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ABSTRACT

The use of medicinal zinc oxide (ZnO) to prevent diarrhoea post-weaning will be banned in the EU from 2022. Therefore, new alternatives are needed to avoid an increase in diarrhoea and higher antibiotic use. A low dietary CP level has shown to lower the frequency of diarrhoea in pigs, due to lower microbial protein fermentation in the colon as well as improved conditions in the small intestine after weaning. The objective of this study was to examine the effect of decreased CP levels post-weaning as an alternative to medicinal ZnO on gut morphology and histopathology. Five hundred and sixty pigs were randomly assigned into one of six groups receiving a two-phase diet from 5.5 to 15 kg: positive control group (PC) with medicinal ZnO and standard levels of protein (19.1–18.4% CP), negative control group (NC) without medicinal ZnO and standard levels of protein (19.1–18.4% CP). The remaining four low protein groups were a low-standard (LS) CP level (16.6–18.4% CP), a low-low (LL) CP level (16.6–16.2% CP), a very low-high (VLH) CP level (14–19.3% CP) and a very low-medium (VLM) CP level (14–17.4% CP). Individual BW was recorded at day 0, 10 and 24 post-weaning, and all antibiotic treatments were recorded. Tissue samples from the small intestine (mid-jejunum) for morphological and histopathologic analysis, organ weights, blood and urine samples were collected at day 10 and 24 post-weaning from a total of 90 sacrificed weaners. The results demonstrated no differences in intestinal morphology between groups, but the histopathology showed a damaged brush border score in VLM and VLH pigs . In addition, a lower blood urea nitrogen in VLM pigs at 24 days was found. The LL and VLM pigs had a significantly decreased average daily gain in the overall trial period compared to PC and NC pigs. Conclusively, intestinal brush border was damaged by the very low protein diet at 24 days post-weaning, but intestinal morphology was unaffected by dietary strategy.

Implications

Different protein strategies were tested to find an alternative to medicinal zinc oxide which is being phased out by 2022. The results demonstrated that feeding a low protein level without medicinal zinc oxide post-weaning damages the intestinal brush border but has no effect on the intestinal surface morphology (villous height, crypt depth, enterocytes and goblet cells). A low protein diet can be part of the dietary solution when phasing out zinc oxide to avoid an increase in diarrhoea-related antibiotics usage.

Introduction

Weaning imposes many physiological, immunological and environmental changes, making this transition very stressful for pigs (Coffey et al., 2000; Heo et al., 2013). Due to different stress factors, there is an increased risk of a lower growth performance, a higher frequency of diarrhoea-related antibiotics treatments and an impaired small intestinal health caused by gut morphological changes at weaning (Mccracken et al., 1999; Boudry et al., 2004).

Medicinal zinc oxide (ZnO) is known to have a reducing effect on diarrhoea post-weaning (Zhu et al., 2017; Wang et al., 2019), a positive effect on growth performance (Li et al., 2001; Starke et al., 2014) and a positive effect on the small intestinal morphology including villi height and crypt depth (Li et al., 2001; Yu et al., 2017). In Denmark, medicinal ZnO (2 500 ppm) is therefore often included in the weaner diet the first 14 days post-weaning to avoid an increase in diarrhoea and to improve growth performance. However, high levels of ZnO have been associated with the development of antibiotics resistant microorganisms (Bednorz et al., 2013), which has led to a ban of medicinal ZnO by 2022 in the European Union (European Medicines Agency, 2016). Removal of medicinal ZnO from the diet imposes a risk of increased diarrhoea post-weaning and can cause an increase in the use of antibiotics.

Several studies have demonstrated that a low CP level results in reduced post-weaning diarrhoea through decreased microbial fermentation of undigested protein (Nyachoti et al., 2006; Bikker et al., 2007;

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Heo et al., 2008). However, the literature is inconclusive on the effect of a low CP level on villi height and crypt depth of the small intestine of weaned pigs. Wu et al. (2015) and Peng et al. (2016) found a reduced villous height to crypt depth ratio (**VCR**) in the small intestine with increasing CP levels (17–23% CP and 14–20% CP, respectively), whereas others report a decreasing VCR with a decreasing CP level (20–14% CP) (Yu et al., 2019). However, no studies have reported on the effect of a low CP level followed by a high or medium CP supply on intestinal morphology.

The primary objective of the present study was to determine the effect of different protein strategies on small intestinal morphology and histopathology without medicinal ZnO. It was hypothesized that the protein strategies with a low CP level in phase 1 (day 1 to 11 post-weaning) would have a positive effect on small intestinal morphology and histopathology.

Material and methods

Animals and experimental design

The study was performed on a subpopulation of 560 weaners from a study by Lynegaard et al. (2021). Briefly, the study was conducted at the Danish Pig Research Centre's experimental unit Grønhøj. The newly weaned pigs (Danbred (Duroc × (Danish Landrace × Yorkshire))) were purchased from a commercial sow herd, with a BW at entry of 5.5–9.0 kg (mean BW of 6.7 kg ± 0.9 kg) and a weaning age of 21–28 days. The study was performed as a randomized block design, where pigs were randomly allocated to one of six dietary treatments divided into a two-phase feeding plan from entry to ~15 kg (Table 1).

The treatments were standard CP levels (191 and 184 g/kg CP) with a standard AA profile in both phases (Tybirk et al., 2018) and allocated 2500 ppm ZnO in phase 1 (PC = positive control); standard CP levels (191 and 184 g/kg CP) with a standard AA profile in both phases (Tybirk et al., 2018) and no added ZnO in phase 1 (NC = negative control); low CP levels of 166 and 184 g/kg (LS = low-standard) with added AA (Lys, Thr, Met, Trp and Val) according to standards and all other AA according to the protective profile in phase 1, and according to a standard AA profile in phase 2 (Tybirk et al., 2018); CP levels of 166 and 162 g/kg (LL = low-low) with added AA (Lys, Thr, Met, Trp and Val) according to standards and all other AA according to the protective profile in phase 1 and 2 (Tybirk et al., 2018); CP levels of 140 and 193 g/kg (VLH = very low-high) with added AA (Lys, Thr, Met, Trp and Val) according to standards and all other AA below standard in phase 1, and according to standard AA profile in phase 2 (Tybirk et al., 2018); and lastly, CP levels of 140 and 174 g/kg (VLM = very low-medium) with added AA (Lys, Thr, Met, Trp and Val) according to standards and all other AA below standard in phase 1 and all AA (−Lys) according to the protective profile in phase 2 (Tybirk et al., 2018).

Table 1
The experimental design of the six dietary groups, their protein and lysine levels for weaned pigs in the two feeding phases.

	Treatment					
Dietary group[1]	PC	NC	LS	LL	VLH	VLM
Medicinal zinc	+	−	−	−	−	−
Phase 1, day 1 to 11						
CP (%)	19.1	19.1	16.6	16.6	14	14
SID[1] CP (g/kg)	167	167	144	144	121	121
SID lysine (g/kg)	12.2[2]	12.2[2]	11.5[3]	11.5[3]	11.5[4]	11.5[4]
Phase 2, day 11 to ~15 kg						
CP (%)	18.4	18.4	18.4	16.2	19.3	17.4
SID CP (g/kg)	160	160	160	140	169	152
SID lysine (g/kg)	11.8[2]	11.8[2]	11.8[2]	11.1[3]	12.3[2]	12.3[5]

[1] PC = positive control group with medicinal zinc oxide; NC = negative control group without medicinal zinc oxide; LS = low-standard protein levels in the three dietary phases; LL = low-low protein levels in the three dietary phases; VLH = very low-high protein levels in the three dietary phases; VLM = very low-medium protein levels in the two dietary phases; SID = standardized ileal digestible, g/kg as fed.
[2] Remaining amino acids according to standard (Tybirk et al., 2018).
[3] Remaining added amino acids (threonine, methionine, tryptophan and valine) according to standards, and all other amino acids according to the "protective profile" (Tybirk et al., 2018).
[4] Remaining added amino acids (threonine, methionine, tryptophan and valine) according to standard levels, and the remaining amino acids below standard (Tybirk et al., 2018).
[5] Remaining amino acids according to the "protective profile" (Tybirk et al., 2018).

Housing and management

Upon arrival, the weaners were divided into pens and balanced by gender (female and barrow) and BW (small, medium and large). The weaners were vaccinated against *Lawsonia intracellularis* (2 ml Enterisol®) and against Porcine Circovirus type 2 (0.5 ml Circovac®, Ceva Animal Health A/S). A weekly batch was divided between two sections, which contained 18 pens each with a holding capacity of 10 pigs. A total of 6 week batches divided into 108 pens were included in this study. Each pen had an individual feeder and a water dispenser. The temperature at day 1 (arrival) was 24 °C and was reduced gradually until day 21 post-weaning, where the temperature was kept at constant equal to 17.5 °C until the end of the experiment.

Experimental feed

The pigs had *ad libitum* access to the experimental diets as dry pelleted feed produced by Danish Agro (Sjølund, Denmark), in an individual Spotmix feeding system using air-assisted transport (SPOTMIX, BoPil A/S, Denmark). The weaner period was divided into a three-phase feeding plan, but only phase 1 and 2 were included in this study: phase 1 (day 1 to 11), phase 2 (day 11 to ~15 kg) and phase 3 (BW ~15 to ~30 kg). The feed changes occurred gradually over a 3-day period, starting at the end of each phase. The shift between phase 1 and 2 was set at 11 days post-weaning to prevent provision of medicinal ZnO later than 14 days post-weaning. The BW of pigs from each pen was weighed continuously, in order to allocate the feed change from phase 2 to 3 at a BW of 15 kg (±1.5 kg). The dietary ingredients and composition can be seen in Supplementary Tables S1 and S2.

Recordings

Piglets from six weekly batches were ear-tagged within 12 h of farrowing before litter equalization at the sow herd, and sex, sow number, litter size and sow parity were recorded. The ear-tag was removed if the pigs were treated with antibiotics in the farrowing unit. Thereby, all ear-tagged pigs that entered the experimental unit were antibiotics-free, and all further diarrhoea treatments were individually recorded. Antibiotic treatments were performed according to the veterinarian's instructions. All ear-tagged pigs were individually weighed on a scale (UWE, Bjerringbro vægte APS, Bjerringbro, Denmark) at day 1, 10 and 24 post-weaning at the end of this experiment.

Post-mortem examinations and samplings

At day 10 and 24 post-weaning, just before transition between feeding phases, 8–10 antibiotics-free pigs were randomly selected from each dietary group and different pens and were sacrificed for sampling (total of 90 pigs). Until day 10, the LS and LL diets were identical as well as the VLH and VLM diets, and 4–5 pigs from each dietary treatment were therefore sacrificed and analysed as one group for LS + LL and one group for VLH + VLM at day 10 post-weaning. Pigs were randomly selected based on a list of criteria: no signs of illness, no antibiotic treatments, weaned at 4 weeks of age and from different pens. Pigs were euthanized using a stun gun, de-bleed from the jugular vein, and two blood samples were collected (EDTA and Serum) in 4 ml tubes

(BD-Plymouth, Plymouth, UK). The EDTA tubes were placed on ice and the serum tubes left at room temperature for 1 to 3 h, until they were centrifuged. The EDTA tubes were centrifuged at 3500 RPM for 15 min and the serum tubes were centrifuged at 3200 RPM for 12 min, before the plasma and serum were collected. Serum samples were kept at −20 °C until further analysis, and plasma samples were kept as a backup at −20 °C.

The urine samples were collected directly from the bladder with a 22-gauge syringe into an Eppendorf tube and frozen at −20 °C until further examination. The entire gastrointestinal tract was extracted, and weight of the stomach, small intestine and colon was recorded both with and without contents. The small intestine was placed on a dissection table and length was recorded in a relaxed state. The mid-jejunum was located and approximately 5–8 cm of tissue was cut off and the sample was gently washed with tap water and placed on dental wax with small pins to preserve and protect the intestinal villi. Samples were stored in 10% neutral buffered formalin (10% formalin, 4% paraformaldehyde and 1–2% methanol; CellPath, UK) solution until further morphological and histological analysis.

Tissue analysis

Tissue samples were prepared for embedding, by cutting the middle part of the sample into slices of 4 cm × 0.3 cm or less and placed into cassettes. The tissue went through a fixation process of a minimum 24 h (Excelsior VIP processor), following dehydration by means of graded ethanol and xylene baths. The samples were sent to ALAB Weterynaria (Warsaw, Poland) for morphological (villi height, crypt depth, VCR), enterocytes height, goblet cells) and histolopathological analysis scoring of intraepithelial lymphocytes (IELs) infiltration, stromal lymphocytes (SL) infiltration, epithelium and brush border score. Intestinal tissue sections of 3–4 μm were placed on microscopic slide and stained by haematoxylin and eosin according to standard procedure. Evaluation was performed using a standard light microscope (Olymbus BX41 and CellsSens software; Olympus Corporation, Tokyo, Japan).

All histological and morphological analyses and evaluations were performed using the following scoring protocol by ALAB Weterynaria (Warsaw, Poland). Lymphatic infiltration of the stromal mucosa and epithelium was evaluated at a magnification of 40 times. The SL infiltration was scored from 0 to 3: 0 = normal (visible single lymphocytes in stromal connective tissue of villous and crypts), 1 = low (is an increased number of lymphocytes that do not obscure and do not damage the stroma structure), 2 = moderate (lymphocytes abundantly infiltrate the stroma, damage the wall of blood vessels and connective tissue fibres, reducing the visibility of stroma structures) and 3 = severe (lymphocyte infiltration completely disrupts and conceals the stroma). The IELs infiltration was defined as a score from 0 to 3: 0 = normal (0–10 IELs/100 enterocytes and the histological picture does not deviate from the norm), 1 = low (10–15 IELs/100 enterocytes and increasing infiltration without damage of the stroma or epithelium, it is not a pathological condition), 2 = moderate (15–20 IELs/100 enterocytes – this level may suggest chronic subclinical inflammation. The infiltration affects the continuity and tightness of the epithelium and may damage the intestinal–blood barrier, it is a weak lymphocyte inflammation, and 3 = severe (>20 IELs/100 enterocytes – this level may indicate a chronic inflammation. Infiltration damages epithelium and the intestinal–blood barrier, it is a moderate lymphocytic inflammation) (ALAB Weterynaria, Warsaw, Poland). The brush border and mucosal epithelium evaluation included the number of lymphoid follicles visible in 10 fields of view at ×4 magnification. Brush border was scored according to a scale of 0 to 4: 0 = no visible, 1 = poorly visible and discontinuous, 2 = moderately visible and discontinuous, 3 = clearly visible and discontinuous and 4 = clearly visible and continuous. Mucosal epithelium was scored according to a scale from 0 to 4: 0 = total destruction, peeling and massive infiltration; 1 = visible structure with rich infiltration and dysplastic changes; 2 = moderate infiltration, decreased epithelial height and correct continuity of the epithelial barrier; 3 = poor infiltration and correct continuity of the epithelial barrier and 4 = epithelium with the correct structure (ALAB Weterynaria, Warsaw, Poland).

Blood and urine analysis

The blood serum was analysed for blood creatinine and blood urea nitrogen (Advia 1800 Chemistry System; Siemens Healthcare Diagnostics, Tarrytown, NY, USA), and urine samples were analysed for creatinine, protein and protein/creatinine ration (Advia 1800 Chemistry System, Siemens Healthcare Diagnostics).

Calculations and statistical analysis

All statistical data analyses were performed in R version 1.0.153 – © 2009–2017. Statistical significance was accepted at $P < 0.05$, with individual pig as the experimental unit. The villi height, crypt depth, VCR, enterocytes height, goblet cells, blood, urine, organ weights and average daily gain (ADG) were analysed in a linear mixed model, with dietary group and day post-weaning as a fixed effects and BW as a covariate. The IELs infiltration score, SL infiltration score, epithelium score and brush border score were analysed in a categorical analysis, with dietary group as a fixed effect and BW as a covariate. Diarrhoea treatments were analysed using a logistic regression model, with dietary group as a fixed effect, BW at entry as a covariate and batch as a random effect. Antibiotic treatments against diarrhoea were presented as the cumulative percentage of diarrhoea treated pigs at day 10 and 24 post-weaning. Results were considered significant at $P < 0.05$. When results were significant, the Tukey–Kramer test was used in pairwise comparison of means adjusting *P*-values. Interactions were tested and insignificant. Sow, weaning age (21 or 28 days) and sex were all tested in the models and excluded if not significant ($P > 0.05$). Pigs sacrificed for samplings were included in growth performance and diarrhoea frequency analysis until the point of removal (day 10 or 24).

Results

Intestinal morphology and histopathology

The effect of dietary treatment on intestinal morphology and histopathology is summarized in Tables 2 and 3. There was no difference between dietary treatments for the morphological parameters (villi height, crypt depth and VCR), enterocytes height, goblet cells ($P > 0.05$)), but crypt depth and VCR were affected by age ($P < 0.05$). There was a tendency for a higher number of goblet cells in VLM at day 24 compared to PC (PC = 3.01, VLM = 4.79; $P = 0.098$). Brush border score was reduced in VLH and VLM at day 24 compared to the PC. Fig. 1 displays the intestinal brush border of (A) PC at day 10, (B) PC at day 24, (C) VLH at day 24 and (D) VLM at day 24. The VLH and VLM had poorly visible and discontinuous brush border compared to PC ($P < 0.05$), which clearly had visible and continuous brush border. Additionally, the odds ratios, confidence intervals and *P*-values for odds ratios can be seen for histopathological parameters in Supplementary Tables S3 and S4.

Blood and urine

The effect of dietary group on blood and urine parameters is presented in Table 4. The VLM pigs had a lower blood urea nitrogen than the VLH pigs at days 24 ($P = 0.024$). Moreover, there was an effect of age in blood creatinine, blood urea nitrogen and urine creatinine ($P < 0.05$) with decreasing effect of age in blood creatinine and an increasing effect of age in blood urea nitrogen and urine creatinine.

Table 2
Effect of the six dietary treatments on small intestinal morphological parameters in weaned pigs.

	Dietary treatment[1,2]												
	PC	NC	LS LL	VLH VLM	PC	NC	LS	LL	VLH	VLM		*P*-value	
Item	Day 10				Day 24						SEM	Diet	Age
n	8	8	8	8	10	10	10	10	10	8			
Intestinal morphology													
Villous height, (μm)	547	536	541	512	449	460	449	402	439	438	34.3	0.803	0.214
Crypt depth, (μm)	210	225	213	208	215	224	235	227	215	217	9.55	0.250	<0.001
VCR[1]	2.59	2.41	2.55	2.47	2.10	2.07	1.93	1.79	2.04	2.03	0.17	0.669	<0.001
Enterocytes height (mm)	34.6	32.3	33.8	33.5	32.2	30.9	31.6	30.4	32.8	32.7	1.40	0.554	0.333
Goblet cells[3]	2.41	3.44	3.14	3.89	3.01^{A}	3.71^{AB}	3.18^{AB}	3.77^{AB}	4.42^{AB}	4.79^{B}	0.56	0.018	0.139

[A,B] Values within a row with different superscripts have a tendency to differ at $P < 0.10$.
[1] PC = positive control, NC = negative control, LS = low-standard CP level, LL = low-low CP level, VLH = very low-high CP level, VLM = very low-medium CP level and VCR = villous height to crypt depth ratio.
[2] LS and LL groups have the same diet until day 10, and VLH and VLM groups have the same diet until day 10.
[3] Goblet cells per 100 enterocytes.

Table 3
Effect of the six dietary treatments on histopathological parameters in weaned pigs.

	Dietary group[1]						SEM	*P*-value[1]
	PC	NC	LS	LL	VLH	VLM		
Day 10[2]								
Brush border score	3.4	3.0	3.1		3.0		1.07	ns
Intraepithelial lymphocytes	1.6	2.0	1.9		1.8		1.08	ns
Stromal lymphocyte score	1.4	1.3	1.0		1.3		0.97	ns
Epithelium score	3.8^{A}	3.1^{AB}	3.1^{AB}		3.0^{B}		1.33	0.063
Day 24								
Brush border score[3]	3.1^{a}	2.5^{ab}	2.7^{a}	2.8^{a}	2.1^{b}	1.9^{b}	1.22	0.075
Intraepithelial lymphocytes[3]	3.4	3.4	3.2	3.4	3.5	3.5	1.01	ns
Stromal lymphocyte score[3]	2.6	2.6	2.5	2.6	2.7	2.8	0.95	ns
Epithelium score[3]	3.1^{A}	2.8^{AB}	3.1^{A}	2.9^{A}	2.4^{B}	2.4^{B}	1.10	0.077

[a,b] Values within a row with different superscripts differ significantly between dietary treatment at $P < 0.05$.
[A,B] Values within a row with different superscripts have a tendency to differ at $P < 0.10$.
[1] PC = positive control, NC = negative control, LS = low-standard CP level, LL = low-low CP level, VLH = very low-high CP level, VLM = very low-medium CP level and ns = not significant.
[2] LS and LL groups have the same diet until day 10, and VLH and VLM groups have the same diet until day 10.
[3] The intraepithelial lymphocyte infiltration and stromal lymphocyte infiltration were defined as a score from 0 to 3. Zero meaning normal (0–10 IELs/100 enterocytes or visible single lymphocytes in stromal connective tissue of villus and crypts, respectively) and 3 meaning severe (20 IELSs/100 enterocytes – this level may indicate chronic inflammation or lymphocyte infiltration completely disrupts and conceals the stroma, respectively). Epithelium and brush border score were characterized as a score from 0 to 4, where 0 is total destruction, peeling and massive infiltration or not visible, respectively, and 4 defines epithelium with the correct structure or a clearly visible and continuous brush border.

Organ measurements

The relative organ weights presented as percentage of BW are summarized in Table 5. Treatment VLH/VLM had a smaller empty colon weight at day 10 ($P < 0.05$). The NC pigs had an increased empty stomach weight at day 24 compared to PC and LL ($P < 0.05$). For all measured organs, there was a difference with age ($P < 0.05$).

Growth performance and diarrhoea treatments

The effect of dietary CP level on ADG and diarrhoea treated pigs is presented in Table 6. The VLM pigs had a lower ADG than the PC and NC pigs in phase 1 ($P < 0.05$), whereas both LL and VLM had a lower ADG in the overall trial period compared to the two control groups ($P < 0.05$). There was no evidence of effect of dietary treatment on the number of antibiotics treated pigs at day 10 or 24 post-weaning ($P > 0.05$).

Discussion

Changes to the villi height and crypt depth are considered as key measurements for the assessment of intestinal development and the effect of nutritional changes (Peng et al., 2016). The literature is inconclusive with regard to the influence of CP levels on villi height and crypt depth. Wu et al. (2015) reported a decreased villi height, an increased crypt depth and a decreased VCR in the jejunum with increasing CP levels from 17–23%, and Peng et al. (2016) found an increased crypt depth and a reduced VCR in the jejunum with increasing CP levels from 14 to 20%. In contrast, another study demonstrated an increased crypt depth and decreased VCR with decreasing CP levels from 20 to 14% (Yu et al., 2019), whereas Bikker et al. (2007) and Heo et al. (2010) found no clear effect of decreasing CP levels (from 21 to 15% and 25 to 19%, respectively) on intestinal morphology. In the current study, the villi height, crypt depth and VCR were not affected by reducing the CP levels from 19 to 17 or 14% at day 10 and 24 post-weaning in antibiotics-free pigs. Additionally, no effect of medicinal ZnO in the PC diet was found in intestinal morphology, which is consistent with previous studies (Hedemann et al., 2006; Lallès et al., 2007; Liu et al., 2014). This suggests that the impact of CP and medicinal ZnO on intestinal morphology is limited under normal physiological conditions, with low levels of disease and no antibiotics usage. On the other hand, crypt depth increased with age in the present study, resulting in a decreased VCR with increasing age, while a previous study reported an effect of age on both villi height and crypt depth in the distal jejunum (Liu et al., 2014). The hypothesis was therefore rejected, as there was neither a positive nor negative effect on intestinal morphology in this study.

The lower brush border score in the VLM and VLH groups at day 24 means that the brush border was poorly visible and discontinuous (Fig. 1). This structure change could be caused by the very low CP level in phase 1, if there was not enough protein to maintain the intestinal epithelium, which is also consistent with the high number of goblet cells in the VLM and VLH groups. Alternatively, the damaged brush border could be due to the drastic change from very low CP level (14%) in phase 1 to a high and medium level (19.3 and 17.4%, respectively) in phase 2. However, as the brush border score was only different from the PC group and not the NC group, it indicates that this difference in brush border score was mainly caused by a turnover effect of the lack of ZnO in phase 1. On the other hand, the structure damage could also result from undigested protein as dietary CP levels play a role in the health status of pig intestine (Nyachoti et al., 2006; Wellock et al.,

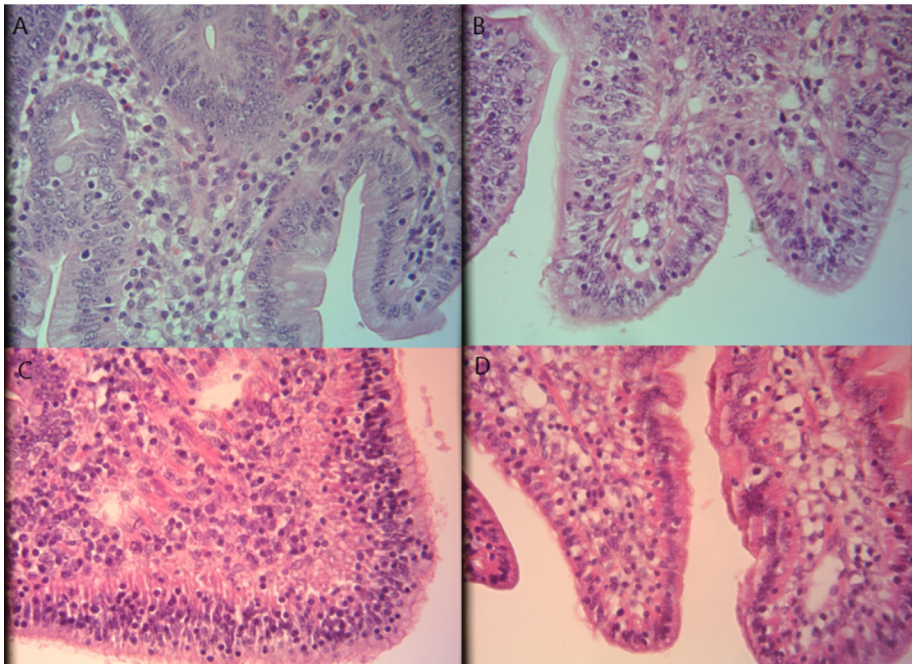

Fig. 1. Electron microscopy observations (40× magnification) of the small intestinal jejunum morphology in pigs at day 10 and 24 post-weaning[1]. The intestinal brush border of PC at day 10 is shown in A, and PC at day 24 in B, the intestinal brush border of VLH at day 24 is shown in C, and brush border score of VLM at day 24 in D. VLH and VLM (C and D) had poorly visible and discontinuous brush border compared to PC (B) ($P < 0.05$), which had clearly visible and continuous brush border. [1]PC = positive control, VLH = very low-high CP level, VLM = very low-medium CP level.

2006). It can be speculated whether the damaged brush border of the VLM and VLH pigs contributes to the poor growth performance, as they have a smaller intestinal surface for nutrient absorption. Additionally, the VLH and VLM pigs had a tendency towards a lower epithelium score at day both day 10 and 24 post-weaning compared with the PC pigs. This further suggests that the intestinal mucosa of these pigs was more damaged, most likely due to the very low CP level. The results therefore contradict the hypothesis of the trial, as a low CP level damaged the intestinal histopathology quantified by brush border score instead of improving it.

Urea is a waste product of protein digestion in pigs which is produced by the liver and filtered out of the blood by the kidneys (Fang et al., 2019). Therefore, blood urea nitrogen can be used to evaluating protein digestibility and be an indicator of protein utilization (Fang et al., 2019). In the current study, blood urea nitrogen was decreased in the antibiotics-free pigs receiving a low CP diet 24 days post-weaning, which is in agreement with previous studies reporting that decreasing protein levels result in a lower blood urea nitrogen (Heo et al., 2008; Zhao et al., 2019). The lower blood urea nitrogen level indicated that the protein and AA degradation in the intestines or liver were reduced, which likely improved protein utilization.

The effect of dietary CP level on growth performance diarrhoea frequency for the overall study was discussed in a previous study where it was found that a low CP level reduced diarrhoea frequency (Lynegaard et al., 2021). The results from the current study on individual ear-tagged pigs support those results. All groups were supplemented with AA to meet standard requirements (Tybirk et al., 2018), but even with an increased CP level in phase three (day 24 to 30 kg), as presented in Lynegaard et al. (2021), the pigs receiving the low CP level in early nursing experienced reduced growth performance.

Table 4
Effect of dietary treatment on blood and urine parameters in pigs at day 10 and 24 post-weaning.

	Dietary treatment[1,2]												
	PC	NC	LS LL	VLH VLM	PC	NC	LS	LL	VLH	VLM		*P*-value[3]	
Item	Day 10				Day 24						SEM	Diet	Age
n	8	8	8	8	10	10	10	10	10	8			
Blood parameters													
Creatinine (μmol/l)	72.0	71.6	80.7	78.9	56.6	57.0	55.1	55.2	55.6	51.7	5.27	0.893	<0.001
Blood urea nitrogen (mmol/l)	0.92	0.99	0.57	0.15	1.29^{ab}	1.29^{ab}	1.30^{ab}	1.11^{ab}	1.52^{a}	0.95^{b}	0.15	0.055	0.003
Urine parameters													
Creatinine (μmol/l)	6055	4392	4857	1952	4125	4622	5901	5207	4192	4326	1419	0.476	0.004
Protein (mg/l)	117	83	94	46	170	112	170	174	209	135	90.6	0.881	0.487
UPC ratio[4]	0.19	0.16	0.21	0.27	0.83	0.17	0.27	0.28	0.48	0.19	0.47	0.697	0.763

[a,b] Values within a row with different superscripts differ significantly between dietary treatment at $P < 0.05$.
[1] PC = positive control, NC = negative control, LS = low-standard CP level, LL = low-low CP level, VLH = very low-high CP level, VLM = very low-medium CP level.
[2] LS and LL groups have the same diet until day 10, and VLH and VLM groups have the same diet until day 10.
[3] No significant interaction between diet and age.
[4] Urine protein/creatinine ratio.

Table 5
Effect of dietary treatment on pig organs at day 10 and 24 post-weaning.

	Dietary treatment[1,2]												
	PC	NC	LS LL	VLH VLM	PC	NC	LS	LL	VLH	VLM		*P*-value[3]	
Item	Day 10				Day 24						SEM	Diet	Age
n	8	8	8	8	10	10	10	10	10	8			
BW (kg)	8.95	9.29	8.31	8.86	12.76	13.0	12.75	13.6	13.03	12.36	–	–	–
Relative organ weight (%)[4]													
Full stomach	4.18	3.45	4.02	4.50	4.76	5.47	4.99	4.39	4.82	4.63	0.50	0.859	0.011
Empty stomach	0.87	0.79	0.82	0.85	0.86^{ac}	1.05^{b}	0.99^{acb}	0.87^{ac}	0.95^{acb}	0.94^{acb}	0.45	0.199	<0.001
Full small intestine	8.54^{A}	8.11	7.18	6.83^{B}	9.47	10.14	9.73	10.05	9.76	8.56	0.49	0.601	<0.001
Empty small intestine	4.95	4.92	4.57	4.26	5.80	6.13	5.80	5.87	5.87	5.47	0.23	0.177	<0.001
Full colon	4.05	3.89	3.81	3.59	5.17	4.92	4.74	5.34	4.99	5.16	0.29	0.577	<0.001
Empty colon	1.87^{a}	1.82^{a}	1.78^{a}	1.38^{b}	1.94	2.0	1.96	2.02	1.93	1.97	0.09	0.086	<0.001
Small intestine length	1.09	1.01	1.04	0.99	0.90	0.85	0.85	0.85	0.89	0.86	0.39	0.618	<0.001

[a,b] Values within a row with different superscripts differ significantly between dietary treatment at $P < 0.05$.
[A,B] Values within a row with different superscripts have a tendency to differ at $P < 0.10$.
[1] PC = positive control, NC = negative control, LS = low-standard CP level, LL = low-low CP level, VLH = very low-high CP level, VLM = very low-medium CP level.
[2] LS and LL groups have the same diet until day 10, and VLH and VLM groups have the same diet until day 10.
[3] No significant interaction between diet and age.
[4] Percentage of BW.

Table 6
Effect of dietary group on individual pig growth performance from 0 to 10 days, from 0 to 24 days and the percentage of diarrhoea treated pigs at day 10 and 24 post-weaning.

Item	Dietary treatment[1]						SEM	*P*-value
	PC	NC	LS	LL	VLH	VLM		
Day 10 (n)	68	191	86	73	70	72	–	–
Day 24 (n)	45	149	71	60	52	56	–	–
BW								
Day 0	6.9	6.7	6.6	6.7	6.6	6.6	–	–
Day 10	8.5^{ac}	8.3^{c}	8.1^{abc}	8.2^{abc}	7.9^{ab}	7.9^{b}	0.19	<0.001
Day 24	13.4^{a}	13.2^{a}	12.7^{ab}	12.3^{b}	12.7^{ab}	12.4^{b}	0.35	<0.001
ADG (g/day)[2]								
Day 0 to 10	189^{ac}	193^{c}	172^{abc}	179^{abc}	160^{ab}	152^{b}	19.3	<0.001
Day 10 to 24	351^{a}	351^{a}	328^{ab}	290^{b}	328^{ab}	304^{b}	18.1	<0.001
Day 0 to 24	290^{a}	288^{a}	268^{ab}	245^{b}	265^{ab}	246^{b}	17.1	<0.001
Diarrhoea treated pigs (%)								
Day 10	1.5	5.6	1.2	5.6	0.0	0.0	2274	0.997
Day 24	18.6	39.7	37.1	33.9	17.8	25.5	0.5	0.140

[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$. Values presented as least square means ± SEM.
[1] PC = positive control, NC = negative control, LS = low-standard CP level, LL = low-low CP level, VLH = very low-high CP level, VLM = very low-medium CP level.
[2] ADG = average daily gain.

Conclusion

Conclusively, some damages were observed in intestinal histopathology (brush border score) but no differences in morphology were detected (villous height, crypt depth, etc.), suggesting that a very low CP level causes some structural damage to the intestinal surface but not enough to alter the morphology.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100188.

Ethics approval

The staff at Grønhøj experimental station looked after the animal continuously and were followed up by visits from the veterinarian. Sick or injured pigs were removed from the trial and treated in separate pens.

Data and model availability statement

None of the data were deposited in any official repository and are available upon request.

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Declaration of interest

The authors declare that funding was received from the Danish pig industry. N.J Kjeldsen and C. F Hansen both work for the Danish pig industry. All authors contributed to analysing and interpreting the data and therefore declare no conflict of interest.

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