SLUTRAPPORT
NR. 2017-145

Mildt varmebehandlet bovint valleproteinkoncentrat som supplement til modermælks-erstatning og human donormælk

Mejeribrugets
ForskningsFond

MAJ 2017

Final report

for collaborative projects funded via
the Danish Dairy Research Foundation (DDRF)

1. Title of the project

MILDT VARMEBEHANDLET BOVINT VALLEPROTEINKONCENTRAT SOM SUPPLEMENT TIL MODERMÆLKSERSTATNING OG HUMAN DONORMÆLK

MILD-HEAT-TREATED BOVINE WHEY PROTEIN CONCENTRATE AS A SUPPLEMENT FOR INFANT FORMULA AND DONOR HUMAN MILK

2. Project manager

Associate Prof. Stine Brandt Bering
Section for Comparative Pediatrics and Nutrition (CPN)
Dept. Veterinary and Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen
Dyrlægevej 68
1870 Frederiksberg C.
Phone: 3533 1092
E-mail: sbb@sund.ku.dk

3. Other project staff

Prof. Per Torp Sangild, CPN (pig studies)
Post doc Yanqi Li, CPN (pig studies)
Post doc Duc Ninh Nguyen, CPN (milk analysis and in vitro studies)
Ass. Prof. Dereck Chatterton, Dept. Food Science (milk bioactivity and analyses)
Ass. Prof. Thomas Thymann, CPN (pig studies)
Anne Staudt Kvistgaard, Arla Foods Ingredients (WPC production)
Anne Heckmann, Arla Foods Ingredients (WPC production)
Post doc Pingping Jiang, CPN (proteome analysis, FACS analyses)

4. Sources of funding

DDRF: 1,626,000 DKK
Arla Foods Ingredients: 1,500,000 DKK
University of Copenhagen: 148,000 DKK

5. Project period

Project period with DDRF funding: 01/2015 – 12/2016

6. Project summary

Human mælk er designet til at tilføre næringsstoffer, men også vækstfaktorer og immun-suppressive faktorer, der beskytter mod inflammation og infektion i nyfødte. Human donormælk (DM) og/eller modermælkserstatning er alternativerne når mors egen mælk ikke er tilgængelig eller nok.

Projektet fokuserer på effekten af mildere processering af DM og mælkeprotein til modermælkserstatninger for øget tarmmodning og beskyttelse mod tarminflammation. Ultraviolet-C bestrålet (UVC) mod konventionel Holder pasteuriseret (HP) DM samt mildere varmebehandlet mod konventionelt bovint valleprotein

(BioWPC) undersøges ved biokemisk karakterisering, effekter på tarmceller samt fysiologisk respons i en grisemodel for tarminflammation i tidligt fødte børn.

UVC og HP DM havde begge reduceret antallet af bakterier i mælken. UVC bevarede samtidig vigtige enzymer, herunder galdesalt stimuleret lipase og alkalisk fosfatase. Derimod reducerede HP laktoferrin niveauet i mælken samt vægtøgningen i nyfødte grise og øgede forekomsten af diarré. Både tarmparametre og mikrobiota data understøttede den positive virkning af UVC mælk.

BioWPC viste en bedre bevaring af flere bioaktive proteiner samt øget fødevaretolerance i nyfødte grise i forhold til WPC. Der var ingen effekt på vægtøgning og tarminflammation. Tarmparametre som villus højde og enzymaktivitet var højere i BioWPC grise, og de havde tendens til bedre sukker absorption, lavere tarmpermeabilitet og øget fysisk aktivitet.

Det kan konkluderes at UVC bestråling som en ny teknologi til at pasteurisere DM bevarer bioaktive faktorer, der er vigtige for tarmudviklingen og immunsystemet i tidligt fødte børn. Ligeledes bevares bioaktive proteiner i BioWPC ved reduceret varmebehandling og øger fødevaretolerance og tarmfunktion. Modermælkserstatning indeholdende BioWPC kan være vigtigt for øget tarmmodning og sundhed i sensitive nyfødte børn.

Human milk is designed to provide nutrition but also growth factors and immunosuppressive factors, which protect against inflammation and infection in newborns. Donor human milk (DM) and/or infant formula are alternatives when mother's own milk is not available or sufficient.

The project focuses on the effect of milder processing of DM and milk protein for infant formula for improved gut maturation and protection against inflammation. Ultraviolet-C irradiation (UVC) against conventional Holder pasteurized (HP) DM and milder heat-treated against conventional bovine whey protein concentrate (BioWPC) is investigated by biochemichal characterization, effects in intestinal cells and physiological response in a pig model of gut inflammation in preterm infants.

UVC and HP DM reduced the concentration of bacteria in the milk, but only UVC preserved important enzyme activities, such as bile salt stimulated lipase and alkaline phosphatase. In contrary, HP reduced milk lactoferrin and weight increase in newborn pigs and increased the incidence of diarrhea. Gut parameters and the microbiota data both supported the beneficial effect of UVC milk.

BioWPC showed improved bioactivity of several proteins and increased food tolerance in newborn pigs compared to WPC. No effect on weight and gut inflammation was observed. Gut parameters such as villus hight and enzyme activity was higher in BioWPC pigs, and they tended to have improved sugar absorption, lower gut permeability and increased physical activity.

In conclusion, UVC irradiation as a new technology to pasteurize DM preserves bioactive factors that are important for gut maturation and the immune system in preterm neonates. Further, bioactive proteins in BioWPC is preserved by reduced heat treatment and improved food tolerance and gut function. Infant formula with bioactive WPC may be important for improved gut maturation and health in sensitive newborn infants.

7. Project aim

Formål

1. At undersøge effekten af mild varmebehandling på bioaktiviteten af sød valleprotein-koncentrat til stimulering af tarmmodning og beskyttelse i for tidligt fødte grise og en tarmcellemodel.
2. At undersøge effekten af Holder og UV-C pasteurisering vs. rå human donor mælk på bioaktiviteten af donormælk til stimulering af tarmmodning og beskyttelse i for tidligt fødte grise og en tarmcellemodel

3. At sammenligne effekten af modermælkserstatning tilsat mildt varmebehandlet sød valleproteinkoncentrat med effekten af human donor mælk på tarmmodning og beskyttelse i for tidligt fødte grise og en tarmcellemodel.

Objectives

1. To investigate the effects of mild heat-treatment on the bioactivity of sweet WPC to stimulate intestinal maturation and protection in preterm newborn pigs and an intestinal cell model.
2. To investigate the effects of raw vs. Holder and UV-C pasteurization on the bioactivity of DM to stimulate intestinal maturation and protection in preterm newborn pigs and an intestinal cell model.
3. To investigate if infant formula containing a mild heat-treated sweet WPC has effects more comparable to DM on intestinal maturation comparing with a conventionally heat-treated WPC in preterm newborn pigs and an intestinal cell model.

8. Background for the project

Human milk is designed to provide optimal nutrition and protection against inflammation and infection by providing growth factors and immunosuppressive factors (1-5). The beneficial actions of milk bioactives are important for all infants, but particularly when the intestine is immature or sensitive to infection, such as in preterm infants that often develop necrotizing enterocolitis (NEC) (6). Availability of mother's milk is often limited after complicated deliveries and premature birth. In these cases, donor human milk (DM) may be the best alternative (7,8). In contrast to mother's own milk, where proteins are intact, DM is pasteurized by Holder pasteurization to prevent transmission of potential contaminants, such as HIV, Human T-lymphotropic virus, and Cytomegalovirus. However, the Holder pasteurization abolishes B and T lymphocytes, and decreases the activity of some bioactive proteins (lipoprotein, lipase, IgM, IgA, IgG, lactoferrin, lysozyme, insulin-like growth factors (IGFs), and IGF binding proteins), while others are less affected (oligosaccharides, gangliosides, cytokines, and growth factors, such as TGF-β and epidermal growth factor) (9-11). It remains unclear whether the reduction in the natural trophic, antibacterial, and immunoregulatory components has health consequences for the sensitive infants and requires investigation in an appropriate *in vivo* model system.

DM is recommended as the best alternative for preterm infants, but infant formula is frequently used, due to the unavailability of DM in many countries. DM also has the disadvantage that fortification with other milk products (protein, minerals) is required to increase the nutritional value for optimal infant growth. For these infants, improving the quality of the infant formula may be life-saving. Immunoglobulins, lactoferrin, lysozyme, lactoperoxidase, TGF-β, EGF, IGFs, and osteopontin-supplemented formulas have been tested and shown to have beneficial effects on intestinal health (12,13). These proteins are all present in whey protein concentrate (WPC), which is the main by-product from the cheese industry and used as an ingredient in infant formula. To produce WPCs, many milk processing techniques are applied, including heat-treatment and spray-drying. These processes cause a significant reduction of many bioactive proteins (14-17). Nevertheless, the possible physiological effects of improving bioactivity of infant formulas are poorly characterized. In previous studies, including the MFF project "Bioactive milk proteins against gut inflammation – characterization of gut effects", we showed that lower heat-treatment and milder filtration improved intestinal effects of acid WPC and bovine colostrum (13). Furthermore, raw bovine milk more efficiently protected and matured the gut than infant formula (18), in line with what has been observed for bovine colostrum. Consequently, reduction

of heat-treatment during WPC production may be an instrument to increase the quality of WPC products used in infant formulas. To follow up on these studies, it is important to investigate to which extent a mild heat-treated WPC product based on sweet whey, can match DM as the clinically relevant diet.

The preterm pig model has shown high sensitivity to differences in the quality of the first enteral feeding (19,20). It will therefore serve as a good model for sensitive newborn infants in the search for diets that are more beneficial in supporting gut maturation and protection. This, coupled with the newborn porcine intestinal epithelial IPEC-J2 cell model (21), will make it possible to investigate the direct interaction between the enterocytes and bioactive milk components, thereby providing evidence for mildly-heat-treated WPC as a potential infant supplement.

Hypotheses for improved intestinal maturation in preterm newborns

1. Mild heat-treated sweet BioWPC is superior conventional WPC in formula.
2. Raw DM is superior to pasteurized DM.
3. Mild heat-treated WPC is more comparable to DM than conventional WPC.

9. Sub-activities in the entire project period

The project consisted of several studies separated into three work packages in which WPC and DM products will be tested (Figure 1).
The DM was tested raw and pasteurized by Holder pasteurization and UV-C treatment, respectively. The mild heat-treated sweet WPC (Bioactive WPC, Arla Foods Ingredients) was tested against the conventionally heat-treated sweet WPC, in which all other parameters and production processes were kept constant (Lacprodan DI-8090, Arla Foods Ingredients).

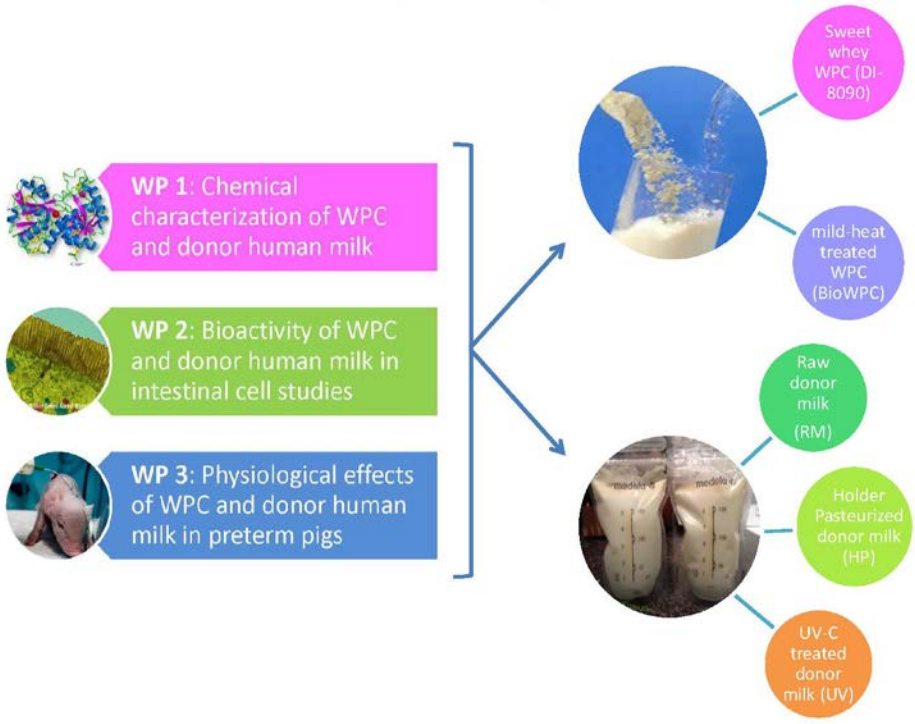


Figure 1 Schematic diagram of the work packages included in the MFF project to investigate the potential for using mildly heat-treated whey-protein concentrate (WPC) as a supplement in infant formula and donor human milk (DM). The work packages indicate the three different levels in which the bioactivity and gut physiological effects of WPC and DM was investigated. The differently treated WPC and DM products outlined were all investigated in each of the three work package.

WP 1: Chemical characterization of WPC and donor human milk products

The WPC and DM products were characterized by a basic chemical characterization as a basis for investigating the bioactive effects *in vitro* and *in vivo* in WP 2

and WP 3. The characterization included determination of total protein content by the BCA assay and separation of proteins using SDS-PAGE before and after centrifugation and filtration. We thereby determined any denatured or aggregated proteins. The stability of some of the bioactive proteins and peptides present, such as TGF-βs, IGFs, EGF, globulins, milk fat globule membrane proteins, lactoferrin, and osteopontin, were identified using Western Immunoblotting.

WP 2: Bioactivity of WPC and DM products in intestinal cell studies

The WPC and DM products were tested for their innate immunosuppressive effect using the intestinal cell line IPEC-J2 as an *in vitro* model for the newborn pig intestinal epithelium. The products were tested for endotoxin content by the LAL endotoxin assay and DM centrifuged for removal of the fat content. In IPEC-J2 cells the WPC and DM samples were incubated in different concentrations with or without co-stimulation with an inflammatory component (lipopolysaccharide (LPS), flagellin, or platelet activating factor) to elicit a strong immune response in the cells. The ability of WPC and DM to reduce the endotoxin response in the cells related to cell viability (cytotoxicity, proliferation, apoptosis), inflammatory pathways (ELISA), cell signaling (Western Blot), and epithelial barrier function (Western Blot) were determined, and correlated to gut epithelial effects in pigs (WP 3).

WP 3: Physiological effects of WPC and donor human milk products in a preterm pig model

The potential stimulating effects of DM and WPC products on intestinal maturation were determined in the preterm pig model of NEC as illustrated in Figure 2 and Figure 3, respectively. Various parameters were analyzed, including intestinal morphology, plasma citrulline level (a biomarker of mucosal mass and function), brush border enzyme activities, galactose and lactose absorption, lactulose/mannitol in urine, and fecal lipid content, fecal short chain fatty acid (SCFA) content and composition, and fecal microbiota. These parameters reflect the intestinal structure and barrier function, digestive and absorptive functions, as well as bacteria colonization. In addition, Western blotting and/or immunohistochemistry were applied to determine the level of proteins related to proliferation and apoptosis in small intestinal tissues. The mRNA expression levels of selected proteins related to the innate immune system in the intestinal tissues and/or circulating blood were analyzed using Fluidigm, and verified by ELISA or Western blotting.

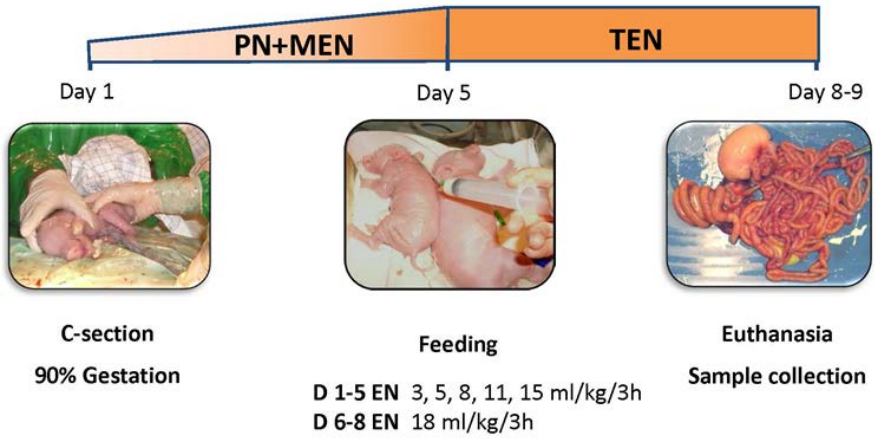


Figure 2 DM study. Fifty-seven preterm pigs from four litters were delivered by caesarean section and received increasing volumes of RM, HP or UVC treated DM as minimal (MEN) or total enteral nutrition (TEN). Parenteral nutrition (PN) was administered with the same dosage and composition to each group to supplement fluid and nutrient intake. Pigs were euthanized on day 8 for collection of blood and organs. The main outcome parameters were growth, diarrhea, necrotizing enterocolitis (NEC), intestinal function, and bacteria in blood and bone marrow.

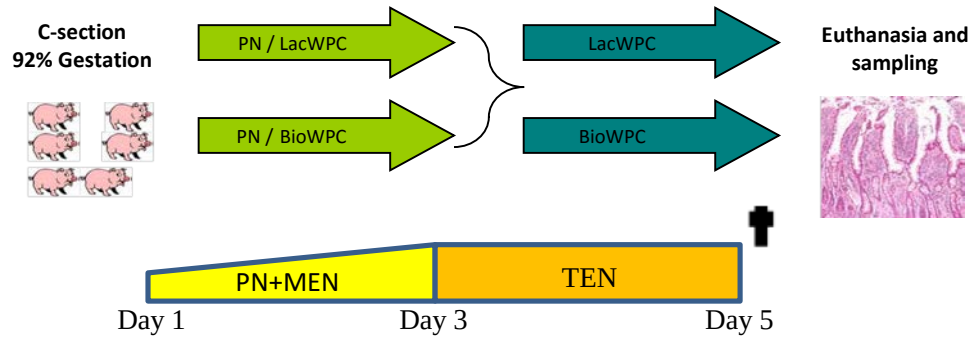


Figure 3 BioWPC study. Ninety-two preterm pigs were delivered from six sows by caesarean section and received increasing volumes of BioWPC or Control LacWPC as minimal (MEN) or total enteral nutrition (TEN). Parenteral nutrition (PN) was administered with the same dosage and composition to each group to supplement fluid and nutrient intake. Pigs were euthanized on day 8 for collection of blood and organs. The main outcome parameters were necrotizing enterocolitis (NEC), intestinal function, and physical activity.

Table 1 shows the time schedule for the project in a Gantt chart.

Table 1 Gantt chart

	2015					2016		
WP 1								
Product characterization								
WP 2								
In vitro cell studies								
Sample analyses								
Data analyses								
Manuscript preparation								
WP 3								
In vivo pig studies								
Tissue analysis								
Data analyses								
Manuscript preparation								

10. Project results

The work packages included analyses related to both DM studies and BioWPC studies. To get the best overview of the results related to the DM products and BioWPC, respectively, these studies are each presented separately with selected data from all WPs together. Methods and results are described in detail in the manuscripts attached.

Donor human milk study

WP1: Chemical characterization of DM products

The levels of some of the measured bioactive proteins in DM are shown in Figure 4. The majority of the bioactive components measured were higher in raw and UVC treated milk compared to HP milk.

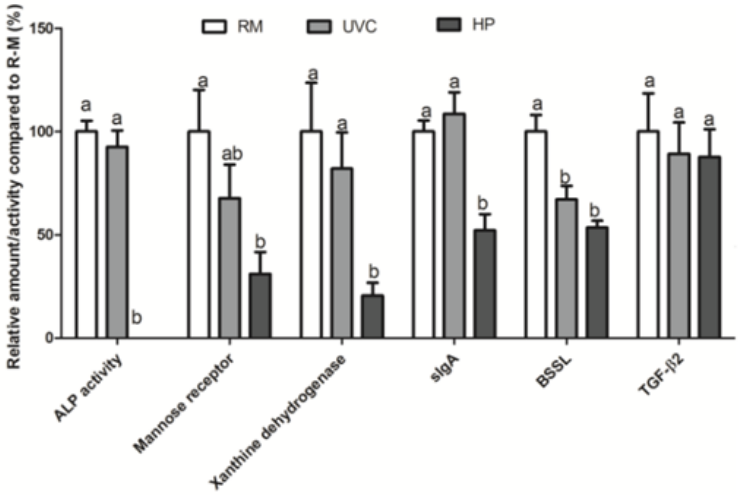


Figure 4 Quantification of proteins in donor human milk. Values are means ± SEM (n = 3). Values not sharing common letters are significantly different (P < 0.05).

The oxidation marker GSH was more pronounced in the RM and HP milk, relative to UVC milk (both P<0.05), whereas the oxidation of vitamin C was more pronounced in both UVC and RM milk compared with HP milk (both P<0.05). The data indicate that UVC treatment does not significantly worsen the oxidative status of the milk.

WP 2: Bioactivity of DM products in intestinal cell studies
The cytotoxicity of DM was increased with increasing concentrations, with HP milk being the least cytotoxic at higher doses. At low doses, raw milk increased proliferation compared to HP milk, with UVC milk in between.

WP 3: Physiological effects of DM products in a preterm pig model
There were no differences between the HP, raw milk and UVC groups in birth weights, weights at death, life-time, overall growth rates, and organ weights or lengths. Pigs fed UVC milk and pigs fed raw milk gained more weight than did those fed HP milk (P < 0.05). Total blood leukocyte and neutrophil counts were higher in pigs fed raw DM than in those fed UVC milk, whereas lymphocyte counts were higher in pigs fed HP milk than in pigs fed UVC milk (both P < 0.05).

Milk treatment did not affect activities of the three measured disaccharidases and dipeptidyl peptidase IV in any of the intestinal regions (Figure 5). In the Dist section, pigs fed UVC milk had or tended to have higher peptidase activities than those fed raw milk (ApN: P < 0.01; ApA: P = 0.07) or HP milk (ApN: P = 0.07). Intestinal monosaccharide absorptive capacity and intestinal permeability as measured by in vivo galactose and lactulose/mannitol tests were similar between groups.

Citrulline is a biomarker for intestinal functionality. On day 5, plasma citrulline levels in HP pigs were lower than that in UVC pigs (P < 0.05) and tended to be lower than that in RM pigs (P = 0.09). The plasma citrulline levels on day 5 were positively correlated with villus heights in three small intestinal regions (Prox: P < 0.001, R^2 = 0.33; Mid: P < 0.01, R^2 = 0.28; Dist: P = 0.09, R^2 = 0.14).

Intestinal concentrations of IL-1β, IL-6, IL-8 were comparable among groups. Relative abundance of β-actin and proliferating cell nuclear antigen did not differ among groups, whereas UVC pigs had, or tended to have, higher level of claudin-4, relative to RM (P < 0.01) and HP pigs (P = 0.08). Gene expression of IL-1β, IL-8, TNF-α, occludin, and claudin-1 did not differ among groups, while gene expression of IL-8 was increased in pigs with osteomyelitis.

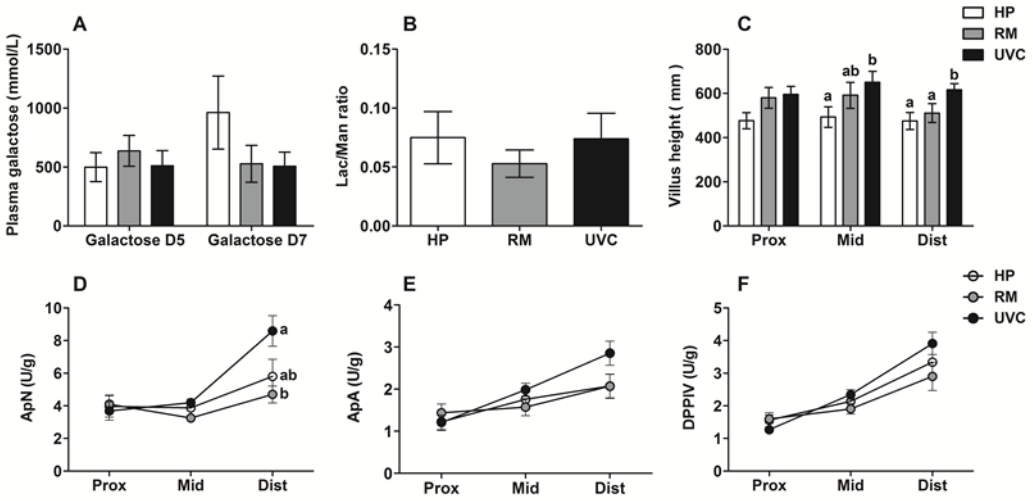


Figure 5 Increments in plasma galactose at 20 min after administration of an oral bolus of galactose on day 5 or the day prior to euthanasia (A). Intestinal permeability in preterm pigs as indicated by the urinary lactulose to mannitol ratio (Lac/Man ratio) measured after oral administration of lactulose/mannitol solution (B). Villus length in the proximal (Prox), middle (Mid), and distal (Dist) regions of the small intestine (C). Activities of 3 brush border peptidases in 3 small intestinal regions: aminopeptidases N (ApN) (D), aminopeptidase A (ApA) (E), and dipeptidyl-peptidase IV (DPPIV) (F). Values are mean ± SEM (n = 17-19/group) and different superscript letters indicate significant differences among groups in the same region ($P < 0.05$).

The total bacterial load in the cecum content did not differ among HP, RM, and UVC pigs. Sequencing data showed that the majority of reads (>99%) were distributed between the phyla Proteobacteria (62.8%) and Firmicutes (37.1%) in cecum content and among Proteobacteria (47.6%), Firmicutes (42.3%) and Bacteroidetes (10%) in mucosal tissues. Relative to RM and HP pigs, the UVC pigs had higher relative abundance of *Enterococcus* in both cecum content and ileal mucosal tissues (all $P < 0.05$, Figure 6). A tendency of lower relative abundance of *Enterobacteriaceae* was observed in the cecum content of UVC pigs compared with RM pigs ($P = 0.072$), with intermediate levels in HP pigs. In ileal mucosal tissues, the bacterial taxa distribution was similar to that in cecum contents, except that *Bacteroides* was only detected in mucosal tissues, while *Ruminococcaceae* was negligible in mucosal tissues relative to that in cecum content.

The total and proximate bacterial areas measured by FISH were not different among the three groups across the three intestinal regions.

Formate, acetate, lactate and succinate were the main short chain fatty acids present in colon contents. Concentrations of lactate in wet feces (mmol/g) differed among groups with higher levels (or tendency) in UVC pigs (52 ± 4) than those in RM pigs (35 ± 5, $P < 0.05$) and HP pigs (41 ± 5, $P = 0.14$), and the other fatty acids (mmol/g) were similar among groups for formate (2.0 ± 0.8), acetate (6.2 ± 1.3), and succinate (2.2 ± 0.9).

In this study, we confirmed that UVC is as effective as HP in eliminating bacteria in DM and better preserves heat-sensitive bioactive components, including some antioxidants. Using preterm pigs as a model for preterm infants, we have demonstrated that the treatment-related differences in the biochemistry of DMs have potential physiological effects in preterm neonates. UVC-treated DM improves growth, intestinal health, and systemic bacterial resistance compared with HP treatment at least in preterm pigs. Our findings suggest that UVC may be an improved method of pasteurizing DM. Nevertheless, the safety of UVC treatment of DM for sensitive preterm infants requires further documentation in vitro and in vivo, for example, regarding the possible effects on stem cells and milk leukocytes.

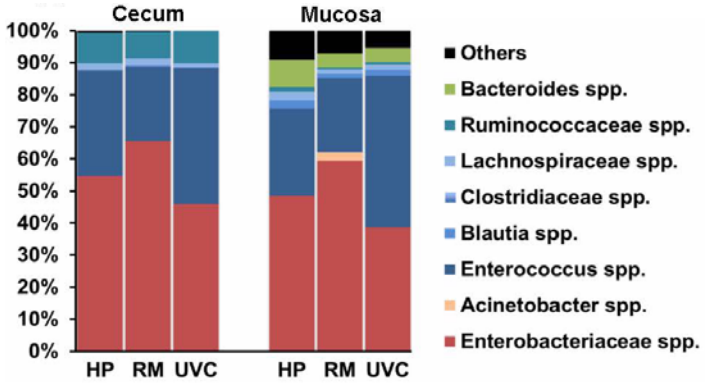


Figure 6 Relative abundance distribution of the major genera in the cecum content and distal mucosal tissue samples (n = 15-19). Dist, distal small intestine; HP, holder pasteurized donor milk; Mid, middle small intestine; Prox, proximal small intestine; RM, raw donor milk; UVC, ultraviolet-C irradiated donor milk.

Bioactive WPC study

WP1: Chemical characterization of WPC products

The level of aggregated protein in BioWPC was negligible, whereas approximate 20% protein was aggregated in ConWPC. LF and BSA levels were higher in BioWPC vs. ConWPC before and after aggregate removal ($P < 0.05$, Figure 7). After aggregate removal, BioWPC also had higher levels of β-lactoglobulin and α-lactalbumin than ConWPC ($P < 0.05$). For growth factors, two WPCs had similar levels of IGF-1 (170-180 ng/g).

WP 2: Bioactivity of WPC products in intestinal cell studies

At low concentrations of 0.01-0.1 g/L, both BioWPC and ConWPC were negligibly cytotoxic, whereas at 1 g/L, cytotoxicity was induced at higher levels by ConWPC than BioWPC (Figure 8A, $P < 0.05$). BioWPC-induced cell proliferation was greater than ConWPC-induced cell proliferation at 0.01 g/L, whereas at 1 g/L, ConWPC decreased cell proliferation ($P < 0.05$, Figure 8B).

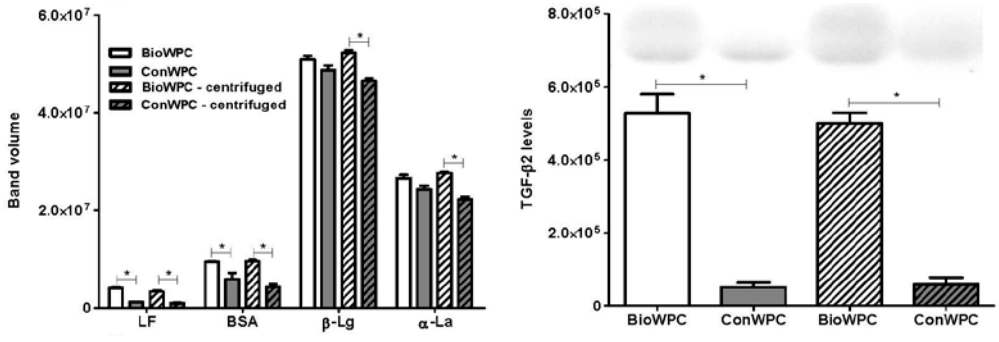


Figure 7 Protein composition in BioWPC and ConWPC with and without aggregate removal by centrifugation analyzed by SDS-PAGE. (A) Relative quantification of protein band volume for LF, BSA, β-Lg, α-La and TGF-β2. Values (means ± SEM, n = 3 for triplicates of sample preparation) indicated by '⋆' differ between each other, $P < 0.05$. BioWPC, bioactive WPC; ConWPC, conventional WPC; β-lg, β-lactoglobulin; α-La, α-lactalbumin; BSA, bovine serum albumin.

WP 3: Physiological effects of WPC products in a preterm pig model

There was no difference between the BioWPC and ConWPC groups in terms of birth weight (1014 ± 46 g), incidence of necrotizing enterocolitis (14/31), and lesion scores in the stomach (1.4 ± 0.2), small intestinal (1.7 ± 0.1), and colon (2.1 ± 0.2). The relative weights of organs and the relative length of the small intestine

did not differ among groups. Interestingly, feeding intolerance was observed in seven pigs in the ConWPC group and none in the BioWPC group.

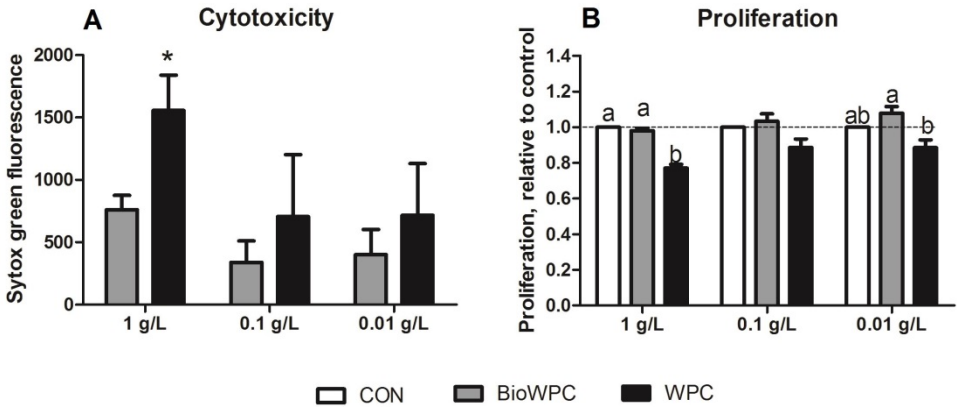


Figure 8 Effects of BioWPC and ConWPC on the cell cytotoxicity (A) and proliferation (B) of IPEC-J2 cells *in vitro*. Values are means ± SEM, n = 3 representing triplicates in three different cell passages. Values indicated by '*' differ between each other, P < 0.05. BioWPC, bioactive WPC; WPC, conventional WPC; CON, control.

Intestinal absorptive functions measured by galactose test on day 3 tended to be higher in the BioWPC group relative to the Con group (P = 0.11, Figure 9A), whereas the function measure by lactose test on day 4 did not differ between two groups. Intestinal permeability measured by lactulose/mannitol test tended to be lower in the Bio group then that in the Con group (P = 0.07, Figure 9B). When it comes to ex vivo brush border enzyme activities, only lactase in Prox showed higher values in the BioWPC group (P < 0.05, Figure 9C).

In the Prox region, pigs in the Bio group had increased villus height and decreased crypt depth compared with the Con group (both P < 0.05). Villus height and crypt depth did not differ between two groups in the Mid and Dist regions (data not shown). Goblet cell density did not differ between two groups neither in the Dist small intestine nor in the colon.

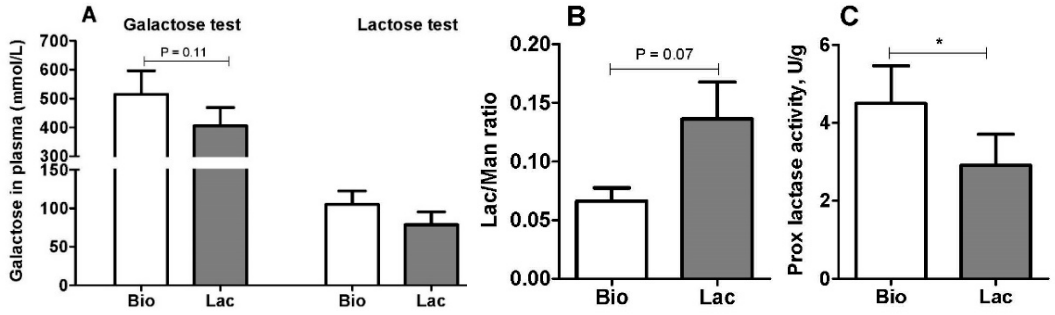


Figure 9 Intestinal digestive and absorptive functions and permeability in pigs. A: Plasma galactose levels at 20 min after administration of oral boluses of galactose solution on day 3 and at 40 min after administration of oral boluses of lactose solution. B: Intestinal permeability presented as measured by the urinary lactulose to mannitol ratio (L/M ratio) after oral administration of lactulose/mannitol solution. C: Brush boarder enzyme activity in the proximal region of small intestine. Values are means ± SEM. Values indicated by '*' differ between each other, P < 0.05. Bio, pigs receiving formula with bioactive WPC; Lac, pigs receiving formula containing conventional Lacprodan.

The physical activity recorded by the motion cameras tended to be higher in the BioWPC group, compared with the ConWPC group (over all P = 0.09, Figure 10A), and was significantly increased during the last day prior to euthanasia (P < 0.05). Further, in the open field test on day 4, BioWPC pigs walked almost twice

the distance of ConWPC pigs (P<0.05, Figure 10B), overall supporting an increased motor activity in Bio pigs.

Higher bioactivity of WPC is achieved by reducing the thermal-treatment. This increases feeding tolerance, intestinal structure and function and physical activity in pigs and may be important, particularly in sensitive newborns.

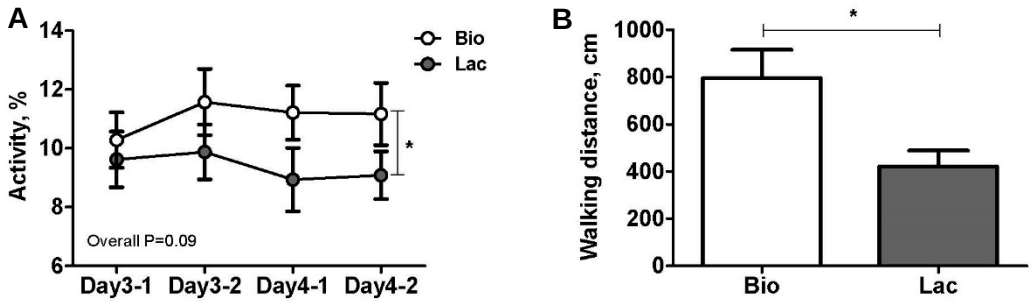


Figure 10 General physical activity. A: Trend of proportion of active time during postnatal day 3 and 4 showing in every half day. B: Walking distance in open field test on day 4. Values are means + SEM. Values indicated by '*' differ between each other, P < 0.05. Bio, pigs receiving formula containing bioactive WPC; Lac, pigs receiving formula containing conventional Lacprodan.

In conclusion, UVC irradiation as a new technology to pasteurize DM preserves bioactive factors that are important for gut maturation and the immune system in preterm neonates. Further, bioactive proteins in BioWPC is preserved by reduced heat treatment and improved food tolerance and gut function. BioWPC thereby comes closer to the beneficial effects of DM as an alternative to mothers milk. Infant formula with bioactive WPC may be important for improved gut maturation and health in sensitive newborn infants that are in need of protein supplementation to mothers own milk or DM for growth or as an alternative to mothers own milk or DM in general.

11. Deviations

11.1 Scientific

Based on the beneficial effects of raw DM on growth, and several intestinal parameters on UVC milk, together with the observed higher bacterial content in the raw milk and osteomyelitis incidence in HP pigs, it was prioritized to make a deep investigation of host responses by blood biochemistry, hematology (leukocytes and neutrophils) and innate immune parameters (cytokine synthesis). These parameters were originally not included in the project but as potential additional parameters. Instead, the detailed investigations of inflammatory pathways and cell signaling in IPEC-J2 cells in WP2 were not prioritized. The decision was made in agreement with the industry partners and with consultancy of the DDRF Board.

11.2 Financial None

11.3 Timetable None

12. The relevance of the results, including relevance for the dairy industry

Overall, we have documented beneficial physiological effects of milk heat-treatment of milk proteins. A new UVC method for mild pasteurization of DM compared to conventional Holder pasteurization has been applied and the product tested in a neonatal pig model. We have documentet that the milder and improved technology confers improved effect on gut function and health in new-

borns. Further, we have documented that milder heat-treatment of WPC also protects bioactive proteins and improve the beneficial functions in the gut of the newborn. Particularly for BioWPC, this has a big industrial potential by providing a high-quality protein supplement on the market for use in the production of infant formula and thereby increase the market potential globally.

The results obtained has significant influence on the further development and production implementation of BioWPC. Nevertheless, there are challenges related to regulatory affairs and production.

The societal related effects are primarily related to improved health of the newborns. With an optimized protein supplement for infant formulas, the infants may show an immediate improved resistance against infections both short and long term.

13. Communication and knowledge sharing about the project

Papers in international journals:

Li Y, Nguyen DN, Ryom K, Andersen AD, Thymann T, Chatterton D, Purup S, Heckmann AB, Bering SB, Sangild PT. *Bioactive whey protein concentrate and lactose stimulate gut function in formula-fed preterm pigs*. J Funct Foods 2017 (submitted).

Li Y, Nguyen DN,de Waard M, Christensen L, Zhou P, Jiang P, Sun J, Bojesen AM, Lauridsen C, Lykkesfeldt J, Dalsgaard TK, Bering SB, Sangild PT. *Pasteurization procedures affect body growth, intestinal structure, and resistance against bacteria in preterm pigs.* J Nutr 2017 (in press).

Easily read papers:

Bering SB, Nguyen DN, Li Y, Chatterton DEW, Heckmann ABL, Sangild PT. *Bioaktive mælkeproteiner styrker tarmsundhed*. Mælkeritidende 2015;10:12-13.

Oral presentations at scientific conferences, symposiums etc.:

Bering SB & Sangild PT. *Protective effects of milk and their species-specificity for the preterm newborn*. Origins and Benefits of Biologically-Active Components of Human Milk, FASEB. Big Sky, Montana, USA, 19-24 July 2015.

Oral presentations at meetings:

Bering SB & Sangild PT. *Ultraviolet-c irradiation of donor human milk improves growth, intestinal function and systemic immunity in preterm pigs*. Medela research meeting. Warsaw 15 April 2015.

Others

Abstracts presented as posters at scientific conferences:

Li Y, Nguyen DN, Thymann T, Chatterton DEW, Kvistgaard AS, Bering SB, Sangild PT. *Bioactive whey protein concentrate and lactose stimulate gut function in preterm pigs.* 48th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). Amsterdam, The Netherlands, 6-9 May 2015.

Li Y, Nguyen DN, de Waard M, Bojesen A, Thymann T, Bering SB, Sangild PT. *Ultraviolet-c irradiation of donor human milk improves growth, intestinal function and systemic immunity in preterm pigs.* 1st Congress of joint European Neonatal Societies (jENS), 16-20 September 2016, Budapest, Hungary.

14. Contribution to master and PhD education

Several master students and PhD students have been involved in the project for specific analytical parts.

MSc student Lars Christensen (Univ. Copenhagen) supported with the animal procedures and performed the microbiota analyses.

MSc student Karina Ryom supported with the animal procedures and data analyses.

MSc student Monica Dagsvik conducted the open field tests on the pigs.

PhD student Marita de Ward (VU University Medical Center, Amsterdam, Netherlands) was involved in designing the donor milk study.

PhD student Jing Sun (Univ, Copenhagen) contributed with the qPCR analyses.

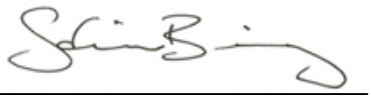
PhD student Anders Brunse supported with the animal procedures.

15. New contacts/projects

An IP application has been filed based on an invention relating to a bioactive sweet WPC for increasing the brain development and thereby cognitive functions particularly in young mammals such as preterm or term infants, toddlers, children or young adults. The present invention further disclose new routes for large-scale production methods of bioactive whey protein concentrates and preparations. Further initiatives for documentation of bioactive WPC effects in the preclinical setting is negotiated with the industry partner.

16. Signature and date

The project is formally finalised when the project manager and DDRF-representative (e.g. steering committee leader) have signed this final report.

Date: 05.05.17 Signature, Project manager: 

Date: _____ Signature, DDRF-representative: _____

Appendix A: References

1. Boyd, Quigley & Brocklehurst (2007) Donor breast milk versus infant formula for preterm infants: systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal* Ed 92: F169-F175.
2. Lucas. & Cole (1990) Breast milk and neonatal necrotising enterocolitis. *Lancet* 336: 1519-23.
3. McGuire & Anthony (2003) Donor human milk versus formula for preventing necrotising enterocolitis in preterm infants: systematic review. *Arch Dis Child Fetal Neonatal* Ed 88: F11-F14.
4. Schanler, Shulman, Lau, Smith & Heitkemper (1999) Feeding strategies for premature infants: randomized trial of gastrointestinal priming and tube-feeding method. *Pediatrics* 103: 434-9.
5. Sisk, Lovelady, Dillard, Gruber & O'Shea (2007) Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. *J Perinatol* 27: 428-33.
6. Sangild, Siggers, Schmidt, Elnif, Bjornvad, Thymann, Grondahl, Hansen, Jensen, et al. (2006) Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* 130: 1776-92.
7. Wight (2001) Donor human milk for preterm infants. *J Perinatol* 21: 249-254.
8. Gregory & Walker (2013) Immunologic Factors in Human Milk and Disease Prevention in the Preterm Infant. *Curr Pediatr Rep* 1.
9. Tully, Jones & Tully (2001) Donor milk: what's in it and what's not. *Journal of Human Lactation: Official Journal of International Lactation Consultant Association.* 17:152-5.
10. Ewaschuk, Unger, Harvey, O'Connor & Field (2011) Effect of pasteurization on immune components of milk: implications for feeding preterm infants. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme.* 36: 175-82.
11. ESPGHAN Committee on Nutrition, Arslanoglu, Corpeleijn, Moro, Braegger, Campoy, *et al.* (2013) Donor human milk for preterm infants: current evidence and research directions. *Journal of Pediatric Gastroenterology and Nutrition.* 57(4): 535-42.
12. Chatterton, Nguyen, Bering & Sangild (2013) Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The International Journal of Biochemistry & Cell Biology.* 45: 1730-47.
13. Li, Ostergaard, Jiang, Chatterton, Thymann, Kvistgaard, *et al* (2013) Whey protein processing influences formula-induced gut maturation in preterm pigs. *The Journal of Nutrition.* 143(12): 1934-42.
14. Chen, Hwang, Liau, Ho, Hong & Mao (2005) Beta-lactoglobulin is a thermal marker in processed milk as studied by electrophoresis and circular dichroic spectra. *J Dairy Sci* 88: 1618-30.
15. Elshereef, Budman, Moresoli & Legge (2006) Fluorescence spectroscopy as a tool for monitoring solubility and aggregation behavior of beta-lactoglobulin after heat treatment. *Biotechnol Bioeng* 95: 863-74.
16. McGuffey, Epting, Kelly & Foegeding (2005) Denaturation and aggregation of three alpha-lactalbumin preparations at neutral pH. *J Agric Food Chem* 53: 3182-90.
17. Wang, Tolkach & Kulozik (2006) Quantitative assessment of thermal denaturation of bovine alpha-lactalbumin via low-intensity ultrasound, HPLC, and DSC. *J Agric Food Chem* 54: 6501-6.
18. Li, Jensen, Chatterton, Jensen, Thymann, Kvistgaard & Sangild (2013) Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets. *Am J Physiol Gastrointest Liver Physiol* (e-pub ahead of print).
19. Bjornvad, Thymann, Deutz *et al* (2008) Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. *Am J Physiol Gastrointest Liver Physiol* 295: G1092-103.
20. Cilieborg, Boye, Thymann, Jensen & Sangild (2011) Diet-dependent effects of minimal enteral nutrition on intestinal function and necrotizing enterocolitis in preterm pigs. JPEN *J Parent Enteral Nutr* 35: 32-42.
21. Schierack, Nordhoff, Pollmann et al. (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochem Cell Biol* 125: 293-305.

The Journal of Nutrition. First published ahead of print March 15, 2017 as doi: 10.3945/jn.116.244822.

The Journal of Nutrition
Nutrition and Disease

Pasteurization Procedures for Donor Human Milk Affect Body Growth, Intestinal Structure, and Resistance against Bacterial Infections in Preterm Pigs[1–3]

Yanqi Li,[4] Duc Ninh Nguyen,[4] Marita de Waard,[6] Lars Christensen,[4] Ping Zhou,[7] Pingping Jiang,[4] Jing Sun,[4] Anders Miki Bojesen,[5] Charlotte Lauridsen,[8] Jens Lykkesfeldt,[5] Trine Kastrup Dalsgaard,[9] Stine Brandt Bering,[4] and Per Torp Sangild[4,10]*

[4]Comparative Pediatrics and Nutrition and [5]Department of Veterinary Disease Biology, University of Copenhagen, Copenhagen, Denmark; [6]Dutch Human Milk Bank, VU University Medical Center, Amsterdam, Netherlands; [7]Department of Neonatology, Shenzhen Bao'an Maternal and Child Health Hospital, Shenzhen, China; [8]Department of Animal Science, Aarhus University, Foulum, Denmark; [9]Department of Food Science, Aarhus University, Aarhus, Denmark; and [10]Department of Pediatrics and Adolescent Medicine, Rigshospitalet, Copenhagen, Denmark

Abstract

Background: Holder pasteurization (HP) destroys multiple bioactive factors in donor human milk (DM), and UV-C irradiation (UVC) is potentially a gentler method for pasteurizing DM for preterm infants.

Objective: We investigated whether UVC-treated DM improves gut maturation and resistance toward bacterial infections relative to HP-treated DM.

Methods: Bacteria, selected bioactive components, and markers of antioxidant capacity were measured in unpasteurized donor milk (UP), HP-treated milk, and UVC-treated milk (all from the same DM pool). Fifty-seven cesarean-delivered preterm pigs (91% gestation; ratio of males to females, 30:27) received decreasing volumes of parental nutrition (average 69 mL · kg^{-1} · d^{-1}) and increasing volumes of the 3 DM diets (n = 19 each, average 89 mL · kg^{-1} · d^{-1}) for 8–9 d. Body growth, gut structure and function, and systemic bacterial infection were evaluated.

Results: A high bacterial load in the UP (6×10^5 colony forming units/mL) was eliminated similarly by HP and UVC treatments. Relative to HP-treated milk, both UVC-treated milk and UP showed greater activities of lipase and alkaline phosphatase and concentrations of lactoferrin, secretory immunoglobulin A, xanthine dehydrogenase, and some antioxidant markers (all $P < 0.05$). The pigs fed UVC-treated milk and pigs fed UP showed higher relative weight gain than pigs fed HP-treated milk (5.4% and 3.5%), and fewer pigs fed UVC-treated milk had positive bacterial cultures in the bone marrow (28%) than pigs fed HP-treated milk (68%) ($P < 0.05$). Intestinal health was also improved in pigs fed UVC-treated milk compared with those fed HP-treated milk as indicated by a higher plasma citrulline concentration (36%) and villus height (38%) ($P < 0.05$) and a tendency for higher aminopeptidase N (48%) and claudin-4 (26%) concentrations in the distal intestine ($P < 0.08$). The gut microbiota composition was similar among groups except for greater proportions of *Enterococcus* in pigs fed UVC-treated milk than in pigs fed UP and those fed HP-treated milk in both cecum contents (20% and 10%) and distal intestinal mucosa (24% and 20%) (all $P < 0.05$).

Conclusions: UVC is better than HP treatment in preserving bioactive factors in DM. UVC-treated milk may induce better weight gain, intestinal health, and resistance against bacterial infections as shown in preterm pigs as a model for DM-fed preterm infants. *J Nutr* doi: 10.3945/jn.116.244822.

Keywords: Preterm neonates, donor human milk, pasteurization, intestinal health, systemic bacterial resistance

Introduction

Preterm birth occurs in ~11% of all live-born infants worldwide (1). Preterm infants are at increased risk of a number of complications because of their immature intestine and immune systems, including feeding intolerance, growth retardation, necrotizing enterocolitis (NEC)[11], and infections. Feeding mother's own milk (MM) improves feeding tolerance, protects against NEC, and reduces late-onset sepsis (LOS) compared with infant

Manuscript received December 8, 2016. Initial review completed January 17, 2017. Revision accepted February 17, 2017.
doi: 10.3945/jn.116.244822.

formula (2). After preterm birth, MM is sometimes insufficient during the first weeks, and donor human milk (DM) is the recommended alternative (3). DM is suggested to provide better protection against feeding intolerance and NEC than infant formula (3), but DM remains inferior to MM with regard to growth, feeding tolerance, and resistance against late-onset sepsis and NEC (4–7). The reduced benefits of DM, relative to MM, may be partly due to the heat treatment used to pasteurize DM before being fed to preterm infants.

Holder pasteurization (HP) is the standard method to remove potential transmissible microbiological contaminants in DM. The method involves heating of bottled milk in a water bath at 62.5°C for 30 min to kill viruses and bacteria. However, the heating process also inactivates immune cells and decreases the concentration and activity of milk enzymes and bioactive proteins that exert a range of trophic, antibacterial, or immunological effects. For example, bile salt–stimulated lipase (BSSL), alkaline phosphatase (ALP), Igs, lactoferrin, lysozyme, and lactoperoxidase are all heat-sensitive proteins (8). In addition, HP may decrease the antioxidant capacity of DM, which may be important to protect against intestinal inflammation and NEC (9). Consequently, UV-C irradiation (UVC) technology has been investigated as a novel pasteurization method to better preserve human milk bioactivity (10, 11). As a nonthermal pasteurization method, UVC destroys microorganisms in milk by damaging DNA rather than by protein denaturation and aggregation. The technique retains the bacteriostatic properties of untreated DM and appears to be as effective in removing vegetative bacteria without inducing a marked loss of bioactive factors, such as BSSL, ALP, lysozyme, lactoferrin, and secretory immunoglobulin A (sIgA) (10).

In the present study, we hypothesized that UVC-treated DM would be comparable to unpasteurized donor milk (UP) and superior to HP-treated DM in promoting growth, intestinal health, and resistance against bacteria in preterm pigs. Selected bioactive components and markers of antioxidant capacity were measured, and the in vitro proliferative effect of UP, UVC-treated milk, and HP-treated milk on intestinal epithelial cells was assessed. Finally, we fed the 3 DM diets to preterm pigs, used as a preclinical model for preterm infants with a very immature gut and immune system (12). Mucosal morphology, digestive function, intestinal integrity, NEC resistance, gut microbiota, and the presence of bacteria in blood and bone marrow were recorded.

Methods

DM preparation and in vitro cytotoxicity and proliferation assays. Sixty liters of frozen DM were purchased from the Danish Milk Bank. Milk from 15 donors, who gave consent for the use for research purposes, was pooled and divided into 3 identical portions. One portion was immediately stored at −20°C, and the other 2 were pasteurized by HP (62.5°C for 30 min) or by UVC at a dose of 4863 J/L as described previously (11). All 3 DM preparations were tested for microbe density (DANAK, Danish Veterinary and Food Administration), and macronutrient concentrations were determined (Human Milk Analyzer). The milk samples were stored at −20°C until feeding to preterm pigs and at −80°C for later biochemical analyses. Skimmed DM (centrifuged at 10,000 × *g* for 15 min at 4°C) samples were prepared for the analysis of selected bioactive components and for in vitro cytotoxicity and proliferation assays with the use of porcine intestinal epithelial cells and the methodology described in **Supplemental Methods**.

Bioactive proteins, enzymes, and antioxidant capacity of DM. Skimmed DM samples corresponding to 15 μg protein (quantified by BCA Protein Assay Kit; Thermo Scientific; n = 3/group) were analyzed by SDS-PAGE for quantification of major proteins. TGF-β2 was analyzed by Western blot by using TGF-β2 antibody (Santa Cruz Biotechnology). The absolute concentrations of human lactoferrin were further quantified by ELISA (Abcam). Activities of total milk lipase (including BSSL) and ALP were quantified by corresponding commercial assays (QuantiChrom Lipase Assay Kit; BioAssay Systems, and ALP assay kit; Abcam). Antioxidant capacity was measured according to the methods described in Supplemental Methods.

Pigs and experimental procedures. All animal procedures were approved by the Danish National Committee on Animal Experimentation. Fifty-seven preterm pigs were delivered from 4 sows by cesarean delivery at 106 d of gestation (Large White Danish × Landrace × Duroc; Askelygaard Farm; full term = 116 ± 2 d). Surgical procedures with an orogastric feeding tube and an umbilical catheter for parental nutrition and sow's plasma (for passive immunization) were performed as described previously (12). The pigs were stratified according to birth weight and sex into 3 groups receiving UP, HP-treated DM, and UVC-treated DM (n = 19/group; ratio of males to females, 30:27). The pigs were color-coded to mask the treatments to pig care-takers and researchers. To mimic the feeding strategy in preterm infants, pigs received gradually increasing volumes of DM from 24 mL · kg^{-1} · d^{-1} at birth to 144 mL · kg^{-1} · d^{-1} on day 6 (increasing by 24 mL · kg^{-1} · d^{-1}), and volumes were kept at this amount until the pigs were killed on days 8–9. To compensate for the small initial enteral volume, pigs also received parenteral nutrition at a rate of 96 mL · kg^{-1} · d^{-1} from birth, gradually reduced to 48 mL · kg^{-1} · d^{-1} on day 5 (decreasing by 12 mL · kg^{-1} · d^{-1}), and continued at this amount until tissue collection. A commercially available parenteral nutrition product (Kabiven; Fresenius Kabi) was used after adjustments as described previously (12). Notably, the DM was fed without nutrient fortification and thereby an enteral supply of milk nutrients suboptimal for maximal growth of both preterm pigs and infants (e.g., ~13 g protein/L DM).

Clinical evaluation and sample collection. Pigs were continually monitored (at least every 3 h) and were euthanized if clinical complications were evident (e.g., severe pain) or killed at the end of the study on days 8–9. Fecal characteristics and degree of dehydration were recorded twice daily according to predefined scoring systems (13). Body weights were measured daily, and percentage of weight gain was calculated according to the formula: (weight on day$_n$ – birth weight)/(birth weight) × 100. Food transit time was measured on day 5 by replacing one scheduled feeding with a chromium-oxide–added bolus feed (6 g/L). The time between the test bolus and the first appearance of green feces was recorded as the transit time. After the pigs were killed, organ weights were measured, and the entire small intestine was evenly divided into 3 regions: proximal small intestine (Prox), middle small intestine (Mid), and distal small intestine (Dist). Macroscopic lesion scores (1–6) were given to the stomach, Prox, Mid, Dist, and colon, depending on the extent and severity of pathological changes such as hyperemia, edema, hemorrhage, pneumatosis, and necrosis (12). Pigs with a score of ≥3 in any of the 4 intestinal regions were identified as having NEC. Intestinal whole-wall and mucosal tissue samples were collected from each region and immediately snap-frozen in liquid nitrogen and stored at −80°C or fixed in paraformaldehyde solution for further analyses. The intact left thigh of each pig was carefully dissected out for later bacterial analysis of the bone marrow.

[1] Supported by the Danish Dairy Research Foundation and Medela AG. Fresenius Kabi donated the parenteral nutrition used in this experiment.

[2] Author disclosures: Y Li, DN Nguyen, M de Waard, L Christensen, P Zhou, P Jiang, J Sun, AM Bojesen, C Lauridsen, J Lykkesfeldt, TK Dalsgaard, and SB Bering, no conflicts of interest. PT Sangild has given a scientific presentation at a meeting organized by Medela AG in Berlin, Germany.

[3] Supplemental Methods, Supplemental Tables 1–6, and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

*To whom correspondence should be addressed. E-mail: pts@sund.ku.dk.

[11] Abbreviations used: ALP, alkaline phosphatase; ApA, aminopeptidase A; ApN, aminopeptidase N; BSSL, bile salt–stimulated lipase; CRP, C-reactive protein; Dist, distal small intestine; DM, donor human milk; FISH, fluorescence in situ hybridization; GSH, reduced glutathione; GSSG, glutathione disulfide; HP, Holder pasteurization; LOS, late-onset sepsis; Mid, middle small intestine; MM, mother's own milk; NEC, necrotizing enterocolitis; Prox, proximal small intestine; sIgA, secretory immunoglobulin A; UP, unpasteurized donor milk; UVC, UV-C irradiation.

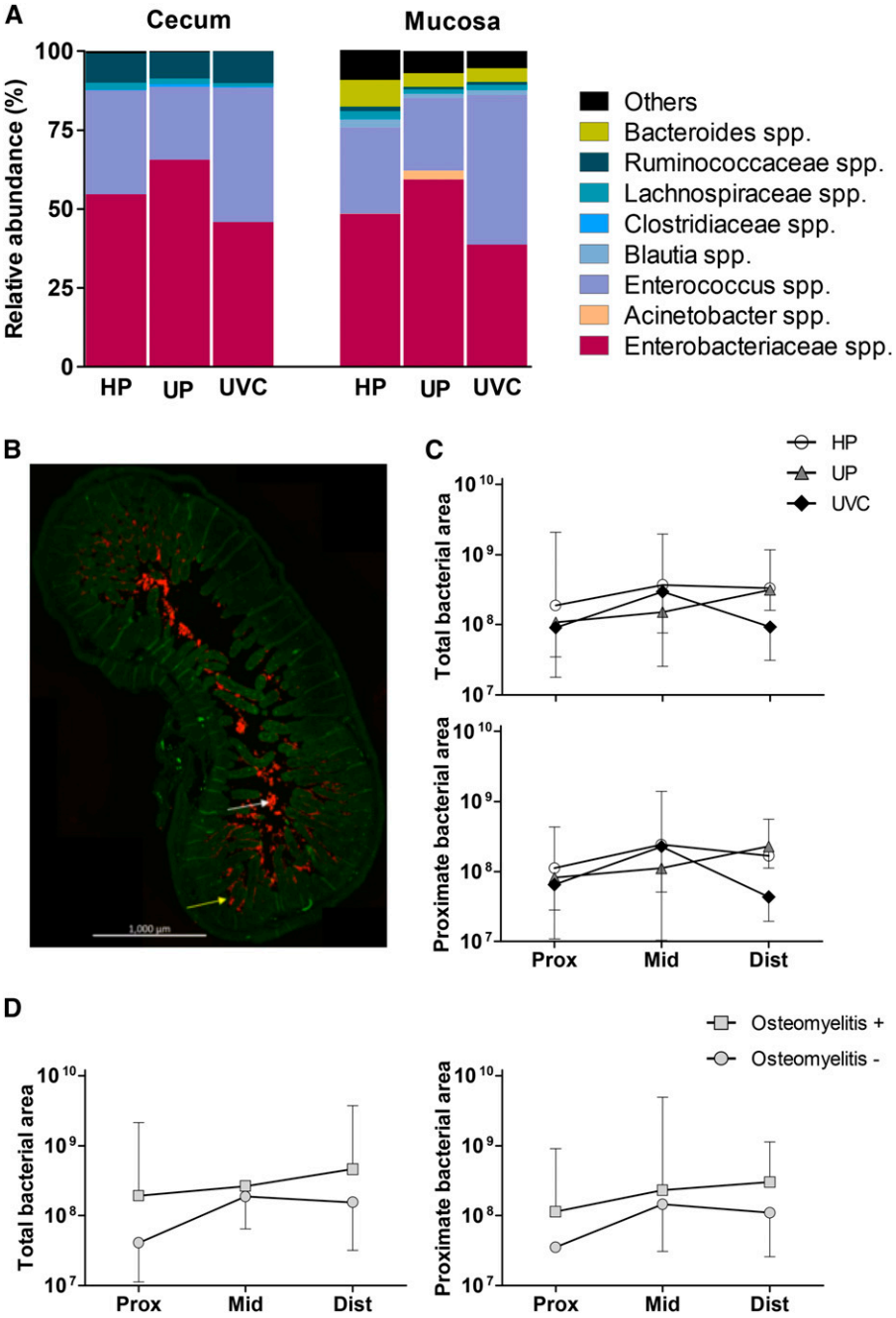


FIGURE 1 Relative abundance distribution of the major genera in the cecum content and distal mucosal tissue samples from neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d (A; n = 15–19). A representative picture of the fluorescence in situ hybridization analysis of intestinal tissues with a general bacterial probe in red fluorescence (B). The red area pointed to by a white arrow is an example of luminal bacteria. The red area pointed to by a yellow arrow is an example of proximate bacteria (bacteria invading the mucosa). The area of proximate bacteria (pixel × pixel) was measured by quantifying the red area invaded by bacteria in the intestinal mucosa. The area of total bacteria (pixel × pixel) was measured by quantifying the red area in both the proximate and luminal area. The area of total bacteria and proximate bacteria between groups (n = 15–16/region and group) and between pigs with osteomyelitis (+; n = 20–21 per region) and without osteomyelitis (−; n = 22/region) in Prox, Mid, and Dist regions (C and D: values are means ± SEMs, n = 20–22). Dist, distal small intestine; HP, Holder pasteurization; Mid, middle small intestine; Prox, proximal small intestine; spp., species; UP, unpasteurized donor milk; UVC, UV-C irradiation.

Blood biochemistry, cell counts, systemic bacteria, and circulating infection markers. Blood biochemistry was analyzed when the pigs were killed (ADVIA 1800 Chemistry System; Siemens). Fresh cardiac blood was collected at the time of death for blood cell counting by an automatic cell counter (ADVIA 2120i Hematology System; Siemens). Systemic bacteria were cultured from aseptic cardiac blood, and homogenized bone marrow samples from left femurs of blood agar and colonies were enumerated, isolated, and identified by using matrix-assisted laser desorption ionization/time-of-flight MS as previously described (14). The total bacterial load and the load of dominant genera are presented as CFU/mL blood and CFU/g bone marrow. Pigs with positive bacterial culture results in the bone marrow were defined as having osteomyelitis. C-reactive protein (CRP) and IL-6 in plasma at the time of death were measured by commercial porcine DuoSet ELISA kits according to the manufacturer's protocols (R&D Systems).

Mucosal structure, brush border enzyme activities, and in vivo intestinal functions. Villus height and crypt depth were measured on hematoxylin and eosin–stained paraformaldehyde-fixed histology slices as previously described (15). Plasma citrulline on day 5 and at the time of death was measured by ultraperformance LC–tandem quadrupole detector MS (Waters) as previously described with minor modifications (16). Activities of 6 brush border enzymes, lactase, maltase, sucrase, aminopeptidase N (ApN), aminopeptidase A (ApA), and dipeptidyl peptidase IV, were analyzed in tissue homogenates (17). Intestinal absorptive function was assessed in vivo by measuring the concentration of plasma galactose after an oral bolus of 10% galactose (15 mL/kg) on day 5 and on the day of death. Intestinal permeability was tested in vivo by giving an oral bolus (15 mL/kg) of 5% lactulose and 5% mannitol to measure the lactulose-to-mannitol ratio in urine collected at the time of death 180 min later. Detailed methods of these in vivo tests have been described previously (18).

Proteins related to inflammation, proliferation, and tight junctions in the Dist. Dist tissues were homogenized in radioimmunoprecipitation assay buffer at 0°C, containing a protease inhibitor cocktail (Sigma-Aldrich) for analysis of the protein levels of IL-1β, IL-6, and IL-8 by

TABLE 1 Macronutrients and bacteria in donor milk[1]

	HP	UP	UVC	*P*
Protein, g/L	13 ± 0.0	13 ± 0.3	13 ± 0.6	0.512
Lipid, g/L	41 ± 0.3[a]	36 ± 2.0[b]	37 ± 0.6[b]	<0.01
Lactose, g/L	95 ± 1.4	96 ± 1.0	97 ± 0.3	0.326
Energy, kcal/L	768 ± 2[a]	728 ± 22[b]	740 ± 3[ab]	<0.05
Bacteria, CFU/mL				
Enterobacteriaceae	<10	1.7×10^5	167	—
Aerobic microorganisms	8600	4.3×10^5	494	—
Thermotolerant coliform bacteria	<10	1400	<10	—
Hemolytic germs	<10	500	<10	—
Presumptive *Bacillus cereus*	<10	<10	<10	—
Coagulase positive staphylococci	<10	6500	<10	—

[1] Data are means ± SEMs unless noted otherwise, n = 3/milk diet analyzed for macronutrients. Labeled means in a row without a common superscript letter differ, $P < 0.05$. HP, Holder pasteurization; UP, unpasteurized donor milk; UVC, UV-C irradiation.

using porcine DuoSet ELISA kits. The relative concentrations of proliferating cell nuclear antigen and claudin-4 were determined by Western blot in the last 3 litters (n = 50 in total), and β-actin was determined for every gel as the loading control (19). The visualized protein bands were digitalized by ImageQuant LAS 4000 digital imaging system (GE Healthcare Life Sciences), and the intensity change was analyzed by ImageJ (NIH). The gene expression of *Tnf* (TNF), *Il1β* (IL-1β), *Il8* (IL-8), *Cldn1* (claudin1), *Ocln* (occludin), and a house-keeping gene was measured by qPCR, and detailed methods are described in Supplemental Methods. All primers used were listed in **Supplemental Table 1**.

Gut microbiota and colonic SCFAs. Total DNA of the cecum content and the Dist mucosal tissue was extracted, and the total bacterial load from the cecum content was quantified by a 7500 Fast Real-time PCR system (Applied Biosystems) by using *Escherichia coli* K12 strain as the reference (20). Microbiota composition in the cecum content and mucosal tissue was determined by using tag-encoded 16S rRNA gene MiSeq-based (Illumina) high-throughput sequencing (21, 22). Quantitative Insight Into Microbial Ecology (version 1.7.0) and UPARSE were used to analyze the sequencing data (23). Total bacterial abundance and bacteria adhering to the intestinal mucosa were analyzed by fluorescence in situ hybridization (FISH) by using paraffin-embedded cross sections from Prox, Mid, and Dist as previously described (14). FISH slides were digitalized by the Axio Scan Z1 Slide Scanner (ZEISS Microscopy). The area of red fluorescence signal (bacterial area) was quantified by Visiopharm software to detect both total bacterial density and the specific bacteria that were in close contact with the mucosa (proximate bacteria, **Figure 1**B). The SCFA concentrations were measured in the colon contents by GC to reflect bacterial fermentation and metabolic activity of the gut microbiota (24).

Statistical analysis. Bioactive components and markers of antioxidant capacity were compared between the 3 DM diets by ANOVA and Tukey's post hoc test (GraphPad Prism 5.0). Circulating CRP and IL-6 concentrations were compared between pigs with and without osteomyelitis or bacteremia by the Mann-Whitney U test in GraphPad. All other data were analyzed by using the software package R (version 3.2.2). Repeated measurements (i.e., weight-gain percentage over time) were analyzed by using the linear mixed-effects model (lmer function) followed by group comparisons by using the lsmeans package. Incidences of NEC, bacteremia, and osteomyelitis were evaluated by multiple logistic regression models (glm function), and other continuous outcome measures were analyzed by using linear models (lm function), followed by Tukey's post hoc test (multcomp function). The above-mentioned models were adjusted for potential confounders, such as birth weight, sex, litter, and life time. The normality and homoscedasticity of the residuals and fitted values were performed for model validation, and data were transformed when required. Data are presented as raw arithmetic means ± SEMs unless otherwise stated. A P value <0.05 was considered statistically significant; a P value ≤0.10 was mentioned as a tendency to an effect. The slight variability in the number of samples for individual parameters was due to the euthanasia of a few clinically

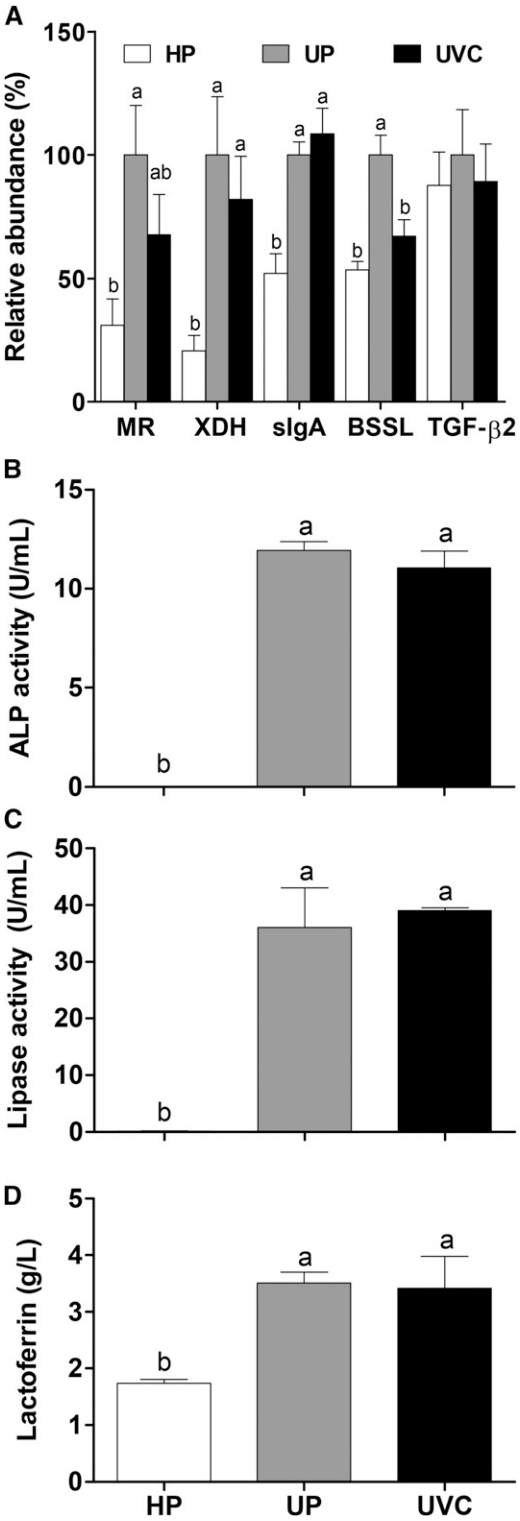


FIGURE 2 Bioactive components in donor human milk as relative abundance to the concentrations in UP (A). Activities of ALP (B) and milk lipase (C), and concentrations of lactoferrin (D) in the 3 differently treated milk samples. Values are means ± SEMs, n = 3. Labeled means without a common letter differ, $P < 0.05$. ALP, alkaline phosphatase; BSSL, bile salt–stimulated lipase; HP, Holder pasteurization; MR, mannose receptor; sIgA, secretory immunoglobulin A; UP, unpasteurized donor milk; UVC, UV-C irradiation; XDH, xanthine dehydrogenase.

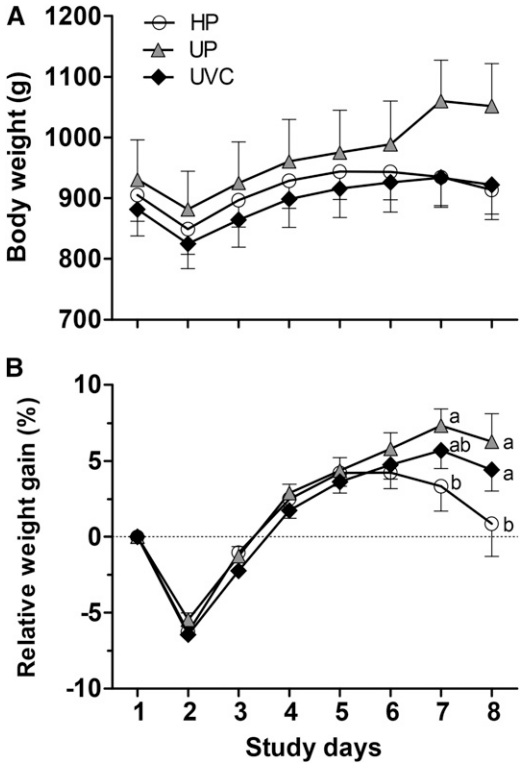


FIGURE 3 Body weight (A) and relative weight gain (B) of neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d. Relative weight gain was calculated according to the formula: relative weight gain (%) = (weight day$_n$ − birth weight)/birth weight × 100. Values are means ± SEMs, n = 18 or 19. Labeled means at a time without a common letter differ, $P < 0.05$. HP, Holder pasteurization; UP, unpasteurized donor milk; UVC, UV-C irradiation.

affected pigs before the planned end of the study combined with the unavailability of some biological samples for a few pigs (e.g., inability to collect urine or gut contents).

Results

Macronutrients, bacteria, bioactive components, and antioxidant markers in DM. The concentrations of protein and lactose were comparable between the 3 types of DM (**Table 1**). However, the levels of lipid and energy were higher in HP-treated milk relative to UP and/or UVC-treated milk (Table 1). Bacterial cultures revealed that UP contained a total concentration of 6.7×10^5 CFU/mL bacteria, and the main groups were Enterobacteriaceae and aerobic microorganisms (Table 1). Both the HP and UVC treatments eliminated most bacteria except a small number of aerobic microorganisms and Enterobacteriaceae still present after treatment (Table 1).

Analyzed by SDS-PAGE, sIgA and xanthine dehydrogenase concentrations were decreased by HP treatment compared with UP and UVC-treated milk. The mannose receptor concentration was lower in HP-treated milk than in UP ($P < 0.05$; **Figure 2**A). The concentration of milk BSSL was decreased in both UVC-treated and HP-treated milk compared with UP ($P < 0.05$). Western blot showed no difference in TGF-β2 abundance between the groups. The activity of ALP and total lipase was completely abolished in HP-treated milk but preserved in UVC-treated milk at the same concentration as UP (Figure 2B, C). Lactoferrin concentration was reduced to 50% in the HP-treated milk compared with UP and UVC-treated milk ($P < 0.01$; Figure 2D). The in vitro cell study showed that DM exerted a limited cytotoxicity effect on epithelial cells at a concentration of 0.01 g/L (data not shown), and cell proliferation was increased by UP compared with control medium ($P < 0.05$; **Supplemental Figure 1**).

The summed reduced glutathione (GSH) and glutathione disulfide (GSSG; GSH+GSSG) and vitamin C concentrations were reduced in the HP-treated milk compared with UP and UVC-treated milk (all $P < 0.05$; **Supplemental Table 2**). Oxidation of GSH (GSSG/GSH+GSSG) was more pronounced in UP and HP-treated milk compared with UVC-treated milk (both $P < 0.05$), whereas the oxidation of vitamin C (measured by dehydroascorbic acid) was more pronounced in both UVC-treated milk and UP samples than in

TABLE 2 Clinical outcomes, brush border enzyme activities, and cecum bacterial load in neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d[1]

	HP	UP	UVC	*P*
Energy intake, kcal · kg^{-1} · d^{-1}	118 ± 0.9^a	114 ± 1.0^c	116 ± 0.8^b	<0.0001
Dehydration, *n* (%)	5 (26)^a	0 (0)^b	2 (11)^{ab}	<0.01
Diarrhea on day 5, *n* (%)	13 (68)^a	5 (26)^b	8 (42)^{ab}	<0.05
Overall diarrhea, *n* (%)	15 (79)	15 (79)	16 (84)	0.928
Food passage time on day 5, h	6 (3–16)	24 (11–56)	10 (4–29)	0.11
NEC, *n* (%)	7 (37)	11 (58)	9 (47)	0.409
SI NEC, *n* (%)	0^b	6 (32)^a	0^b	<0.0001
Average lesion score	1.5 ± 0.1^b	2.1 ± 0.2^a	1.5 ± 0.1^b	<0.01
Plasma galactose day 5, mmol/L	255 (200–703)	336 (235–1090)	265 (158–889)	0.567
Plasma galactose day 7, mmol/L	326 (163–1363)	254 (177–534)	293 (168–758)	0.893
Ratio of lac:man in urine	0.07 ± 0.02	0.05 ± 0.01	0.07 ± 0.02	0.713
Intestinal enzyme activity, U/g				
Maltase	4.78 ± 0.93	3.52 ± 0.34	3.87 ± 0.47	0.343
Sucrase	0.73 ± 0.14	0.62 ± 0.13	0.52 ± 0.09	0.220
Lactase	20.3 ± 6.1	22.3 ± 5.2	13.6 ± 2.9	0.628
DPPIV	1.56 ± 0.22	1.58 ± 0.14	1.27 ± 0.09	0.180
Cecum bacteria, ×10^8 copies/g	9.2 (3.1–18.6)	13.7 (4.8–31.9)	8.4 (4.2–16.2)	0.464

[1] Values are means ± SEMs or medians (IQRs) unless otherwise noted; n = 13–19. Labeled means in a row without a common superscript letter differ, $P < 0.05$. DPPIV, dipeptidyl peptidase IV; HP, Holder pasteurization; lac:man, lactulose:mannitol; NEC, necrotizing enterocolitis; SI, small intestine; UP, unpasteurized donor milk; UVC, UV-C irradiation.

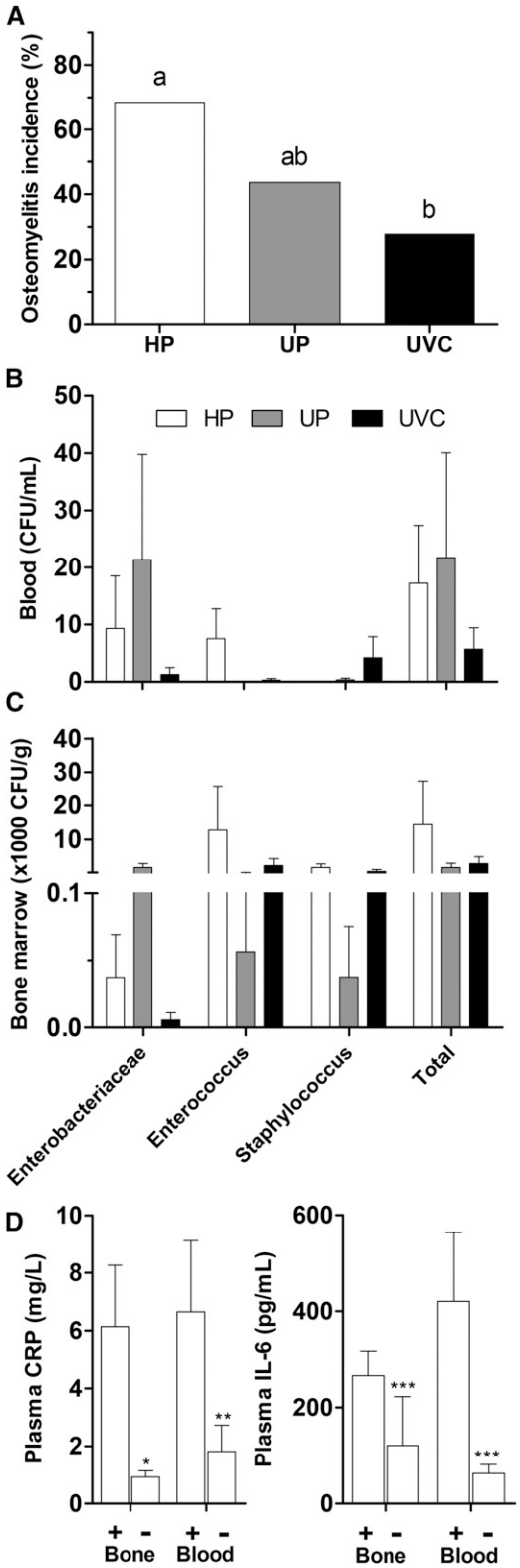


FIGURE 4 Incidence of osteomyelitis in neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d (A), total bacterial load and the predominant family and genera in blood (B) and bone marrow (C), and plasma C-reactive protein and IL-6 concentrations at study end in those with (+) and without (−) osteomyelitis or bacteremia (D). Values are percentages (A) or means ± SEMs, n = 16–19 (B), n = 16–19 (C), n = 17–34 (D). Labeled means without a common letter differ, $P < 0.05$. *,**,***,****Different from + in either bone marrow or blood, respectively: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. CRP, C-reactive protein; HP, Holder pasteurization; UP, unpasteurized donor milk; UVC, UV-C irradiation.

HP-treated milk (both $P < 0.05$). Lipid oxidation measured by malondialdehyde was higher in UVC-treated milk than that in UP ($P < 0.05$) with intermediate values for HP-treated milk. Another lipid oxidation marker, hexanal, was markedly higher in HP-treated milk than in UP or UVC-treated milk (both $P < 0.05$).

Clinical outcomes. There were no significant differences between the HP, UP, and UVC groups in birth weights, weights at death (**Figure 3**A), life time, overall growth rates, and organ weights or lengths (**Supplemental Table 3**). By the end of the study, pigs fed UVC-treated milk and pigs fed UP had gained more weight than did those fed HP-treated milk ($P < 0.05$; Figure 3B). Two pigs fed HP-treated milk and 3 pigs fed UP were euthanized 1–2 d before they were scheduled to be killed because of dehydration or clinical signs of NEC or sepsis. Pigs fed HP-treated milk had a higher incidence of diarrhea on day 5 (**Table 2**), but by the end of the experiment the mean diarrhea scores were similar. The intestinal transit time on day 5 was faster in pigs fed HP-treated milk than in pigs fed UP ($P < 0.05$) and was intermediate in pigs fed UVC-treated milk, supporting the clinical evaluation of diarrhea. Dehydration was observed more frequently in pigs fed HP-treated milk than in pigs fed UP ($P < 0.05$) with intermediate values for pigs fed UVC-treated milk throughout the study period. Bloody diarrhea occurred in 11 of 57 pigs with no differences between groups and no differences in overall NEC incidence. NEC lesions in the small intestine were observed only in pigs fed UP, and the mean intestinal NEC score was higher in pigs fed UP than that in pigs fed HP- or UVC-treated milk (both $P < 0.05$, Table 2).

Blood biochemistry, cell counts, systemic bacteria, and circulating infection markers. Blood biochemistry variables were generally similar between pigs fed UP and pigs fed UVC-treated milk, and some variables showed higher values in pigs fed HP-treated milk than in those fed UP or UVC-treated milk (**Supplemental Table 4**). At the time of death, total blood leukocyte and neutrophil counts were higher in pigs fed UP than in those fed UVC-treated milk, whereas lymphocyte counts were higher in pigs fed HP-treated milk than in pigs fed UVC-treated milk (both $P < 0.05$; **Supplemental Table 5**). Other hematological parameters did not differ between groups. The incidence of bacteremia at the time of death was similar between groups (33% across groups), whereas the incidence of osteomyelitis was higher in pigs fed HP-treated milk than in those fed UVC-treated milk and intermediate in pigs fed UP ($P < 0.05$; **Figure 4**A).

Enterobacteriaceae, *Enterococcus*, and *Staphylococcus* were the main bacterial family and genera found in both blood and bone marrow (Figure 4B, C). In the bone marrow of pigs fed UP, the gram-negative Enterobacteriaceae appeared dominant, whereas in pigs fed HP-treated milk and pigs fed UVC-treated milk the gram-positive *Enterococcus* and *Staphylococcus* were more dominant, although the differences were not statistically significant (Figure 4C). Both plasma CRP and IL-6 concentrations were increased in pigs with bacteremia or osteomyelitis (all $P < 0.05$; Figure 4D).

Mucosal structure, brush border enzyme activities, and in vivo intestinal function. In the Prox intestine, villi were shorter in pigs fed HP-treated milk than in pigs fed UP and those fed UVC-treated milk ($P < 0.05$; **Figure 5**A), whereas in the Mid and Dist regions, pigs fed HP-treated milk showed decreased villi length compared with those fed UVC-treated milk ($P < 0.05$) with intermediate values in pigs fed UP. On day 5, plasma

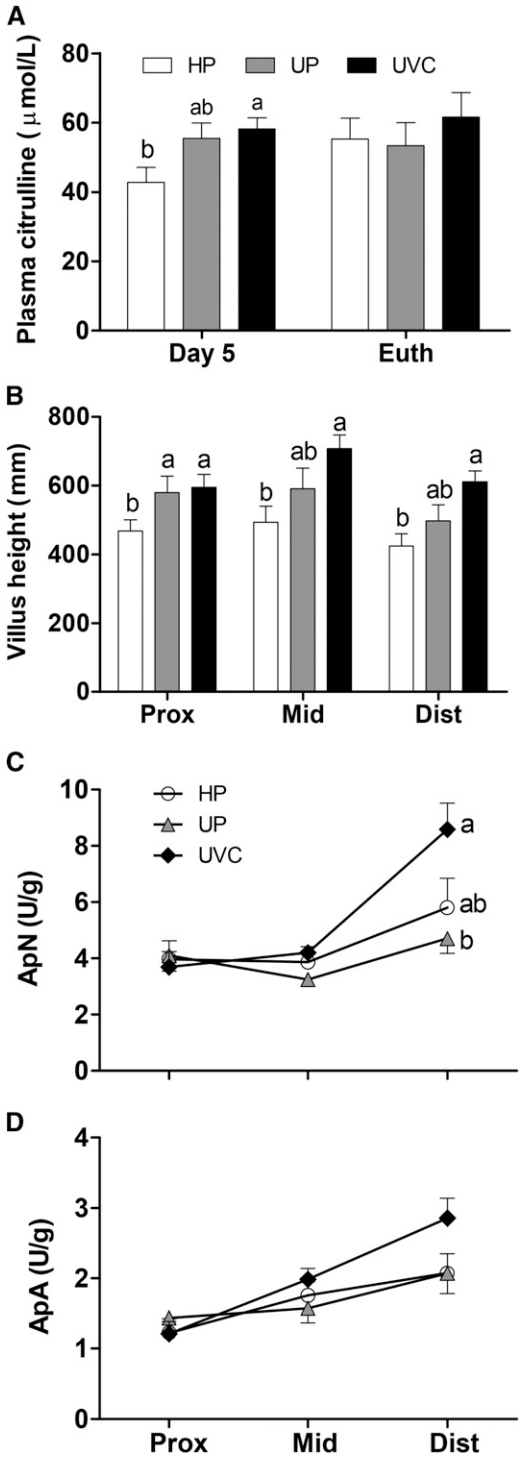


FIGURE 5 Concentrations of plasma citrulline on day 5 and at study end (A), villus height (B), and activities of ApN (C) and ApA (D) in Prox, Mid, and Dist of neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d. Values are means ± SEMs, n = 17–19. Labeled means at a time or for a region without a common letter differ, $P < 0.05$. ApA, aminopeptidase A; ApN, aminopeptidase N; Dist, distal small intestine; Euth, euthanization; HP, Holder pasteurization; Mid, middle small intestine; Prox, proximal small intestine; UP, unpasteurized donor milk; UVC, UV-C irradiation.

citrulline concentrations in pigs fed HP-treated milk were lower than in those fed UVC-treated milk ($P < 0.05$) and tended to be lower than in pigs fed UP ($P = 0.09$), although concentrations were similar at the time of death (Figure 5B). The plasma citrulline concentrations on day 5 were positively correlated with villus heights, especially in the most proximal intestinal regions (Prox: $P < 0.001$, $R^2 = 0.33$; Mid: $P < 0.01$, $R^2 = 0.28$; Dist: $P = 0.09$, $R^2 = 0.14$). Diet type did not affect activities of the 3 measured disaccharidases and dipeptidyl peptidase IV in any of the intestinal regions (Table 2) or the peptidase activities in Prox and Mid regions (Figure 5C, D). In the Dist section, pigs fed UVC-treated milk had or tended to have higher peptidase activities than those fed UP (ApN: $P < 0.01$; ApA: $P = 0.07$) or HP-treated milk (ApN: $P = 0.07$; Figure 5C, D). Intestinal monosaccharide absorptive capacity and intestinal permeability as measured by in vivo galactose and lactulose/mannitol tests were similar between groups (Table 2).

Proteins related to inflammation, proliferation, and tight junctions in the Dist. Concentrations of IL-1β, IL-6, and IL-8 in the Dist region did not differ significantly between the groups (**Figure 6**A–C). The relative abundance of β-actin and proliferating cell nuclear antigen was comparable between the groups (data not shown), but pigs fed UVC-treated milk tended to have a higher concentration of claudin-4 than the figs UP ($P < 0.01$) and those fed HP-treated milk ($P = 0.08$; Figure 6D). Interestingly, when pigs with osteomyelitis were compared with those without, the protein concentrations of IL-6 and IL-8 were elevated, and the concentration of claudin-4 was decreased in the Dist (all $P < 0.05$; Figure 6B–D). None of these protein levels differed between pigs with and without bacteremia. Gene expression for *Il1β*, *Il8*, *Tnf*, *Ocln*, and *Cldn* did not differ among the groups, although gene expression of *Il8* was increased in pigs with osteomyelitis (**Supplemental Table 6**).

Gut microbiota and colonic SCFAs. Total cecum bacterial load measured by qPCR did not differ between the groups (Table 2). Compared with pigs fed UP and those fed HP-treated milk, the pigs fed UVC-treated milk had a higher relative abundance of *Enterococcus* in both cecum content and ileal mucosal tissues (all $P < 0.05$; Figure 1A). A tendency to a lower relative abundance of Enterobacteriaceae was observed in the cecum content of pigs fed UVC-treated milk compared with those fed UP ($P = 0.07$) with intermediate levels in pigs fed HP-treated milk (Figure 1A). The total and proximate bacterial areas measured by FISH were not different between the 3 groups across the 3 intestinal regions (Figure 1C). Interestingly, the total and proximate bacterial areas in Prox and Dist tended to be higher in pigs with osteomyelitis than in uninfected pigs ($P = 0.07$ and 0.09, respectively; Figure 1D). This difference was not detected between pigs with and without bacteremia (data not shown). Formate, acetate, lactate, and succinate were the main SCFAs present in colon contents. Concentrations of lactate in wet feces differed between the groups with higher levels in pigs fed UVC-treated milk (52 ± 4 mmol/g) than in those fed UP (35 ± 5 mmol/g, $P < 0.05$) and intermediate values in pigs fed HP-treated milk (41 ± 5 mmol/g). The remaining FAs were similar between the groups for formate (2.0 ± 0.8 mmol/g across groups), acetate (6.2 ± 1.3 mmol/g), and succinate (2.2 ± 0.9 mmol/g).

Discussion

Our results suggest that UVC treatment has potential as a superior technology to pasteurized DM for preterm newborns. The UVC method reduced the bacterial load in DM as effectively as the standard HP method but preserved more milk enzymes and bioactive proteins, including lipase, ALP, sIgA, and lactoferrin,

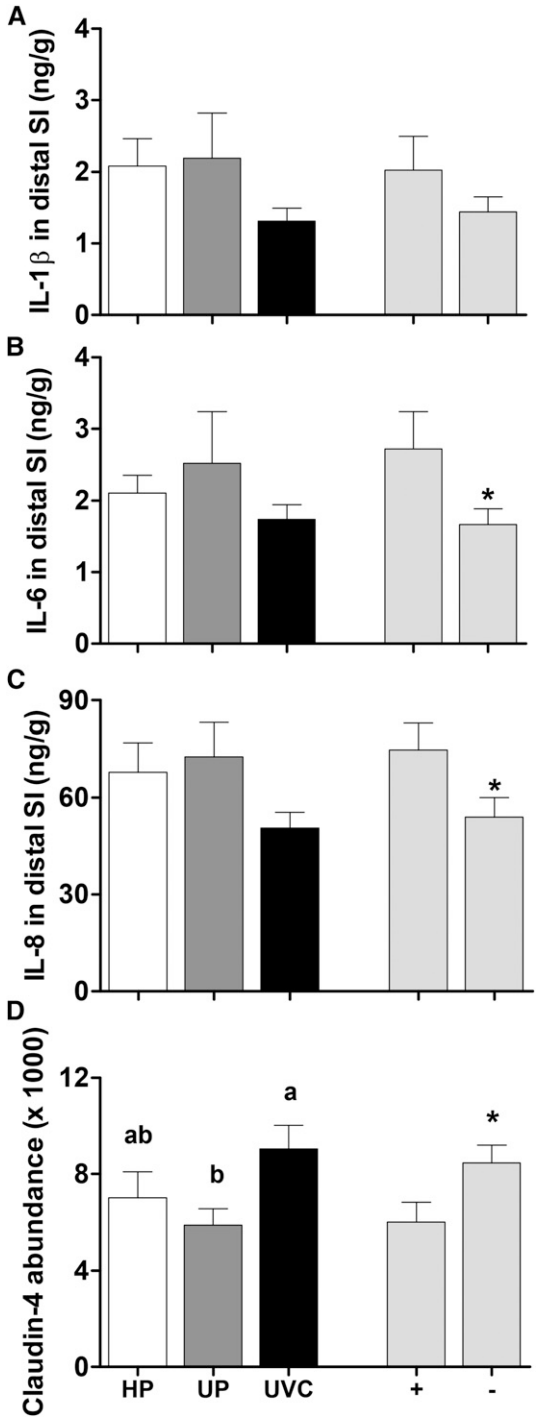


FIGURE 6 Distal SI concentrations of IL-1β (A), IL-6 (B), IL-8 (C), and claudin-4 (D) in neonatal pigs fed UP, HP-treated human milk, or UVC-treated human milk for 8–9 d and between pigs with (+) and without (−) positive bacteria in bone marrow. Values are means ± SEMs, $n = 15-28$. Labeled means without a common letter differ, $P < 0.05$. *Different from +, $P < 0.05$. HP, Holder pasteurization; SI, small intestine; UP, unpasteurized donor milk; UVC, UV-C irradiation.

supporting earlier findings (10, 11). Antioxidant capacity was also preserved by UVC to some extent. In preterm pigs, UVC-treated milk improved body growth, intestinal mucosa structure, and resistance to systemic bacteria compared with HP-treated milk.

For the antioxidant capacity of DM, a higher proportion of GSSG out of the total GSH+GSSG contents, together with lowered total vitamin C, indicates an impaired antioxidant capacity of DM after HP treatment. However, higher ratios of dehydroascorbic acid to total vitamin C in UVC-treated milk and UP indicate more exposure to oxidative stress, probably because of UVC and the high bacterial load in UP. The retained activity of vitamin C may have quenched the increased exposure to oxidative stress in UVC-treated milk and UP. Further, the markedly higher hexanal concentration in HP-treated milk indicates more lipid oxidation after this treatment. Finally, the higher malondialdehyde concentration in UVC-treated milk is consistent with studies showing that malondialdehyde is more sensitive to UV radiation than longer alkanals (25).

Human milk lipase, especially BSSL, may play an important role for fat absorption in preterm infants who may suffer from reduced lipase secretion from the immature stomach and pancreas. Reduced fat absorption, weight gain, and linear growth have also been documented for preterm infants fed pasteurized MM compared with infants fed fresh milk (4), and growth was restored by supplementing the milk with BSSL (26). Thus, the slightly improved body growth in pigs fed UVC-treated milk and those fed UP toward the end of the present study, despite lower lipid content in HP-treated milk, may be explained by better preservation of milk lipase activity. Still, the unfortified DM used in this study was not designed to fulfill the nutritional requirements of preterm pigs, and growth rates are not directly comparable with those in DM-fed infants during the first weeks after birth. Impaired gut function and increased tissue protein catabolism in the HP group, as indicated by increased blood creatinine, creatine phosphokinase, and amino acid transferases, may also play a role. Related to this, decreased villus height in the proximal intestine of pigs fed HP-treated milk may be explained by a reduction in heat-sensitive trophic factors in HP-treated DM, such as lactoferrin and insulin-like growth factors (8). In a previous preterm-pig study (27), shorter villi were observed after feeding a thermally processed bovine-milk product with reduced concentrations of trophic factors (lactoferrin, TGF-β, insulin-like growth factor-1), but NEC sensitivity was not affected. Likewise, the overall NEC incidence was not affected by pasteurization method in this study, and incidence was similar to that in a previous study of HP-treated DM in which NEC resistance and growth rates were reduced relative to preterm pigs fed a more nutrient-dense, bioactive diet of bovine colostrum (28).

The pigs fed UP had similar weight gain as those fed UVC-treated milk but showed more signs of systemic infection and intestinal inflammation. These signs were supported by higher white blood cell counts, neutrophil counts, and intestinal NEC incidence and decreased villus heights, claudin-4 levels, and intestinal aminopeptidase activity. These results probably relate to the relatively high bacterial load in UP, including Enterobacteriaceae. Contaminated milk may be a direct infection risk for preterm infants, predisposing to NEC and/or sepsis (29, 30), which highlights the need for optimized pasteurization of DM. Further studies are required to document differences in the biological effects of pasteurization when unpasteurized DM contains limited or no bacteria.

The pigs fed UVC-treated milk had lower incidence of osteomyelitis than those fed HP-treated milk despite the fact that all pigs were handled similarly and all had vascular catheters inserted. Pigs with positive bacterial culture results in the bone marrow and blood had elevated plasma CRP and IL-6 concentrations. This supports the theory that bacteria found in these samples indeed originated from the pigs and not from contamination during the handling of samples. In contrast to blood cultures, the bone marrow cultures reflect the infection status during a longer time period because of the accumulation of bacteria at the metaphyseal ends of the long bones. This indicates that UVC-treated milk may

decrease the continuous risk of bacterial translocation from the gut. The increased mucosa-associated bacteria, elevated proinflammatory cytokines, and decreased claudin-4 in pigs with osteomyelitis indicate impaired barrier function (31, 32) and suggest that the bacteria found in the bone marrow may originate mainly from the intestine. In our previous studies, the incidence of osteomyelitis was 94% for preterm pigs fed a commercial infant formula for 5 d (14) and 60% for preterm pigs fed pasteurized DM for 9 d (J Sun, Y Li, PT Sangild, unpublished results, 2017). Interestingly, treatment with oral antibiotics reduced the incidence of osteomyelitis from 94% to 19% in formula-fed preterm pigs, whereas systemic antibiotics had negligible effects (14). These results indicate that efforts to decrease bacterial translocation from the immature intestine may be very important in preventing systemic infections and sepsis in preterm neonates.

The mechanism whereby UVC-treated milk reduces bacterial translocation may relate to its higher concentrations of antimicrobial proteins and peptides such as sIgA, lysozyme, and lactoferrin. The sIgA is a well-known protective component in MM, and enteral supplementation of human sIgA protects the binding of microorganisms to the intestinal epithelium, which inhibits translocation (33). In preterm newborns, the production of endogenous antibacterial lysozyme is developmentally impaired, indicating the importance of exogenous lysozyme from milk (34). In pigs infected with enterotoxigenic *E. coli*, supplementation with human lysozyme induced faster recovery from infection, improved gut function, and reduced inflammation (35). Oral administration of lactoferrin, a trophic, antimicrobial, and immune-modulatory milk protein, reduced LOS in preterm infants (36) and reduced intestinal infections and bacterial translocation in rats (37, 38). Lactoferrin has also been shown to improve the intestinal barrier in rats via upregulation of intestinal sIgA secretion and production of tight junction proteins, including claudin-4 (39).

In our study, the concentrations of mucosal proinflammatory cytokines and mucosal bacterial density did not differ between the groups. Still, the pigs fed UVC-treated milk were inclined to have decreased IL-1β and IL-8 (P = 0.05 and P = 0.12, respectively) and a reduced proximate bacterial area in the Dist (P = 0.13) compared with the pooled values from the pigs fed HP-treated milk and those fed UP, which were similar.

The differences in blood biochemistry values between the groups support the clinical findings. In preterm infants, sepsis is a risk factor for acute kidney injury and is correlated with increased serum creatinine (40). Higher concentrations of creatinine and minerals (e.g., magnesium) in the pigs fed HP-treated milk may therefore indicate potential kidney damage related to more exposure to systemic bacteria and toxins. In addition, blood creatinine is mainly produced from muscle degradation, and its higher concentration in pigs fed HP-treated milk may also reflect increased mobilization of muscle protein to compensate for decreased nutrient absorption (41). Higher concentrations of creatine phosphokinase and aspartate aminotransferase suggest more muscle proteolysis in the pigs fed HP-treated milk, partly explaining the lower weight gain after HP treatment of DM.

Bacterial sequencing revealed that Enterobacteriaceae and *Enterococcus* were the most dominant taxa found in both the cecum and distal mucosa across the 3 groups. This colonization pattern is similar to that in preterm infants with increased concentrations of facultative anaerobes (e.g., Enterobacteriaceae) and reduced concentrations of strict anaerobes (e.g., *Bacteroides*) (42). Enterobacteriaceae were more abundant in pigs fed UP, reflecting their higher density in the UP and thus shaping of the early gut colonization by the first milk diet. Interestingly, only the DM pasteurized by UVC and not HP was able to affect the early bacterial colonization. Potentially, the colonization pattern induced by UVC-treated DM better reflects the pattern after exclusive feeding with MM, which typically has more lactic acid–producing bacteria (e.g., *Enterococcus*) and less Enterobacteriaceae (43). It remains unclear from our study if the relatively high load of bacteria in our untreated DM (6×10^5 CFU/mL) was responsible for the apparent negative effects observed for some variables in pigs fed UP.

In this study, we confirmed that UVC is as effective as HP in eliminating bacteria in DM and better preserves heat-sensitive bioactive components, including some antioxidants. Using preterm pigs as a model for preterm infants, we have demonstrated that the treatment-related differences in the biochemistry of DMs have potential physiological effects in preterm neonates. UVC-treated DM improves growth, intestinal health, and systemic bacterial resistance compared with HP treatment at least in preterm pigs. Our findings suggest that UVC may be an improved method of pasteurizing MM and DM. Nevertheless, the safety of UVC treatment of DM for sensitive preterm infants requires further documentation in vitro and in vivo, for example, regarding the possible effects on stem cells and milk leukocytes. It is also important to note that preterm infants often receive a combination of fresh MM and pasteurized DM, and this may reduce the potential damaging effects of heat pasteurization in clinical neonatology. Regardless, it is now time to initiate a randomized controlled trial on different treatments of human milk for preterm infants. Such a study could use the combined NEC and LOS incidence as the primary outcome and markers of intestinal health and body growth as secondary outcomes.

Acknowledgments

We thank Elin Skytte, Kristina Møller, Jane Povlsen, Karina Ryom, Stanislava Pankratova, Lukasz Krych, Caroline Nebel, and Joan Frandsen for technical support with animal procedures and laboratory analyses; Karen Simmer for important inspiration to do this study; Anne Olin for collecting and analyzing the donor milk; and Lukas Christen from Carag AG for his assistance in the UVC treatment of donor milk. YL, DNN, SBB, and PTS designed the research; YL, DNN, MdW, LC, and PZ conducted the pig experiment; YL, DNN, LC, PJ, JS, AMB, CL, JL, and TKD conducted the lab analysis; YL and DNN analyzed the data; YL wrote the manuscript; and PTS had primary responsibility for final content. All authors contributed to and approved the final manuscript.

References

1. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller A, Narwal R, Adler A, Garcia CV, Rohde S, Say L, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 2012;379:2162–72.
2. Menon G, Williams TC. Human milk for preterm infants: why, what, when and how? Arch Dis Child Fetal Neonatal Ed 2013;98:F559–62.
3. ESPGHAN Committee on Nutrition; Arslanoglu S, Corpeleijn W, Moro G, Braegger C, Campoy C, Colomb V, Decsi T, Domellof M, Fewtrell M, et al. Donor human milk for preterm infants: current evidence and research directions. J Pediatr Gastroenterol Nutr 2013;57:535–42.
4. Andersson Y, Savman K, Blackberg L, Hernell O. Pasteurization of mother's own milk reduces fat absorption and growth in preterm infants. Acta Paediatr 2007;96:1445–9.
5. Montjaux-Régis N, Cristini C, Arnaud C, Glorieux I, Vanpee M, Casper C. Improved growth of preterm infants receiving mother's own raw milk compared with pasteurized donor milk. Acta Paediatr 2011;100:1548–54.

6. Cossey V, Vanhole C, Eerdekens A, Rayyan M, Fieuws S, Schuermans A. Pasteurization of mother's own milk for preterm infants does not reduce the incidence of late-onset sepsis. Neonatology 2013;103:170–6.
7. Dritsakou K, Liosis G, Valsami G, Polychronopoulos E, Skouroliakou M. Improved outcomes of feeding low birth weight infants with predominantly raw human milk versus donor banked milk and formula. J Matern Fetal Neonatal Med 2016;29:1131–8.
8. Ewaschuk JB, Unger S, Harvey S, O'Connor DL, Field CJ. Effect of pasteurization on immune components of milk: implications for feeding preterm infants. Appl Physiol Nutr Metab 2011;36:175–82.
9. Silvestre D, Miranda M, Muriach M, Almansa I, Jareño E, Romero FJ. Antioxidant capacity of human milk: effect of thermal conditions for the pasteurization. Acta Paediatr 2008;97:1070–4.
10. Christen L, Lai CT, Hartmann B, Hartmann PE, Geddes DT. The effect of UV-C pasteurization on bacteriostatic properties and immunological proteins of donor human milk. PLoS One 2013;8:e85867.
11. Christen L, Lai CT, Hartmann B, Hartmann PE, Geddes DT. Ultraviolet-C irradiation: a novel pasteurization method for donor human milk. PLoS One 2013;8:e68120.
12. Sangild PT, Thymann T, Schmidt M, Stoll B, Burrin DG, Buddington RK. Invited review: the preterm pig as a model in pediatric gastroenterology. J Anim Sci 2013;91:4713–29.
13. Andersen AD, Sangild PT, Munch SL, van der Beek EM, Renes IB, Van Ginneken C, Greisen GO, Thymann T. Delayed growth, motor function and learning in preterm pigs during early postnatal life. Am J Physiol Regul Integr Comp Physiol 2016;310:R481–92.
14. Nguyen DN, Fuglsang E, Jiang P, Birck MM, Pan X, Kamal SB, Pors SE, Gammelgaard PL, Nielsen DS, Thymann T, et al. Oral antibiotics increase blood neutrophil maturation and reduce bacteremia and necrotizing enterocolitis in the immediate postnatal period of preterm pigs. Innate Immun 2016;22:51–62.
15. Li Y, Jensen ML, Chatterton DE, Jensen BB, Thymann T, Kvistgaard AS, Sangild PT. Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets. Am J Physiol Gastrointest Liver Physiol 2014;306:G81–90.
16. Demacker PN, Beijers AM, van Daal H, Donnelly JP, Blijlevens NM, van den Ouweland JM. Plasma citrulline measurement using UPLC tandem mass-spectrometry to determine small intestinal enterocyte pathology. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:387–92.
17. Shen RL, Thymann T, Ostergaard MV, Stoy AC, Krych L, Nielsen DS, Lauridsen C, Hartmann B, Holst JJ, Burrin DG, et al. Early gradual feeding with bovine colostrum improves gut function and NEC resistance relative to infant formula in preterm pigs. Am J Physiol Gastrointest Liver Physiol 2015;309:G310–23.
18. Thymann T, Burrin DG, Tappenden KA, Bjornvad CR, Jensen SK, Sangild PT. Formula-feeding reduces lactose digestive capacity in neonatal pigs. Br J Nutr 2006;95:1075–81.
19. Jiang P, Sangild PT, Siggers RH, Sit W, Lee C, Wan JM. Bacterial colonization affects the intestinal proteome of preterm pigs susceptible to necrotizing enterocolitis. Neonatology 2011;99:280–8.
20. Hansen CH, Holm TL, Krych L, Andresen L, Nielsen DS, Rune I, Hansen AK, Skov S. Gut microbiota regulates NKG2D ligand expression on intestinal epithelial cells. Eur J Immunol 2013;43:447–57.
21. Pyndt Jørgensen B, Hansen JT, Krych L, Larsen C, Klein AB, Nielsen DS, Josefsen K, Hansen AK, Sørensen DB. A possible link between food and mood: dietary impact on gut microbiota and behavior in BALB/c mice. PLoS One 2014;9:e103398.
22. Østergaard MV, Shen RL, Støy AC, Skovgaard K, Krych Ł, Leth SS, Nielsen DS, Hartmann B, Bering SB, Schmidt M, et al. Provision of amniotic fluid during parenteral nutrition increases weight gain with limited effects on gut structure, function, immunity, and microbiology in newborn preterm pigs. JPEN J Parenter Enteral Nutr 2016;40:552–66.
23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.
24. Canibe N, Hojberg O, Badsberg JH, Jensen BB. Effect of feeding fermented liquid feed and fermented grain on gastrointestinal ecology and growth performance in piglets. J Anim Sci 2007;85:2959–71.
25. Breusing N, Grune T, Andrisic L, Atalay M, Bartosz G, Biasi F, Borovic S, Bravo L, Casals I, Casillas R, et al. An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples. Free Radic Res 2010;44:1203–15.
26. Casper C, Carnielli VP, Hascoet JM, Lapillonne A, Maggio L, Timdahl K, Olsson B, Vågerö M, Hernell O. rhBSSL improves growth and LCPUFA absorption in preterm infants fed formula or pasteurized breast milk. J Pediatr Gastroenterol Nutr 2014;59:61–9.
27. Li Y, Ostergaard MV, Jiang P, Chatterton DE, Thymann T, Kvistgaard AS, Sangild PT. Whey protein processing influences formula-induced gut maturation in preterm pigs. J Nutr 2013;143:1934–42.
28. Rasmussen SO, Martin L, Ostergaard MV, Rudloff S, Li Y, Roggenbuck M, Bering SB, Sangild PT. Bovine colostrum improves neonatal growth, digestive function and gut immunity relative to donor human milk and infant formula in preterm pigs. Am J Physiol Gastrointest Liver Physiol 2016;311:G480–91.
29. Widger J, O'Connell NH, Stack T. Breast milk causing neonatal sepsis and death. Clin Microbiol Infect 2010;16:1796–8.
30. Ng PC, Lewindon PJ, Siu YK, Wong W, Cheung KL, Liu K. Bacterial contaminated breast milk and necrotizing enterocolitis in preterm twins. J Hosp Infect 1995;31:105–10.
31. Lamb-Rosteski JM, Kalischuk LD, Inglis GD, Buret AG. Epidermal growth factor inhibits Campylobacter jejuni-induced claudin-4 disruption, loss of epithelial barrier function, and Escherichia coli translocation. Infect Immun 2008;76:3390–8.
32. Clark JA, Gan H, Samocha AJ, Fox AC, Buchman TG, Coopersmith CM. Enterocyte-specific epidermal growth factor prevents barrier dysfunction and improves mortality in murine peritonitis. Am J Physiol Gastrointest Liver Physiol 2009;297:G471–9.
33. Maxson RT, Jackson RJ, Smith SD. The protective role of enteral IgA supplementation in neonatal gut origin sepsis. J Pediatr Surg 1995;30:231–3.
34. Coutinho HB, da Mota HC, Coutinho VB, Robalinho TI, Furtado AF, Walker E, King G, Mahida YR, Sewell HF, Wakelin D. Absence of lysozyme (muramidase) in the intestinal Paneth cells of newborn infants with necrotising enterocolitis. J Clin Pathol 1998;51:512–4.
35. Cooper CA, Brundige DR, Reh WA, Maga EA, Murray JD. Lysozyme transgenic goats' milk positively impacts intestinal cytokine expression and morphology. Transgenic Res 2011;20:1235–43.
36. Manzoni P, Rinaldi M, Cattani S, Pugni L, Romeo MG, Messner H, Stolfi I, Decembrino L, Laforgia N, Vagnarelli F, et al. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birthweight neonates: a randomized trial. JAMA 2009;302:1421–8.
37. Edde L, Hipolito RB, Hwang FF, Headon DR, Shalwitz RA, Sherman MP. Lactoferrin protects neonatal rats from gut-related systemic infection. Am J Physiol Gastrointest Liver Physiol 2001;281:G1140–50.
38. Sherman MP, Bennett SH, Hwang FF, Yu C. Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and Lactobacillus GG. Biometals 2004;17:285–9.
39. Wu J, Chen J, Wu W, Shi J, Zhong Y, van Tol EA, Tang Q, Cai W. Enteral supplementation of bovine lactoferrin improves gut barrier function in rats after massive bowel resection. Br J Nutr 2014;112:486–92.
40. Iacobelli S, Bonsante F, Ferdinus C, Labenne M, Gouyon JB. Factors affecting postnatal changes in serum creatinine in preterm infants with gestational age <32 weeks. J Perinatol 2009;29:232–6.
41. Kim JC, Mullan BP, Hampson DJ, Pluske JR. Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract. Br J Nutr 2008;99:1217–25.
42. Arboleya S, Solis G, Fernandez N, de los Reyes-Gavilan CG, Gueimonde M. Facultative to strict anaerobes ratio in the preterm infant microbiota: a target for intervention? Gut Microbes 2012;3:583–8.
43. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. Early Hum Dev 2012;88 Suppl 1:S41–9.

Elsevier Editorial System(tm) for Journal of Functional Foods

Manuscript Draft

Manuscript Number:

Title: Bioactive whey protein concentrate and lactose stimulate gut function in formula-fed preterm pigs

Article Type: Full Length Article

Keywords: Whey protein concentrate, lactose, maltodextrin, milk processing, intestinal health, preterm piglet model

Corresponding Author: Professor Per T Sangild,

Corresponding Author's Institution: University of Copenhagen

First Author: Yanqi Li

Order of Authors: Yanqi Li; Duc N Nguyen; Karina Ryom; Anders D Andersen; Thomas Thymann; Dereck Chatterton; Stig Purup; Anne B Heckmann; Stine B Bering; Per T Sangild

Abstract: Optimal milk diets are essential for the health of newborn infants, especially preterm infants with an immature intestine. Infant formula remains inferior to maternal milk but the mechanisms behind this are unclear. We hypothesized that reduced contents of non-milk carbohydrate (i.e. maltodextrin) and reduced thermal-treatment of whey protein concentrate (WPC) would improve formula quality in preterm pigs, a model for preterm infants. In Experiment 1, a WPC product with higher lactoferrin and IgG levels improved intestinal structure and lactose digestion, predominantly in a lactose-dominant formula. In Experiment 2, a WPC produced by reduced thermal-treatment contained higher bioactivity (increased lactoferrin and TGF-β2 and stimulation of proliferative effects in vitro). This resulted in increased feeding tolerance, intestinal structure and function and physical activity in preterm pigs, relative to a control WPC. Gently processed formula with lactose as the main carbohydrate may support gut maturation and health in sensitive, formula-fed neonates.

Suggested Reviewers: Bo Lonnerdal
bllonnerdal@ucdavis.edu

Janet Berrington
j.e.berrington@newcastle.ac.uk

Randal Buddington
rbddngtn@memphis.edu

Highlights

- Whey protein concentrate (WPC) is rich in bioactive proteins and is a functional ingredient in infant formulas
- Industrial processing destroys bioactive proteins in WPC, which can be improved by reducing the temperature of thermal-treatment
- Bioactive WPC produced by reduced thermal-treatment contains higher levels of bioactive proteins, such as lactoferrin and TGF-β
- When used together with lactose as the main carbohydrate source, bioactive WPC stimulates intestinal health in formula-fed preterm piglets, a model for sensitive newborns.

Bioactive whey protein concentrate and lactose stimulate gut function in formula-fed preterm pigs

Yanqi Li[1], Duc Ninh Nguyen[1], Karina Ryom[1], Anders D. Andersen[1], Thomas Thymann[1], Dereck E. W. Chatterton[2], Stig Purup[3], Anne B. Heckmann[4], Stine B. Bering[1], Per T. Sangild [1 *]

[1] Comparative Paediatrics and Nutrition, University of Copenhagen, Copenhagen, Denmark; [2] Department of Food Science, University of Copenhagen, Denmark; [3] Department of Animal Science, Aarhus University; [4] Arla Foods Ingredients, Denmark

Short title: Bioactive WPC and lactose for sensitive newborns

Key words: Whey protein concentrate, lactose, maltodextrin, milk processing, intestinal health, preterm neonates, piglet model

*Corresponding author: Professor Per T. Sangild, Section of Comparative Paediatrics and Nutrition, Faculty of Health and Medical Sciences, University of Copenhagen, Dyrlægevej 68, DK-1870 Frederiksberg C, Denmark, Tel: +45 35 33 26 98, Fax: +45 3533 2483, email: psa@sund.ku.dk

Abstract

Optimal milk diets are essential for the health of newborn infants, especially preterm infants with an immature intestine. Infant formula remains inferior to maternal milk but the mechanisms behind this are unclear. We hypothesized that reduced contents of non-milk carbohydrate (i.e. maltodextrin) and reduced thermal-treatment of whey protein concentrate (WPC) would improve formula quality in preterm pigs, a model for preterm infants. In Experiment 1, a WPC product with higher lactoferrin and IgG levels improved intestinal structure and lactose digestion, predominantly in a lactose-dominant formula. In Experiment 2, a WPC produced by reduced thermal-treatment contained higher bioactivity (increased lactoferrin and TGF-β2 and stimulation of proliferative effects *in vitro*). This resulted in increased feeding tolerance, intestinal structure and function and physical activity in preterm pigs, relative to a control WPC. Gently processed formula with lactose as the main carbohydrate may support gut maturation and health in sensitive, formula-fed neonates.

1. Introduction

Preterm birth occurs for 5-15% of all live-born infants worldwide and is the second most common cause of death in children less than 5 years (Blencowe et al., 2012). Optimal nutrition is essential in preventing morbidities in this highly sensitive population. Relative to infant formulas, human milk feeding decreases necrotising enterocolitis (NEC) and sepsis, and stimulates intestinal maturation (Moro et al., 2015). However, infant formula, especially preterm formula, is often used for preterm infants when mother´s own milk or donor human milk is absent or insufficient. Formulas may be specifically designed for preterm infants to fulfil their special nutrient requirements, e.g. relatively high protein and mineral needs for growth (Hay & Hendrickson, 2016). Preterm infants also have reduced intestinal lactase activity (Shulman, Wong, & Smith, 2005) and to avoid lactose maldigestion, a mixture of lactose and glucose polymers (e.g. maltodextrins) is commonly used as the carbohydrate source in many preterm formulas. Whey protein is used as the main protein source due to its high digestibility, bioavailability and bioactive effects (e.g. trophic, immuno-modulatory, anti-inflammatory, anti-bacterial) in infants, relative to casein and vegetable proteins (Patel, 2015).

Although preterm infants fed formulas have similar growth rate as infants fed fortified human milk, their morbidity (NEC, sepsis) remains higher (Menon & Williams, 2013). This may partly be due to the use of non-milk based ingredients (e.g. maltodextrins) and heavily processed WPCs. In preterm pigs, a maltodextrin-based formula predisposes to NEC, induces intestinal atrophy, reduces brush-border enzyme activities and aldohexose absorption and changes the gut bacterial composition, relative to a lactose-based formula (Thymann et al., 2009). The WPC used in infant formulas is produced from sweet whey or acid whey after various technological processes, including pasteurization, fractionation and spray drying (Li et al., 2013). These processes can cause denaturation, aggregation, and redistribution of bioactive proteins in WPC and thus reduce the levels of these in infant formulas (Chatterton, Nguyen, Bering, & Sangild, 2013). In preterm piglets, WPCs produced by altered filtration steps and reduced thermal-treatment result in improved intestinal maturation compared with a conventional WPC (Li et al., 2013). However, it is unknown

whether the preservation of WPC bioactivity can be achieved by only reducing the thermal-treatment with no alteration on filtration steps.

On this background it is possible that infant formula better supports the growth and maturation of the immature intestine, when the carbohydrate content is based on lactose (rather than maltodextrin) and when the protein is based on gently produced WPC. Hence, we hypothesized that the use of WPCs produced by reduced thermal-treatment in a formula with increased percentage of lactose would improve intestinal maturation in a highly diet-sensitive model of preterm infants in preterm pigs. In Experiment 1, we tested if two WPCs, one with high-bioactivity (WPC Hi) and one with low-bioactivity WPC (WPC Lo) in either a lactose-dominant or a maltodextrin-dominant formula would affect intestinal health differently. In Experiment 2, we tested if reduced thermal-processing preserved the bioactivity of a WPC product and if this bioactive WPC (BioWPC) improved intestinal health, feeding tolerance and physical activity, relative to a conventional WPC (ConWPC). We measured intestinal morphology, digestive and absorptive functions, and inflammatory conditions as the indicators for intestinal health in the experiments.

2. Material and methods

2.1. Pigs, diets and experimental design

Ninety-two preterm pigs were delivered from six sows by caesarean section at 105 d gestation (Large White × Danish Landrace × Duroc, Gadstrup Farm, Roskilde, Denmark; term = 116 ± 2 days). An oro-gastric feeding tube (6F, Portex, Smiths Medical, St Paul, MN, USA) was placed into the stomach for enteral nutrition (EN) and a vascular catheter (4F, Portex) was inserted in the umbilical artery for parental nutrition (PN). Pigs were reared in temperature-regulated individual incubators with oxygen supply and given maternal plasma at 16 ml/kg during the first 24 h after birth to achieve passive immunological protection. All pigs received parenteral nutrition (PN) for the first 2 days, as previously described (Sangild et al., 2013). Pigs also received their respective milk diets as minimal enteral nutrition (MEN; 24-40 ml/kg/d) for the first two days and as full enteral nutrition (120 ml/kg/d) from day 3 to euthanasia. The studies were approved by the National Committee on Animal Experimentation in Denmark.

In Experiment 1, 61 pigs were stratified according to birth weight and gender into 4 enteral feeding groups: 1) lactose-dominant (lactose/maltodextrin: 3/1) formula containing WPC Hi (Lac-hi, *n*=15); 2) lactose-dominant formula containing WPC Lo (Lac-lo, *n*=15); 3) maltodextrin-dominant (maltodextrin/lactose: 3/1) containing WPC Hi (Mal-hi, *n*=15) and 4) maltodextrin-dominant formula containing WPC Lo (Mal-lo, *n*=16). In Experiment 2, 31 pigs were stratified into two feeding groups: 1) lactose-dominant formula containing BioWPC (Bio, *n*=16) and 2) lactose-dominant formula containing ConWPC (Con, *n*=15). The six milk formulas were made by mixing the following ingredients and contained similar macronutrient compositions and energy levels (Table 1). The WPCs (WPC Hi, WPC Lo, BioWPC and ConWPC) and lactose (Variolac 960) were obtained from Arla Foods Ingredients (AFI, Viby J., Denmark), maltodextrin (Ross Polycose) from Abbott Nutrition (Columbus, USA) and lipid (Liquigen and Calogen), vitamins and minerals (SHS Seravit) from Nutricia (Allerød, Denmark).

2.2. WPC products and their analyses

The four WPCs were produced from pooled bovine milk obtained from Danish dairy cows (Danish Red and Danish Holstein-Friesian). WPC Hi and WPC Lo used in Experiment 1 were similar acid whey products, containing about 80% whey protein. The ConWPC and BioWPC products used in Experiment 2 were produced from sweet whey by similar processing procedures with the only difference being the temperature of pasteurization (Fig. 1). In Experiment 1, LF and IgG were selected as bioactive markers and measured using commercial kits for bovine LF (ELISA, Bethyl Laboratories, Montgomery, TX, USA) and bovine IgG (radial immunodiffusion kit, The Binding Site, San Diego, CA, USA), respectively (analysed by Eurofins Steins Laboratorium, Holstebro, Denmark). In Experiment 2, protein aggregation, protein compositions and levels of LF, IgG, IGF-I, TGF-β1 and TGF-β2 were measured as indicators of bioactivity.

The level of total protein was measured by a BCA protein assay (Thermo Scientific, Slangerup, Denmark), before and after removal of the aggregated protein (centrifuged at 15000 × g, 4^{o}C for 30 min). WPCs with or without centrifugation (15 µg protein in each sample) were loaded onto 15% SDS-PAGE gels, using non-reducing conditions for major protein analysis. TGF-β2 was measured by Western blot (TGF-β2 antibody obtained from Sc-90, Santa Cruz Biotechnology, CA, USA) before and after removal of aggregated protein using reducing condition. The concentrations of LF and IgG were analysed by Eurofins as described above and the concentrations of IGF-1, TGF-β1 and TGF-β2 were analysed without removal of aggregates by TR-IFMA and ELISA assays as previously described (Purup, Vestergaard, O Pedersen, & Sejrsen, 2007; Sejrsen, Pedersen, Vestergaard, & Purup, 2001).

2.3. Clinical evaluation and sample collection

Pigs were continually monitored and euthanized if clinical symptoms, such as severe pain, infection, or NEC appeared. All remaining pigs were euthanized for tissue collection on day 5. Feeding intolerance was monitored at each feeding and defined as withholding or reducing the amount of

planned enteral feeding volume due to vomiting or abdominal distension. After anaesthesia (a mixture of zolazepam/tiletamin/xylazine/ketamine; Boehringer Ingelheim, Copenhagen, Denmark), cardiac blood was collected into heparin- or EDTA-containing tubes and subsequently pigs were euthanized with an intracardiac injection of pentobarbitone sodium (60 mg/kg). Individual weights of internal organs and the length of the small intestine were recorded. Whole-wall tissue samples of proximal (Prox), middle (Mid) and distal (Dist) small intestine were taken and immediately snap-frozen in liquid nitrogen and stored at -80°C or fixed in paraformaldehyde solution for further analyses. A 1-cm segment of Dist tissue obtained 10 cm prior to the ileo-cecal junction and another 1-cm segment of colon tissue from the colonic apex region were fixated in Clarke's solution for later goblet cell quantification. According to the degree of pathological changes (e.g. hyperemia, hemorrhage, pneumatosis, necrosis), macroscopic lesion scores (1–6) were assigned to stomach, Prox, Mid, Dist and colon regions (Sangild et al., 2013). Pigs with a score of three or above in any of the Prox, Mid, Dist and colon regions were classified as having NEC.

2.4. *In vivo* gut functions and *ex vivo* brush border enzyme activities

To determine the intestinal digestive and absorptive capacity, the increment of plasma galactose in response to oral boluses of galactose and lactose was measured. On day 3, before full EN, pigs were given a bolus (15 ml/kg) of 5% galactose via the oro-gastric feeding tubes. Heparinized blood samples were taken through the umbilical artery catheter before and 20 min after administration of the bolus. On day 4, pigs were given a bolus (15 ml/kg body weight) of 10% lactose and blood was sampled into heparin-containing tubes prior to and at 20 min and/or 40 min after administration of the bolus. Concentrations of galactose in plasma were measured by spectrophotometry, as described previously (Thymann et al., 2006). To test intestinal permeability, pigs received an oral bolus (15 ml/kg body weight) containing 5% lactulose and 5% mannitol 3 h prior to euthanasia. Post-mortem urine samples were taken to measure the concentrations of lactulose and mannitol, as described previously (Blood, Ingle, Allison, Davies, & Hill, 1991). The ratio between lactulose and mannitol concentrations in urine was calculated to determine the intestinal permeability.

Snap-frozen tissue samples from Prox, Mid and Dist were homogenized in 1.0% Triton X-100 (10 ml/g tissue) using gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA, USA). After centrifugation (2000 x g, 10 min, 4 °C), the supernatant was isolated and used for determining the brush border enzyme activities. The activities of lactase, maltase, sucrase, aminopeptidase N (ApN), aminopeptidase A (ApA) and dipeptidyl-peptidase IV (DPPIV) in the homogenates were analysed by spectrophotometry using corresponding sugars and peptides as substrates, as described previously (Sangild, Sjöstrom, Norén, Fowden, & Silver, 1995).

2.5. Intestinal morphology and goblet cell density

Paraformaldehyde-fixed tissues from Prox and Dist were embedded in paraffin, sectioned (3 µm), mounted on slides and stained with hematoxylin and eosin. Digital histology images were obtained with a camera-attached light microscope (Ortho-plane, Leitz, Germany). Mean villus height (µm) and crypt depth (µm) were measured (Image J 1.44p, National Institutes of Health, Bethesda, MD, USA) on the digital images for ten representative vertically well-oriented villus-crypt axes in each region. An average of 10 measurements in each region was used as the representative villus height or crypt depth for each pig. Clarke's solution-fixed Dist intestine as well as colon samples were embedded in paraffin, sectioned (2 µm), mounted on slides and stained with Alcian Blue-Periodic Acid Shiff (AB-PAS) for evaluation of goblet cell density. Slides were visualized using a light microscope (20X lens, Olympus BX45TF, Tokyo, Japan) equipped with a camera (Olympus). STEPanizer (Tschanz, Burri, & Weibel, 2011) was used to visualize the tunica mucosa, including the goblet cells. Goblet cell density was calculated as the fractional area of the total tunica mucosa that was covered by goblet cells (lamina muscularis mucosa excluded).

2.6. Physical activity

Physical activity was recorded by continuous video surveillance using infrared cameras installed over each incubator and connected to an HD recorder with built-in motion detection. The digital output for each camera allowed recording of the status of the individual piglets as being either

active or resting. With the PIGLWin application (Ellegaard Systems, Faaborg Denmark), the proportion of time when pigs were active was automatically registered for every hour. The cameras were turned off during any handling of the pigs. Activity recording was performed from total enteral feeding commenced on day 3 at 9:00am and ended just before pigs were euthanized on day 5 at 9:00am. The proportion of active time was analysed from means of recordings covering the day- (from 9:00am to 9:00pm) and night-time (from 9:00pm to 9:00am the next day), respectively.

On day 4, spontaneous motor activity was evaluated in an open field arena (1.20 m × 1.20 m), with a video camera mounted from the ceiling (bird's eye view) during a 3-min recording period. From these recordings, piglet movements were tracked and analyzed using EthoVision XT10 (Noldus Information Technology, Wageningen, Netherlands) providing information on distance travelled inside the arena. Pigs that were clinically ill on day 4 were excluded from the open field test.

2.7. Effects of WPCs on cytotoxicity and proliferation of porcine immature IECs in vitro

The porcine IPEC-J2 cell line (DSMZ, Braunschweig, Germany) derived from the jejunum of a newborn pig, was used to test the effects of WPCs *in vitro*. Cells were cultured between passage 5-25 in advanced DMEM/F12 medium supplemented with 2% heat-inactivated fetal bovine serum, 40 U/ml penicillin, 40 µg/ml streptomycin and 2 mM Glutamax (all from Life Technologies, Nærum, Denmark), at 37°C and 5% CO_2. Centrifuged BioWPC and ConWPC solutions at 10 g/L were prepared, as mentioned above, and sterile-filtered (0.2 µm) and diluted in serum-free culture medium to reach protein concentrations of 1, 0.1 and 0.01 g/L.

For cell cytotoxicity assays, the cells were seeded in 96 well plates at 2×10^4 cells/well and allowed to attach for 24 h. BioWPC and ConWPC at 1, 0.1 and 0.01 g/L concentrations were mixed with Sytox green (5 µM, Life Technologies), a non-membrane permeable dye, which binds to extracellular DNA, and incubated with IECs for 6 h. Fluorescence intensity was then measured at 485/520 nm (excitation/emission). Cell cytotoxicity was calculated by the fluorescence intensity of treatments subtracted by that of serum-free medium alone. For cell proliferation, cells were seeded

in 96 well plates at 2×10^4 cells/well, allowed to attach for 24 h prior to treatments with BioWPC and ConWPC at 1, 0.1 and 0.01 g/L for 48 h. Cell proliferation was quantified by Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Nacka, Sweden) according to the manufacturer's instructions.

2.8. Statistical analysis

Levels of proteins in WPCs were analysed by student t-test (GraphPad Prism 5.0, La Jolla, CA, USA). Data of cell cytotoxicity and cell proliferation were analysed by a linear model with treatment and cell passage as fixed factors followed by *post hoc* Tukey test (JMP, SAS Institute, Cary, USA). Binary data, such as NEC incidence, were evaluated using logistic regression in R (version 3.1.1). NEC lesion scores were analysed using non-parametric analysis with the *nparcomp* package. Continuous outcomes were compared among groups using general linear model with adjustments, e.g. sow, sex and birth weight. Repeated measurement of daily physical activity was analysed using the *lmer* function for mixed modeling as repeated measures and comparisons were made with the lsmeans package. To confirm the validity of the modelled data, residuals and fitted values were assessed for normality and variance homogeneity, which for some outcomes required log-transformation of data before modelling. Resulting P-values were evaluated at a 5% significance level. The *multcomp* single-step method was used to adjust P values for multiple comparisons within each outcome measure and point of measurements. Data are presented as raw arithmetic mean and SEM, unless otherwise stated.

3. Results

3.1. WPC bioactivity markers and cytotoxicity and proliferation effecs *in vitro*

In Experiment 1, the levels of native LF and IgG were higher in WPC hi than those in WPC lo by 6 and 3 times, respectively (Table 1). In Experiment 2, the level of aggregated protein in BioWPC was negligible, whereas approximate 20% protein was aggregated in ConWPC. The levels of native LF and IgG were 3 and 1.3 times higher respectively, in BioWPC compared with ConWPC (Table 1). By non-reducing SDS-PAGE, LF and bovine serum albumin levels were found to be higher in BioWPC versus ConWPC before and after aggregate removal (all $P < 0.05$, Fig. 2A-B). After aggregate removal, BioWPC had higher levels of β-Lg, α-La and TGF-β2 (all $P < 0.05$, Fig. 2B-C) than ConWPC. Without aggregate removal, levels of TGF-β2 did not differ between BioWPC and ConWPC by western blotting (Fig. 2C) and concentrations of IGF-1, TGF-β1 and TGF-β2 measured by ELISA were also similar in the two WPCs (ConWPC: 180 ng/g, 22.3 ng/g and 229.1 ng/g; BioWPC: 170 ng/g, 22.1 ng/g and 210.4 ng/g).

At low concentrations (0.01-0.1 g/L), both BioWPC and ConWPC had negligible cytotoxic activity while at higher levels (1 g/L) cytotoxic activity was 2-fold higher for ConWPC than for BioWPC ($P < 0.05$). Relative to the medium control, BioWPC-induced cell proliferation was greater than for ConWPC-induced cell proliferation at low concentrations (0.01 g/L, 1.1- vs. 0.9-fold relative to control medium, respectively, both $P < 0.05$), whereas at higher levels (1 g/L), ConWPC decreased cell proliferation relative to medium control (0.8-fold vs. control medium, $P < 0.05$).

3.2. Experiment 1: Clinical outcomes and intestinal effects

There was no difference among groups with respect to life span before euthanasia (90.2 ± 1.6 h), birth weight (955 ± 33 g), NEC incidence (31/60) and lesion scores in the stomach (2.5 ± 0.3), small intestine (2.1 ± 0.2) and colon (2.9 ± 0.3). There were no differences between groups for intestinal circumference (13 ± 0.5 cm, pooled values across groups), intestinal length (322 ± 10

cm/kg), or relative weight of internal organs (g/kg body weight) for heart (8.1 ± 0.2), lungs (23.6 ± 0.8), kidneys (10.6 ± 0.3), spleen (1.9 ± 0.1), liver (29.1 ± 0.7), stomach (8.8 ± 0.5), small intestine (31.3 ± 0.7) and colon (15.0 ± 0.6). Only the groups fed lactose-dominant formulas (Lac-hi and Lac-lo) had a positive mean daily weight gain, but the group differences were not statistically significant (Fig. 3A). The increments in plasma galactose 20 min after the galactose bolus, and 20 min and 40 min after the lactose bolus were all higher in Lac-hi than in Lac-lo ($P < 0.05$) and there was no difference between Mal-hi and Mal-lo (Fig. 3B). Intestinal permeability measured by the lactulose to mannitol ratio in urine did not differ among groups (0.19 ± 0.03). Villus height differed in Prox between Lac-hi and Lac-lo groups ($P<0.05$), while the differences between the two maltodextrin-dominant groups only tended to show difference ($P=0.11$, Fig. 3C). The average lactase activity across the three small intestinal regions was higher for the Lac-hi pigs, relative to Lac-lo pigs ($P<0.05$), but no difference was found between the Mal-hi and Mal-lo groups (Fig. 3D). The activities of the other five brush-border enzymes did not differ between Mal-hi and Mal-lo or between Lac-hi and Lac-lo across the three small intestinal regions (data not shown).

3.3. Experiment 2: Clinical outcomes, intestinal structure and function, and physical activity

There was no difference between the Bio and Con groups in terms of birth weight (1014 ± 46 g), small intestinal NEC incidence (5/31), colon NEC incidence (11/31) and lesion scores in the stomach (1.4 ± 0.2), small intestine (1.7 ± 0.1) and colon (2.1 ± 0.2). There were no differences between groups for intestinal circumference (12.0 ± 0.5 cm, pooled values across groups), intestinal length (299 ± 11 cm/kg) or relative weight of internal organs (g/kg body weight) for heart (8.3 ± 0.2), lungs (20.1 ± 0.6), kidneys (10.7 ± 0.3), spleen (2.0 ± 0.1), liver (33.2 ± 0.8), stomach (7.1 ± 0.3), small intestine (29.8 ± 1.0) and colon (13.0 ± 0.7). Interestingly, feeding intolerance was observed in seven pigs for the Con group (44%) and none in the Bio group ($P < 0.01$).

Intestinal absorptive functions measured by galactose test on day 3 tended to be higher for the Bio group, relative to the Con group ($P = 0.13$), whereas the digestive and absorptive function measured by the lactose test on day 4 did not differ between groups (Table 2). Intestinal

permeability measured by the lactulose/mannitol test tended to be lower in the Bio group than in the Con group (P = 0.07, Table 2). Brush border lactase activity was higher in Bio than Con pigs in both Prox and Mid regions (both P < 0.05), while no difference was found in the Dist intestine (Table 2). The activities of the other five enzymes measured did not differ between groups across three small intestinal regions (Table 2).

In the Prox region, pigs in the Bio group had increased villus height and decreased crypt depth compared with Con pigs (both P < 0.05, Table 2). Villus height and crypt depth did not differ between the two groups in the Mid and Dist regions. Goblet cell density did not differ between the two groups, neither in the Dist nor in the colon (Table 2). Pigs that were scored completely healthy (intestinal NEC score 1) had higher goblet cell density in this region, relative to pigs with higher scores (4.1±0.5 versus 2.4±0.4%, P < 0.05).

The physical activity recorded by the motion cameras tended to be higher in the Bio group, compared with the Con group (over all P=0.09, Fig. 4A), and was significantly increased during the last day prior to euthanasia (P<0.05, Fig. 4A). Further, in the open field test on day 4, Bio pigs walked almost twice the distance of Con pigs (P<0.05, Fig. 4B), which is in line with the observation above regarding overall increased physical activity in Bio pigs.

4. Discussion and conclusions

Newborn infants, and especially preterm infants, have an immature intestine that is highly sensitive to the quality of enteral feeding. Optimal feeding provides protection against luminal bacteria and stimulates intestinal maturation via trophic and immuno-modulatory factors. Maternal milk and donor human milk are generally accepted as the first and second choices for preterm infants but when these are not available or sufficient, high-quality preterm formula must be provided as an alternative. A high grade preterm formula consists of a balanced nutrient composition, thus fulfilling the needs of a fast-growing, immature infant. Moreover, it should also contain the right combination of ingredients with the most preserved bioactivity to protect infants with an immature gut and immune system. Our findings in preterm pigs reveal that WPCs with different levels of bioactive ingredients exert different effects on the immature intestine and that these effects are affected by other formula ingredients, such as lactose and maltodextrin. Specifically for a sweet-whey based WPC, we demonstrated that bioactivity was preserved by reducing processing temperature. The observed *in vivo* effects in this study were rather modest, compared with other diet- and microbiota interventions in preterm pigs that have shown more prominent effects of intact milk and colostrum versus formula (Bjornvad et al., 2008; Li et al., 2014) or gut microbiota manipulation (Birck et al., 2016; Jensen et al., 2014). Still, we conclude from our two experiments that WPCs with different bioactivity do affect intestinal function and physical activity in preterm pigs and that these effects may be affected by other ingredients used (e.g. maltodextrin or lactose).

Lactose maldigestion in preterm infants has been suggested to contribute to feeding intolerance, poor growth and even NEC in preterm infants (Kien, 1990; Shulman et al., 2005). At 34 weeks gestation, intestinal lactase activity (hydrolysing lactose to glucose and galactose) in preterm infants only reaches 30% of the activity present in term infants. At the same gestational age, maltase and sucrase-isomaltase, the main enzymes catalysing hydrolysis of maltodextrin, may already reach 70% of the activity in term infants (Antonowicz, Chang, & Grand, 1974). Therefore, manufacturers of formulas for preterm infants typically replace 60% of the lactose with

maltodextrin (glucose polymers produced from hydrolysis of starch) to account for the low lactase activity (Commare & Tappenden, 2007). However, a systematic review showed no evidence of benefit from adding lactase to formulas for preterm infants (Tan-Dy & Ohlsson, 2013). A few studies showed increased weight gain and feeding tolerance in preterm infants in response to low-lactose or lactase-supplemented formulas (Erasmus, Ludwig-Auser, Paterson, Sun, & Sankaran, 2002; Griffin & Hansen, 1999), but regardless, there is no direct evidence that maltodextrin-based formulas better stimulate intestinal maturation and NEC resistance than lactose-based formulas. Furthermore, human milk remains the most tolerable and beneficial feeding for preterm infants and human milk contains mainly lactose as the digestible carbohydrate source, and at higher levels than in most other mammals. While excessive lactose maldigestion and fermentation would be a problem, moderate amounts of non-digested lactose entering the colon may promote the growth of beneficial microorganisms (e.g. bifidobacteria and lactobacillus) and inhibit the growth of opportunistic pathogenic bacteria (Chichlowski, De Lartigue, German, Raybould, & Mills, 2012; Hay & Hendrickson, 2016; Heyman, 2006).

In preterm piglets, we previously showed that a maltodextrin-based formula, with trace amount of lactose, resulted in more NEC and reduced intestinal functions markedly (Thymann et al., 2009). In the current study, a maltodextrin-dominant formula, with 25% lactose, resulted in a similar NEC incidence as a lactose-dominant formula with 25% maltodextrin, but both formulas led to 50% NEC. This value was intermediate between the NEC incidences observed after complete maltodextrin- and lactose-based formulas, respectively (91 and 27%) suggesting that even as little as 25% maltodextrin of the carbohydrate fraction in formulas is enough to induce intestinal damage and NEC, at least in preterm piglets. This may be due to changes in the gut microbiota and excessive fermentation caused by maltodextrin, potentially stimulating an inflammatory response (Thymann et al., 2009). Interestingly, when we examined the bioactivity of the two WPCs on intestinal structure and function (e.g. villus height, galactose absorption, lactose digestion and absorption), the stimulating effect of WPC Hi was predominantly present with the lactose-dominant

formula. This underlines the importance of not only preserving bioactivity of WPC, but also improving other base macronutrient composition, when optimizing infant formula.

Based on the results of Experiment 1, we chose the lactose-dominant formula as the base to investigate whether WPC produced by reduced thermal-treatment possessed more bioactivity than a conventional WPC. Whey ingredients are typically derived from cheese production as a by-product, and therefore the raw material of WPC has already been subjected to several steps of pasteurization from milk to cheese milk and whey. The cumulative effects of several steps of heat-treatment cause denaturation and aggregation of bioactive proteins. Similar to one of our previous studies (Nguyen, Sangild, Li, Bering, & Chatterton, 2016), we demonstrated in this study that a lower processing temperature prevented protein aggregation, preserveed the levels of marker bioactive proteins (e.g. LF and TGF-β2) and improved the proliferative effects *in vitro*. Our earlier study on WPC processing (Li et al., 2013) also showed differences in IGF-I and IgG levels between bioactive and control WPCs, which did not differ in the current study. Possibly because in the previous study, the control WPC had an additional filtration step, following which proteins with high molecular weight were removed to a large extent, including the IGFs attached to IGF-binding proteins and immunoglobulins (Elfstrand, 2002; Kamau, Cheison, Chen, Liu, & Lu, 2010).

Feeding intolerance is a clinical measurement that is commonly used in the clinics to indicate the level of intestinal maturation and diet tolerability in preterm infants (Fanaro, 2012). In our study, feeding intolerance was only observed in the Con group. The increased villus height, brush-border lactase activity and better intestinal integrity in the Bio group may better explain the feeding tolerance for this group although other factors may be involved also (e.g. the enteric nervous system). Milk whey proteins (e.g. LF) have previously been shown to increase epithelial proliferation, attenuate epithelial apoptosis, stimulate hexose transport and support intestinal integrity (Ben-Lulu et al., 2012; Nguyen, Jiang, et al., 2016; Zhang et al., 2012). Consistent with this, our *in vitro* cell proliferation assay showed an increased proliferative effect of BioWPC, which may related to the preserved bioactive proteins.

The BioWPC induced increased home cage physical activity and walking distance in the open field test. These variables have previously been shown to characterize the impaired activity and neurodevelopment of preterm versus term piglets (Andersen et al., 2016), in piglets fed formula versus natural milk (Cao et al., 2015), and in NEC versus healthy pigs, especially for intestinal NEC lesions (Cao et al., 2016). Although the incidence of small intestinal NEC was low in the Bio and Con groups (2-3 animals in each group), the reduced physical activity in Con pigs might be due to general discomfort following feeding intolerance and intestinal inflammation, and an early sign of small intestinal NEC development in this group. The extent to which other body functions (e.g. neuromuscular function, brain development) could be involved remains speculative.

The increased distance travelled in the open field test support the notion that the Bio group had better general motor functions and/or exploratory behaviour than Con pigs. In a previous study, preterm 1-3 week-old pigs travelled shorter distances and spent more time in the centre of the test cage, indicating reduced exploration, relative to term piglets (Andersen et al., 2016). In this study, our open field tests were performed already on day 4, making it less likely that the differences could be a direct diet-induced maturation of the distant organ functions (e.g. brain), although this cannot be excluded. It is likely that the reduced digestive discomfort in Bio animals is the most likely explanation for their increased physical activity. However, the immature state of the intestine and other organ systems, such as the brain, following preterm birth may make milk quality particularly important for preterm infants, acting via the immature gut-brain axis. Even in more mature rats and pigs, LF has been shown to cross the blood-brain barrier (Chen et al., 2015; Harada et al., 1999; Kamemori et al., 2008), allowing direct activation of LF receptors. Dietary LF supplementation to term piglets from day 3-38 showed improved neurodevelopment and cognition, probably via up-regulated neurotrophic factors and polysialylation (Chen et al., 2015). Dietary LF and other bioactive milk components may also modulate central nervous system by modulating the gut microbiota, intestinal inflammation and the gut immune system (Cong, Henderson, Graf, & McGrath, 2015; Ramel & Georgieff, 2014; Rose, Vassar, Cahill-Rowley, Hintz, & Stevenson,

2015). Longer-term studies are warranted to investigate whether the increased short-term physical activity stimulated by BioWPC is related to direct or indirect effects on neurodevelopment and behaviour after birth.

We demonstrated that the carbohydrate source influences the biological effects of different types of WPCs present in the same formula on intestinal health in preterm pigs. High bioactivity of WPC can be achieved by only reducing the thermal-treatment, which could increase feeding tolerance, intestinal structure and function and physical activity, particularly in sensitive newborns. Lactose-based formulas containing WPC with maximal preservation of bioactive proteins could be important to support intestinal maturation and general health in sensitive newborn infants.

Conflict of interest

Anne B. Heckmann is an employee of Arla Foods Ingredients. Yanqi Li was employed at Arla Foods Ingredients when Experiment 1 was performed. Yanqi Li, Thomas Thymann, Stine B. Bering and Per T. Sangild have given scientific presentations at meetings organized by Arla Foods Ingredients. The rest of the authors declared no conflict of interests.

Acknowledgements

We thank Monica Dagsvik, Jane Povlsen, Ping Zhou, Lars Christiansen and Anders Brunse for their support with the animal procedures. We also appreciate the technical assistances from Elin Skytte Kristina Møller and Kasper Vrangstrup Poulsen.

Funding

This work was funded by the NEOMUNE project from the Innovation Fund Denmark, the Danish Dairy Research Foundation, and Arla Foods Ingredients, Denmark.

References

Andersen, A. D., Sangild, P. T., Munch, S. L., van der Beek, E. M., Renes, I. B., Ginneken, C. van, … Thymann, T. (2016). Delayed growth, motor function and learning in preterm pigs during early postnatal life. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *310*(6), R481-92.

Antonowicz, I., Chang, S. K., & Grand, R. J. (1974). Development and distribution of lysosomal enzymes and disaccharidases in human fetal intestine. *Gastroenterology*, *67*(1), 51–8.

Ben-Lulu, S., Pollak, Y., Mogilner, J., Bejar, J., G. Coran, A., Sukhotnik, I., … Hickman, J. (2012). Dietary Transforming Growth Factor-Beta 2 (TGF-β2) Supplementation Reduces Methotrexate-Induced Intestinal Mucosal Injury in a Rat. *PLoS ONE*, *7*(9), e45221.

Birck, M. M., Nguyen, D. N., Cilieborg, M. S., Kamal, S. S., Nielsen, D. S., Damborg, P., … Thymann, T. (2016). Enteral but not parenteral antibiotics enhance gut function and prevent necrotizing enterocolitis in formula-fed newborn preterm pigs. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *310*(5), G323–G333.

Bjornvad, C. R., Thymann, T., Deutz, N. E., Burrin, D. G., Jensen, S. K., Jensen, B. B., … Sangild, P. T. (2008). Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *295*(5), G1092-103.

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., … Lawn, J. E. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: A systematic analysis and implications. *The Lancet*, *379*(9832), 2162–2172.

Blood, J., Ingle, A. R., Allison, N., Davies, G. R., & Hill, P. G. (1991). Rapid enzymatic method for the measurement of mannitol in urine. *Annals of Clinical Biochemistry*, *28 (Pt 4)*, 401–6.

Cao, M., Andersen, A. D., Li, Y., Thymann, T., Jing, J., & Sangild, P. T. (2016). Physical Activity and Gastric Residuals as Biomarkers for Region-Specific NEC Lesions in Preterm Neonates.

Neonatology, *110*(4), 241–247.

Cao, M., Andersen, A. D., Van Ginneken, C., Shen, R. L., Petersen, S. O., Thymann, T., … Sangild, P. T. (2015). Physical activity level is impaired and diet dependent in preterm newborn pigs. *Pediatric Research*, *78*(2), 137–144.

Chatterton, D. E. W., Nguyen, D. N., Bering, S. B., & Sangild, P. T. (2013). Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *International Journal of Biochemistry and Cell Biology*, *45*(8), 1730–1747.

Chen, Y., Zheng, Z., Zhu, X., Shi, Y., Tian, D., Zhao, F., … Wang, B. (2015). Lactoferrin Promotes Early Neurodevelopment and Cognition in Postnatal Piglets by Upregulating the BDNF Signaling Pathway and Polysialylation. *Molecular Neurobiology*, *52*(1), 256–269.

Chichlowski, M., De Lartigue, G., German, J. B., Raybould, H. E., & Mills, D. A. (2012). Bifidobacteria isolated from infants and cultured on human milk oligosaccharides affect intestinal epithelial function. *Journal of Pediatric Gastroenterology and Nutrition*, *55*(3), 321–7.

Commare, C. E., & Tappenden, K. a. (2007). Development of the infant intestine: Implications for nutrition support. *Nutrition in Clinical Practice*, *22*(2), 159–173.

Cong, X., Henderson, W. A., Graf, J., & McGrath, J. M. (2015). Early Life Experience and Gut Microbiome: The Brain-Gut-Microbiota Signaling System. *Advances in Neonatal Care : Official Journal of the National Association of Neonatal Nurses*, *15*(5), 1–10.

Elfstrand, L. (2002). Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. *International Dairy Journal*, *12*(11), 879–887.

Erasmus, H. D., Ludwig-Auser, H. M., Paterson, P. G., Sun, D., & Sankaran, K. (2002). Enhanced weight gain in preterm infants receiving lactase-treated feeds: A randomized, double-blind, controlled trial. *The Journal of Pediatrics*, *141*(4), 532–537.

Fanaro, S. (2012). Strategies to improve feeding tolerance in preterm infants. *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of*

Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, *25 Suppl 4*(December), 54–6.

Griffin, M. P., & Hansen, J. W. (1999). Can the elimination of lactose from formula improve feeding tolerance in premature infants? *The Journal of Pediatrics*, *135*(5), 587–592.

Harada, E., Sugiyama, A., Takeuchi, T., Sitizyo, K., Syuto, B., Yajima, T., & Kuwata, T. (1999). Characteristic transfer of colostral components into cerebrospinal fluid via serum in neonatal pigs. *Biology of the Neonate*, *76*(1), 33–43.

Hay, W. W., & Hendrickson, K. C. (2016). Preterm formula use in the preterm very low birth weight infant. *Seminars in Fetal and Neonatal Medicine*.

Heyman, M. B. (2006). Lactose Intolerance in Infants, Children, and Adolescents. *Pediatrics*, *118*(3).

Jensen, M. L., Thymann, T., Cilieborg, M. S., Lykke, M., Mølbak, L., Jensen, B. B., … Sangild, P. T. (2014). Antibiotics modulate intestinal immunity and prevent necrotizing enterocolitis in preterm neonatal piglets. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *306*(1).

Kamau, S. M., Cheison, S. C., Chen, W., Liu, X.-M., & Lu, R.-R. (2010). Alpha-Lactalbumin: Its Production Technologies and Bioactive Peptides. *Comprehensive Reviews in Food Science and Food Safety*, *9*(2), 197–212.

Kamemori, N., Takeuchi, T., Sugiyama, A., Miyabayashi, M., Kitagawa, H., Shimizu, H., … Harada, E. (2008). Trans-endothelial and trans-epithelial transfer of lactoferrin into the brain through BBB and BCSFB in adult rats. *J Vet Med Sci*, *70*(3), 313–315.

Kien, C. L. (1990). Colonic fermentation of carbohydrate in the premature infant: Possible relevance to necrotizing enterocolitis. *The Journal of Pediatrics*, *117*(1), S52–S58.

Li, Y., Jensen, M. L., Chatterton, D. E. W., Jensen, B. B., Thymann, T., Kvistgaard, A. S., & Sangild, P. T. (2014). Raw bovine milk improves gut responses to feeding relative to infant formula in preterm piglets. *American Journal of Physiology. Gastrointestinal and Liver*

Physiology, *306*(1), G81-90.

Li, Y., Ostergaard, M. V, Jiang, P., Chatterton, D. E. W., Thymann, T., Kvistgaard, A. S., & Sangild, P. T. (2013). Whey protein processing influences formula-induced gut maturation in preterm pigs. *The Journal of Nutrition*, *143*(12), 1934–1942.

Menon, G., & Williams, T. C. (2013). Human milk for preterm infants: why, what, when and how? *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *98*(6), F559-62.

Moro, G. E., Arslanoglu, S., Bertino, E., Corvaglia, L., Montirosso, R., Picaud, J.-C., … Ziegler, E. E. (2015). XII. Human Milk in Feeding Premature Infants: Consensus Statement. *Journal of Pediatric Gastroenterology and Nutrition*, *61 Suppl 1*(3), S16-9.

Nguyen, D. N., Jiang, P., Stensballe, A., Bendixen, E., Sangild, P. T., & Chatterton, D. E. W. (2016). Bovine lactoferrin regulates cell survival, apoptosis and inflammation in intestinal epithelial cells and preterm pig intestine. *Journal of Proteomics*, *139*, 95–102.

Nguyen, D. N., Sangild, P. T., Li, Y., Bering, S. B., & Chatterton, D. E. W. (2016). Processing of whey modulates proliferative and immune functions in intestinal epithelial cells. *Journal of Dairy Science*, *99*(2), 959–69.

Patel, S. (2015). Functional food relevance of whey protein: A review of recent findings and scopes ahead. *Journal of Functional Foods*, *19*, 308–319.

Purup, S., Vestergaard, M., O Pedersen, L., & Sejrsen, K. (2007). Biological activity of bovine milk on proliferation of human intestinal cells. *The Journal of Dairy Research*, *74*(1), 58–65.

Ramel, S. E., & Georgieff, M. K. (2014). Preterm nutrition and the brain. *World Review of Nutrition and Dietetics*, *110*, 190–200.

Rose, J., Vassar, R., Cahill-Rowley, K., Hintz, S., & Stevenson, D. (2015). Neonatal Biomarkers of Inflammation: Correlates of Early Neurodevelopment and Gait in Very-Low-Birth-Weight Preterm Children. *American Journal of Perinatology*, *33*(1), 071–078.

Sangild, P. T., Sjöstrom, H., Norén, O., Fowden, A. L., & Silver, M. (1995). The Prenatal Development and Glucocorticoid Control of Brush-Border Hydrolases in the Pig Small

Intestine. *Pediatric Research*, *37*(2), 207–212.

Sangild, Thymann, T., Schmidt, M., Stoll, B., Burrin, D. G., & Buddington, R. K. (2013). The preterm pig as a model in pediatric gastroenterology. *Journal of Animal Science*, *91*, 4713–6359.

Sejrsen, K., Pedersen, L. O., Vestergaard, M., & Purup, S. (2001). Biological activity of bovine milk. Contribution of IGF-I and IGF binding proteins. *Livestock Production Science*, *70*(1–2), 79–85.

Shulman, R. J., Wong, W. W., & Smith, E. O. (2005). Influence of changes in lactase activity and small-intestinal mucosal growth on lactose digestion and absorption in preterm infants. *The American Journal of Clinical Nutrition*, *81*(2), 472–9.

Tan-Dy, C. R. Y., & Ohlsson, A. (2013). Lactase treated feeds to promote growth and feeding tolerance in preterm infants (Review). *Cochrane Database of Systematic Reviews (Online)*, (2), 1–21.

Thymann, T., Moller, H. K., Stoll, B., Stoy, A. C. F., Buddington, R. K., Bering, S. B., … Burrin, D. G. (2009). Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs. *AJP: Gastrointestinal and Liver Physiology*, *297*(6), G1115–G1125.

Thymann, Burrin, D. G., Tappenden, K. A., Bjornvad, C. R., Jensen, S. K., Sangild, P. T., … Mcburney, M. I. (2006). Formula-feeding reduces lactose digestive capacity in neonatal pigs. *British Journal of Nutrition*, *95*(6), 1075.

Tschanz, S. A., Burri, P. H., & Weibel, E. R. (2011). A simple tool for stereological assessment of digital images: the STEPanizer. *Journal of Microscopy*, *243*(1), 47–59.

Zhang, T., Wang, Y., Ban, R., Tong, L., Qiao, H., Lao, H., … Zhang, F. (2012). Oral administration of lactoferrin attenuates intestinal ischemia-reperfusion injury in rats. *European Surgical Research. Europaische Chirurgische Forschung. Recherches Chirurgicales Europeennes*, *49*(2), 99–106.

Table 1. Macronutrient compositions and levels of lactoferrin and IgG

	Experiment 1				Experiment 2	
	Lac-hi	Lac-lo	Mal-hi	Mal-lo	Bio	Con
Energy, *kJ/L*	4487	4487	4508	4508	4495	4504
Protein, *g/L*	79	79	79	79	79	79
Lactoferrin, *mg/L*	134.3	23.7	134.3	23.7	264.6	88.4
IgG, *g/L*	1.9	0.68	1.9	0.68	5.2	4.0
Carbohydrate, *g/L*	62.5	62.5	62.5	62.5	62	62
Lactose, *g/L*	47.5	47.5	15	15	47	47
Maltodextrin, *g/L*	15	15	47.5	47.5	15	15
Fat, *g/L*	57	57	57	57	57	57

The macronutrient compositions of the formulas were calculated based on the product specification provided by the manufacturers. Lac-hi, lactose-dominant formula containing WPC Hi; Lac-lo, lactose-dominant formula containing WPC Lo; Mal-hi, maltodextrin-dominant formula containing WPC Hi; Mal-lo, maltodextrin-dominant formula containing WPC Lo; WPC Hi, whey protein concentrate with high bioactivity; WPC Lo, whey protein concentrate with low bioactivity; Bio, formula containing bioactive WPC; Con, formula containing conventional WPC; IgG, immunoglobulin G

Table 2. Intestinal structure and functions in Experiment 2 [1]

	Bio	Con	*P* value
Plasma galactose d3, *μmol/L*[2]	549 (221-741)	310 (257-496)	0.13
Plasma galactose d4, *μmol/L*[2]	96 (86-138)	81 (67-94)	0.52
Lac/man ratio	0.07±0.01	0.14±0.03	0.07
Distal goblet cell density	3.5±0.5%	3.3±0.5%	0.81
Colon goblet cell density	9.3±1.1%	11.0±1.7%	0.84
Maltase, *U/g*	1.7±0.1	1.8±0.1	0.66
Sucrase, *U/g*	0.37±0.01	0.35±0.01	0.06
ApA, *U/g*	1.7±0.3	1.8±0.2	0.75
ApN, *U/g*	4.7±1.0	4.2±0.6	0.39
DPPIV, *U/g*	1.6±0.2	1.4±0.2	0.38
Proximal SI			
Lactase, *U/g*	4.2±1.0	2.9±0.8	0.01
Villus height, *μm*	458±54	344±37	0.04
Crypt depth, *μm*	83±3	93±4	0.02
Middle SI			
Lactase, *U/g*	9.4±2.0	7.4±1.4	0.02
Villus height, *μm*	659±105	588±68	0.37
Crypt depth, *μm*	83±3	84±3	0.63
Distal SI			
Lactase, *U/g*	10.4±2.8	14.1±2.6	0.18
Villus height, *μm*	517±64	520±54	0.93
Crypt depth, *μm*	88±4	87±3	0.99

[1] Data are means ± SEM unless noted otherwise, *n* = 13-16 per group. [2]Data are medians with interquartile range. ApA, aminopeptidase A; ApN, aminopeptidase N; Bio, BioWPC-containing formula; Con, conventional WPC-containing formula; DPPIV, dipeptidylpeptidase IV; lac/man, lactulose/mannitol; SI, small intestine

Figure Legends

Fig. 1. Schematic diagram displaying the production flow of bioactive WPC and conventional WPC for Experiment 2. BioWPC, bioactive WPC; Bio, BioWPC-containing formula; Con, ConWPC-containing formula; ConWPC, conventional WPC; WPC, whey protein concentrate.

Fig. 2. Protein composition in BioWPC and ConWPC, with and without aggregate removal by centrifugation, as analyzed by SDS-PAGE. (A) Representative SDS-PAGE gel. (B) Relative quantification of protein band volume for LF, BSA, β-Lg and α-La. (C) TGF-β2 and a representative Western blot band image of TGF-β2 monomers (12.5 kDa). Values (means ± SEM, n = 3 for triplicates of sample preparation) indicated by '*' differ between each other, $P < 0.05$. BioWPC, bioactive WPC; ConWPC, conventional WPC; β-lg, β-lactoglobulin; α-La, α-lactalbumin; BSA, bovine serum albumin; LF, lactoferrin.

Fig. 3. Outcomes of Experiment 1. A: Daily weight gain. B: Plasma galactose levels at 20 min after administration of oral boluses of galactose solution on day 3 and at 20 min and 40 min after administration of oral boluses of lactose solution. C: Villus height in the proximal region of small intestine. D: Brush-border enzyme activity in the proximal region of small intestine. Values are means ± SEM. Values indicated by '*' differ between each other, $P < 0.05$. Gal-test, galactose test; Lac-hi, lactose-dominant formula containing WPC Hi; Lac-lo, lactose-dominant formula containing WPC Lo; Lac-test, lactose test; Mal-hi, maltodextrin-dominant formula containing WPC Hi; Mal-lo, maltodextrin-dominant formula containing WPC Lo; WPC Hi, whey protein concentrate with high bioactivity; WPC Lo, whey protein concentrate with low bioactivity.

Fig. 4. General physical activity of piglets. **A**: Proportion of active time during postnatal days 3 and 4 showing. B: Walking distance in the open field test on day 4. Values are means + SEM. Values indicated by '*' differ from the other group, $P < 0.05$. Bio, formula containing bioactive WPC; Con,

formula containing conventional WPC; Day3-D, day-time on day 3; Day4-D, day-time on day 4; Day3-N, night-time on day 3; Day4-N, night-time on day 4.

Figure 1

Click here to download Figure: Figure 1.pdf

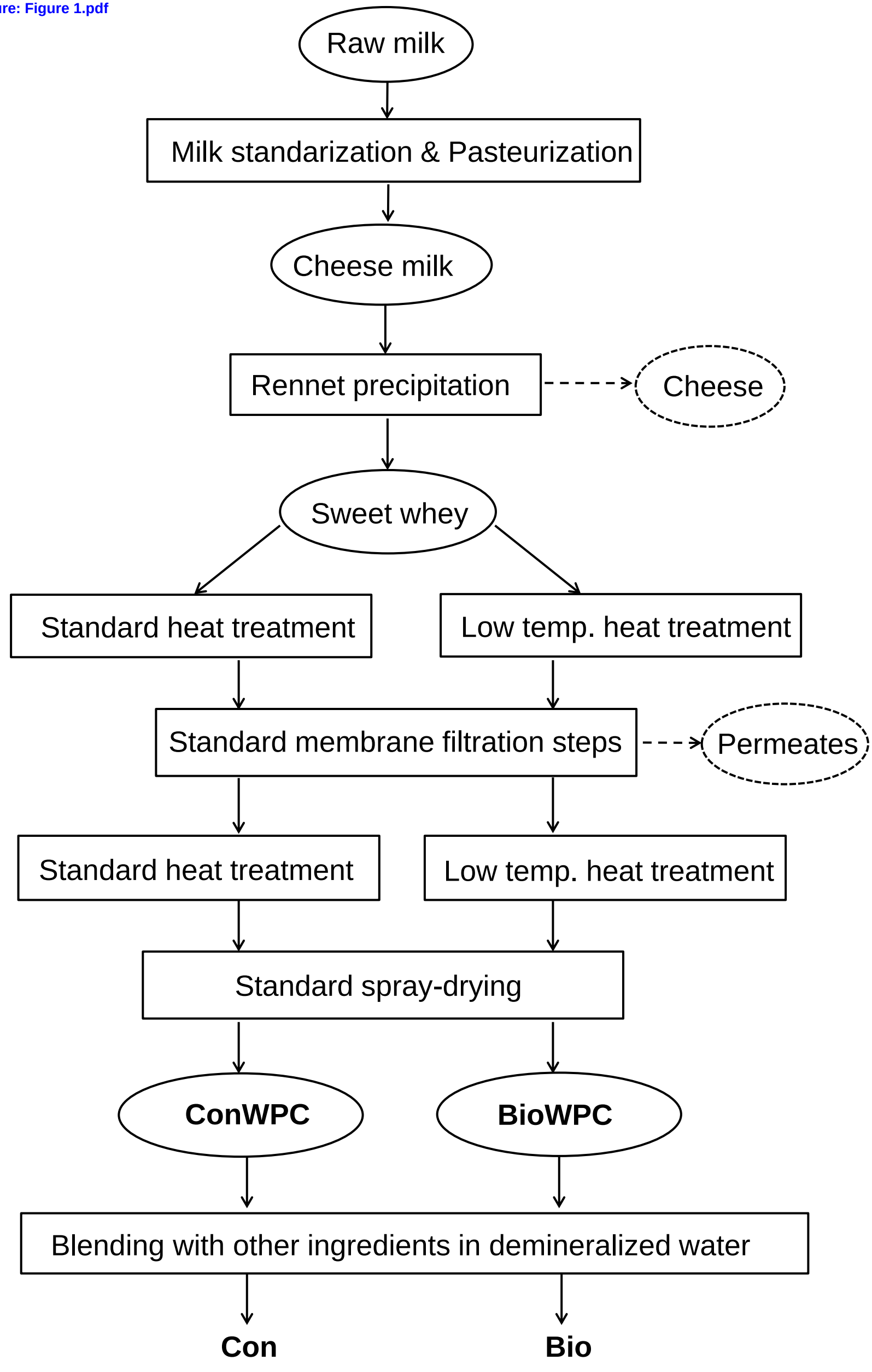


Figure 2

Click here to download Figure: Figure 2_new.pdf

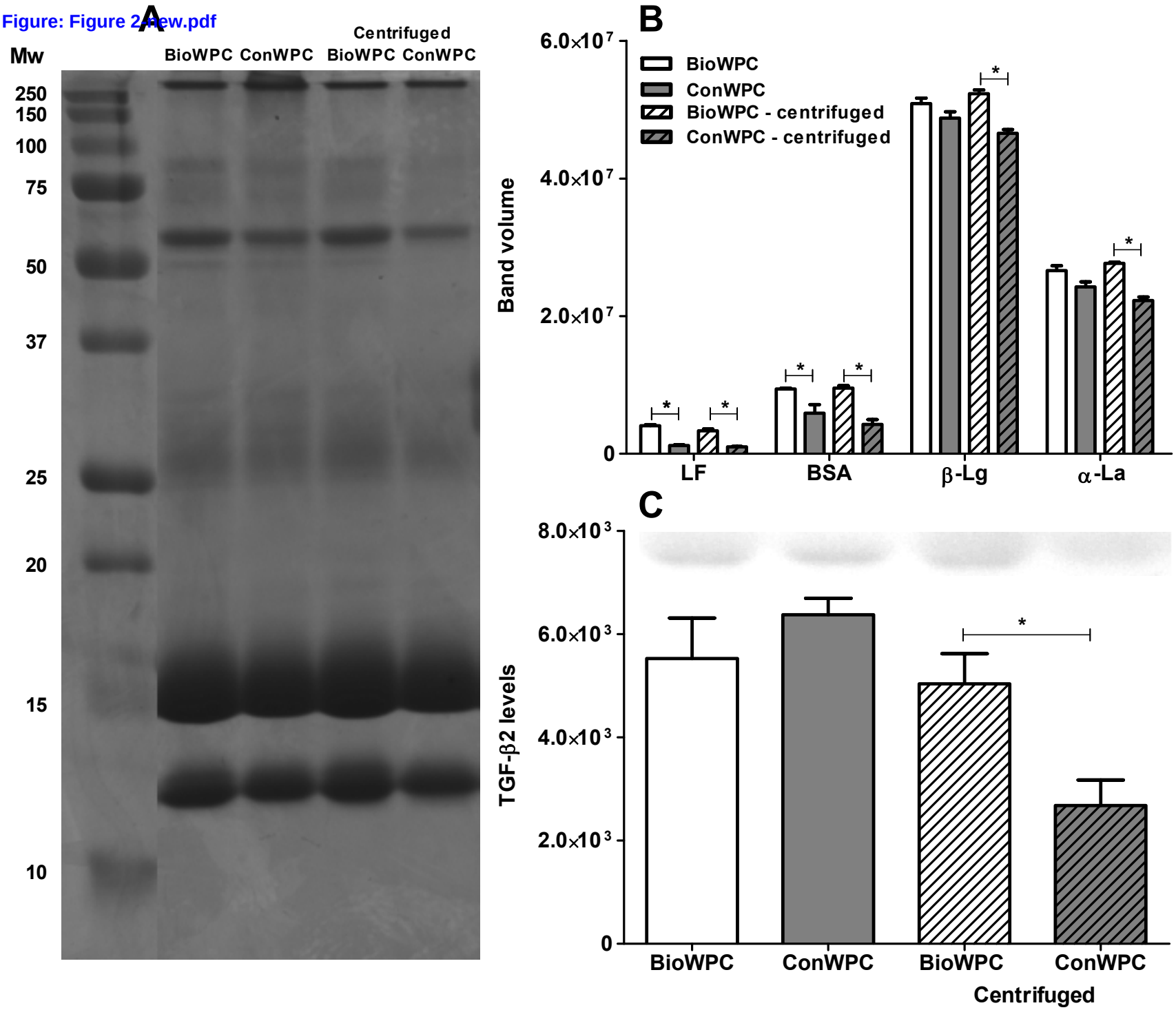


Figure 3

Click here to download Figure: Figure 3.pdf

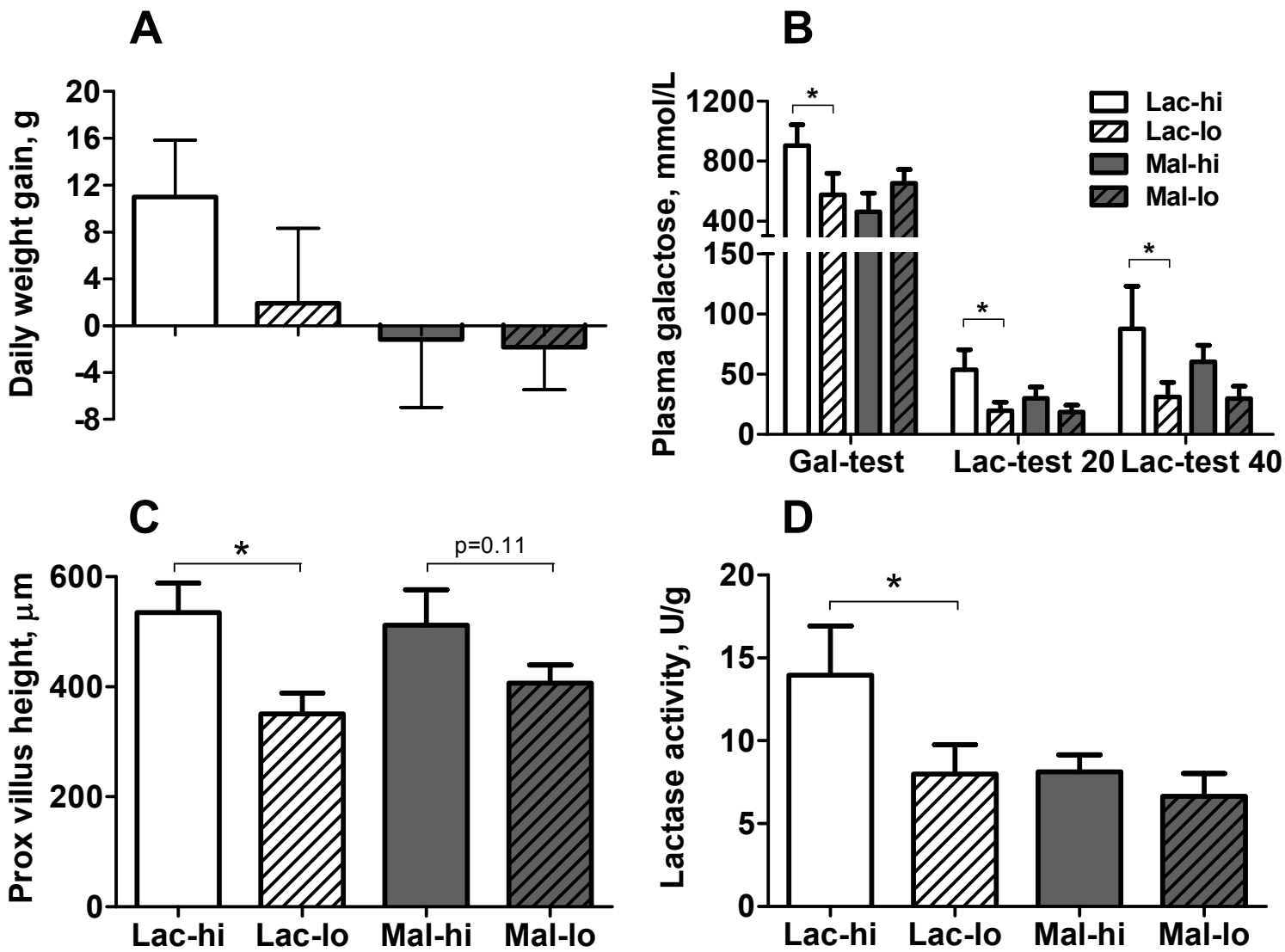
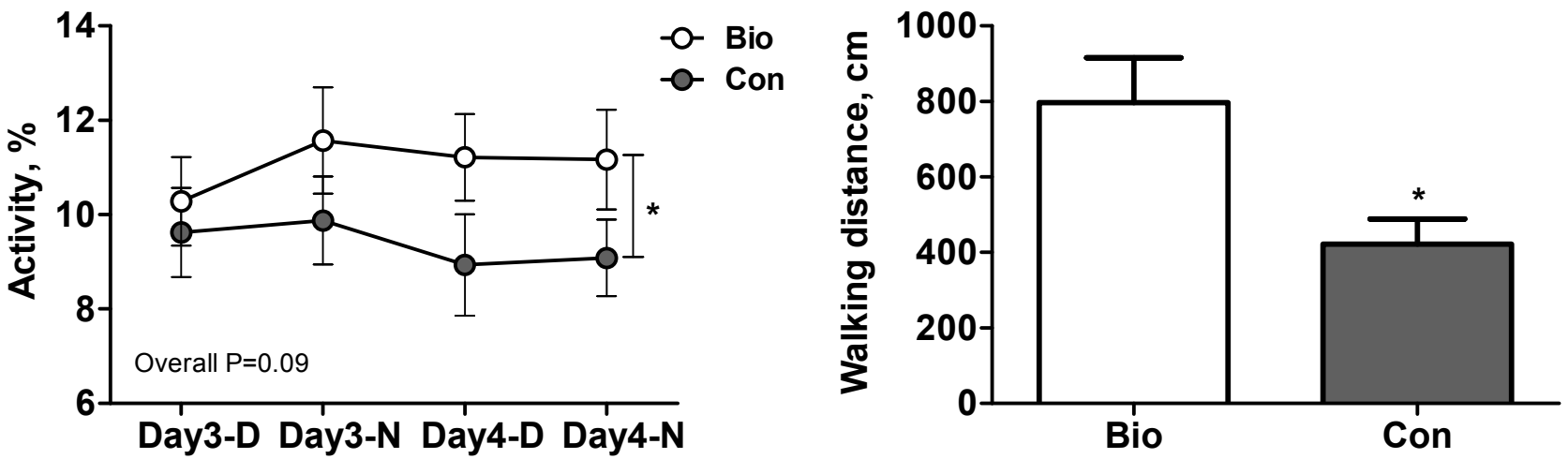


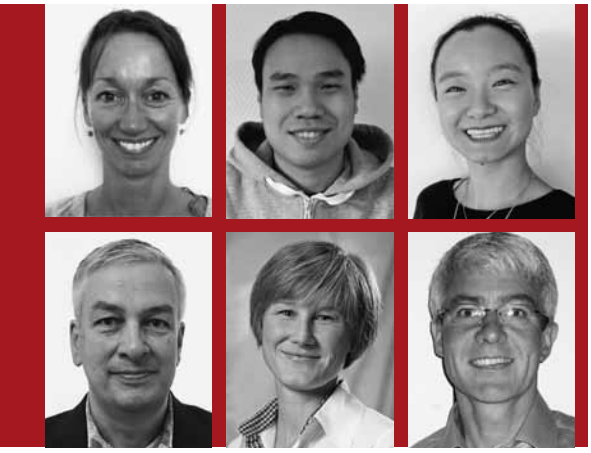
Figure 4

Click here to download Figure: Figure 4.pdf



Bioaktive mælkeproteiner **styrker tarmsundhed**

Mildere varmebehandling af mælkeproteiner styrker den fysiologiske modning og beskyttelse af tarmen i nyfødte og kan øge kvaliteten af modermælkserstatninger.



Af lektor Stine B. Bering[1], post.doc. Duc Ninh Nguyen[1], post.doc. Yanqi Li[1], lektor Dereck E.W. Chatterton[1,2,] Nutrition Scientist Anne B. Lau Heckmann[3] og professor Per T. Sangild[1]
1: Sektion for Komparativ Pædiatri og Ernæring, Institut for Klinisk Veterinær- og Husdyrvidenskab.
2: Institut for Fødevarevidenskab, Københavns Universitet.
3: Arla Food Ingredients, Viby

Mælk som den første ernæring

Som den første føde til nyfødte er brystmælk designet til, foruden at være den basale ernæringskilde, at modne tarmen og beskytte mod patogene bakterier og fødevareantigener. Dette gøres ved brystmælks høje indhold af vækstfaktorer og immunstimulerende stoffer, der beskytter tarmen mod inflammation og infektion. Til forskel fra brystmælk, som indtages friskt og ubehandlet og dermed med intakte bioaktive proteiner, er bioaktiviteten af mælkeproteiner nedsat i valleproteinkoncentrater (WPC) produceret bl.a. til modermælkserstatninger. WPC er baseret på komælk, og den nedsatte bioaktivitet er et resultat af pasteurisering og spray-tørring i fremstillingsprocessen. En mildere behandling af WPC-produkter under fremstillingen vil forventes at øge bioaktiviteten af mælkeproteiner til stimulering af tarmsundhed og restitution fra tarmsygdomme i nyfødte. Herved forbedres specifikke aktive ingredienser og kan således optimere modermælkserstatninger foruden at øge tarmsundheden hos både børn og voksne generelt.

Forskningsprojekt om bevaret bioaktivitet

I et nyligt afsluttet forskningsprojekt, støttet af Mejeriernes ForskningsFond samt Arla Foods Ingredients, har vi ved biokemisk analyse undersøgt om mindsket varmebehandling forhindrer nedbrydningen af bioaktive mælkeproteiner og vækstfaktorer. Derudover er det undersøgt, om bioaktiviteten af WPC samt specifikke mælkeproteiner øges ved lavere varmebehandling, herunder proteinerne laktoferrin og vækstfaktoren TGF-β. Dette blev undersøgt i en tarmcellemodel samt i vores unikke og internationalt anerkendte grisemodel for følsomme nyfødte børn.

Øget fysiologisk effekt af mælkeproteiner

Resultaterne fra celle og grisestudier viser, at anvendelse af en mildere varmebehandlingsteknologi ved fremstilling af WPC-produkter mindsker proteinaggregering og derved øger bioaktiviteten af mælkeproteiner og den positive effekt i tarmen sammenlignet med almindelig WPC i modermælkserstatning. Laktoferrin og TGF-β var medvirkende til, gennem specifikke signaleringsveje i tarmcellerne, at opretholde balance og ligevægt i tarmen og beskytte mod inflammation ved at stimulere tarmreparation i nyfødte. Selv om laktoferrin

Kort resumé
Mælk indeholder bioaktive komponenter, der modner og beskytter tarmen mod infektioner. Dette er specielt afgørende for nyfødte, særligt når tarmfunktionen er nedsat og følsom over for infektioner. Mildere varmebehandling og spray-tørringsteknologi til produktion af WPC som ingrediens i modermælkserstatning har vist sig at være vigtig for at bevare bioaktiviteten og øge den fysiologiske beskyttelse i tarmen. Dermed er det bioaktive WPC et bedre alternativ end en standard WPC til brug i modermælkserstatning. I et nyt projekt skal det undersøges, om mildere pasteurisering af human donormælk på samme måde kan optimere bioaktiviteten samt om en modermælkserstatning med bioaktiv WPC er et reelt alternativ hertil.
(Foto: Colourbox)

viste flere anti-inflammatoriske effekter var en høj dosis af laktoferrin dog skadelig i den nyfødte tarm, og en nøje optimering af laktoferrin som tilskud til modermælkserstatning er derfor nødvendig. En mildere varmebehandling af WPC er derfor lovende for bevaring af bioaktive proteiner såsom laktoferrin og TGF-β i modermælkserstatninger til styrkelse af tarmens immunsystem. Dette vil øge kvaliteten af WPC og dermed øge bioaktiviten af mælkeproteiner i modermælkserstatninger sammenlignet med standard WPC.

Er bioaktiv WPC lige så godt som donormælk?

Modermælkserstatning og human donormælk er alternativerne, når moderens egen mælk ikke er tilgængelig, eller når der er behov for at give tilskud af ekstra protein for optimal vækst såsom efter for tidlig fødsel. Donormælk er det foretrukne alternativ i klinikken til de særligt følsomme nyfødte, men ligesom modermælkserstatning er donormælk pasteuriseret, hvilket formentlig nedsætter bioaktiviteten af disse vigtige mælkeproteiner. Derudover er der ofte behov for at supplere moden donormælk med ekstra protein, da den indeholder lavere mængder protein end mælk fra mødre, der har født for tidligt.

Forskningen er bl.a. foretaget i projektets unikke og internationalt anerkendte grisemodel for følsomme nyfødte børn.

Nyt forskningsprojekt om WPC og donormælk

Et nyt forskningsprojekt støttet af Mejeriernes ForskningsFond, Arla Foods Ingredients samt Medela, et firma specialiseret i ammeprodukter, skal fortsætte studierne i skånsomt varmebehandlede bioaktive WPC-produkter samt donormælk. Vi dokumenterede, at bioaktiv WPC er bedre end konventionelt WPC i en modermælkserstatning til at beskytte og stimulere tarmen i nyfødte. Vi vil nu gerne undersøge, om effekten af det bioaktive WPC er på højde med effekten af donormælk, samt hvilken betydning pasteurisering af donormælk har til sammenligning. Projektet inkluderer undersøgelse af optimeret bioaktiv WPC samt mildere pasteuriseringsmetoder til human donormælk, herunder UV-C pasteurisering i forhold til konventionel Holder-pasteurisering. Produkterne vil, foruden den biokemiske karakterisering, blive testet både i celle- og grisestudier i forhold til de rå produkter.

Perspektiver for bioaktiv WPC

Resultaterne af det nye projekt vil give os et indblik i betydningen af de alternative ernæringsregimer som modermælkserstatning og human donormælk i forhold til den optimale ernæring med moderens egen mælk, mens det tidligere projekt fokuserede på forbedringen i forhold til den konventionelle modermælkserstatning. Vi forventer, at projektet får betydning både industrielt, ved at sætte fokus på vigtigheden af skånsom behandling af mælkeproteiner ved industriel produktion, samt klinisk, ved at vurdere effekten af bioaktiv WPC og optimere de nødvendige alternativer til moderens egen mælk. I første omgang vil dette have relevans særligt for følsomme nyfødte som eksempelvis de for tidligt fødte børn, men hermed også kunne danne basis for øget tarmsundhed hos normalt fødte børn samt hos voksne patienter med eksempelvis korttarmssyndrom eller patienter i behandling med kemoterapi. ■