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Supplementing formula-fed piglets with a low molecular weight fraction of bovine colostrum whey results in an improved intestinal barrier^{1,2}

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ABSTRACT: To test the hypothesis that a low molecular weight fraction of colostrum whey could affect the morphology and barrier function of the small intestine, 30 3-d-old piglets (normal or low birth weight) were suckled ($n = 5$), artificially fed with milk formula ($n = 5$), or artificially fed with milk formula with a low molecular weight fraction of colostrum whey ($n = 5$) until 10 d of age. The small intestine was sampled for histology (haematoxylin and eosin stain; anti-KI67 immunohistochemistry) and enzyme activities (aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV, lactase, maltase, and sucrase). In addition, intestinal permeability was evaluated via a dual sugar absorption test and via the measurement of occludin abundance. Artificially feeding of piglets reduced final BW ($P < 0.001$), villus height ($P < 0.001$), lactase ($P < 0.001$), and dipeptidylpeptidase IV activities ($P < 0.07$), whereas crypt depth ($P < 0.001$) was increased.

No difference was observed with regard to the permeability measurements when comparing artificially fed with naturally suckling piglets. Supplementing piglets with the colostrum whey fraction did not affect BW, enzyme activities, or the outcome of the dual sugar absorption test. On the contrary, the small intestines of supplemented piglets had even shorter villi ($P = 0.001$) than unsupplemented piglets and contained more occludin ($P = 0.002$). In conclusion, at 10 d of age, no differences regarding intestinal morphology and permeability measurements were observed between the 2 BW categories. In both weight categories, the colostrum whey fraction affected the morphology of the small intestine but did not improve the growth performances or the *in vivo* permeability. These findings should be acknowledged when developing formulated milk for neonatal animals with the aim of improving the performance of low birth weight piglets.

Key words: bovine colostrum, epithelial barrier, low birth weight, neonatal piglets

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INTRODUCTION

Interest in the physiological functions of bioactive substances present in colostrum and milk has increased in recent years (Bendixen et al., 2011). Bovine colostrum has been used as an alternative for fetal bovine serum to support growth in rat (Thoreux

et al., 1996; Playford et al., 1999), human (Takeda et al., 2004; Purup et al., 2007), and swine intestinal cells (Roselli et al., 2007). Additionally, various studies have confirmed that orally fed bioactive substances such as IGF-1 (Burrin et al., 1996; Houle et al., 1997), epidermal growth factor (Zijlstra et al., 1994), leptin (Wolinski et al., 2003), ghrelin (Kotunia et al., 2006), insulin (Shulman, 1990), and arginine (Kim and Wu, 2007) affect small intestinal growth and maturation of piglets. The positive effect of milk-born substances could be particularly important for smaller piglets because several studies reported that intrauterine growth restriction prevented the development and maturation of the small intestine, hampering nutrient utilization

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and barrier function (Xu et al., 1994; Wang et al., 2005, 2010; D'Inca et al., 2010).

Most of these milk-derived bioactive factors are small proteins or polypeptides, which are found in the whey fraction of colostrum or mature milk (Madureira et al., 2007). Surprisingly, these milk-born factors are poorly studied when they are administered simultaneously or in their natural matrix. Previous in vitro research using intestinal porcine epithelial jejunal cells pointed out that the low molecular weight fraction of bovine colostrum seems promising in stimulating growth of enterocytes and in maintaining integrity. The hypothesis tested in the current study was that supplementing formula-fed piglets with a low molecular weight fraction of colostrum, stimulates small intestinal growth, improves intestinal barrier function and digestive enzyme activities, and, thus, positively affects BW gain and the functional maturation of the gut.

MATERIAL AND METHODS

Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by the Ethical Committee of Animal Experimentation, University of Antwerp, Belgium.

Preparation of the Colostral Whey Fraction

Bovine colostrum powder (Biofiber- Damino A/S, Gesten, Denmark) was reconstituted to 10% (wt/vol) in saline (NaCl, 0.9%) and centrifuged ($10,000 \times g$ for 30 min at 4°C) to separate the cream from the skim milk. Colostral whey was prepared by adding rennin (R 7751; Sigma-Aldrich, Steinheim, Germany) to milk and removing the curds by centrifugation ($3,000 \times g$ for 30 min at 4°C). The resulting whey was microfiltrated (0.2- μ m pore filter; Schleicher and Schuell, Dassel, Germany). Clear whey was further ultrafiltrated using a ultrafiltration concentrator with a filter device of 50 kDa (Vivacell 250; Sartorius Stedim, Vilvoorde, Belgium). The colostrum whey filtrates (CWF), and not residues, were used for further experiments.

Analysis of the Colostral Whey Fraction

The protein content of the colostral whey fraction was determined (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Before digestion, 100 μ g of the ultrafiltrated sample was precipitated overnight at -20°C by adding 6 volumes of ice-cold acetone. After centrifugation, the supernatant was removed and the protein pellet was resuspended in 50mM Tris-HCl/6M urea/5mM DTT/10% β -mercaptoethanol (25 μ L/100 μ g protein) at a pH of 8.7.

For the denaturation and reduction process, all samples were incubated at 65°C for 1 h. Subsequently, proteins were diluted in 50 mM Tris-HCl/1 mM CaCl_2 (75 μ L/100 μ g protein) and then alkylated by adding 200 mM iodoacetamide (10 μ L/100 μ g protein) for 1 h at room temperature. Finally, proteomics-grade modified trypsin (Roche, Mannheim, Germany) was added at a 30:1 protein-to-enzyme. After incubation at 37°C for 18 h the digestion was stopped by freezing the fractions at -80°C.

Peptides were separated based on hydrophobicity by using a reverse phase C18 column on a microcapillary HPLC system (Agilent 1100 Series; Agilent Technologies, Waldbronn, Germany). Ten micrograms of peptides were injected on a Zorbax 300SB-C18 guard column (0.3 $\times$ 5 mm; particle size 3.5 μ m; Agilent Technologies) serially connected with a Zorbax 300SB-C18 analytical column (0.3 $\times$ 150 mm; particle size 3.5 μ m; Agilent Technologies). Solvent A contained 0.1% formic acid (FA) in water, while solvent B contained 0.1% FA in 90% acetonitrile (ACN). Then, ACN gradient was applied using the capillary pump with a constant flow rate at 6 μ L/min: 5 to 55% B in 56.7 min, increase quickly to 90% B over 3.3 min, persistent 90% B for 5 min, 85% B for 5 min, and back to equilibrating conditions of 3% B. Starting from min 5 till min 51.7 of the chromatographic run, 350 spots (800 nL/spot) were spotted (Opti-TOF MALDI-Target; Applied Biosystems, Inc., Foster City, CA). Afterward, each spot was covered with matrix (2 mg/mL α -cyano-hydroxy cinnamic acid in 70% ACN; internal calibrant: 63 pmol/mL human [Glu¹]-fibrinopeptide B) using an external syringe pump at a flow rate of 6 μ L/min (800 nL/spot).

Spotted fractions were analyzed using a matrix assisted laser desorption/ionization (MALDI; AB4800 Proteomics Analyzer; Applied Biosystems, Inc.). The MALDI-ToF MS-analysis (reflectron mode; laser intensity, 3,200; 25 $\times$ 20 laser shots per spot; mass range 800–3,000 Da) was performed first, after which precursors with a signal-to-noise (S/N) ratio above or equal to 100 were selected. The MALDI-ToF/ToF MS/MS-analysis was performed on these selected precursors, and a maximum of 50 unique precursors per spot were selected for fragmentation, beginning with the precursors with the lowest S/N ratio. These precursors were ionized (laser intensity: 4,200; 25 $\times$ 20 laser shots per spot) and fragmented in a collision cell (1kV collision) with air.

Acquired MS/MS spectra were screened against the mammalian Swiss-Prot database (Version: 57.1, www.uniprot.org) using the MASCOT search engine (Matrix Science; Version 2.1.03, Matrix Science Inc., Boston, MA) with *Bos Taurus* being the only criterion. Carbamidomethylation of Cys was listed as fixed modification, and oxidation of Met was listed as a variable modification. A maximum of 2 missed cleavages

of trypsin was tolerated. The mass tolerance was set to 0.5 Da for the precursors and 0.2 Da for the fragment ions. The MudPIT scoring algorithm of MASCOT was used. Scaffold (Version Scaffold_3_00_03; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Amino acid compositions were determined by using the freeware tool (ProtParam; <http://web.expasy.org/protparam>) based on the accession number of each identified protein.

Animal Experiment

Crossbred piglets [(Pietrain × (Finnish Yorkshire × Belgian Landrace))] were obtained from a commercial farm. From 15 litters, pairs of low birth weight (LBW; 0.96 ± 0.06 kg at birth) and normal birth weight (NBW; 1.49 ± 0.12 kg at birth) piglets were selected at birth and allotted to 3 experimental treatments at 3 d of age. Piglets with a BW below 1 kg were considered to be LBW, whereas those with a BW close to the mean of the litter were considered to be NBW. Each experimental group consisted of 10 sex-matched piglets, i.e., 2 littermate piglets (LBW and NBW) from 5 different litters. The LBW and NBW piglets remained with the sow and suckled until d 10 (SOW; $n = 5$), weaned at 3 d of age and fed formula until d 10 (FOR; $n = 5$), or weaned at 3 d of age and fed formula with CWF supplement (SUP; $n = 5$). After transporting from the farm, piglets of the FOR and SUP group were group-housed, without separating littermates, in a commercial brooder. All formula-fed (FOR and SUP) piglets were ad libitum fed using a semiautomatic milk dispenser. All piglets learned to use the feeding system within 6 h. The formulated milk used in this study was a complete milk replacer for young piglets (Piggylac; Nuscience group, Drongen, Belgium), allowing rearing piglets in absence of the sow. Supplemented piglets received the CWF once a day via gavage at a dose of 10 mL/kg BW. The AA composition of the CWF fraction is presented in Table 1. Piglets in all experimental groups had free access to water, while no solid feed was available.

Permeability Measurements

To measure in vivo gut permeability, the piglets were dosed intragastrically with 0.75 g lactulose and 0.3 g mannitol/kg BW (Sigma-Aldrich, Steinheim, Germany) 4 h before euthanasia (Bjarnason et al., 1995; Kansagra et al., 2004; Bjornvad et al., 2008). Urine was collected by cystocentesis at the time of euthanasia. Concentrations of lactulose and mannitol in urine were measured as a percentage of urine recovery using an enzymatic spectrophotometric method (Behrens et

Table 1. Total amino acid content of the colostrum whey fraction

Item	Content, %
Ala	6.37
Arg	4.81
Asn	3.93
Asp	5.46
Cys	1.98
Gln	5.23
Glu	7.17
Gly	6.66
His	2.14
Ile	4.50
Leu	8.01
Lys	7.21
Met	1.47
Phe	3.40
Pro	6.58
Ser	7.38
Thr	6.12
Trp	1.55
Tyr	3.00
Val	7.02

al., 1984; Blood et al., 1991). Piglets were euthanized by the intraperitoneal injection of an overdose sodium pentobarbital (200 mg/kg) followed by exsanguination.

Tissue Collection

After euthanasia, the intestinal tract was flushed and rinsed and internal organs were weighed. The lengths of small and large intestines were recorded. The entire small intestine was equally divided into a proximal, middle, and distal part. Samples were taken from each small intestinal segment. One set of samples was immediately frozen in liquid N and stored at -80°C . Another set was immersed for 2 h in 4% paraformaldehyde solution at room temperature (0.1 M, pH 7.4).

Morphometric Analysis and Enzyme Activities

After fixation, samples were rinsed with PBS (pH 7.4, PBS) for 24 h and routinely processed to paraffin blocks. Transverse paraffin sections (5 μm) were conventionally stained with haematoxylin-eosin. The thickness of the tunica muscularis and tela submucosa, the length and width of the intestinal villi, crypt depth and the percentage of crypt fission were measured at a magnification of 100x. Villus length and crypt depth were measured, for each tissue block, in 30 well-orientated villi and crypts (Olympus BX 61, analysIS Pro; Olympus Belgium, Aartselaar, Belgium). Crypt fission is expressed as the percentage of crypts having a split or fissure in their

base. The percentage of crypt fission was determined in at least 100 crypts in a total of 10 systematically at random sections (Olympus BX 41; Olympus Belgium). Enzyme activities (aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV, lactase, maltase, and sucrase) of homogenized small intestinal samples were measured according to Michiels et al. (2012). Enzyme activity levels were expressed as units per gram wet tissue.

Immunohistochemical Staining

After antigen retrieval with citrate (pH 6; Dakocytomation, Glostrup, Denmark) for the detection of mitotic cells via anti-KI67 protein (Tanaka et al., 2011) or with proteinase K (DakoCytomation; Glostrup, Denmark) for the detection of occludin, paraffin sections (5 μ m) were incubated with 3% H₂O₂ and 20% normal goat serum. Subsequently, sections were incubated overnight at 4°C with monoclonal mouse antihuman Ki67 antibody (1/25, DakoCytomation) diluted in 50 mM Tris(hydroxymethyl) aminomethane-buffered saline solution enriched with 1% bovine serum albumin (TBS/BSA) or with polyclonal rabbit antioccludin (1/250; Invitrogen, Camarillo, CA) diluted in TBS enriched with 0.3% Triton-X-100 (Sigma, St. Louis, MO). After washing in 50 mM TBS (pH 7.4), sections were incubated with biotinylated goat antimouse antibody (for anti-KI67 protein) or with biotinylated goat-antirabbit antibody (for occluding; 1/200 diluted in TBS/BSA; DakoCytomation) for 30 min at room temperature. Following rinsing with TBS, sections were incubated for 30 min with streptavidin-conjugated horseradish peroxidase (1/200 diluted in TBS/BSA; DakoCytomation) at room temperature. After rinsing with TBS and demineralized water, proliferating cells and the tight junction protein occludin were visualized with 3,3'-diaminobenzidinetetrahydrochloride and tissues were counterstained with haematoxylin. The number of immunoreactive cells for KI67 was expressed as a percentage of the total number of nuclei per crypt. At least 30 well-orientated crypts were counted in the proximal, middle, and distal part of the small intestine of each animal.

Protein Extraction and Western Blot

Protein levels of occludin were measured by Western immunoblotting. Frozen tissues were homogenized in lysis buffer [50 mM Tris, 150 mM NaCl, 1% (wt/vol) Nonidet P40, 0.5% (wt/vol) deoxycholate, and protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany)] and subjected to 10 to 15% SDS-PAGE (Biorad Labs, Hercules, CA) under reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes. Nonspecific protein binding was blocked with 5% (wt/vol) nonfat dry milk in

Tris-buffered saline with Tween 20. Membranes were incubated with a rabbit polyclonal antibody to occludin (Invitrogen, Merelbeke, Belgium; 1/1,000). After washing, blots were incubated with an antirabbit secondary antibody conjugated to biotin (DakoCytomation; 1/1,000). After incubation with avidin/biotinylated horseradish peroxidase (DakoCytomation), detection of bound antibodies was performed using a chemiluminescence system (Super Signal West Femto; Thermo Scientific) according to the manufacturer's instructions. A normalized optical density was obtained by dividing the protein density with the density of the loading control β -actin (Sigma-Aldrich, Steinheim, Germany). Band intensities were quantified by densitometry (GeneSnap and GeneTools Software; Syngene, Cambridge, UK).

Statistical Analysis

The effects of the piglet feed and BW on the different anatomical, physiological, and enzymatic characteristics were assessed by fitting linear mixed models. The BW, feed, and (if applicable) region were included as fixed effects, as well as the 2-way interactions between them. The dependence between observations within the same litter and (if applicable) within the same individual was taken into account by including random intercept and random slope terms into the regression model. The fixed effect model was simplified in a stepwise backward way.

The fixed effects were tested by performing an F-test between the larger model and the reduced model, adjusting the number of degrees of freedom using the Kenwardroger method. In case the final model included a significant effect of region or feed, a post-hoc test was performed to test which of the categories had a difference with a Tukey correction for multiple comparisons. All statistical calculations were performed in the software package R version 2.13.1. Mixed models were fit using the lme4 package. The F-test with Kenwardroger correction was performed using the package pbkrtest, and the posthoc test with Tukey correction was performed as implemented in the multcomp package.

RESULTS

Composition of the Colostral Whey Fraction

The protein content of the colostral whey fraction was 284.5 μ g/mL. Plotting the MS/MS spectra of this fraction (< 50 kDa) against the mammalian Swiss Prot Database with *Bos Taurus* as organism being the only criterion, revealed with a high probability (over 80%) that this fraction contained α -S1 casein, α -S2 casein, β -lactoglobulin, β -casein, β -2-microglobulin, κ -casein, fibrinogen α chain, complement C3, α -lactalbumin, se-

Table 2. Body weight and size and weight of digestive organs in piglets^{1,2}

Item	Normal birth weight			Low birth weight			SEM	P-value		
	SOW	FOR	SUP	SOW	FOR	SUP		Birth weight	Piglet feed	Interaction
Birth weight, kg	1.49	1.49	1.49	0.96	0.96	0.96	0.09	< 0.001	–	–
BW, kg	3.77 ^a	2.74 ^b	2.63 ^b	2.40 ^a	1.79 ^b	1.74 ^b	0.19	< 0.001	< 0.001	0.11
Stomach weight, g/kg	5.89 ^a	7.64 ^b	7.60 ^b	6.49 ^a	9.05 ^b	8.68 ^b	0.63	0.01	< 0.001	0.66
Small intestinal weight, g/kg	36.72	33.70	44.15	36.97	40.36	43.00	3.23	0.22	0.13	0.05
Small intestinal length, cm/kg	169.9 ^a	212.3 ^b	251.2 ^b	222.1 ^a	288.9 ^b	309.7 ^b	16.4	< 0.001	< 0.001	0.56
Colon weight, g/kg	7.02 ^a	9.79 ^b	11.72 ^b	6.55 ^a	10.81 ^b	13.14 ^b	2.32	0.73	< 0.001	0.12
Colon length, cm/kg	26.41 ^a	36.06 ^b	46.48 ^c	41.07 ^a	49.40 ^b	59.53 ^c	2.95	< 0.001	< 0.001	0.93
Caecum weight, g/kg	0.93 ^a	1.03 ^a	1.69 ^b	0.93 ^a	1.20 ^a	1.68 ^b	0.16	0.33	0.003	0.23
Liver weight, g/kg	27.41	28.25	28.65	29.54	27.80	29.43	1.35	0.39	0.70	0.61
Pancreas weight, g/kg	1.71	2.78	2.31	1.72	2.10	2.44	0.38	0.45	0.09	0.33
Spleen weight, g/kg	2.34 ^a	1.71 ^b	1.69 ^b	2.35 ^a	1.88 ^b	1.84 ^b	0.18	0.45	0.003	0.89

^{a-c}Within normal or low birth weight, means not sharing the same superscript letter are different ($P < 0.05$).

¹SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, and SUP = weaned at 3 d of age and fed formula with colostrum whey filtrates until d 10.

²Values are presented as means and pooled SEM ($n = 5/\text{treatment}$).

rum amyloid A, polymeric immunoglobulin receptor, cathelicidin-1, cathelicidin-2, fibroblast growth factor-binding protein 1, haemoglobin subunit α , and zinc finger protein 746. The AA composition of the proteins from this fraction is presented in Table 1.

Piglets' Growth and Anatomy of the Gastrointestinal Tract

Because average daily weight gain was lower in artificially fed piglets compared to those that suckled the sow, they had lower final BW ($P < 0.05$; Table 2). Consequently, gross morphometric data were expressed relative to the final BW. Birth weight and feed-related differences were found with regard to the relative size of the digestive organs. Artificially reared piglets had heavier ($P < 0.05$) stomach and colon ($P < 0.05$), whereas spleen weight ($P = 0.05$) was decreased compared to the SOW treatment. Cecum weight ($P < 0.05$) was greater for piglets on the SUP treatment compared to the other treatments. Small intestinal ($P < 0.05$) and colon lengths ($P < 0.05$) were greater for artificially fed pig-

lets compared to those that suckled the sow. Moreover, SUP piglets had a longer colon compared to FOR piglets ($P < 0.05$). The LBW piglets showed heavier stomach weights ($P = 0.01$), whereas the small intestine and colon ($P < 0.001$) were longer compared to NBW piglets.

Structural and Functional Characteristics of the Small Intestine

The microscopic analysis of the small intestine mainly displayed feed-related differences. Artificial feeding was associated with a marked decrease in villus height ($P < 0.05$) and increase in crypt depth ($P < 0.05$) compared to sow feeding (Table 3). Piglets on the SUP treatment had shorter villi ($P < 0.05$) compared to piglets on the FOR treatment. In addition, the percentage of crypt fission was reduced ($P < 0.05$). There was a birth weight $\times$ feed interaction in the tela submucosa ($P < 0.001$). Fitting separate models for LBW and NBW piglets showed that in the LBW group, there was an effect of feed, with the SUP treatment having a greater mean value compared to

Table 3. Small intestinal morphological characteristics and proliferation index in piglets^{1,2}

	Normal birth weight			Low birth weight			SEM	P-value		
	SOW	FOR	SUP	SOW	FOR	SUP		Birth weight	Piglet feed	Interaction
Crypt depth, μm	78.36 ^a	119.67 ^b	135.47 ^b	74.07 ^a	118.85 ^b	136.20 ^b	1.57	0.39	< 0.001	0.80
Crypt fission, %	4.42 ^a	5.08 ^a	2.54 ^b	5.02 ^a	4.13 ^a	2.23 ^b	0.52	0.71	< 0.001	0.42
Villus height, μm	490.8 ^a	419.5 ^b	309.0 ^c	487.3 ^a	361.9 ^b	253.9 ^c	6.7	0.03	< 0.001	0.39
Villus width, μm	89.06	85.04	89.27	83.38	87.96	85.85	1.09	0.09	0.82	0.36
Tela submucosa, μm	73.75	102.63	92.83	70.45 ^a	64.99 ^a	82.10 ^b	2.72	–	–	< 0.001
Tunica muscularis, μm	94.19	76.96	81.90	91.34	75.65	68.41	2.31	0.13	0.11	0.27
Proliferation index, %	42.55	43.66	47.33	37.87	44.85	46.57	3.90	0.56	0.16	0.73

^{a-c}Within normal or low birth weight, means not sharing the same superscript letter are different ($P < 0.05$).

¹SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, and SUP = weaned at 3 d of age and fed formula with colostrum whey filtrates until d 10.

²Values are presented as means and pooled SEM ($n = 5/\text{treatment}$). No main and interaction effects of region.

Figure 1. (1) The KI67 proliferating cells (red brown) are visible in the lamina propria and are predominantly seen in the crypts of the distal small intestine of a piglet of 10 d old that suckled the sow. Scale bar = 100 μ m. (2) Occludin-immunoreactivity (red) is located at the apicolateral side of the enterocytes of the (distal) small intestinal villi and crypts of a piglet of 10 d old that suckled the sow. Scale bar = 50 μ m. See online version for figure in color.

the 2 other dietary treatments. No effects of dietary treatment were observed in the NBW group. No birth weight, feed-, or region-related differences were observed for villus width, tunica muscularis thickness, and KI67 proliferating index in the small intestine. The KI67 immuno-

reactive proliferating epithelial cells were predominantly located in the crypt region (Fig. 1.1).

The small intestinal enzyme activities mainly displayed region-related differences, except for lactase (Fig. 2.1). Sucrase had greater ($P < 0.05$) activities in the proximal compared to the middle and distal part of the

Figure 2. Small intestinal enzyme activities [(1) lactase, (2) sucrase, (3) maltase, (4) aminopeptidase A, (5) aminopeptidase N, and (6) dipeptidylpeptidase IV] in small intestinal regions of piglets. White bars represent piglets that remained with the sow and suckled until d 10, striped bars represent piglets weaned at 3 d of age and fed formula until d 10, and hatched bars represent piglets weaned at 3 d of age and fed formula with colostrum whey filtrates until d 10. Values represent means and SEM; $n = 5/\text{treatment}$. Means with different superscript letters are different, $P < 0.05$. An asterisk refers to a difference from the SOW treatment, $P < 0.05$.

small intestine (Fig. 2.2), whereas maltase activities decreased from proximal towards to distal part of the small intestine ($P < 0.05$; Fig. 2.3). For aminopeptidase A, an effect of region was observed, with the distal part of the small intestine having a greater ($P < 0.05$) activity compared to the middle part (Fig. 2.4), with the difference between the proximal and distal small intestine showing a trend ($P = 0.09$). Aminopeptidase N activity increased ($P < 0.05$) towards the distal part of the small intestine (Fig. 2.5). An interaction was observed between region and feed for dipeptidylpeptidase IV ($P < 0.001$; Fig. 2.6). Splitting the data into proximal, middle, and distal regions showed that in the proximal region, there was no difference in dipeptidylpeptidase IV activity among the 3 dietary treatments. In the middle and distal part of the

small intestine, suckling the sow led to a greater value than the 2 other dietary treatments. Additionally, lactase activity was greater in piglets suckled the sow compared to artificially fed piglets ($P < 0.05$; Fig. 2.1).

Intestinal Permeability

No statistical differences between the birth weights or among feed treatments were observed for the urinary lactulose-to-mannitol ratio (Table 4). For lactulose, no effects of feed and birth weight were found. On the contrary, the greatest levels of mannitol were recovered in the urine of SOW piglets ($P < 0.05$). An effect of feed treatment was found for the occludin expression ($P < 0.05$), with the SUP diet having a greater value compared to the other 2 dietary

Table 4. Lactulose/mannitol and occludin expression ratio in piglets^{1,2}

Item	Normal birth weight			Low birth weight			SEM	<i>P</i> -value		
	SOW	FOR	SUP	SOW	FOR	SUP		Birth weight	Piglet feed	Interaction
Occludin expression	0.19 ^a	1.64 ^a	12.62 ^b	0.18 ^a	0.79 ^a	18.53 ^b	3.14	0.42	< 0.001	0.59
Lactulose, mmol/L	27.95	5.06	2.68	24.84	3.81	7.42	9.83	0.45	0.15	0.27
Mannitol, mmol/L	69.57 ^a	6.80 ^b	ND ^b	52.64 ^a	55.18 ^b	5.15 ^b	16.60	0.48	< 0.001	0.08
Lactulose/mannitol	0.48	0.77	ND	0.39	0.15	0.80	0.23	0.20	0.78	0.15

^{a,b}Means not sharing the same superscript letter are different ($P < 0.05$).

¹SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, and SUP = weaned at 3 d of age and fed formula with colostral whey filtrates until d 10.

²Values are presented as means and pooled SEM ($n = 5/\text{treatment}$).

treatments. The correlation between the lactulose levels and occludin expression was very weak (Spearman correlation, -0.34). The immunohistochemical staining against occludin revealed that the protein was located at the apico-lateral side of the enterocytes (Fig. 1.2).

DISCUSSION

The ingestion of colostrum is believed to play an essential role in gut growth and development via the direct and indirect effects of various bioactive components on the gut epithelium (Zabielski, 1998; Xu et al., 2002). Both in vitro (Playford et al., 1999; Purup et al., 2007; Roselli et al., 2007) and in vivo studies (Simmen et al., 1990; Playford et al., 1999) have shown that colostrum contributes to cell proliferation and differentiation and mediates several changes in the gut during the postnatal period. Various milk-born factors stimulate the proliferative capacity of the intestinal epithelium, which is visible as crypt hyperplasia or crypt fission or both (Park et al., 1997; Berlanga-Acosta et al., 2001; Cummins et al., 2006; Slupecka et al., 2010), and is a prerequisite for increasing the area for nutrient absorption. Although the value of single milk-born substances on piglets' intestinal growth and maturation has been well established (Jaeger et al., 1990; Burrin et al., 1996; Sauer et al., 2011), only a few studies investigated the value of a combination of these components in their natural matrix.

Early weaning on a milk replacer resulted in shorter villi, reduced lactase activities, and increased crypt depths. Similar changes in small intestinal architecture are described at weaning on solid feed (Hampson, 1986; Miller et al., 1986; Marion et al., 2002; Van Dijk et al., 2002), irrespective of the weaning age. In a previous study, no variation in villus lengths or crypt depths were observed between 7-d-old formula-fed and suckling piglets (Biernat et al., 2001); but in contrast to our study, those piglets were bottle fed. Earlier studies unanimously reported the weaning process itself to affect, irrespective of the age at weaning, brush border enzymes activities (Pluske et al., 1997). Usually, a reduction of enzyme activities is observed in the early post-weaning period

(Tang et al., 1999; Lackeyram et al., 2010), whereas elevated enzyme activities are observed following the recovery of villus heights 1 to 2 wk after weaning (Van Dijk et al., 2002). In a previous study, Thymann and co-workers suggested that formula feeding resulted in insufficient lactase activity, which is in accordance with our previous study (Thymann et al., 2006). Similar to in our study, at 10 d of age, BW of artificially fed piglets were lower compared to sow-reared pigs (Wolinski et al., 2003). The reduced absorptive capacity (shorter villi and lesser mannitol absorption) and reduced functional capacity (lesser lactase activity) in the small intestine of artificially reared piglets contributes to their reduced body weights. No differences (organ weights, intestinal morphology, enzyme activities, and permeability) between both birth weight categories could be detected. Boudry et al. (2011) made a similar observation. The finding that LBW piglets responded similarly to artificial rearing as NBW piglets illustrates that a more in depth analysis of the composition of the milk formula is needed to find out which factor improves the growth performance of the LBW piglets. In this respect, our study attempted to investigate the effect of a fraction of colostral whey (bovine) that promoted enterocyte proliferation in vitro and improved the barrier functioning of the intestine.

The proteome analysis of the < 50 kDa fraction of colostral whey corresponds to earlier descriptions of the bovine whey proteome (Le et al., 2011; Senda et al., 2011; Nissen et al., 2012; Chatterton et al., 2013). Proteins with a documented effect on intestinal health (e.g., α -lactalbumin and cathelicidin; Chatterton et al., 2013) were present in the fraction that was supplemented to the piglets. However supplementing CFW did not affect the BW at d 10. Nevertheless, the addition of CWF induced changes in gut architecture. Namely, CWF decreased villus length and crypt fission percentage. Park et al. (1997) stated that crypt fission rate is indeed decreased by epidermal growth factor, a growth factor that was absent or present below detectable levels in our fraction. No effect was observed on the proliferation index, which differs from our in vitro trials that showed a positive effect of CWF on the proliferation

of IPEC-J2 cells. Previous studies have confirmed that the addition of colostrum (Klagsbrun, 1980; Hironaka et al., 1997; Playford et al., 1999; Lee et al., 2007; Purup et al., 2007) or whey (Takada et al., 1996; Guimont et al., 1997) to cell cultures results in increased cell proliferation. Moreover, no changes in brush border enzyme activities were observed in the supplemented and unsupplemented piglets. Treatment with CWF resulted in mannitol levels below the detection limit (NBW piglets) or very low levels (LBW piglets). This is an indication for decreased transcellular transport, an observation that can be explained by the reduced absorptive area and related to the shortening of the villi. Although lactulose levels did not differ between the dietary treatments, occludin expression was increased in the supplemented group. Barrier function is maintained by, among others, tight junctions, in which occludin is an important integral membrane protein (Furuse et al., 1993). The epithelial barrier forms the first line of defense within the intestinal lumen and prevents the passage of pathogens, toxins and other antigens. In our in vitro study, the addition of CWF reduced the drop in resistance that was observed after the addition of basal medium. Likewise, in our in vivo study, occludin expression was increased in piglets that had received the colostrum whey fraction. In an earlier study, it was demonstrated that bovine colostrum protects swine intestinal cells against increased membrane permeability caused by enterotoxigenic *Escherichia coli* (Roselli et al., 2007). Previous studies have shown that several proteins, which were identified in our fraction, maintain epithelial barrier integrity in both cell cultures and in vivo experiments (Hashimoto et al., 1998; Izumi et al., 2009; Otte et al., 2009). Besides proteins, individual AA may have a specific role in maintaining intestinal barrier function (Wu, 1998; Wang et al., 2009). The role of L-Gln in reducing intestinal permeability has been thoroughly described (Rapin and Wiernsperger, 2010). The L-Gln maintains epithelial barrier integrity in cell cultures (Groschwitz and Hogan, 2009) and in vivo (Ewaschuk et al., 2011; Jiang et al., 2011). Additionally, other AA such as Arg (Viana et al., 2010), citrulline (Batista et al., 2012), and Thr (Mao et al., 2011) improve gut barrier integrity.

The formation of tight junctions is not only influenced by alimentary components but also by intestinal microbiota and inflammatory processes (Arrieta et al., 2006; Groschwitz and Hogan, 2009). As a result, our findings need to be corroborated by further experiments, in which these variables are taken into account.

In conclusion, at the age of 10 d, no morphological and functional differences could be observed between the NBW and LBW piglets. Moreover, the supplementation of a low molecular weight fraction of bovine colostrum whey to piglets fed on a milk replacer did not

stimulate weight gain, digestive enzyme activities, or in vivo permeability, but resulted in structural changes of the small intestine. The reduced villus lengths and the increased expression of occludin strangely did not have a functional impact. These findings should be acknowledged when developing formulated milk with the aim of improving the survival and performance of especially piglets with low birth weight.

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