeding sow's colostrum compared with formula feeding to preterm pigs down-regulate early intestinal lesions and IL-1 β levels (37) and here we show that colostrum from a different species has the same effect. These differences were not observed in the expression of *IL1B* and *IL8*, and actually, a tendency toward higher expression of *IL1B* was observed in COLOS pigs compared with FORM pigs. The differences in mRNA and protein levels may be explained by differences in onset of the expression of the gene and the following secretion of the protein (39). The correlation between the expression of *IL1B*, *IL6* and *IL8* and the total mean NEC severity, suggests a close relationship between the expression of these inflammatory mediators and the degree of intestinal inflammation, more than a relation with the type of diet. Bacterial overgrowth is associated with the progression of feeding-induced inflammatory lesions in preterm pigs (38), which was also observed here by the positive correlation between mean NEC severity and bacterial abundance score. We confirmed that the density of the microbiota appears relatively independent of the nature and amount of enteral feeding (38). However, the tendency towards higher total amount of OA in the colon of FORM pigs is an indication of a higher microbial activity in the colon.

The processing conditions employed to prepare the colostrum products used in this study, did not seem to influence the biological effect of POW and POWPAS compared with COLOS, despite the observed changes in the protein fraction. Interestingly, no differences were observed between COLOS and POW products, indicating that the type of spray drying used in this commercial production of colostrum powder is gentle in contrast to pasteurization, which resulted in a higher degree of protein aggregation. Furthermore, the concentration of especially TGF- β 2 was reduced by pasteurization, while spray drying reduced the concentration of TGF- β 1 in POW and POWPAS compared with COLOS. The concentration of IGF-I were not affected by pasteurization or spray drying. This suggests that significant amounts of the investigated proteins remain unaffected and could account for the biological effects observed to be protective against NEC. This is in agreement with a recent review on the effect of pasteuriza-

tion on immune component of donor milk showing that despite a reduction of bioactive factors after pasteurization, human milk still retains its beneficial effects (40). Furthermore, several other non-protein bioactive factors are present in milk. For example polyunsaturated fatty acids (PUFA), which are not affected by Holder pasteurization (41) and thus also may be preserved during high temperature, short time pasteurization as performed in this study. Long-chain polyunsaturated fatty acids have shown to reduce the incidence of NEC and intestinal inflammation in a neonatal rat model, and *TLR4* and *PAFR* expression in IEC-6 cells have been blocked by PUFA supplementation (42, 43). Further studies in the preterm pig model of NEC could include groups receiving BC products exposed to pasteurization or spray drying at lower or higher temperatures to further explore the effects of bioactive factors after heat treatment on intestinal repair and NEC, and the mechanism behind the effects could be studied *in vitro*. The gamma-irradiation used in this study did not appear to lead to significant denaturation of colostrum proteins as indicated by a study comparing gamma-irradiated colostrum from this study and non-irradiated colostrum from the same pool of colostrum (unpublished data).

We conclude that the beneficial effects of colostrum feeding in preterm pigs are not altered notably by spray drying or pasteurization. BC products, modified to meet the nutritional requirements of the preterm infant, may therefore serve as valuable alternatives to infant formula during the first critical days after birth if mother's milk is not available for the preterm neonate. Studies in preterm infants are required to investigate if BC could serve as a safe and effective alternative, when mother's milk is unavailable.

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FIGURE LEGENDS

FIGURE 1: Mean NEC severity scores across the five gut regions based on macroscopic tissue evaluation (A), distal small intestinal villus heights (B), activity of brush-border enzymes: maltase (C), lactase (D), dipeptidylpeptidase IV (DPPIV) (E), aminopeptidase A (ApA) (F) and aminopeptidase N (ApN) (G) across the three small intestinal regions, and values for IL-1 β (H) and IL-8 (I) protein levels in distal small intestinal tissue from preterm pigs. All data are presented as means \pm SEM, and means not sharing the same superscript letters are significantly different ($P < 0.05$).

FIGURE 2: Arterial plasma levels of galactose after an oral bolus of galactose given before (0 h, pooled values, horizontal lines) as well as 6 h and 30 h after initiation of the enteral feeding period (means \pm SEM) in FORM pigs (white), COLOS pigs (light gray), POW pigs (dark gray) and POWPAS pigs (black). Means not sharing the same superscript letters within the same time point differ significantly ($P < 0.05$).

FIGURE 3: FISH analysis showing representative cross sections of distal small intestine from preterm pigs: bacterial abundance score (red signal) = 1, no or very few microcolonies and distal NEC score = 1, no or minimal focal hyperaemic gastroenterocolitis (A), and bacterial abundance score = 5, extensive colonization and distal NEC score = 5 (B).

FIGURE 4: Total concentration and the concentration of the dominating OA in stomach (A) and colon contents (B) in FORM pigs (white), COLOS pigs (light gray), POW pigs (dark gray) and POWPAS pigs (black) (means \pm SEM). Different superscript letters indicate significant differences ($P < 0.05$).

FIGURE 5: Effect of increasing amounts of whey prepared from COLOS (light gray), POW (dark gray) and POWPAS (black). Values are least-squares means \pm SEM and significant differences within concentrations of whey are indicated by different letters, $P < 0.05$.

FIGURE 6: Effects of spray drying and pasteurization on protein aggregation in colostrum determined by SDS-page. Untreated total colostrum proteins: lane 1, COLOS; lane 2, POW; lane 3, POWPAS; and proteins remaining in the supernatant following removal of aggregated proteins by centrifugation at $13,000 \times g$ for 15 min: lane 4, COLOS; lane 5, POW; lane 6 POWPAS. M: Molecular weight marker.

TABLES

Table 1. Primer sequences and reaction conditions for quantitative real-time PCR analysis.

Gene	Sequence (5' - 3')	Amplicon length	Ta (°C) ^a	MgCl ₂ (mM) ^b
<i>B2M</i>	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	70	62	1.5
<i>ACTB</i>	F: CTACGTCGCCCTGGACTTC R: GCAGTCGTAGCTCTTCTCC	76	62	1.5
<i>HPRT1</i>	F: AACTGGCAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71	60	1.5
<i>IL1B</i>	F: CCAAAGAGGGACATGGAGAA R: GGGCTTTTGTCTGCTTGAG	123	59	3.0
<i>IL6</i>	F: GCAGTCACAGAACGAGTGGA R: CAGGCTGAACTGCAGGAAAT	82	59	3.0
<i>IL8</i>	F: GAAGAGAAGCTGAGAAGCAACA R: TTGTGTTGGCATCTTTACTGAGA	99	60	3.0
<i>IL10</i>	F: CTGCCTCCCACTTTCTCTTG R: TCAAAGGGGCTCCCTAGTTT	95	60	1.5
<i>DEFB4A</i>	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99	60	1.5
<i>TLR4</i>	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145	60	3.0
<i>TNF</i>	F: CCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	92	62	1.5
<i>SAA</i>	F: TAAAGTGATCAGCAATGCCAAA R: TCAACCCTTGAGTCTCCAC	96	60	3.0

^a Annealing temperature.

^b MgCl₂ is the total concentration of MgCl₂ in the reaction mix.

Table 2. Relative expression of inflammatory markers in the distal small intestine¹.

Gene	FORM	COLOS	POW	POWPAS
<i>IL1B</i>	0.39 ± 0.11	1.00 ± 0.66	0.31 ± 0.25	0.05 ± 0.01
<i>IL6</i>	0.24 ± 0.09	1.00 ± 0.68	0.30 ± 0.19	0.15 ± 0.03
<i>IL8</i>	1.12 ± 0.38	1.00 ± 0.56	0.61 ± 0.25	0.50 ± 0.20
<i>IL10</i>	1.23 ± 0.34	1.00 ± 0.33	1.50 ± 0.95	1.02 ± 0.29
<i>DEFB4A</i>	1.71 ± 0.61	1.00 ± 0.27 ^a	2.12 ± 1.03	2.30 ± 0.51 ^b
<i>TLR4</i>	1.40 ± 0.32	1.00 ± 0.18	1.23 ± 0.39	1.08 ± 0.19
<i>TNF</i>	0.83 ± 0.13	1.00 ± 0.18	2.12 ± 1.12	1.39 ± 0.35
<i>SAA</i>	1.44 ± 0.38	1.00 ± 0.43	2.76 ± 1.10	1.12 ± 0.50

¹Data are presented as mean ± SEM. Means within each gene not sharing the same superscript letters are significantly different ($P < 0.05$).

FIGURE 1

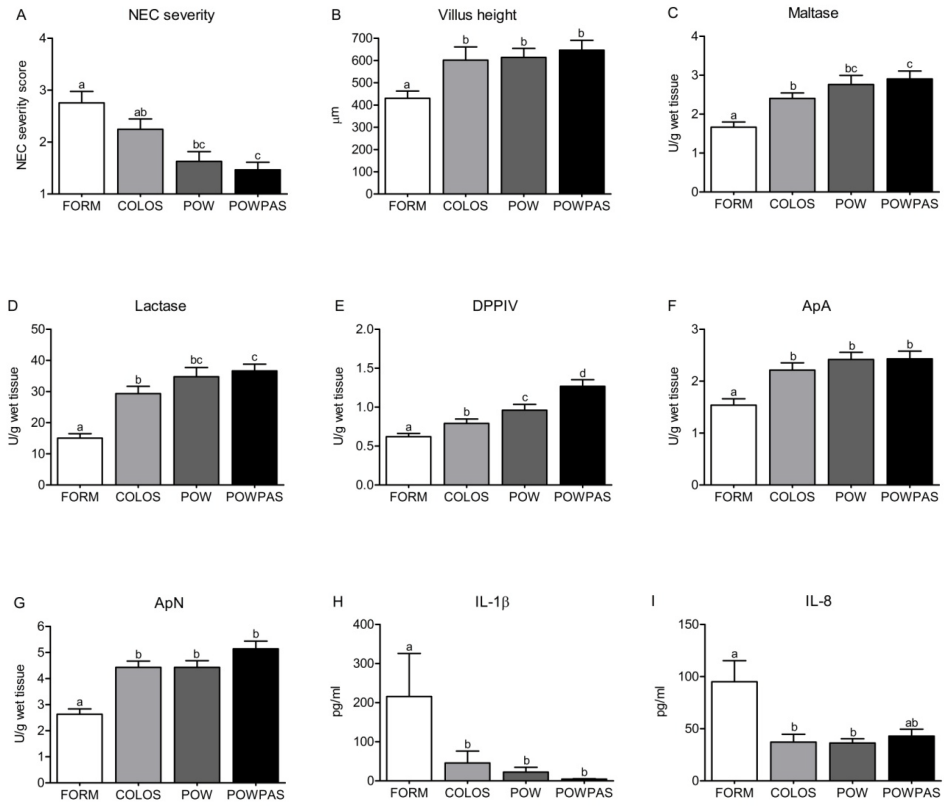


FIGURE 2

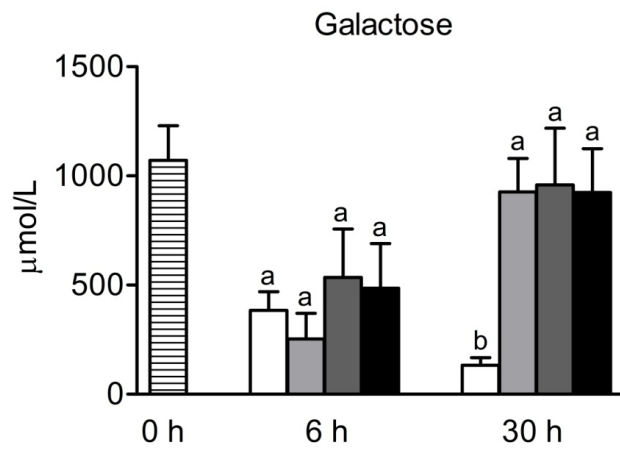


FIGURE 3

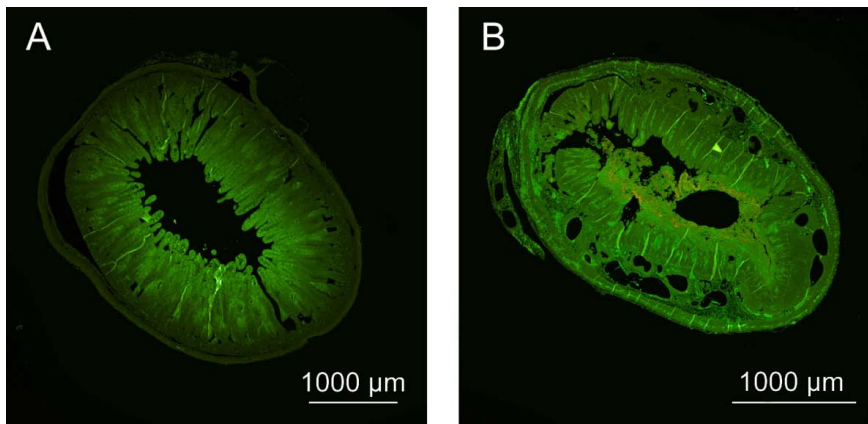


FIGURE 4

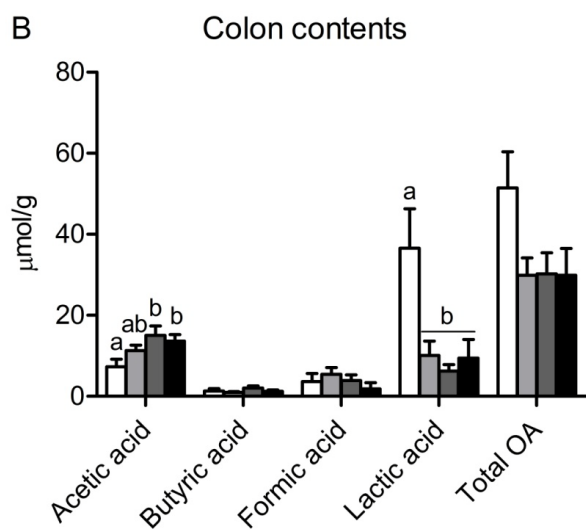
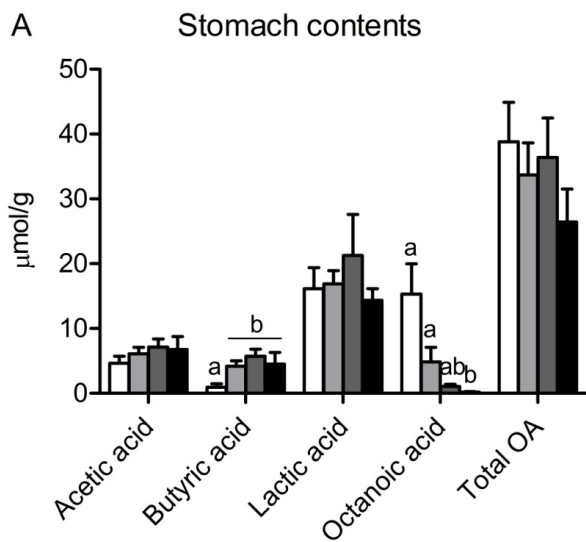


FIGURE 5

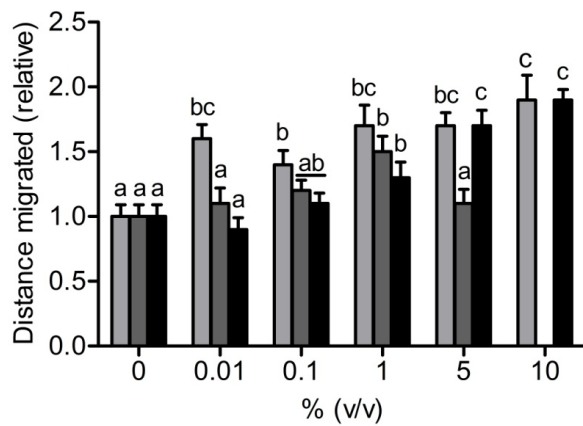
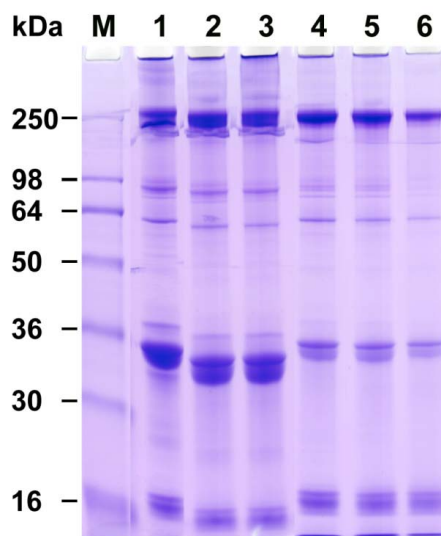


FIGURE 6



Paper III

Gene expression analysis of the IPEC-J2 cell line as a simple system for the inflammation-sensitive preterm intestine

Ann Cathrine F. Støy, Peter M. H. Heegaard, Per T. Sangild, Mette V. Østergaard,
Kerstin Skovgaard

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TITLE PAGE

Gene expression analysis of the IPEC-J2 cell line as a simple system for the inflammation-sensitive preterm intestine

Ann Cathrine F. Støy^{1*}, Peter M. H. Heegaard¹, Per T. Sangild², Mette V. Østergaard², Kerstin Skovgaard¹

¹ Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, DK-1870 Frederiksberg C, Denmark

² Department of Human Nutrition, University of Copenhagen, DK-1958 Frederiksberg C, Denmark

*Corresponding author

Ann Cathrine Findal Støy, Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark, Phone: +45 35886259, Fax: +45 35886001, E-mail: acfst@vet.dtu.dk

Abstract

The IPEC-J2 cell line was studied as a simplified system for investigating responses of the newborn intestinal epithelium to diet. Specifically the preterm intestine is sensitive to diet-induced inflammation.

Gene expression in IPEC-J2 cells stimulated for 2 h with milk formula (CELL-FORM), colostrum (CELL-COLOS) or growth medium (CELL-CONTR), and in distal small intestinal samples from preterm pigs fed milk formula (PIG-FORM) or colostrum (PIG-COLOS) was investigated. Expression analysis of epithelial- and immune response-related genes was performed using high throughput quantitative PCR.

Nineteen genes were expressed in IPEC-J2 cells, while 28 genes were expressed in intestinal samples. Principal component analysis of all expressed genes - except reference genes - discriminated IPEC-J2 cells from intestinal samples. Additionally, pigs, fed either colostrum or milk formula, were divided into 2 distinct groups, whereas no diet-dependent effects were seen for IPEC-J2 cells. Expression differences of *DEFB1*, *CXCL10*, *IL1RN* and *ALPI* were found for PIG-FORM and PIG-COLOS, while only *IL8* were up regulated in CELL-FORM compared with CELL-CONTR.

The lacking responses of IPEC-J2 cells to diets known to affect inflammatory markers in the preterm intestine, indicate that careful considerations must be made prior to analysis and interpretation of diet-induced effects on gene expression in this system.

Key words: animal model, gene expression, IPEC-J2 cell line, necrotizing enterocolitis

Introduction

The intestine is the site for nutrient digestion and absorption, but also a major immunological defense barrier that recognizes and responds to external antigens. In addition to the gut mucosal immune system, intestinal epithelial cells (IECs) are involved in the initiation and coordination of the intestinal immune response by production of signaling molecules including cytokines and chemokines [1]. The IPEC-J2 cell line originates from the jejunum of an unsuckled neonatal pig [2] and can be used as a simple *in vitro* system to investigate the IECs immune response in newborns. It has also been used to investigate host-pathogen interactions and immune responses with relevance for human and swine intestinal diseases [2-7]. IPEC-J2 cells are very similar to IECs with microvilli and tight junctions as well as expression and production of cytokines, defensins, TLRs, and mucins [2,5,8].

Necrotizing enterocolitis (NEC) is a serious gastrointestinal disease in preterm infants, arising from a combined result of prematurity, abnormal bacterial colonization, and enteral feeding. In particular, feeding with milk formula predisposes to NEC, whereas mother's milk is protective [9,10]. We set out to evaluate the IPEC-J2 cell line as an *in vitro* system that could resemble the neonatal intestine and support a well-established preterm pig model of NEC, which has shown to be a valuable model for investigation of diet-induced effects [11-14]. In the preterm pig model of NEC, milk formula-fed preterm pigs have more NEC lesions compared with preterm pigs fed porcine or bovine colostrum, rich in growth- and immunomodulatory factors [11,15,16].

Compared with animal models, cell line studies are less cost intensive, associated with no ethical concerns and provide a highly-controlled simple system to investigate isolated factors, for example diet, on the IECs response. The IPEC-J2 cell line is a promising system for *in vitro* studies of innate immune functions of perinatal IECs in response to dietary stimuli. In this study, we evaluated the potential of IPEC-J2 as an *in vitro* system to study diet-induced effects on the perinatal intestine. We analyzed and compared expression of epithelial- and immune response-related genes in the IPEC-J2 cell line and in preterm pig intestinal tissue.

Materials and methods

IPEC-J2 cell line study

IPEC-J2 cells [2] were maintained in Dulbecco's modified eagle medium-Ham's F-12 (1:1) supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL), pyruvate (1 mM), and L-glutamine (2 mM) (growth medium, Sigma-Aldrich, Brøndby, Denmark) in a humidified atmosphere of 5% CO₂ at 37 °C. Passaging was performed approximately once a week and cells between passages 82–85 were used in the experiment. Prior to the experiment, the cell culture was found to be free of mycoplasma.

For the experiment, cells were transferred to Corning Transwell-COL collagen coated-membrane (0.4 µm pore size; Sigma-Aldrich) and grown in growth medium supplemented with epidermal growth factor (5 mg/mL; Sigma Aldrich) and insulin-transferrin-selenium (5 µg/mL of each; Sigma-Aldrich) until stable trans epithelial electric resistance values, measured with an EVOM - Epithelial Voltohmmeter (World Precision Instruments, Berlin, Germany), were reached after approximately 12 d. The cells were stimulated for 2 h in three different diet-solutions: growth medium alone (CELL-CONTR, n = 4), growth medium containing 1% gamma-irradiated bovine colostrum (CELL-COLOS, n = 4), or growth medium containing 1% milk formula (CELL-FORM, n = 4). These conditions were selected based on results from pre-experiments testing cell viability under different concentration of colostrum and milk formula, and periods of time. The colostrum and milk formula were identical to those in the pig study (see below). The lipid fraction and cellular debris were removed by centrifugation at 3500 rpm for 20 min at 4 °C. After stimulation, the cells were gently washed twice with Dulbecco's phosphate buffered saline (D8537, Sigma-Aldrich), harvested and stored at -80 °C.

Preterm pig study

Nineteen preterm pigs were delivered from four sows by Caesarean section (Large White × Danish Landrace × Duroc, Askelygaard Farm, Roskilde, Denmark) at 105–107 d of gestation (90–92% gestation). Procedures for Caesarean section and nursing of the preterm pigs followed a standard protocol [11,12]. The pigs were given total parenteral nutrition through a vascular catheter (4 mL/kg/h advancing to 6 mL/kg/h) for 2 d based on Nutriflex Lipid Plus (Braun, Melsungen, Germany) and adjusted in nutrient composition to meet the requirements of pigs [11]. Hereafter, pigs were stratified according to birth weight into two enteral nutrition groups: gamma-irradiated (1 x 10 kGy, Sterigenics, Espergærde, Denmark) bovine colostrum (PIG-COLOS, n = 6; kindly donated by Biofiber-Damino A/S, Gesten, Denmark) or milk formula (PIG-FORM, n = 13; 80 g Pepdite, 70 g Maxipro, and 75 g Liquigen per L of water, all products kindly donated by Nutricia, Allerød, Denmark). Bovine colostrum was collected from the first milking after parturition and diluted in tap water to obtain the same dry matter content as in the milk formula (~15 g/L). The products were stored at -20 °C and warmed to body temperature in a water bath before feeding to the pigs (feeding dose interval: 15 mL/kg

body weight/3 h). Pigs were euthanized according to earlier protocols [15] within the following 45–50 h after initiation of enteral nutrition dependent on NEC development. Full thickness tissue samples from the distal small intestinal regions were immediately snap-frozen in liquid nitrogen and stored at -80 °C. All animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

Gene expression analysis

Total RNA from IPEC-J2 cells was extracted using RNeasy Mini kit (Qiagen, Ballerup, Denmark) and on-column DNase treated using RNase-free DNase set (Qiagen) according to manufacturer's protocol. Distal intestinal tissue was homogenized and total RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen) and on-column DNase treated using RNase-free DNase set (Qiagen) according to the manufacturer's protocol. Purity of extracted total RNA was assessed using UV absorption spectrums including OD 260/280 and OD 260/230 ratios on a Nanodrop ND-1000 spectrophotometer (Saveen Biotech, Aarhus, Denmark), and total RNA was quantified at OD 260. Quality (integrity) of extracted total RNA was determined using on-chip electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Nærum, Denmark). A RNA integrity number was assigned to each sample using the 2100 Expert software (Agilent Technologies, v.B.02.01).

Extracted RNA was converted into cDNA by reverse transcription of 500 ng total RNA using the QuantiTECT Reverse Transcription kit (Qiagen), containing a mix of random primers and oligo-dT, according to the manufacturer's instructions. cDNA was diluted 1:7 in low EDTA TE-buffer (VWR - Bie & Berntsen, Herlev, Denmark) prior to pre amplification. Pre amplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems, Nærum, Denmark). Stocks of 200 nM primer mix were prepared combining equal concentration of all primers used in the present study (Table 1), and in the following the genes will be denoted by their gene symbol (Table 1). TaqMan PreAmp Master Mix (5 µl) were mixed with 2.5 µl 200 nM stock primer mix and 2.5 µl diluted cDNA, and incubated at 95 °C in 10 min followed by 16 cycles of 95 °C in 15 sec and 60 °C in 4 min. Pre amplified cDNA was diluted at least 1:4 in low EDTA TE-buffer (VWR – Bie & Berntsen) before qPCR.

Primers were designed using Primer3 (<http://frodo.wi.mit.edu/>) as described in [17], and synthesized at TAG Copenhagen (Copenhagen, Denmark). Primer sequences and amplicon length are shown in Table 1. Primer amplification efficiencies and dynamic range were acquired from standard curves constructed from dilution series of highly responding samples. Melting curves were inspected for all primer assays and agarose gel electrophoresis and sequencing of most amplicons was performed to ensure primer specificity.

Quantitative PCR (qPCR) was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm Corporation, San Francisco, CA, USA), combining 48 samples with 48 primer sets for 2304 simultaneous qPCR reactions. Reaction mix was prepared using the following components for each of the 48 sample: 3 µl ABI TaqMan Gene Expression Master Mix (Applied Biosystems), 0.3 µl 20X DNA Binding Dye Sample Loading Reagent (Fluidigm Corpo-

ration), 0.3 μ l 20X EvaGreen (Biotium, VWR - Bie & Berntsen), and 0.9 μ l low EDTA TE Buffer (VWR - Bie & Berntsen). Reaction mix (4.5 μ l) was mixed with 1.5 μ l pre amplified cDNA. Primer mix for each of the 48 primer sets was prepared using 2.3 μ l 20 μ M specific primer (forward and reverse), 2.5 μ l 2X Assay Loading Reagent (Fluidigm Corporation) and 0.2 μ l low EDTA TE-buffer (VWR - Bie & Berntsen). Reaction mix, including cDNA (5 μ l) and primer mix (5 μ l), was dispensed and loaded into the integrated Fluidic Circuit of the Dynamic Array using the IFC Controller (Fluidigm Corporation). After loading, the Dynamic Array was placed in the BioMark real-time PCR instrument (Fluidigm Corporation) and the following cycle parameter was used: 2 min at 50 $^{\circ}$ C, 10 min at 95 $^{\circ}$ C, followed by 35 cycles with denaturing for 15 s at 95 $^{\circ}$ C and annealing/elongation for 1 min at 60 $^{\circ}$ C. Melting curves were generated to confirm a single PCR product for each reaction (from 60 $^{\circ}$ C to 95 $^{\circ}$ C, increasing 1 $^{\circ}$ C/3 s). Reactions were performed in duplicates (cDNA replicates). No-template controls were included to indicate potential problems with non-specific amplification or sample contaminations. Quantification cycle (Cq) was acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm Corporation) and exported to GenEx5 (MultiD Analyses AB, Göteborg, Sweden).

Data analysis and statistics

Data pre-processing, normalization, relative quantification and statistics were performed using GenEx5 (MultiD Analyses AB). Data were pre-processed as follows; 1. Data were corrected for PCR efficiency for each primer assay individually; 2. Inter Dynamic Array variation was compensated by using several highly stable samples as inter Dynamic Array calibrators; 3. Hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*) and beta-actin (*ACTB*) were found to be the most stably expressed reference genes in the present study using both GeNorm [18] and NormFinder [19] thus, the geometric mean of these two genes were used to normalize all samples in GenEx5; 4. Average of cDNA technical repeats was performed after reference gene normalization but before Cq were transformed to linear scale (relative quantities). In rare cases of high standard deviation between the two cDNA replicates one or both of the samples or the primer assay were excluded from the analysis based on visually inspection of fluorescence- and melting curves. To visualize differential gene expression, relative expression for all samples was calculated relative to the sample with the lowest expression for each primer set in the group of samples tested. Data was \log_2 transformed prior to t-test, ANOVA, and Principal Component Analysis (PCA). Gene expression was considered significantly different if the P-value < 0.05 and fold change > 2.0.

Results

An overview of genes expressed in preterm pigs intestinal tissue and IPEC-J2 cells is presented in the heat map (Figure 1), based on color coding of the expression level before pre processing. Within the dynamic range, 28 epithelial- and immune response related genes were

expressed in pig intestinal tissue, and of these, 19 genes were expressed in IPEC-J2 cells. No genes were expressed only in IPEC-J2 cells.

In PIG-COLOS, *DEFB1* was more highly expressed, while *CXCL10*, *IL1RN* and *ALPI* were less expressed than in PIG-FORM intestinal tissue (Figure 2A). In IPEC-J2 cells, no diet-induced differences were seen in the expression of *DEFB1*, *CXCL10*, and *IL1RN*, while *ALPI* was not expressed. However, *IL8* expression was significantly higher in CELL-FORM compared with CELL-CONTR with intermediate expression in CELL-COLOS (Figure 2B).

When comparing CELL-FORM and CELL-COLOS with PIG-FORM and PIG-COLOS in a PCA two major groups were identified (Figure 3), discriminating gene expression profiles of pig intestinal tissue from that of IPEC-J2 cells. Furthermore, a discrimination between PIG-COLOS and PIG-FORM was generally achieved by PCA, while no discrimination between CELL-COLOS and CELL-FORM was observed. As expected, major differences in gene expression were seen between IPEC-J2 cells and pig intestinal tissue, which accounted for the clear clustering in the PCA.

Transcripts coding for *CXCL10*, *CCL5* and *SLC5A1* was found at a higher level in pig intestinal tissue compared with IPEC-J2 cells; in contrast, expression of *CLDN3*, *DEFB1*, *IL1RN*, *IL6*, *MUC1* and *OCN* was lower in intestinal tissue compared with IPEC-J2 cells.

Discussion

In this study, the IPEC-J2 cell line was evaluated as a possible *in vitro* system to investigate IECs gene expression in relation to dietary effects on the preterm intestine. To our knowledge, this is the first demonstration of *CCL5*, *CD14*, *CXCL10*, *IL1RN*, *PAFAH1B1*, *SLC5A1* expression in IPEC-J2 cells. The expression of *DEFB1*, *DEFB4A*, *IL6*, *IL8*, *IL18*, *MUC1*, *NFKBIA*, *NFKB1*, *OCN*, *TLR4*, *TGFB1* and *TNFAIP3* has been reported previously [2-5,8,20]. However, a lack of *TGFB1* expression has also been reported in infection studies [2]. Finally, we confirmed the lack of expression of *CCL2* [8] and *MUC2* [2] in IPEC-J2 cells. With the presence of microvilli and tight junctions [2], the IPEC-J2 cell line is a suitable system to simulate immune functions of IECs. Still, it remains questionable if IPEC-J2 is an optimal system to investigate diet-related effects since the gene expression differences found in intestinal tissue could not be demonstrated in the IPEC-J2 cell line.

It was possible to discriminate between the gene expression profiles of the intestinal tissue from preterm pigs in different diet groups, as evident from the PCA. Four genes were differentially expressed between pigs fed milk formula or colostrum. *DEFB1*, encoding the antimicrobial protein defensin beta1 [21], was up regulated in PIG-COLOS compared with PIG-FORM, which suggests that colostrum may stimulate the host antimicrobial response. The expression of *ALPI*, in addition to *CXCL10* and *IL1RN* was down regulated in PIG-COLOS compared with PIG-FORM. *CXCL10* is involved in T lymphocyte activation and induction of chemotaxis toward infected tissues [22], and *IL1RN*, coding for the IL-1 receptor antagonist has previously been found to show similar expression patterns as *CXCL10* in viral lung infection of pigs (Personal communication, Kerstin Skovgaard). None of these four genes were

differentially expressed in IPEC-J2 cells. However, the expression of *IL8*, coding for the potent neutrophil chemoattractant IL-8, was higher in CELL-FORM and CELL-COLOS relative to CELL-CONTR. Thus, colostrum and milk formula might induce a certain level of pro-inflammatory response in IPEC-J2 cells.

Of the genes up regulated in IPEC-J2 cells relative to pig intestinal tissue, *MUC* and *DEFIB* stood out as highly expressed, with fold change differences of more than a 1000 (data not shown). These differences were probably due to the heterogeneous mixture of cells in the intestine, while the IPEC-J2 cell line is a homogenous sample consisting of IECs only.

In vitro cell systems provide the opportunity to investigate the interaction between a limited numbers of factors in a standardized setting. We found, the IPEC-J2 cells and the intestinal tissue clustered in two very distinct groups in the PCA of gene expression patterns. Future studies should include gene expression analysis of intestinal samples from term born pigs, to further determine if the differences in gene expression were due to the difference in maturational state.

Conclusion

The IPEC-J2 cell line provides the possibility to investigate the interaction between a limited numbers of factors in a standardized setting. However, differences in the gene expression and in gene responsiveness to nutritional factors were seen between intestinal tissue from preterm pigs and the IPEC-J2 cell line, and therefore careful considerations must be made prior to analysis and interpretation of diet-induced effects on gene expression in the IPEC-J2 cell line.

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Conflicts of Interest

The authors claim to have no conflicts of interest in the context of this work.

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FIGURE LEGENDS

FIGURE 1: Heat map showing the Cq value of samples from the distal small intestine of preterm pigs and IPEC-J2 cells. No-template control (NTC). A high gene expression correspond to a low Cq value (yellow), while a low gene expression correspond to a high Cq value (black).

FIGURE 2: Relative fold changes (means \pm SEM) of genes expressed significantly differently in intestinal tissue of COLOS pigs versus FORM pigs (A), and between different IPEC-J2 cell treatment groups: CELL-CONTR, CELL-COLOS and CELL-FORM (B). Relative expression for all samples was calculated relative to the sample with the lowest expression within each gene in the group of samples tested. Different superscript letter within each gene indicate significant differences ($P < 0.05$).

FIGURE 3: Principal component analysis of expression data from distal small intestinal tissue from preterm pigs, PIG-FORM (white circles) and PIG-COLOS (black circles), and IPEC-J2 cells, CELL-FORM (white squares) and CELL-COLOS (black squares).

TABLES

Table 1. Gene symbol, forward and reverse primer sequences, and amplicon length.

Gene symbol	Sequence (5' - 3')	Amplicon length
<i>ACTB</i>	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76
<i>ALPI</i>	F: TCCCAGACATAACAACGTGGA R: GGTCTGGTAGTTGGCCTTGA	90
<i>AOAH</i>	F: GTAATGGCATTGGGGTGTC R: TCTCCAGCAAAATGATCC	97
<i>APOA1</i>	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTCACC	86
<i>CCL2</i>	F: GCAAGTGCCTAAAGAAGCAGTG R: TCCAGGTGGCTTATGGAGTC	103
<i>CCL3</i>	F: CCAGGTCTTCTCTGCACCAC R: GCTACGAATTTGCGAGGAAG	90
<i>CCL5</i>	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCTCCATCCTAGC	121
<i>CD14</i>	F: GGGTTCCTGCTCAGATTCTG R: CCCACGACACATTACGGAGT	164
<i>CD40</i>	F: TGAGAGCCCTGGTGGTTATC R: GTCCTTGGTCACCTTTCTG	90
<i>CD163</i>	F: CACATGTGCCAACAAAATAAGAC R: CACCACCTGAGCATCTTCAA	130
<i>CD200</i>	F: TCCCCAGGAAGTTTGTATTG R: CCATGGTTCTTGCTGAAGGT	84
<i>CLDN3</i>	F: ATCGGCAGCAGCATTATCAC R: ACACCTTGCCTGCATCTGG	94
<i>CRP</i>	F: GGTGGGAGACATTGGAGATG R: GAAGGTCCCACCAGCATAGA	85
<i>CXCL10</i>	F: CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTGTTTCGAGGA	141
<i>C3</i>	F: ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG	76
<i>DEFB1</i>	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109
<i>DEFB4A</i>	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99
<i>FGG</i>	F: GAATTTTGGCTGGGAAATGA R: CAGTCCTCCAGCTGCACTCT	86
<i>HP</i>	F: ACAGATGCCACAGATGACAGC R: CGTGCGCAGTTTGTAGTAGG	105
<i>HPRT1</i>	F: ACACTGGCAAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71
<i>IL1B</i>	F: CCAAAGAGGGACATGGAGAA R: GGGCTTTTGTCTGCTTGAG	123
<i>IL1RN</i>	F: TGCCTGTCCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC	90
<i>IL6</i>	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTCCCTTA	116

Continued on next page

Table 1, Continued from previous page

Gene symbol	Sequence (5' - 3')	Amplicon length
<i>IL8</i>	F: TTGCCAGAGAAATCACAGGA R: TGCATGGGACACTGGAAATA	78
<i>IL10</i>	F: CTGCCTCCCACTTTCTCTTG R: TCAAAGGGGCTCCCTAGTTT	95
<i>IL18</i>	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100
<i>IFNG</i>	F: CCATTCAAAGGAGCATGGAT R: TTCAGTTTCCCAGAGCTACCA	76
<i>ITIH41</i>	F: ATGACAGCAAGCGAACAGTG R: GGGGATCCCTCTTGGAATC	85
<i>ITIH42</i>	F: AGGCCCTCACCATATCACAG R: GTTGCCATCCAGGACTGTTT	110
<i>LBP</i>	F: CCCAAGGTCAATGATAAGTTGG R: ATCTGGAGAACAGGGTCGTG	83
<i>LCT</i>	F: CACTCAAAGCTGTGCAGGAC R: GGATCCTTGGCAGAGAAGTG	144
<i>MUC1</i>	F: GGATTTCTGAATTGTTTTGCAG R: ACTGTCTTGAAGGCCAGAA	116
<i>MUC2</i>	F: GCACGTCTGCAACAAGGAC R: CAAAGCCCTCCAGGCAGT	125
<i>NFKBIA</i>	F: GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTAGACACTTTCC	85
<i>NFKB1</i>	F: CTCGCACAAGGAGACATGAA R: GGGTAGCCAGTTTTTGTCA	97
<i>OCN</i>	F: CGGTGAGAAGATTGGCTGAT R: TTTCAAAAGGCCTGGATGAC	100
<i>PAFAH1B1</i>	F: GCAAAGTGGCTACTGTGTGAAG R: GCACAGTCTGGTCATTGGAA	113
<i>PTGS2</i>	F: AGGCTGATACTGATAGGAGAAAACG R: GCAGCTCTGGGTCAAACCTC	100
<i>RPL13A</i>	F: ATTGTGGCCAAGCAGGTA R: AATTGCCAGAAATGTTGATGC	76
<i>SAA</i>	F: TAAAGTGATCAGCAATGCCAAA R: TCAACCCTTGAGTCCTCCAC	96
<i>SFTPA1</i>	F: CATGGGTGTCCTCAGTTTCC R: CATCAAAAGCGACTGACTGC	86
<i>SLC5A1</i>	F: CTGCAAGAGAGTCAATGAGGAG R: CCGGTTCCATAGGCCAACT	99
<i>TF</i>	F: CTCAACCTCAAACTCCTGGAA R: CCGTCTCCATCAGGTGGTA	82
<i>TGFB1</i>	F: GCAAGGTCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG	97
<i>TLR2</i>	F: GTTTTACGGAAATTGTGAAACTG R: TCCACATTACCGAGGGATT	136
<i>TLR4</i>	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145
<i>TNF</i>	F: CCCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	92
<i>TNFAIP3</i>	F: CCCAGCTTCTCTCATGGAC R: TTGGTTCTCTGCCGTCTCT	113

FIGURE 1

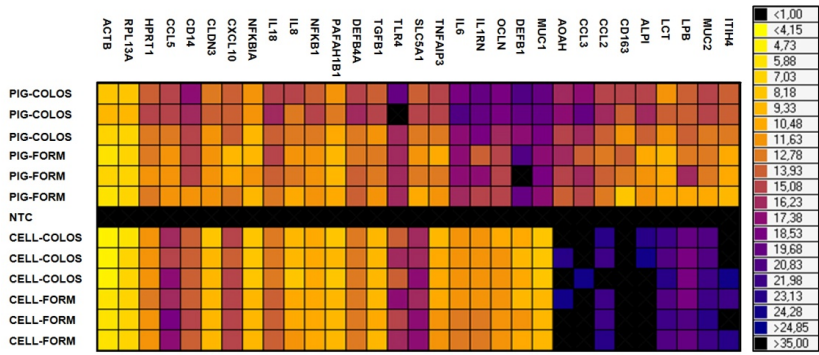


FIGURE 2

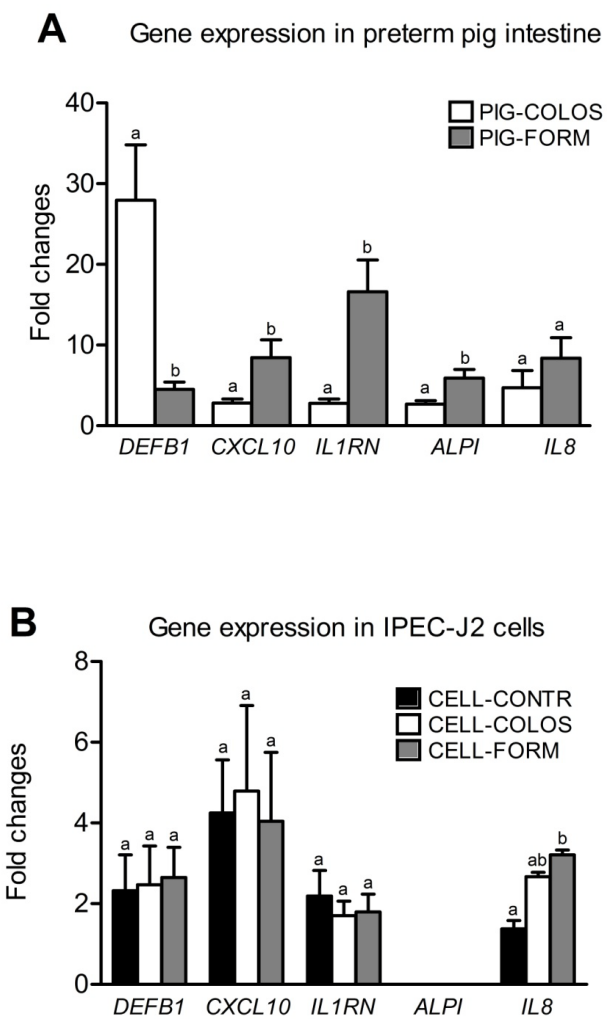
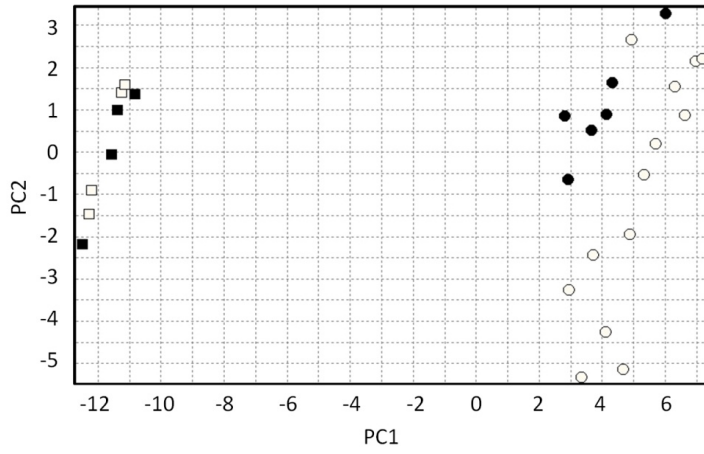


FIGURE 3



Paper IV

Total bacterial and *C. perfringens* abundance is associated with necrotizing enterocolitis in preterm pigs, and *C. perfringens* induces gene expression changes in IPEC-J2 cells

Ann Cathrine F. Støy, Camilla L. Delègue, Thomas Thymann, Per T. Sangild, Peter M. H. Heegaard, Kerstin Skovgaard, Sarmauli Manurung, Lars Mølbak

Manuscript in preparation

Transcriptome analysis will be included in the manuscript before submission

TITLE PAGE

Total bacterial and *C. perfringens* abundance is associated with necrotizing enterocolitis in preterm pigs, and *C. perfringens* induces gene expression changes in IPEC-J2 cells

Short title: Abundance of total bacterial and *C. perfringens* is associated with NEC

Ann Cathrine F. Støy^{1*}, Camilla L. Delègue¹, Thomas Thymann², Per T. Sangild², Peter M. H. Heegaard¹, Kerstin Skovgaard¹, Sarmauli Manurung¹, Lars Mølbak³

¹ Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, DK-1870 Frederiksberg C, Denmark

² Department of Human Nutrition, University of Copenhagen, DK-1958, Frederiksberg C, Denmark

³ Animal Health and Nutrition Innovation, Chr. Hansen A/S, Hørsholm, Denmark

*Corresponding author

Ann Cathrine Findal Støy, Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark, Phone: +45 35886259, Fax: +45 35886001, E-mail: acfst@vet.dtu.dk

ABSTRACT

Increased bacterial and *C. perfringens* subsp. abundance has been associated with necrotizing enterocolitis (NEC) in preterm infants and pigs determined mainly by conventional culturing and semi-quantitative molecular methods.

Therefore, we aimed to determine if a quantitative association between NEC and total bacteria and/or *C. perfringens* in a preterm pig model could be observed. The abundance of total bacteria and *C. perfringens* measured as pg DNA/reaction in distal small intestinal samples of 58 preterm pigs were determined using quantitative real-time PCR (qPCR). Furthermore, the effect of increased numbers of *C. perfringens* type A (multiplicity of infection (MOI) = 0–50) on host gene expression of 48 epithelial- and immune response related genes in IPEC-J2 cells was analyzed using high through-put reverse transcription qPCR.

The abundance of total bacterial and *C. perfringens* DNA was significantly higher in pigs suffering from NEC than in healthy pigs. Transcripts coding for *IL8*, *CCL5*, *IL1RN*, *NFKBIA* and *TNFAIP3* were up regulated in MOI = 10 compared with MOI = 0, and a following decrease in the expression of *IL8*, *CCL5*, *IL1RN* was observed at MOI = 20. *TNFAIP3* and *NFKBIA* expressions were higher at MOI = 20 compared with MOI = 0. The expression of *SLC5A1* was highest in MOI = 0 and decreased with increasing MOI. At MOI = 50, the RNA was highly degraded.

In conclusion, this study showed a quantitative association between NEC and total bacterial and *C. perfringens* abundance, and induction of changes in host gene expression with increasing numbers of *C. perfringens* type A were observed in IPEC-J2 cells.

Keywords: necrotizing enterocolitis, quantitative PCR, microbiota, *C. perfringens*, animal model, IPEC-J2 cell line.

Introduction

The intestinal microbiota is a contributing factor to the development of the severe gastrointestinal disease necrotizing enterocolitis (NEC) in preterm infants. Bacterial overgrowth and increased abundance of several specific bacterial members of the enteric microbiota have been shown to be associated with NEC in preterm infants and pigs (1-8). One of the pathogens associated with NEC in preterm infants and pigs is *C. perfringens* type A (1;6;9).

In a healthy intestine, the mucosal layer prevents close contact between host cells and bacteria, but in preterm infants, the function and restoration of the mucosal layer may be impaired (10). This leads to intestinal frailty that may allow opportunistic pathogens of the resident microbiota to invade the intestinal epithelium. This was shown in a study in preterm pigs, in which *C. perfringens* invaded the intestinal tissue in preterm pigs with NEC, while *C. perfringens* was located at the tissue surface in healthy pigs (11).

Bacterial production of toxins and metabolites such as a too high level of butyrate may contribute to tissue necrosis (12;13). Toxin production may be a contributing factor to the more rapid progression of NEC and the more extensive gangrene seen in *Clostridium*-associated NEC compared with NEC associated with *Klebsiella*, *E. coli*, or *B. fragilis* (6;8). An *in vitro* study showed a rapid up regulation of several toxins from *C. perfringens* type C when the bacteria were in close contact with Caco-2 cells (12). Furthermore, tissue necrosis may also be caused by excessive carbohydrate fermentation, and *Clostridium* spp., including *C. perfringens* type A, have been implicated in the formation of cecal NEC lesions possibly through excessive production of butyrate as seen in an experimental model of NEC (13). Furthermore, the gas forming capacity of *Clostridium* spp. may contribute to an increased development of pneumatosis intestinalis than observed with the involvement of other bacteria (3;6).

To quantitatively establish if an association between bacterial abundance and/or *C. perfringens* and NEC can be observed, we aimed to quantify the amount of total bacteria and *C. perfringens* type A using quantitative real-time PCR (qPCR). Intestinal tissue samples from 58 preterm pigs were included in this study and analyzed using quantitative real-time PCR (qPCR). To investigate the effects of *C. perfringens* type A on host gene expression patterns in intestinal epithelial cells, IPEC-J2 cells, originating from the jejunum of an unsuckled neonatal pig (14), were stimulated with increasing numbers of *C. perfringens* type A.

Materials and methods

Preterm pig study

Fifty-eight preterm pigs from two studies (Study I, n = 31, (15;16)) and (Study II, n = 27 (17)) were included in this study. The pigs were delivered by Caesarean section from four sows (Large White × Danish Landrace × Duroc; Askelygaard, Roskilde or Research Station Sjælland II, Denmark) at 105–107 d of gestation (~92%). Procedures for Caesarean section, rearing, euthanasia and tissue collection were described in details previously (15-17). The pigs were given total parenteral nutrition (TPN, prepared as described in (18)) for 36 h. Hereafter,

pigs within each study were randomly assigned into enteral nutrition groups (n = 6, across studies; feeding dose 15 ml/kg body weight/3 h) according to birth weight.

Enteral feeding groups from Study I included pigs fed milk formula with lactose (n = 11), milk formula with maltodextrin (n = 11), or milk formula with a ratio of casein to whey of 60:40 (n = 9). The diets were formulated using Seravit, Liquigen-medium-chain triglyceride and Calogen long chain triglycerides (Nutricia, Allerød, Denmark); Variolac, Lacprodan alpha 15 and Miprodan (ARLA Foods Ingredient, Viby, Denmark); and Polycose (Abbott Nutrition, Columbus, Ohio, USA) (15;16).

Enteral feeding groups from Study II included pig fed porcine colostrum (n = 5) plus peptone-water placebo, milk formula plus peptone-water placebo (n = 9), milk formula plus a probiotic mixture of *B. animalis* (DSM 15954) and 4 *Lactobacillus* species: *acidophilus* (DSM 13241), *casei* (ATCC 55544), *pentosus* (DSM 14025), and *plantarum* (DSM 13367) (n = 13, kindly donated by Chr. Hansen, Hørsholm, Denmark). The milk formula was prepared from Pepdite, Maxipro and Liquigen-medium-chain triglyceride (all products kindly donated by SHS International, Liverpool, UK). Peptone-water placebo and probiotic boluses were administered (2 mL/kg body weight) every 6 h during the TPN period and every 3 h during the enteral feeding period. Detailed information regarding the composition of the formula and preparation of the probiotic mixture has been given previously (17).

Pigs were euthanized upon NEC development or after 40–48 h of enteral feeding if no signs of NEC were observed. The procedures for euthanasia and tissue collection have been described previously (15;17). The presence of NEC was determined using a NEC severity score (ranging from 1–6) evaluating the degree of NEC lesion in the gastrointestinal tract as described previously (15;17). A score of minimum three indicated a case of NEC. For qPCR, full thickness tissue samples of approximately 3 cm of the distal small intestinal region without luminal content were snap frozen in liquid nitrogen and stored at -80 °C until DNA extraction. Across the two studies, 34 pigs developed NEC (S-NEC); 19 pigs from Study I (S1-NEC) and 15 pigs from Study II (S2-NEC). Twenty-four pigs showed no signs of NEC (S-HEAL); 12 pigs from Study I (S1-HEAL) and 12 pigs from Study II (S2-HEAL). All animal studies were approved by the National Committee on Animal Experimentation, Denmark.

qPCR analyses of total bacterial and *C. perfringens* abundance

DNA from full thickness tissue samples was extracted using the QIAamp DNA mini kit (Qiagen, Wst Sussex, UK) according to the manufacturer's instructions with minor modifications. Quantification of total bacteria was performed according to (19), and quantification of *C. perfringens* was performed according to (20), both with minor modifications. For total bacterial quantification 25 µl reaction mix consisted of 5 µl template (5 ng DNA/µl), 12.5 µL 2 x TaqMan Universal PCR Master Mix (Applied Biosystems, Nærum, Denmark), 0.50 µL of each primer: universal forward primer 8FM (5'-AGAGTTTGATCMTGGCTCAG-3'), and *B. longum* forward primer 8FB (5'-AGGGTTCGATTCTGGCTCAG-3), with universal reverse

primer Bact515R (5'-TTACCGCGGCKGCTGGCAC-3') and 0.25 μ L TaqMan probe Bact338K (5'-FAM/CCA KACTCCTACGGGAGGCAGCAG/TAMRA-3').

For *C. perfringens* quantification, 25 μ l reaction mix consisted of 5 μ l template (5 ng DNA/ μ l), 10 μ l of a 2 x Taqman Universal PCR Master Mix (Applied Biosystems), 2 μ l of bovine serum albumin (2.5 mg/ml, Applied Biosystems), 1 μ l 20 μ M of each primers CP165F (5'-CGC ATA ACG TTG AAA GAT GG-3') and CP269R (5'-CCT TGG TAG GCC GTT ACC C-3'), and 1 μ l TaqMan probe CP187F (5'- FAM/TC ATC ATT CAA CCA AAG GAG CAA TCC/TAMRA-3', 2 μ M). Primers and probes were synthesized at DNA Technology A/S, Aarhus, Denmark.

All qPCR reactions were performed on a RotorGene 3000 Detection System (Corbett Life Science, Sydney, Australia) under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 60 °C for 45 s, 65 °C for 15 s, and 72 °C for 15 s for quantification of total bacteria, and 95 °C for 10 min, 45 cycles of denaturation at 94 °C for 10 s, 55°C for 20 s, and 70 °C for 10 s for *C. perfringens* quantification. Standard curves were generated from DNA extracted from *C. perfringens* (NCTC 10240, National Veterinary Institute) ranging over six tenfold dilutions from the limit of detection (0.1 pg DNA/ μ l for total bacteria and 0.0125 pg DNA/ μ l for *C. perfringens*). The cycle threshold was determined using the Rotor-Gene 3000 data analysis software (Corbett Life Science) using the Auto-Find Threshold function. Both Dynamic Tube Normalisation and Noise Slope Correction functions were activated and a “best-fit” regression line was generated. All standards and samples were run in triplicates, and every qPCR reaction plate included one no-template control in triplicate. The results were calculated as pg DNA/reaction.

Infection study in IPEC-J2 cells

From an overnight culture of *C. perfringens* type A (NCTC 10240, National Veterinary Institute) a single bacterial colony was inoculated into brain heart infusion broth (SSI diagnostics) and incubated 16 h at 37 °C under anaerobic conditions. The bacterial culture was centrifuged at 4500 rpm for 5 min, washed twice in Dulbecco's phosphate buffered saline (PBS, Sigma-Aldrich, Brøndby, Denmark) and resuspended in growth medium without penicillin and streptomycin.

IPEC-J2 cells (14) were maintained in growth medium containing Dulbecco's modified eagle medium (DMEM)-Ham's F-12 (1:1) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), pyruvate (1 mM), and L-glutamine (2 mM), all purchased from Sigma-Aldrich, in a humidified atmosphere of 5% CO₂ at 37 °C. Passaging was performed once a week and cells from passage 83 were used in the experiment. Prior to the experiment, the cell culture was found to be free of mycoplasma.

For the experiment, IPEC-J2 cells were grown in 6-well plates (growth area 9.5 cm², Corning Costar cell culture plates, Sigma-Aldrich) until confluence were achieved after 4–5 days (8.5 \times 10⁶ cells). Twenty-four hours prior to the experiment, IPEC-J2 cells were incubated in growth medium without penicillin and streptomycin. IPEC-J2 cells were infected with *C. per-*

fringens at multiplicity of infection (MOI) = 0, 10, 20 and 50 and incubated for 2 h in a humidified atmosphere of 5% CO₂ at 37 °C. OD600 measurement of bacterial cultures was used to determine MOI.

To collect IPEC-J2 cells, the supernatant from two wells was combined to harvest enough cells for RNA extraction. The remaining monolayers were washed with pre warmed PBS, and harvested with 1 x trypsin:EDTA followed by a gentle wash with pre warmed growth medium and pooled with the supernatant containing the detached cells. The collected IPEC-J2 cells were centrifuged for 10 min at 1000 rpm and 4 °C, the supernatant removed completely, and the cells were immediately stored at -80 °C.

Gene expression analysis of IPEC-J2

For quantitative reverse transcription real-time PCR (RT-qPCR) of IPEC-J2 gene expression, total RNA from IPEC-J2 cells was extracted using RNeasy Mini kit (Qiagen) and on-column DNase treated using RNase-free DNase set (Qiagen) according to manufacturer's protocol. RT-qPCR was performed as described previously (21). After assessment of the quantity and quality of extracted total RNA, it was converted into cDNA by reverse transcription of 500 ng total RNA using the QuantiTECT Reverse Transcription kit (Qiagen), containing a mix of random primers (Table 1, TAG Copenhagen, Copenhagen, Denmark) and oligo-dT, according to the manufacturer's instructions. cDNA was diluted 1:7 in low EDTA TE-buffer (VWR - Bie & Berntsen, Herlev, Denmark) prior to pre amplification performed as previously described (21). qPCR was performed in cDNA duplicates and no-template controls were included to indicate potential problems with non-specific amplification or sample contaminations. Quantification cycle (C_q) was acquired using the Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm Corporation) and exported to GenEx5 (MultiD Analyses AB, Göteborg, Sweden).

Data analysis and statistics

The amount of *C. perfringens* and total bacteria were analyzed using a non-parametric Mann Whitney test, and correlation analyses were performed using Spearman correlation according to (22) in GraphPad Prism 5 (Version 5.02, La Jolla, CA, USA). Results are presented as means with standard error of mean (SEM), and $p < 0.05$ was used as the critical level of significance.

For RT-qPCR of the gene expression response in IPEC-J2 cells, normalization, relative quantification and statistics were performed using GenEx5 (MultiD Analyses AB). Data were pre-processed as follows: 1. Data were corrected for PCR efficiency for each primer assay individually; 2. Inter Dynamic Array variation was compensated for by using several highly stable samples as inter Dynamic Array calibrators; 3. Hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*) and beta-actin (*ACTB*) were found to be the most stably expressed reference genes in the present study using both GeNorm (23) and NormFinder (24) thus, the geometric mean of these two genes was used to normalize all samples in GenEx; 4. Average

of cDNA replicates was calculated after reference gene normalization but before Cq was transformed to linear scale (relative quantities). In rare cases of high standard deviation (> 1) between the two cDNA replicates, one or both of the samples or the primer assay were excluded from the analysis based on visually inspection of fluorescence- and melting curves. To visualize differential gene expression, relative expression for all samples was calculated relative to the average expression for each gene. For graphical presentation, the normalized mean for the MOI = 0 group was set to one, and the normalized mean for MOI = 10 and MOI = 20 was displayed as fold change compared to the normalized mean for the MOI = 0 group. Data were \log_2 transformed prior to t-test and ANOVA. Gene expression was considered significantly different if $p < 0.05$ and fold change > 2.0 .

Results

Total bacterial and *C. perfringens* abundance in intestinal tissue from preterm pigs

The amount of total bacteria was significantly higher in S1-NEC compared with S1-HEAL, while no difference was observed between S2-NEC and S2-HEAL (Figure 1A). Across studies, the amount of total bacteria was significantly higher in S-NEC than in S-HEAL (Figure 1A). The amount of *C. perfringens* was significantly higher in pigs suffering from NEC than healthy pigs when analyzing the studies individual or combined (Figure 1B). A positive correlation between the amount of total bacteria and *C. perfringens* in Study I ($r = 0.749$, $p < 0.001$), Study II ($r = 0.741$, $p < 0.001$) and across studies was observed ($r = 0.708$, $p < 0.001$).

Gene expression response in IPEC-J2 cell upon *C. perfringens* type A infection

The degradation of RNA measured by RIN increased with increasing MOI, from MOI = 0 to MOI = 20 (Figure 2A). A RIN for MOI = 50 was only obtainable for one replicate indicating a high degree of RNA degradation in this group (Figure 2A). This was supported by a higher Cq value (lower expression) for the reference genes before normalization in the MOI = 50 group relative to the three other groups (Figure 2B). After normalization, the level of all genes in the MOI = 50 group was decreased compared with the other groups indicating that the reference genes could not compensate for the RNA degradation. Therefore, in the following statistical analysis the MOI = 50 group was excluded. Furthermore, one MOI = 10 replicate was excluded due to a very low RIN number. 6 genes were differently expressed between the different MOI groups (Figure 2C). The expression of *IL8*, *CCL5*, *IL1RN*, *NFKBIA* and *TNFAIP3* was up regulated in MOI = 10 compared with MOI = 0. Furthermore, *TNFAIP3* and *NFKBIA* expression was higher at MOI = 20 compared with MOI = 0, while the expression of *IL8*, *CCL5*, *IL1RN* decreased at MOI = 20, to levels comparable with MOI = 0. The expression of *SLC5A1* was highest in MOI = 0 and decreased with increasing MOI.

Discussion

To our knowledge, this is the first demonstration of an association between the amount of total bacteria and *C. perfringens* and NEC in a preterm pig model of NEC based on quantitative measurements. The association between the amount of total bacteria and NEC in Study I has been observed previously using FISH (unpublished data). The association between *C. perfringens* and NEC observed in Study II was previously found by terminal restriction fragment length polymorphism, a semi-quantitative analysis (17). In contrast, the association between *C. perfringens* and NEC observed in Study I has not previously been observed by terminal restriction fragment length polymorphism analysis or by fluorescence *in situ* hybridization (FISH) (unpublished data). These results, using different methods to analyze the intestinal microbiota, indicate that quantitative methods should be preferred over semi-quantitative methods when analyzing the complex intestinal microbiota.

Bacterial overgrowth has also been observed in previous studies in preterm pigs (1;2) and thus support the observations seen in this study. Thus, the bacterial load may at some point reach a threshold that will stress the intestine and concomitant with immaturity of intestinal function and structure may lead to intestinal damage and NEC. It could therefore be speculated that the association between a specific bacteria and NEC (5-8;25) is a cause of a general increase in total bacteria. However, the role of increasing numbers of specific pathogens like *C. perfringens* Type A in the induction of intestinal injury may still be significant, as observed in this study, in which increasing numbers of *C. perfringens* Type A lead to increased RNA degradation. The involvement of *C. perfringens* Type A in NEC development may be due to production of metabolites like butyrate (13) and up regulation of toxins especially when in close contact with epithelial cells (12) possible through the Agr-like quorum sensing system as seen for *C. perfringens* type C (26). However, no positive correlation between *C. perfringens* α -toxins and NEC severity and mortality rate has been established in preterm infants (6).

Increasing numbers of *C. perfringens* type A in this study were associated with decreased integrity of the RNA measured by RIN and increasing Cq values of reference genes in IPEC-J2 cells after 2 hours incubation. This supports a study by Vidal *et al.* (12), who found that 3 hours of infection with *C. perfringens* type C at MOI = 20 was enough to induce morphological changes in Caco-2 cells, while 3 h incubation lead to a cytotoxic effect. In this study, RNA was degraded at MOI = 50. This however, was not reflected in changes in cell morphology in a chamber study using the same experimental setup as in this study, in which IPEC-J2 cells appeared to have a normal cell morphology at MOI = 50 (unpublished data). The present study furthermore showed that changes in intestinal epithelial gene expression is observed already at MOI = 10, where the cells still maintained their RNA integrity.

The expression of the pro-inflammatory factors *IL8* and *CCL5* in addition to the anti-inflammatory factors *IL1RN*, *IkBA* and *TNPAIP3* was up regulated at MOI = 10 compared with MOI = 0. The up regulation of both pro- and anti-inflammatory factors in response to NEC has also been observed in preterm infants (27;28) and pigs (Personal communication, A.

C. F. Støy). With increased MOI and RNA degradation, the gene expression level of the sodium-glucose cotransporter, *SGLT1*, decreased. The sodium-glucose transporter-1 has been found to be involved in cytoprotection of intestinal epithelial cells via anti-apoptotic signaling during intestinal stress (29;30;30;31). Thus, the decrease in *SLC5A1* may be partly involved in a decrease in anti-apoptotic signaling leading to cell damage.

Future studies should include experiments on the effect of probiotic bacteria on the IPEC-J2 cell line. Probiotic bacteria given as supplement to milk formula has in Study II shown to reduce the incidence and severity of NEC, and the number of *C. perfringens* compared with preterm pigs fed milk formula and placebo (17). The commensal and beneficial bacteria may reduce bacterial overgrowth of specific opportunistic pathogens and reduce the risk of close contact between these bacteria and host cells, and thus decrease the risk of intestinal injury and NEC. However, the use of probiotics to reduce NEC in preterm infants needs to be investigated further (32).

In conclusion, the microbiota may play a significant role in the late pathogenesis of NEC since a quantitative association between the amount of total bacteria and *C. perfringens* type A was observed with NEC development, and gene expression changes were induced in host cells *in vitro* by *C. perfringens* type A.

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FIGURE LEGENDS

FIGURE 1: Association between NEC and the abundance of total bacteria (Study I, n = 26; Study II, n = 23, across studies, n = 49, (B)) and the abundance of *C. perfringens* (Study I, n = 31; Study II, n = 24, across studies, n = 58, (A)) determined by qPCR. Results are presented as means \pm SEM, and means within each study not sharing the same superscript letters indicate significant differences: a and b, $p < 0.01$ and c and d, $p < 0.001$. Y-axis is log 10 scale.

FIGURE 2: RNA integrity number (RIN) (A), Cq values before normalization for reference genes in the four groups (B) and relative fold changes of gene significantly different expressed between treatment groups (C) in IPEC-J2 cells stimulated with MOI = 0,10 and 20. Results are presented as means \pm SEM, and means not sharing the same superscript letters indicate significant differences, $p < 0.05$.

TABLES

Table 1. Gene symbol, forward and reverse primer sequences, and amplicon length.

Gene symbol	Sequence (5' - 3')	Amplicon length
<i>ACTB</i>	F: CTACGTGCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76
<i>ALPI</i>	F: TCCCAGACATACAACGTGGA R: GGTCTGGTAGTTGGCCTTGA	90
<i>AOAH</i>	F: GTAATGGCATTGGGGTGTC R: TCTCCCAGCAAAATGATTCC	97
<i>APOA1</i>	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTCACC	86
<i>CCL2</i>	F: GCAAGTGTCTAAAGAAGCAGTG R: TCCAGGTGGCTTATGGAGTC	103
<i>CCL3</i>	F: CCAGGTCTTCTCTGCACCAC R: GCTACGAATTTGCGAGGAAG	90
<i>CCL5</i>	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCTCCATCCTAGC	121
<i>CD14</i>	F: GGGTTCCTGCTCAGATTCTG R: CCCACGACACATTACGGAGT	164
<i>CD40</i>	F: TGAGAGCCCTGGTGGTTATC R: GCTCCTTGGTCACCTTTCTG	90
<i>CD163</i>	F: CACATGTGCCAACAAAATAAGAC R: CACCACCTGAGCATCTTCAA	130
<i>CD200</i>	F: TCCCCAGGAAGTTTTGATTG R: CCATGGTCTTGCTGAAGGT	84
<i>CLDN3</i>	F: ATCGGCAGCAGCATTATCAC R: ACACCTTGCACCTGCATCTGG	94
<i>CRP</i>	F: GGTGGGAGACATTGGAGATG R: GAAGGTCCACCAGCATAGA	85
<i>CXCL10</i>	F: CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTTCGAGGA	141
<i>C3</i>	F: ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG	76
<i>DEFB1</i>	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109
<i>DEFB4A</i>	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99
<i>FGG</i>	F: GAATTTTGGCTGGGAAATGA R: CAGTCTCCAGCTGCACTCT	86
<i>HP</i>	F: ACAGATGCCACAGATGACAGC R: CGTGCGCAGTTTGTAGTAGG	105
<i>HPRT1</i>	F: ACCTGGCAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71
<i>IL1B</i>	F: CCAAAGAGGGACATGGAGAA R: GGGCTTTTGTCTGCTTGAG	123
<i>IL1RN</i>	F: TGCCTGTCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC	90
<i>IL6</i>	F: TGGGTTCAAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	116
<i>IL8</i>	F: TTGCCAGAGAAAATCACAGGA R: TGCATGGGACACTGGAATA	78
<i>IL10</i>	F: CTGCCTCCCACTTCTCTTG R: TCAAAGGGGCTCCCTAGTTT	95
<i>IL18</i>	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100
<i>IFNG</i>	F: CCATTCAAAGGAGCATGGAT R: TTCAGTTTCCCAGAGCTACCA	76

Continued on next page

Table 1, Continued from previous page

Gene symbol	Sequence (5' - 3')	Amplicon length
<i>ITIH41</i>	F: ATGACAGCAAGCGAACAGTG R: GGGGATCCCTCTTGGTAATC	85
<i>ITIH42</i>	F: AGGCCCTCACCATATCACAG R: GTTGCCATCCAGGACTGTTT	110
<i>LBP</i>	F: CCCAAGGTCAATGATAAGTTGG R: ATCTGGAGAACAGGGTCGTG	83
<i>LCT</i>	F: CACTCAAAGCTGTGCAGGAC R: GGATCCTTGGCAGAGAAGTG	144
<i>MUC1</i>	F: GGATTCTGAATTGTTTTGCGAG R: ACTGTCTTGGAAAGGCCAGAA	116
<i>MUC2</i>	F: GCACGTCTGCAACAAGGAC R: CAAAGCCCTCCAGGCAGT	125
<i>NFKBIA</i>	F: GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTTAGACACTTTCC	85
<i>NFKB1</i>	F: CTCGCACAAGGAGACATGAA R: GGGTAGCCAGTTTTTGTCA	97
<i>OCLN</i>	F: CGGTGAGAAGATTGGCTGAT R: TTTCAAAGGCCTGGATGAC	100
<i>PAFAH1B1</i>	F: GCAAAGTGGCTACTGTGTGAAG R: GCACAGTCTGGTCATTGGAA	113
<i>PTGS2</i>	F: AGGCTGATACTGATAGGAGAAACG R: GCAGCTCTGGGTCAAACCTC	100
<i>RPL13A</i>	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76
<i>SAA</i>	F: TAAAGTGATCAGCAATGCCAAA R: TCAACCCTTGAGTCCTCCAC	96
<i>SFTP1A1</i>	F: CATGGGTGTCCTCAGTTTCC R: CATCAAAAGCGACTGACTGC	86
<i>SLC5A1</i>	F: CTGCAAGAGAGTCAATGAGGAG R: CCGGTTCCATAGGCAAAC	99
<i>TF</i>	F: CTC AACCTCAA AACTCCTGGAA R: CCGTCTCCATCAGGTGGTA	82
<i>TGFB1</i>	F: GCAAGGTCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG	97
<i>TLR2</i>	F: GTTTTACGGAAATTGTGAAACTG R: TCCACATTACCGAGGGATTT	136
<i>TLR4</i>	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145
<i>TNF</i>	F: CCCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	92
<i>TNFAIP3</i>	F: CCCAGCTTTCTCTCATGGAC R: TTGGTTCTTCTGCCGTCTCT	113

FIGURE 1

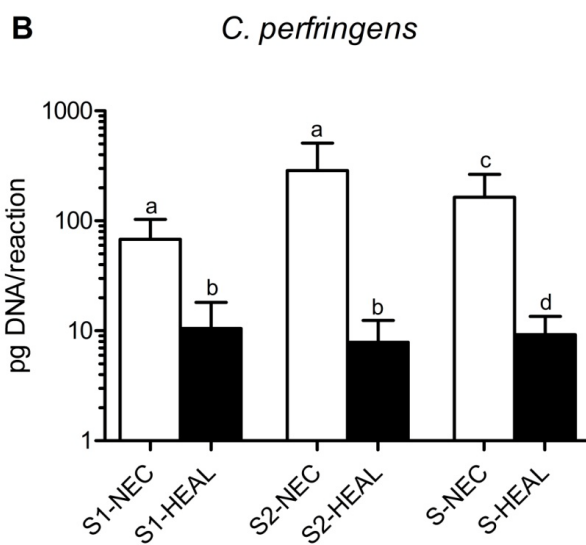
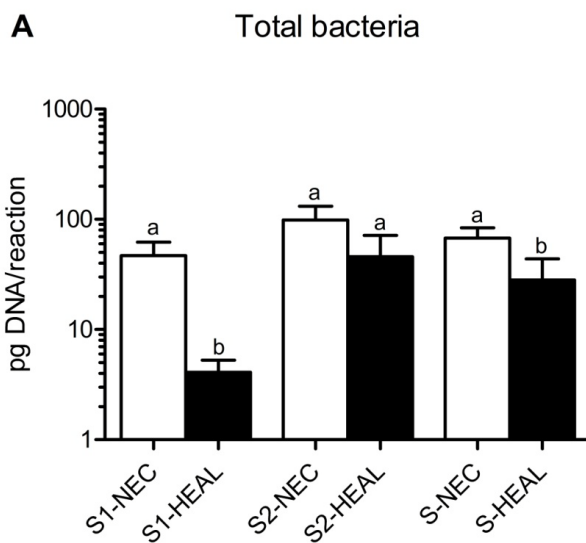
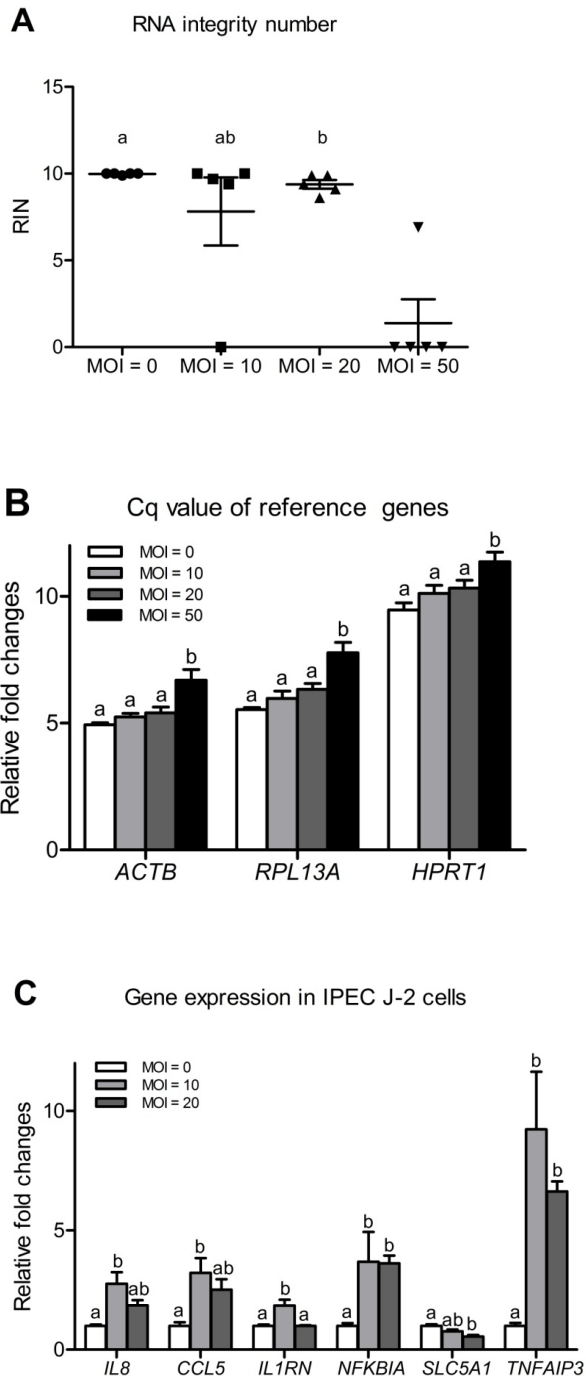


FIGURE 2



Conference presentation: Work in progress

Bovine colostrum with hyperimmunity against Clostridia

Ann Cathrine F. Støy¹, Per T. Sangild², Peter M. H. Heegaard¹

¹ National Veterinary Institute, Technical University of Denmark, 27 Bülowsvej, DK-1790 Copenhagen V, Denmark

² Department of Human Nutrition, University of Copenhagen, 30 Rolighedsvej, DK-1958 Frederiksberg C, Denmark

This work was selected for poster presentation at the “Alternatives to Antibiotics” Symposium in Paris, 25–28 September 2012.

This work in progress aims to investigate a possible preventive effect of hyperimmune colostrum against *Clostridium*-associated NEC. Until now, the work includes production of hyperimmune colostrum against *C. perfringens* and confirmation of hyperimmunity.

Abstract

Bacteria from the *Clostridium* group are associated with gastrointestinal diseases in both infants and adults. Intestinal overgrowth with *C. perfringens* is commonly associated with necrotizing enterocolitis (NEC) in preterm infants and pigs, and *C. difficile* plays a key role in antibiotics-associated diarrhea in adult patients. Studies in preterm pigs suggest that bovine colostrum reduces the risk and severity of NEC.

Here, we investigate the effect of bovine hyperimmune colostrum against *C. perfringens*. The hyperimmune colostrum was prepared by immunizing late gestation pregnant cows (n = 5) 4 times 14 days apart beginning 60 days before expected calving with COVEXIN® 8A (*C. perfringens* Type A, C and D toxoid; *C. Chauvoei*; *C. novyi* (*oedematiens* type B) toxoid; *C. septicum* toxoid; and *C. tetani* toxoid). Colostrum from the first milking was collected (H-COLOS). This was compared with colostrum from non-immunized control cows (COLOS, n = 5). All cows received a standard vaccine (Rotavec Corona Vet). Western blot was used to confirm the presence of immunoreactivity in colostrum from immunized cows, using antigens extracted from *C. perfringens* NCTC 10240 and control species: *C. difficile* O27, *E. coli* ATCC 25922 and *L. sakei* DMS 20017.

We confirmed hyper-immunity of the H-COLOS towards *C. perfringens* compared with COLOS, but no clear difference in band intensity or number of bands was observed towards *C. difficile*, *E. coli* and *L. sakei* between H-COLOS and COLOS.

The results show that we were able to produce hyperimmune bovine colostrum towards *C. perfringens*. However, the practical use of hyperimmune colostrum products for preterm infants remains questionable due to the diversity in bacterial species associated with NEC.

Introduction

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease in preterm infants in which members of the intestinal microbiota, including *C. perfringens*, have found to be implicated (1-5). *C. perfringens*-associated NEC results in a higher risk of pneumatosis intestinalis, and leads to a more severe and rapid disease course than NEC associated with *Klebsiella*, *E. coli*, or *B. fragilis* (2;3;6).

The pathogenicity of *C. perfringens* is most likely due to toxin production and a short chain fatty acid profile with a high level of butyrate. An *in vitro* study has shown that the toxin production is up regulated when *C. perfringens* type C is in close contact with Caco-2 cells (7). Furthermore, a short chain fatty acid profile with a high level of butyrate from fermentation by *Clostridium* species including *C. perfringens* type A has been implicated in the formation of cecal NEC lesions in an experimental model of NEC (8). Normally, the mucosal layer prevents contact between host cells and bacteria. However, in a damaged and stressed intestinal environment, opportunistic pathogens like *C. perfringens* may invade the intestinal epithelium and become virulent. Therefore, a crucial step in reducing the risk of *C. perfringens*-associated NEC is to prevent a close contact between the bacterium and its toxins and the intestinal epithelial cells.

Colostrum, rich in bioactive factors, reduces the risk and severity of NEC compared with infant formula in preterm pigs (9-11), and could serve as an alternative for preterm infants when mother's milk is not available. Immunoglobulin found in high concentration in bovine colostrum, approximately 80 g/L in the first milking after parturition (12), could provide passive immunity by neutralization of microbes and their toxins by agglutination in the intestinal lumen (13-15). This could prevent a close contact between *C. perfringens* and intestinal epithelial cells during intestinal frailty.

Studies have shown that hyperimmune colostrum preparations may prove to be beneficial against infections caused by *E. coli* (16) and rotavirus (17). Therefore, we aimed to investigate if bovine colostrum with increased activity toward *C. perfringens* could be produced by immunizing late-gestational cows with a commercial clostridium vaccine.

Materials and methods

Immunization of cows and collection of colostrum

Five cows were immunized with COVEXIN® 8A containing *C. perfringens* Type A, C and D toxoid; *C. Chauvoei*; *C. novyi* (*oedematiens* type B) toxoid; *C. septicum* toxoid; and *C. tetani* toxoid (Pfizer, Ballerup, Denmark). The cows were immunized 4 times 14 days apart beginning 60 days before expected calving (H-COLOS). Five cows were not immunized and their colostrum was used as control colostrum (COLOS). Cows from both groups received a standard vaccine containing rotavirus, coronavirus and *E. coli* (Rotavec Corona Vet, Intervet, Denmark). After calving, colostrum from the first milking was collected and stored at -20 °C. Before analysis, colostrum was centrifuged at 3500 rpm for 20 min at 4 °C to remove the fat fraction and cellular debris.

Confirmation of hyperimmunity by western blot

Antigens were extracted from overnight cultures of *C. perfringens* NCTC 10240 (National Veterinary Institute, Frederiksberg C, Denmark), and control species *C. difficile* O 27 (Kindly donated by Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark), *E. coli* ATCC 25922 (National Veterinary Institute, Frederiksberg C, Denmark), and *L. sakei* DSM 20017 (Kindly donated by The Faculty of Life Sciences, Copenhagen University, Frederiksberg, Denmark). *C. perfringens*, *C. difficile* and *E. coli* were grown in brain hearth infusion (BHI) broth (Oxoid, Greve, Denmark), while *L. sakei* were grown in deMan, Rogosa and Sharpe (MRS) broth (Oxoid) under anaerobic conditions at 37 °C.

The antigen extraction was done essentially as described by Crichton *et al.* (18). Briefly, an overnight culture of each bacterium was centrifuged at $3,000 \times g$ for 20 min at 4 °C to harvest the bacterial cells. The cell pellet was washed three times in Dulbecco's phosphate buffered saline (0.137 M NaCl, 0.003 M KCl, 0.001 M CaCl₂·2H₂O, 0.0005 M MgCl₂·6H₂O, 0.006 M Na₂HP0₄·2H₂O and 0.0015 M KH₂P0₄, pH = 7.3). The pellet was resuspended in 0.04 M disodium phosphate buffer (pH 8.9) and sonicated on ice for 15 min (U50 control IKA labortechnik, Staufen, Germany). The antigen extracts were stored at -80 °C.

The protein concentration of the antigen extracts was determined on a Nanodrop ND-1000 UV-spectrophotometer (Saveen Biotech, Århus, Denmark) and diluted to 1 mg/mL in NuPAGE LDS sample buffer (4X, Life technologies, Nærum, Denmark). The diluted antigen suspensions were run on NuPAGE 12% Bis-Tris Gels (Life technologies) with NuPAGE MES SDS Running Buffer (20X, Life technologies) for one hour at 200 V and blotted onto PVDF membranes (Immobilon-P, Millipore) by electrophoresis at 150 mA for one hour. The nitrocellulose membranes were blocked with wash buffer (0.05 M Tris, 0.25 M NaCl, with 0.4% Tween 20, pH 8.6) for ten minutes. The membranes were incubated with colostrum products diluted 1:100 in sample buffer for one hour at room temperature or NuPAGE LDS sample buffer (4X, Life technologies) as control. The blots were washed 3 times in TBS buffer for five minutes. The blots were incubated with streptavidin-alkaline phosphatase

(S2890, Sigma-Aldrich, Brøndby, Denmark) and biotinylated sheep anti-bovine IgG-heavy chain antibody (A10-118, Nordic BioSite ApS, Copenhagen, Denmark) and incubated one hour at room temperature. The blots were washed 3 times in wash buffer with 2% Tween 20 (1:5) for ten minutes. Bound antibodies were detected using BCIP/NBT Tablets (Roche Diagnostics A/S, Hvidovre, Denmark), and the reaction stopped with ion exchanged water. Magic Mark (Life technologies) was used as molecular weight marker.

Results

Western blot confirmed increased activity towards *C. perfringens* in H-COLOS indicated by increased intensity of bands compared with COLOS. No clear difference in band intensity or number of bands was observed towards *C. difficile*, *E. coli* and *L. sakei* (Figure 1).

Discussion

Hyperimmunity of H-COLOS towards *C. perfringens* type was evident due to the differences in intensity in bands on the western blot. The increased activity towards *C. perfringens* NCTC 10240, a *Clostridium* type A species, may be due to a specific activity of antibodies in H-COLOS towards toxins remaining from the culture when the antigens were extracted, since the vaccine only contained *C. perfringens* Type A, C and D toxoid, but no *C. perfringens* whole cells. *C. Chauvoei* was included in the vaccine as the only whole cell bacterium, and the increased activity of H-COLOS towards *C. perfringens* may also be caused by cross-reactivity between these two *Clostridium* species. No clear difference in band intensity or number of bands was observed between H-COLOS and COLOS towards *C. difficile*, *E. coli* and *L. sakei*. The presence of bands against the bacteria on the western blot for both H-COLOS and COLOS may be due to a general production of antibodies towards the general bacterial population in the cow's immediate environment (19). Furthermore, *E. coli* antigens were part of the standard vaccination program, and thus also contributing to the activity towards this bacterium in the colostrum.

The clinical use of hyperimmune colostrum products for preterm infants is questionable. Several other bacteria besides *C. perfringens* have been associated with NEC including *E. coli* (1;3), *Klebsiella* (1;3), *Enterobacter* (1), *Enterococcus* species (20), *S. fecalis*, *S. aureus* and *B. fragilis* (3), and in other cases no association between NEC and a specific bacterial species have been observed (6;21-24). Furthermore, colostrum has proven to decrease the risk of NEC in preterm pigs compared with infant formula (9-11). Therefore, production of colostrum with hyper-immunity towards one specific bacterium of several possible bacteria involved in NEC may not be cost-effective. For preterm infants not able to receive mother's milk, which is the optimal nutrition for infants (25), donor milk seems to be the best alternative for these patients, since it decreases the risk of NEC compared with infant formula (26). However, milk formula is a lifesaving alternative when mother's milk or human donor milk is not available (27). The effect and safety of bovine colostrum products for preterm infants still needs to be investigated.

Hyperimmune colostrum may prove valuable for the prevention and treatment of diseases caused by specific known pathogens (16;17) including antibiotics-associated diarrhea caused by *C. difficile* that has become a more severe disease with increased incidence and severity (28). Different colostrum preparations with hyperimmunity towards *C. difficile* toxins have *in vivo* and *in vitro* shown an effect against *C. difficile* diarrhea and the cytotoxic effects of *C. difficile* toxins (29;30). Furthermore, it may also prove to be effective in the prevention of recurrences of *C. difficile* diarrhea in humans (31). However, the efficacy and safety of hyperimmune products for human patients needs to be investigated further.

In this study, an increased level of antibodies against *C. perfringens* was observed in the hyperimmune colostrum; however, the practical use of hyperimmune colostrum products for preterm infants remains questionable due to the diversity in bacterial species associated with NEC and the cost-effectiveness of such a product. In contrast, hyperimmune colostrum may prove to be useful in the prevention and treatment of diseases caused by known pathogens.

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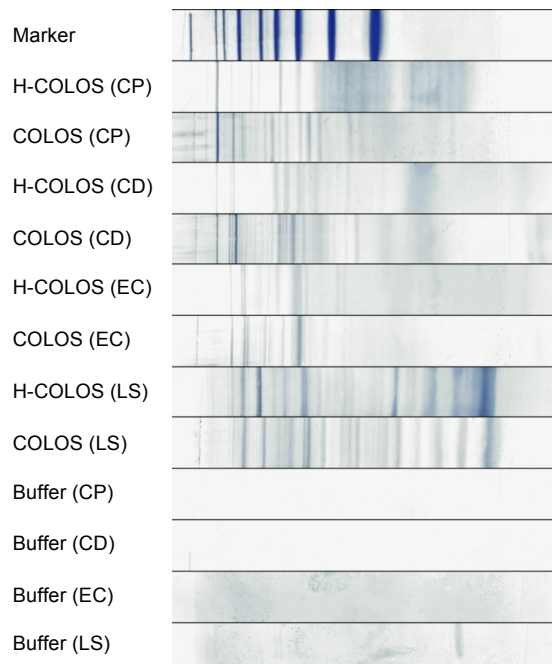
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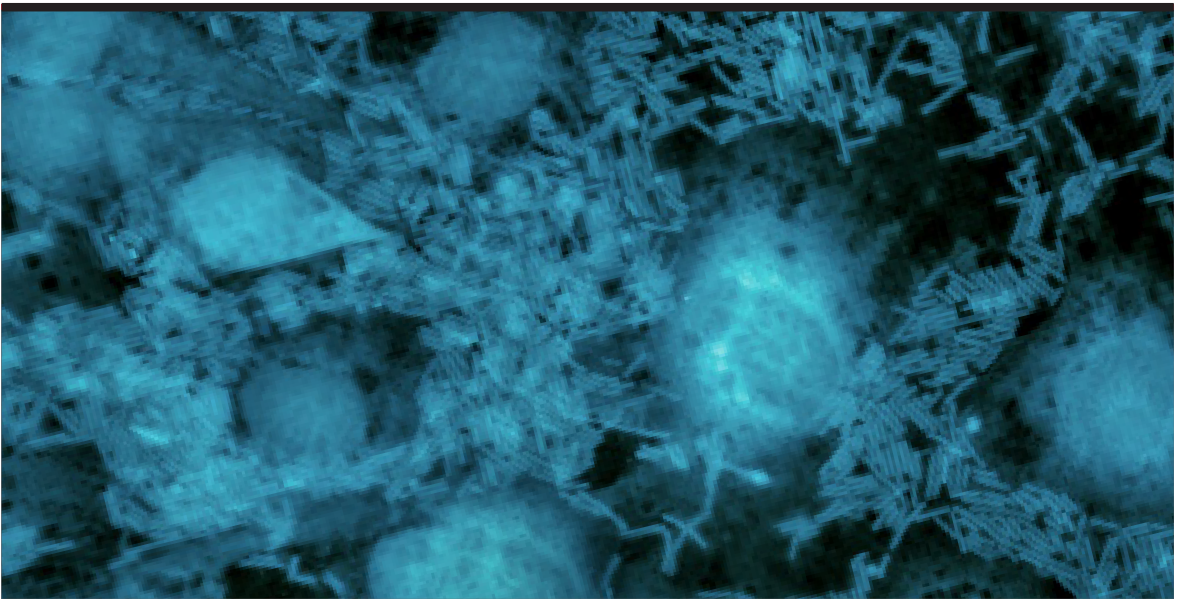
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FIGURE LEGENDS

FIGURE 1: Western blot comparing the activity towards *C. perfringens* (CP), *C. difficile* (CD), *E. coli* (EC) and *L. sakei* (LS) in H-COLOS and COLOS.

FIGURE 1





Innate Immunology Group
Section for Immunology and Vaccinology
National Veterinary Institute
Technical University of Denmark
Bülowsvej 27
1870 Frederiksberg C
Denmark

www.vet.dtu.dk

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