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Bioaktive mælkeproteiner mod tarminflammation

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1. Projektets titel

BIOACTIVE MILK PROTEINS AGAINST GUT INFLAMMATION
- characterization of gut effects

2. Projektleder

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4. Projektperiode

January 2011 – September 2014

5. Projektresume

Projektet fokuserer på bevaring af bioaktivitet af proteiner i valleproteinkoncentrater (WPC) produceret ved lav varmebehandling (bioaktivt WPC) til beskyttelse mod tarminflammation hos nyfødte samt til generel tarmsundhed.

Vi har tidligere vist et højere indhold af laktoferrin (LF) og vækstfaktoren TGF-β2 in bioaktivt WPC sammenlignet med standard WPC. Ydermere har vi vist flere fysiologiske effekter af bioaktivt WPC, LF og TGF-β2 i tarmceller samt effekt af LF i en grisemodel for nyfødte børn.

I indeværende periode er det i tarmceller påvist at LF dosis spiller en vigtig rolle i interaktionen med tarmceller ved at regulere LF absorption, cellulær energi metabolisme, celledeling og celledød. Baseret på data indsamlet i projektet er vi nu i stand til at foreslå specifikke mekanismer for hvordan en lav (op til 1 g/L) og høj (10 g/L) dosis af LF influerer forskelligt på tarmceller samt hvad dette betyer for tyndtarmens fysiologi og udvikling af tarminflammation og necrotiserende enterocolitis (NEC) i for tidligt fødte børn. En lav dosis af LF binder til LF receptorer på tarmcellerne og bliver absorberet normalt i cellerne hvor de indgår i cellernes basale energimetabolisme og beskyttelse mod oxidativt stress. I modsætning hertil vil en høj dosis af LF ud over at binde til LF receptoren på tarmceller også binde til receptorer der er centrale i aktiveringen af det innate immunsystem som respons på patogene bakterier. Aktivering af disse receptorer fører til nedsat energi metabolisme, nedsat celledeling samt nedsat beskyttelse mod stress faktorer og opregulering af proteiner involveret i celledød. Dette vil dermed modsat en lav dosis føre til initiering af immunrespons svarende til et inflammatorisk respons. Disse data stemmer godt overens med vores tidligere observationer i grisemodellen for NEC, hvor en høj dosis af LF øgede NEC severiteten. Dermed understøtter og forklarer vores seneste studier specifikt i celler de negative effekter af en høj dosis af LF vi tidligere har dokumenteret i grisemodellen samtidig med at de indikerer positive effekter af en lavere dosis.

6. Projektets formål

Heat treatments applied to milk proteins, e.g. pasteurization are a prerequisite ensuring that pathogens are effectively destroyed, especially in products used in infant nutrition. Spray-drying is another thermal process applied to preserve proteins. These treatments can denature and aggregate proteins affecting particular milk components. Effects of lower heat processing will be studied on the stability and efficacy of bioactive milk proteins when compared to conventionally heat-treated milk proteins in countering inflammation in the intestine.

7. Projektets delaktiviteter i hele projektperioden

WP 1: SDS PAGE, Western Immunoblotting, Fractionation, and Cell Culture
Proteins will be separated using SDS-PAGE before and after centrifugation, thereby allowing determination of any denatured or aggregated proteins. Additionally, aggregated proteins will be detected using size exclusion chromatography. The stability of selected bioactive proteins and peptides present at low concentrations, such as and transforming growth factor β2 (TGF-β2), EGF, globulins, milk fat globule membrane proteins, lactoferrin (LF), and osteopontin, will be identified using Western Immunoblotting coupled to ECL-chemiluminescent detection. Selected fractions will be tested for their immunosuppressive effect using the intestinal porcine epithelial cell line PsIc1 as an *in vitro* model for the newborn pig intestinal epithelium. The cells will be stimulated with endotoxin such as lipopolysaccharide (LPS) and co-incubated with the selected protein fractions. The bioactivity of the differentially treated milk proteins will thereby be determined in terms of immunomodulatory effects by measuring pro- and anti-inflammatory cytokine expression and production by real-time qPCR and ELISA, respectively.

WP 2: *In vitro* and *in vivo* gastrointestinal digestion on different milk protein preparations
Gastric juice isolated as an aspirate from a piglet stomach under fasting conditions will be used to study the digestibility of the different milk preparations, thereby simulating the conditions in the infant stomach *in vitro*. Furthermore, different milk protein preparations will be administered to piglets by nasogastric feeding and aspirates will be sampled from the nasogastric tube at timed intervals following administration of a bolus of different treated milk proteins for *in vivo* digestion. Samples will be analyzed by SDS-PAGE, Western Immunoblotting, RP-HPLC, and mass spectrometry, and digestibility thereby assessed. Acid treatment can be used to dissociate growth factors from their respective binding proteins. Therefore, this methodology can be used to look at release of growth factors during the early stages of digestion. This could also provide information of whether the pH in the stomach of the preterm piglet is low enough to release the active growth factors. This would be an important factor in determining if the growth factors are biologically active in preterm neonates and therefore could prevent intestinal diseases.

WP 3: Small intestinal health as affected by different milk protein preparations
Different heat-treated milk preparations will be administered to preterm piglets, and at timed intervals following feeding, tissue samples will be excised from the small intestine, and effects on up- and down-regulation of tissue proteins will be determined (using proteomics techniques). A well-established preterm pig model of necrotizing enterocolitis (NEC) is used as a sensitive model of intestinal inflammation. Hence, we increase the response to dietary treatment and are better able to identify specific preparations that have high bioactivity and immunosuppressive effects *in vivo*. Following homogenization of the tissue, proteins will

be isolated using 2-D gels and treated with trypsin, and the released peptides will be identified using mass spectrometry (MALDI-TOF MS-MS). Bioinformatics software will be used to relate groups of up- and down-regulated proteins to potential bioactivity. In addition to a proteomics approach, we will also measure a series of structural, functional, microbiological immunological parameters, as described in our previous works.

Gantt chart: See Figure 6 below.

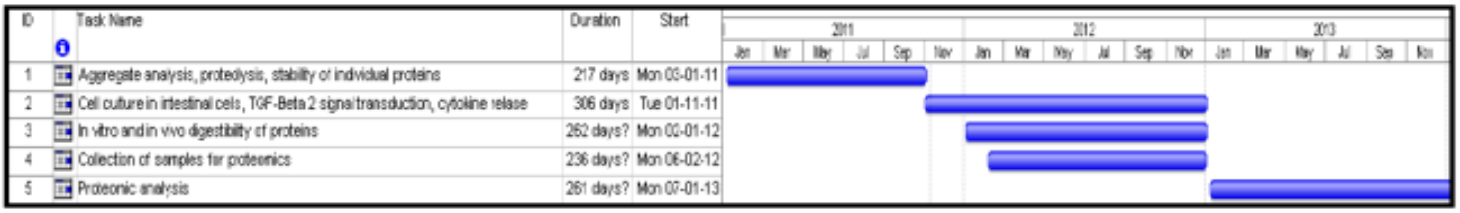

Figure 6 Gantt diagram of the project.

8. Projektets resultater/faglige forløb i perioden

In the previous phases, we reported the abundant amounts of lactoferrin (LF) and transforming growth factor β2 (TGF-β2) in low-heat treated WPCs, compared to standard heat treated WPCs. Furthermore, we demonstrated several effects of low-heat treated WPCs, bovine LF (bLF) and TGF-β2 in the intestinal epithelial cells (IECs) PsIc1, and effects of TGF-β2 and LF in preterm pigs. We also reported data from the proteomics study regarding effects of TGF-β2 on the proteome changes in PsIc1 cells.

In this final period, we report data from the proteomic study where the porcine intestinal epithelial cells PsIc1 were treated with various concentrations of LF (0, 0.1, 1 and 10 g/L). This will further elucidate the mechanisms of any detrimental effects of high doses (10 g/L) of bLF-enriched formula in preterm pigs and the dose-dependent effects of bLF on the inflammatory signaling pathways in PsIc1 cells, as previously reported.

WP1: SDS PAGE, Western Immunoblotting, Fractionation, and Cell Culture

Completed

WP2: ***In vitro* and *in vivo* gastrointestinal digestion on different milk protein preparations**

Completed

WP3: Small intestinal health as affected by different milk protein preparations

Dose-dependent effects of LF in PsIc1 cells by proteomic approach

Methods

A high-throughput approach of LC-MS-based proteomics combined with iTRAQ labeling was applied. PsIc1 cells were cultured until 90-95% confluency and maintained in serum-free medium for 24 h prior to stimulation with bLF at different doses of 0, 0.1, 1 and 10 g/L (LF0, LF0.1, LF1 and LF10, respectively) in triplicate for another 24 h. These doses were equivalent to 0, 1.25, 12.5 and 125 µM of LF. Thereafter, the IECs were washed, and collected, and lyzed by sonication. Extracted proteins were precipitated before trypsin digestion overnight. Obtained peptides were labeled with iTRAQ tags. After labeling, the 4-plexed samples were dried and analyzed by LC-MS/MS. Resulting raw files were analyzed using Thermo Proteome Discoverer (Thermo Scientific) and MaxQuant LFQ. Protein

abundance in bLF treated cells was reported relative to that in the common reference sample. Data were searched against Uniprot Sus scrofa and Bos taurus protein reference proteome databases.

Results

More than 1,200 proteins were identified in at least 3 plexes, which were used for statistical analysis. bLF and 193 porcine proteins showed differences in abundance among the four treatment groups. After applying a cutoff threshold of 1.2-fold for up-regulation and 0.85-fold for down-regulation, a total of 122 porcine proteins and bLF were differentially expressed (63 up-regulated and 60 down-regulated proteins). Information about the 123 identified proteins, including their accession number, % coverage, number of unique peptides, protein score, and fold change are shown in Tables 1 and 2, and stratified into groups, based on their biological functions. The major protein functions regulated by bLF were related to metabolic processes (40 proteins), DNA and RNA binding and processing (26 proteins), signal transduction (16 proteins), and proliferation, apoptosis and cell death (11 proteins). Due to the large number of differentially expressed proteins, the bLF uptake and a series of proteins involved in four different cellular events, including cell proliferation and cell death, energy metabolism, hypoxia inducible factor 1 (HIF-1) pathway, and aminoacyl-tRNA ligase activity, were selected for a detailed focus.

Table 1 Up-regulated proteins by bLF (0.1-10 g/L) in PsIc1 cells. LF0.1, LF1 and LF10 indicate cells treated with 0.1, 1 and 10 g/L of bLF.

Access. number	Protein name	% coverage	Unique peptides	Score	Fold change vs. LF0			Overall P
					LF0.1	LF1	LF10	
Lactoferrin uptake								
P24627	Lactoferrin	38.3	22	1989	1.93	3.09	3.27	0.012
Proliferation, apoptosis and cell death								
F1SJB5	Annexin 1	37.9	12	1031	1.10	1.15	1.21	0.013
K7GP58	Apoptosis-inducing factor, isoform 3	12.2	4	117	0.93	0.93	1.31	0.008
P00795	Cathepsin D	21.7	6	412	1.35	1.37	1.28	0.018
F1RTY6	Peptidyl-prolyl cis-trans isomerase d	18.7	5	101	1.26	1.19	1.88	0.011
Q9TSX9	Peroxiredoxin-6	42	7	342	2.55	2.32	2.69	0.005
Metabolic process								
Q6RWA7	Acyl-CoA desaturase	7.8	3	64	1.74	1.35	1.25	0.014
P12026	Acyl-CoA-binding protein	14.9	1	38	1.39	1.41	1.59	0.004
Q710C4	Adenosylhomocysteinase	16.2	4	224	1.29	1.23	1.52	0.022
F1SG41	Aldehyde dehydrogenase	6.6	2	74	1.00	1.10	1.72	0.025
F1S0N2	ATP-citrate synthase isoform 2	9.4	1	127	1.49	1.45	2.03	0.050
F1RTA1	Canalicular multispecific organic anion transporter 2, isoform 4	5.2	4	115	1.24	1.04	1.06	0.030
F1RPD2	Cytochrome b-c1 complex subunit	13.7	3	466	1.20	1.17	0.94	0.039
F1SEV8	Elongation factor 1-delta isoform 2	14.8	2	466	1.03	0.98	1.39	0.011
I3LGP6	GDP-l-fucose synthase	3.5	1	64	1.20	1.16	1.36	0.048
F1RGS2	Glucosylceramidase	11.6	4	109	1.10	1.19	1.32	0.039
F1SJS0	Malonyl-acyl carrier protein	9.9	1	63	1.26	1.08	1.00	0.045
B8XSJ9	Monoglyceride lipase	6.6	1	31	1.07	1.22	1.12	0.044
F1RTV5	Multifunctional protein ADE2	6.5	3	168	1.21	1.11	1.68	0.032
F1SUZ2	Nuclear pore complex protein nup98-nup96	2.9	4	91	1.13	1.07	1.71	0.011
F1SHL9	Pyruvate kinase	38.7	19	1666	1.78	1.52	2.23	0.002
F1RUV5	Pyruvate carboxylase	9.4	4	122	1.23	1.10	0.95	0.012
F1RK10	Succinyl-CoA ligase subunit β	26.4	7	245	1.39	1.27	0.91	0.000
F1SE08	Serine palmitoyltransferase 2	5.3	2	52	1.38	1.17	1.09	0.008
I3LL84	UDP-glucose 4-epimerase	2.9	1	42	1.29	1.24	1.62	0.039
Signal transduction								
F1SM07	COP9 signalosome complex subunit 8	16.8	2	99	0.98	1.15	1.31	0.002
F1RYY5	EGF receptor kinase substrate 8	11.4	4	116	1.16	1.23	1.52	0.044
I3LUM9	Eukaryotic translation initiation factor 2 subunit 3	21.4	7	110	1.18	1.23	1.89	0.022
F1RHJ2	Hepatoma-derived growth factor	35.4	6	102	1.55	1.74	1.56	0.010
F1RT62	Integrin α3	6	6	130	0.98	0.96	1.31	0.034
I3LLW7	Nodal modulator 2 isoform 2	7.2	1	127	1.47	1.51	1.34	0.037
F1RMX2	Prenylated Rab acceptor protein 1	8.1	1	50	0.98	1.15	1.64	0.040
F1RHM8	Ran-specific GTPase-activating protein	22.3	4	175	2.64	2.29	3.31	0.007
F1SJ46	Secretory carrier-associated membrane protein 2	7	1	42	1.02	1.03	1.41	0.043
I3LC80	Semaphorin-7a isoform 2 (CD108)	8	2	157	1.16	1.38	1.00	0.003
F1S8G6	Small glutamine-rich tetratricopeptide repeat-containing protein α	5.4	1	69	1.10	1.12	1.58	0.015
F1SLU6	Ubiquitin carboxyl-terminal hydrolase	14.3	8	313	1.95	1.84	2.51	0.027
Metal ion binding and transport								
P60902	Protein S100-A10	28.9	4	55	1.47	1.29	1.03	0.007

F1SCI9	Syntaxin-binding protein 2	5.1	2	58	1.11	1.08	1.31	0.005
F2Z5H8	Transcription factor btf3 isoform 2	29.1	4	242	1.81	1.73	2.41	0.029
DNA and RNA binding and processing								
I3LGJ5	182 kda tankyrase-1-binding protein	20.6	23	1051	1.09	1.07	1.28	0.009
I3LP78	60s ribosomal protein L9	19.3	3	361	1.57	1.48	2.42	0.043
F1S1X3	Asparagine-tRNA ligase	9.1	4	274	1.60	1.56	2.73	0.023
F1S8H5	DNA-(apurinic or apyrimidinic site) lyase	8.2	3	119	1.88	1.84	2.59	0.007
F1SII4	Glycine-tRNA ligase	18.3	11	295	1.39	1.42	1.80	0.029
F2Z5B4	Heterogeneous nuclear ribonucleoprotein a3	25.9	6	171	1.26	1.54	1.27	0.036
I3LAT6	Isoleucine-tRNA ligase	11.3	2	69	1.15	1.14	1.64	0.048
I3LVK3	Leucine-tRNA ligase	11.5	6	257	1.34	1.42	1.91	0.015
Q8MIZ3	m7GpppX diphosphatase	7.4	2	44	1.20	1.39	1.63	0.001
I3LMJ1	Non-histone chromosomal protein HMG-14	15.7	2	310	2.59	2.68	2.19	0.001
I3LJQ2	Nucleolar complex protein 3 homolog	1	1	76	0.87	0.92	1.22	0.030
I3L6I8	Probable rRNA-processing protein EBP2	9.9	2	105	0.97	0.85	1.22	0.038
P10775	Ribonuclease inhibitor	14.9	5	173	1.74	1.69	2.16	0.029
F1S5Z3	Serine--tRNA ligase	11.5	3	191	1.18	1.14	1.35	0.007
F1S0R7	tRNA (cytosine -c)-methyltransferase	10.3	4	192	2.03	1.79	2.73	0.007
F1SAP4	Tryptophan-tRNA ligase	11.2	3	123	1.46	1.33	1.55	0.007
F1RSH4	U3 small nucleolar rna-associated protein 18	6.1	1	72	0.98	1.04	1.21	0.013
Cytoskeleton and cell mobility								
F1SMN5	Filamin-c	18.1	25	1965	1.26	1.24	1.46	0.021
F1S0L1	Keratin type i cytoskeletal 14	27.8	4	704	0.96	0.89	1.25	0.041
F1SV88	MARCKS-related protein	56.3	5	378	1.39	1.61	1.88	0.030
F1RIV6	Mical-like protein 2, isoform 4	2.34	1	29	1.22	1.14	1.48	0.045
Q767M0	Phostensin	7.4	3	73	1.13	1.11	1.21	0.007
F1SP56	Protein nap homolog 3a	11.7	1	65	0.86	0.90	2.02	0.006

Number of unique peptides, % coverage and protein score of each identified protein were reported in a representative plex with the highest number of unique peptides among the 4 analyzed plexes.

Table 2 Down-regulated proteins by bLF (0.1-10 g/L) in PsIc1 cells. LF0.1, LF1 and LF10 indicate cells treated with 0.1, 1 and 10 g/L of bLF.

Access. number	Protein name	% coverage	Unique peptides	Score	Fold change vs. LF0			Overall P
					LF0.1	LF1	LF10	
Proliferation, apoptosis and cell death								
I3LKI5	7-dehydrocholesterol reductase	1.3	1	30	1.07	0.94	0.69	0.015
F1SGS9	Catalase	6.8	3	65	0.77	0.76	0.55	0.008
K7GST0	Cd63 antigen isoform 3	21.4	3	112	0.80	0.74	0.31	0.024
F1RQZ0	Granulins	3.8	2	41	0.99	0.73	0.64	0.004
F1REX8	Huntingtin-interacting protein 1	2.5	1	75	1.06	1.04	0.72	0.027
F1SDQ3	Transmembrane protein 214	4.7	2	62	0.94	0.95	0.45	0.006
Metabolic process								
F1S2U6	Acyl- :lysophosphatidylglycerol acyltransferase 1	2.3	1	30	1.01	1.06	0.73	0.015
F1SBN7	ATP synthase f complex subunit	21.9	4	286	1.00	0.93	0.71	0.004
I3L9F1	Basigin Isoform 2	17.9	4	123	1.06	0.95	0.85	0.020
F1S937	Beta-1,3-galactosyltransferase 8	5.9	2	50	0.87	0.75	0.67	0.046
P00889	Citrate synthase	18.5	7	373	1.05	0.97	0.76	0.032
F1RPD4	Cytochrome b-c1 complex subunit	19.6	1	292	0.83	0.70	0.55	0.001
F1S1F3	Cytochrome c oxidase assembly protein 3,	38.7	4	159	0.78	0.71	0.68	0.047
F1RR45	Carnitine acetyltransferase	11.2	4	184	0.88	0.85	0.70	0.005
F1SC47	Delta-1-pyrroline-5-carboxylate synthase	20.5	13	342	1.08	1.00	0.75	0.008
F1SN47	Glutaminase kidney isoform 3	6.8	5	59	0.90	0.81	0.91	0.007
I3LVG4	GrpE protein homolog	31.8	6	120	0.84	0.83	0.72	0.014
I3L7C2	Lanosterol synthase	11.9	3	84	1.13	0.97	0.81	0.039
F1SR72	Long-chain-fatty-acid ligase 3	4.4	2	89	0.91	0.87	0.56	0.020
I3LIC1	Microsomal glutathione s-transferase 2	26.4	1	91	0.84	0.91	0.96	0.032
F1SIS9	NADH dehydrogenase	5.9	1	36	1.06	0.82	0.52	0.049
I3LJH3	Pyrroline-5-carboxylate reductase	53.9	3	250	0.89	0.79	0.72	0.031
F1S8R2	Pyrroline-5-carboxylate reductase	26.9	4	406	0.94	0.92	0.80	0.025
K7GMN8	Pyruvate dehydrogenase E1	19.5	5	210	1.13	1.03	0.75	0.010
F1SUP2	Signal peptidase complex subunit 2	15.5	2	32	0.75	0.68	0.75	0.043
F1SNZ7	Succinyl-CoA ligase subunit α	13.3	4	51	1.06	1.03	0.75	0.015
Q29551	Succinyl-CoA:3-ketoacid coenzyme A transferase 1	12.3	5	171	1.03	0.96	0.81	0.012
Signal transduction								
I3LAU7	Feline leukemia virus subgroup c receptor-related protein 2 isoform 2	5.9	2	36	0.83	0.71	0.58	0.020
I3LHV8	Homolog subfamily b member 1	2.7	1	56	0.91	0.95	0.79	0.032
F1SDZ8	Protein sel-1 homolog 1	7.2	2	167	0.96	0.89	0.84	0.017
I3L5C0	Vesicle-associated membrane protein-associated protein A	10.2	1	55	0.86	0.68	0.49	0.036
Metal ion binding and transport								
I3LLQ8	AFG3-like protein 2	12.4	5	155	0.89	0.80	0.50	0.044
F1S086	Aspartate glutamate carrier 1	17.2	2	80	1.08	0.85	0.62	0.040
I3LTD9	High affinity cationic amino acid transporter 1	19.5	3	132	0.84	0.84	0.67	0.009
I3LDY2	Isopentenyl-diphosphate delta-isomerase 1	6.3	1	40	1.01	0.90	0.59	0.019
F1RN44	Lysosome-associated membrane glycoprotein 1	4.6	2	38	0.88	0.78	0.66	0.055
Q27HK4	Metaxin-1	8.2	2	57	0.83	0.82	0.90	0.004
F1RYY8	Mitochondrial glutamate carrier 1	12.7	4	59	0.85	0.70	0.47	0.016

F1SSK4	Mitochondrial import inner membrane translocase subunit Tim9	21.4	2	79	0.88	0.83	1.01	0.010
F1RP76	Sciellin	15.2	2	40	0.86	0.77	0.70	0.020
D7RK08	Transferrin receptor protein	11.7	8	311	1.02	0.82	0.75	0.006
F1SB09	Vesicle-trafficking protein sec22b	32.1	5	101	0.86	0.92	0.68	0.038
F1SDY8	Zinc finger ccch domain-containing protein 14	7.7	3	79	0.93	0.89	0.75	0.014
DNA and RNA binding and processing								
F1SID4	26s protease regulatory subunit 6a	12.1	5	73	0.95	0.81	1.09	0.031
F1RIC1	Coiled-coil domain-containing protein 86	6.6	2	109	0.76	0.78	0.67	0.026
D3K5N7	Embryonic lethal, abnormal vision	17.2	5	142	0.85	0.88	1.18	0.013
F1RX74	Heterogeneous nuclear ribonucleoproteins a2 b1 (hnRNP a2/b1)	23.8	1	268	0.75	0.63	0.56	0.000
F1SSN5	Kinectin	18.7	19	511	0.88	0.87	0.74	0.029
F1SBU7	Lon protease homolog	13.1	10	311	0.83	0.81	0.77	0.023
F1S8T1	Nucleolar 130 kDa protein	23.6	11	370	0.78	0.72	0.64	0.028
I3LRT6	Peptidyl-prolyl cis-trans isomerase	5.4	2	36	0.89	0.93	0.64	0.023
I3LPQ4	Ribosomal RNA small subunit methyltransferase nep1	18.4	3	62	1.02	0.92	0.84	0.005
Cytoskeleton and cell mobility								
I3LJA8	Collagen alpha-2 chain	12.4	7	530	0.81	0.72	0.55	0.040
F6Q4Y6	F-actin-capping protein subunit alpha-1	28	5	375	1.00	1.08	0.82	0.008
F1S0J8	Keratin type i cytoskeletal 19	66.3	12	10106	0.84	0.76	0.68	0.003
F1SNQ1	Tight junction protein zo-1	3.6	2	56	0.76	0.81	1.10	0.015
I3LIL4	Unconventional myosin-ic	14.3	9	423	0.87	0.84	0.79	0.010
Others								
A1XQR7	Protein QIL1	22.9	2	102	1.05	0.96	0.83	0.032
F1S3M6	Sterile alpha motif domain-containing protein 3	1.1	1	21	1.04	0.95	0.79	0.000
F1SQC6	Transmembrane protein 111	10	2	113	0.90	0.82	0.65	0.004

Number of unique peptides, % coverage and protein score of each identified protein were reported in a representative plex with the highest number of unique peptides among the 4 analyzed plexes.

LF stimulation increased the intracellular uptake of LF

Intracellular bLF levels increased in LF0.1, LF1 and LF10 compared with the control LF0, measured by both SDS-PAGE and proteomics, indicating an increased cellular uptake of bLF following cell stimulation (Fig. 1). In addition, compared with LF0.1, LF1 had a 1.5-fold higher intracellular bLF level. This was not increased further at 10 g/L (Fig. 1B).

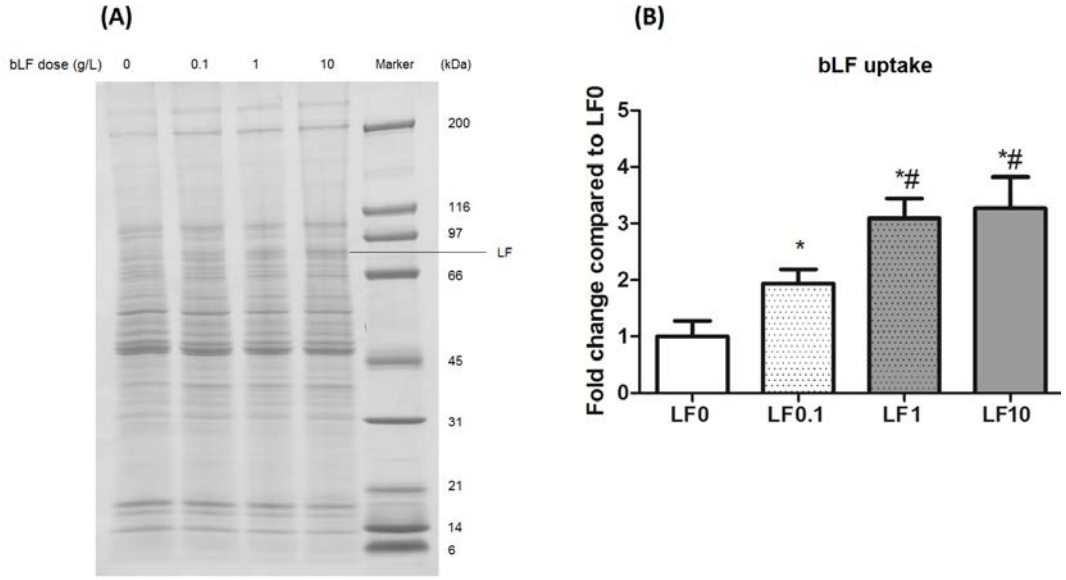

Figure 1 Lactoferrin (LF) detected in porcine IECs by SDS-PAGE (A) and by quantification from iTRAQ-LCMS-based proteomics following treatment with LF 0, 0.1, 1 and 10 g/L for 24 h (B). * and #: $P < 0.05$ compared with LF0 and LF 0.1, respectively.

LF regulated proteins involved in cell proliferation and cell death

A series of 10 proteins involved in the regulation of proliferation, apoptosis and cell death were differentially expressed by bLF, especially at the high dose (10 g/L, Fig. 2A). Four of them, including apoptosis inducing factor (AIF), annexin-1, cyclophilin-40 and cathepsin D, are proteins playing roles in the early phase of apoptosis and cell death. Among these, AIF, annexin-1 and cyclophilin-40 were only elevated by 10 g/L bLF, whereas cathepsin D was up-regulated by both LF0.1, LF1 and LF10. Among three proteins having protective effects against cell death, catalase and huntingtin-interacting protein 1 (HIP1) were down-regulated in only LF10 whereas all bLF doses up-regulated peroxiredoxin-6. The catalase level in LF10 was also lower than that in LF0.1. Three proteins involved in cell proliferation including CD63, granulins and 7-dehydrocholesterol reductase (7DHCR) were down-regulated in LF10 to lower levels than LF0.1 and LF1, whereas they were not affected by LF0.1 and LF1, compared with LF0.

LF regulated cellular energy metabolism

bLF stimulation regulated the levels of a series of 12 proteins involved in cellular energy metabolism (Fig. 2B). Two proteins involved glycolysis (pyruvate kinase, UDP-glucose 4-epimerase), six proteins of TCA cycle (pyruvate carboxylase, pyruvate dehydrogenase, citrate synthase, succinyl-CoA ligase subunit α and β, and succinyl-CoA: 3 keto acid coenzyme A transferase 1), three proteins of electron transport (NADH dehydrogenase, cytochrome b-c1 complex subunit and cytochrome c), and one protein participating in ATP-forming reactions (ATP f complex subunit) were differentially expressed. These pathways are all involved in ATP synthesis. Both moderate and high doses of bLF (1 and 10 g/L) elevated the two proteins involved in glycolysis, but 10 g/L bLF up-regulated them to higher levels than 0.1 and 1 g/L. bLF at 10 g/L down-regulated all the 10 proteins involved in the TCA cycle, electron transport chain and ATP synthesis. The down-regulated levels in LF10 were lower than those in LF0.1 and LF1 for eight out of the ten proteins (except citrate synthase and cytochrome c). LF1 did not affect these energy metabolism-related proteins, compared with LF0. In contrast, 0.1 g/L bLF elevated levels of pyruvate dehydrogenase of the TCA cycle.

LF up-regulated HIF-1 pathway

bLF differentially expressed three proteins involved in the HIF-1 inflammatory /hypoxia pathway (Fig. 2C) including ubiquitin carboxyl-terminal hydrolase (UCH), DNA-(apurinic or apyrimidinic site) lyase (Apex/Ref-1), and heterogeneous nuclear ribonucleoprotein a2/b1 (hnRNP a2/b1). UCH and Apex/Ref-1, two protein facilitating HIF-1 pathway, were up-regulated by all bLF doses, with higher levels in LF10 than in LF0.1 and LF1. In contrast, hnRNP a2/b1, which is an inhibitor of the HIF-1 pathway, was down-regulated to lower levels by 10 g/L bLF than by the control and lower doses.

LF up-regulated aminoacyl-tRNA ligases

Six cytoplasmic aminoacyl-tRNA ligases, which are involved in the initial step of protein synthesis, were regulated by bLF (Fig. 2D). Glycine, isoleucine, and tryptophan tRNA ligases were up-regulated by all three doses of bLF, and bLF at 10 g/L led to a further increase in these protein levels compared with lower doses. In addition, the other three ligases including asparagine, leucine and serine -tRNA ligases were only up-regulated in LF10, but not in LF0.1 or LF1. The level of serine-tRNA ligase in LF10 was also higher than that in LF0.1 and LF1.

Conclusion

This study demonstrates, for the first time, that the dose of bLF plays an important role when interacting with IECs by regulating bLF uptake, cellular energy metabolism, proliferation and cell death. Combining current data and our previous data on the effects of bLF on cytokine secretion and signaling pathways in PsIc1 cells, we propose mechanisms of how low and high doses of bLF act on IECs (Fig. 3). These mechanisms may influence the physiology of the small intestine, and play roles in intestinal inflammation and NEC in preterm neonates. Low doses (0.1-1 g/L) of bLF facilitate the binding between bLF and LfR, leading to receptor internalization and bLF uptake (Fig. 3A). These may be applied to all stimulated bLF molecules so that no other bLF-induced inflammatory signaling pathways occur. These events also lead to ERK activation, up-regulation of proteins involved in energy metabolism and protection against oxidative stress. The endpoints may be cell proliferation and maintenance of cellular homeostasis. In contrast, when high doses of bLF (10 g/L) are present, some bLF molecules are used for binding to LfR, leading to internalization and bLF uptake, whereas the remaining proportion of bLF may facilitate inflammatory signaling via binding to TLR-4 or TNFR (Fig. 3B). The latter facilitates a series of activities including ERK inhibition, HIF-1 and NF-κB activation, down-regulation of proteins involved in energy metabolism, proliferation and protection against stress, and up-regulation of proteins playing roles in cell death. The endpoints of these events may be inflammatory responses, decreased cell proliferation, and increased cell death. These results correspond well with our previous *in vivo* findings in the preterm pig model of neonatal NEC, where 10 g/L of bLF enriched infant formula did not protect against NEC, but rather tended to exacerbate NEC lesions. The evidence found in the current *in vitro* study may support and explain the adverse effects of excessive doses of bLF.

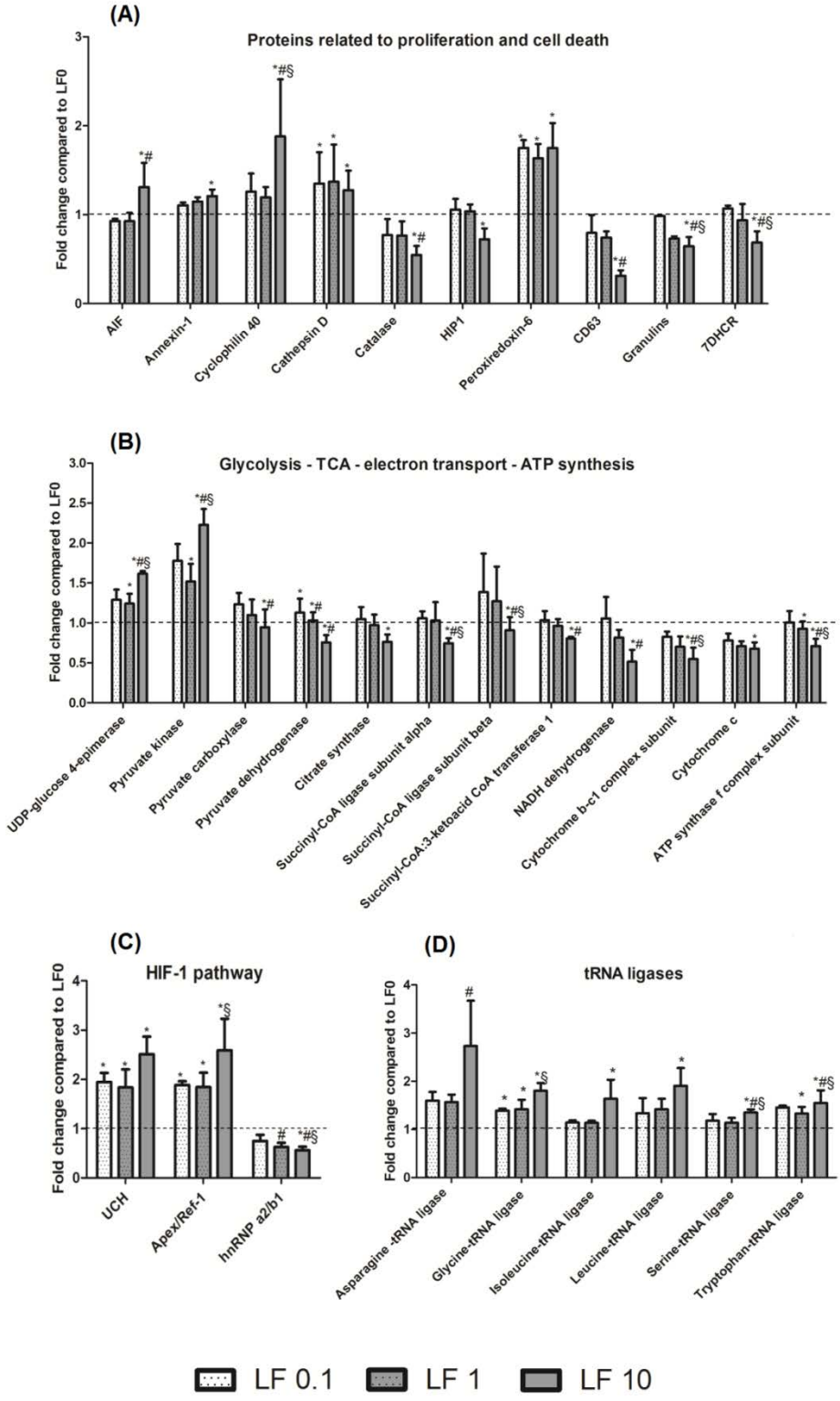

Figure 2 Fold change (compared with LF0) of proteins involved in cell proliferation and cell death (A), in energy metabolism (B), in the HIF-1 pathway (C), and aminoacyl-tRNA ligase activity (D) by bLF with different doses for 24 h.*, #, and §: $P < 0.05$, relative to LF0, LF0.1 and LF1, respectively. AIF: apoptosis inducing factor; HIP1: Huntingtin interacting protein 1; 7DHCR:7-dehydrocholesterol reductase; UCH: ubiquitin carboxyl-terminal hydrolase; hnRNP a2/b1: heterogeneous nuclear ribonucleoprotein a2/b1.

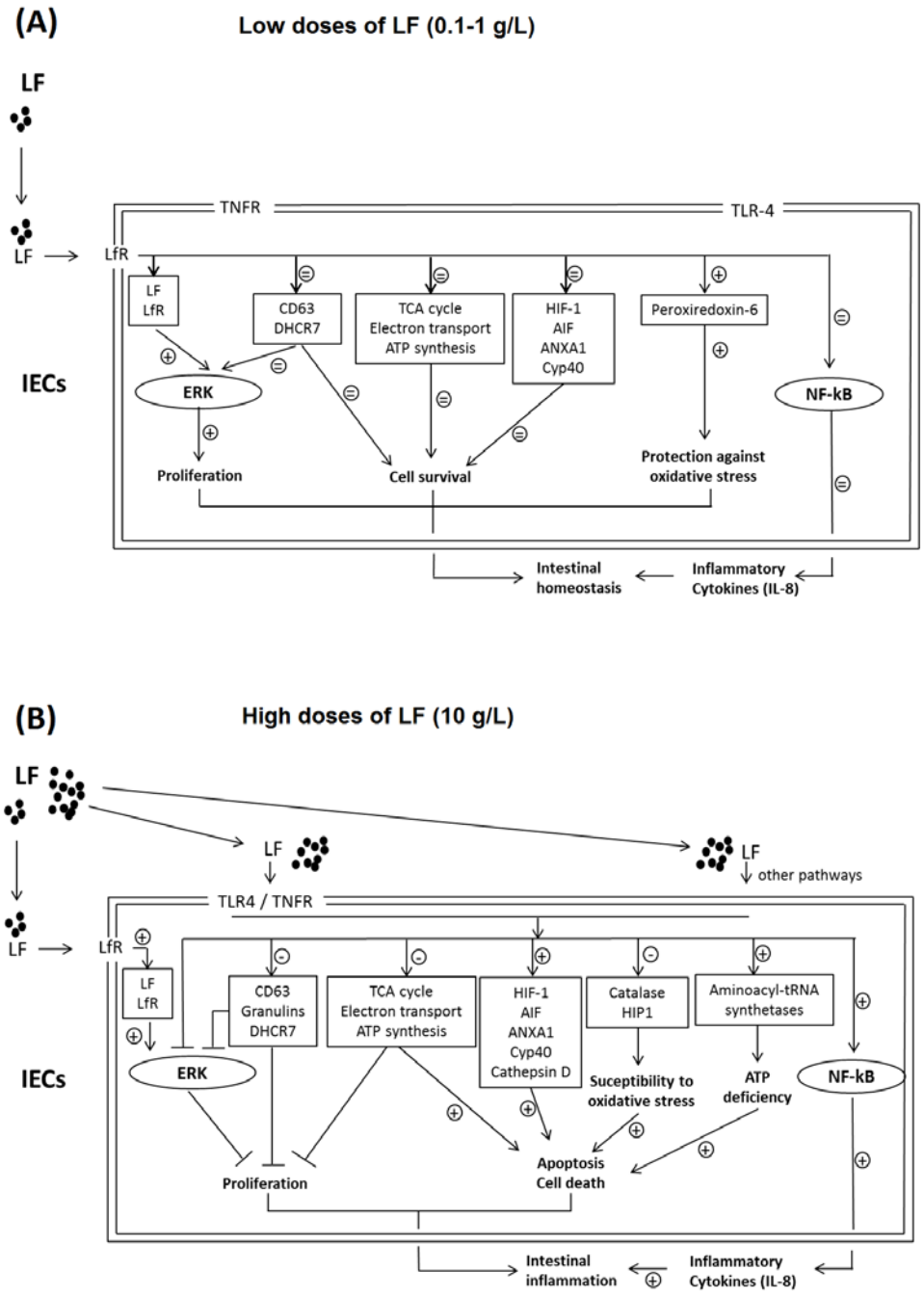

Figure 3 Proposed mechanisms of how low and high doses of bLF influence IEC cellular activities leading to different inflammatory states of the small intestine *in-vivo*. Low doses of bLF (0.1-1 g/L) stimulate LF uptake and ERK activation, up-regulate proteins involved in energy metabolism and protection against stress, thereby leading to cellular and intestinal homeostasis (A). In contrast, high doses of bLF (10 g/L) inhibit ERK, activate HIF-1 and NF-κB secreting inflammatory cytokines, down-regulate proteins involved in cell proliferation and protection against stress, and up-regulate proteins involved in cell death, thereby resulting in intestinal inflammation (B). "+", "-", and "=" indicate increased, decreased and consistent signaling, respectively. "---|" indicates inhibition of signaling.

9. Afvigelser

9.1 Fagligt None

9.2 Økonomisk None

9.3 Tidsplan None

10. Planer for næste halvår

We plan to finalize and submit the manuscript of the LF proteomic study.

11. Formidling og vidensdeling vedr. projektet

Nguyen, D. N., Li, Y., Sangild, P. T., Bering, S. B., & Chatterton, D. Effect of bovine lactoferrin on the immature porcine intestine. *British Journal of Nutrition*. **2014**. *111*:321-331.

Nguyen, D. N., Sangild, P. T., Østergaard, M. V., Bering, S. B., & Chatterton, D. TGF-β2 and endotoxin interact to regulate interleukin 8 levels in the immature porcine intestine. American journal of Physiology- Gastrointestinal and liver physiology. **2014**. 307: G689-G699

Nguyen, D. N., Sangild, P. T., Bering, S. B., & Chatterton, D. Processing of whey modulates proliferative and immune functions in intestinal epithelial cells. Manuscript submitted to Food Chemistry.

Nguyen, D. N., Jiang, P., Jacobsen, J., Sangild, P. T., Bendixen, E. & Chatterton, D. Protective effects of transforming growth factor-β2 in intestinal epithelial cells by regulation of proteins associated with stress and endotoxin responses. Manuscript submitted to PLOS ONE, and under revision.

Nguyen, D. N., Jiang, P., Sangild, P. T., Stensballe, A., Bendixen, E. & Chatterton, D. Bovine lactoferrin regulates cell survival, cell death and energy metabolism in intestinal epithelial cells. Manuscript in preparation.

12. Nye kontakter

None