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Determination of the optimal level of dietary zinc for newly weaned pigs: A dose-response study

Sally V. Hansen [1], Natalja P. Nørskov [1], Jan V. Nørgaard [1], Tofuko A. Woyengo [1], Hanne D. Poulsen [1] and Tina S. Nielsen [1,*]

[1] Department of Animal Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark
* Correspondence: tinas.nielsen@anis.au.dk

Simple Summary: Piglets have a very low feed intake immediately after weaning. We hypothesis that the EU legislated maximum dietary zinc concentration (150 mg zinc/kg diet) will increase the risk of zinc deficiency after weaning. Zinc deficiency includes symptoms such as impaired growth and increased risk of diarrhoea. However, high dietary zinc concentration has an antimicrobial effect on the bacteria and increases the risk of antimicrobial resistance. The findings of this study showed the growth followed a quadratic pattern (turning point at approximately 1400 mg zinc per kg diet). The risk of diarrhoea increased up to 60% for pigs that had a blood zinc concentration that decreased after weaning. It required up to 1,121 mg zinc per kg diet to maintain the blood zinc concentration after weaning. There was no evidence for an antimicrobial effect when feeding pigs a diet with up to 1601 mg zinc per kg diet.

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Abstract: One hundred and eighty individually housed piglets with initial body weight 7.63 ± 0.98 kg (28 days of age) were fed a diet containing either 153, 493, 1,022, 1,601, 2,052 or 2,407 mg Zn/kg (added Zn as ZnO) from day 0-21 post weaning, to determine the optimal level of Zn for weaned piglets. Body weight, feed intake and faecal scores were recorded and blood and faecal samples collected. Dietary Zn content quadratically affected both feed intake and gain the first two weeks, with approximately 1400 mg Zn/kg diet and a Zn intake of 400 mg/day as the optimal. The relative risk of diarrhoea increased up to 60% at d 7 and 14 if serum Zn status dropped below the weaning level (767 µg/L) and it required approximately 1,100 mg Zn/kg (166 mg Zn/day) during week 1 to maintain the weaning serum Zn status. Blood markers of intestinal integrity (D-lactate and diamine oxidase) was unaffected by dietary Zn and intermediate dietary Zn levels did not affect the faecal numbers of total bacteria, Lactobacilli and E. coli bacteria compared to 153 mg Zn/kg. These results indicate that the requirement for Zn in newly weaned piglets may be substantially higher than currently assumed.

Keywords: Serum zinc; Growth performance; Diarrhoea; Zinc oxide; Intestinal integrity

1. Introduction

Zinc (Zn) is an essential micronutrient necessary for multiple structural and biological functions, including enzyme function, DNA and RNA metabolism, protein synthesis, gene expression, cell proliferation and differentiation and cell-mediated immunity [1,2]. Thus, Zn is essential for normal growth and development. All body tissues contain Zn, but Zn stores are small and only up to approximately 15% of the whole-body Zn can be mobilised during insufficient Zn intake [3-5]. Skin lesions or parakeratosis are well-known clinical symptoms of long-term Zn deficiency in both pigs and humans [6-9], whereas reduced feed intake may be a more short-term sign of Zn deficiency [10-12]. Moreover, human studies link Zn deficiency to diarrhoea [13,14]. Zinc deficiency impairs function of the immune system and compromises intestinal function [15,16], which may increase the

risk of entero-viral pathogen infections, triggering mal-absorption and mal-excretion of nutrients and electrolytes, thereby leading to diarrhoea [17]. However, the exact pathophysiological mechanism linking diarrhoea to Zn deficiency is yet to be elucidated. Overall, adequate daily Zn intake is essential for optimal production and health. According to NRC, a Zn intake of 26.6-46.8 mg/day for piglets in the weight interval between 5 to 11 kg is recommended [18]. However, these recommendations are calculated estimates not based on results from Zn dose-response studies in weaned piglets [18]. Furthermore, the NRC recommendations on Zn supply have not been revised since 1979, despite that modern pigs genetic potential for growth has increased substantially over the years [19]. This renders it likely that also the Zn requirement has increased since 1979.

Moreover, from June 2022, the maximal allowed dietary Zn level for weaned piglets in the EU will be 150 mg/kg diet [20,21]. With a dietary Zn level of 150 mg/kg, a newly weaned pig of 7 kg should consume 312 g feed/day to achieve the current daily recommended Zn intake of 48.6 mg/day [18]. However, several studies report that daily feed intake during the first week post-weaning is low and may range from <50 to 235 g/day [22-25]. In this feed intake range, the dietary Zn concentration should optimally be 113-936 mg/kg to achieve NRC's current recommended daily Zn intake. At the 150 mg Zn/kg diet there is a chance that many low feed-intake pigs will be undersupplied with Zn during the first period PW.

Generally, nutrient requirement can be estimated through dose-response experiments by measuring many biological endpoints including production and biochemical parameters that the nutrient of interest affects. Thus, the adequate level of a nutrient depends on the endpoint as some endpoints are more sensitive to deprivation of the nutrient than others [26,27]. In humans, linear growth is the only recommended functional indicator of Zn requirement, because increased growth as a result of Zn supplementation can only be interpreted as an indication of pre-existing Zn deficiency [28]. Similar statement have been made about Zn deficiency in pigs, as it will impair growth performance [29]. Some dose-response studies have investigated the Zn requirement in weaned pigs [30,31], but these experiments included an acclimatisation period of one to two weeks and the results can therefore not be used to estimate the Zn requirement immediately PW. Furthermore, these studies were not designed to study effects on growth and feed intake. In the current dose-response study the aim was to determine the optimal dietary level of Zn for pigs the first three weeks PW. The diet was supplemented with the most common source of added Zn in diets (zinc oxide; ZnO) and the endpoints were feed intake, growth performance, serum Zn status, faecal scores, blood biomarkers of intestinal integrity and faecal microbial composition. Especially the latter was intended to illuminate when and if supra-nutritional levels of dietary Zn was supplied, since Zn-induced modifications of the gut microbial community have been used to distinguish between nutritional and pharmacological effects [29]. It was hypothesized that 150 mg Zn/kg diet is insufficient to provide newly weaned pigs the currently recommended 46.8 mg Zn/day due to low feed intake but also that the optimal Zn supply immediately post-weaning may be substantially higher than what NRC recommendations currently indicate.

2. Materials and Methods

2.1. Animals, housing, and experimental diet

The experiment was carried out with 180 crossbred ([Danish Landrace × Yorkshire] × Duroc) piglets (90 males and 90 females) obtained from a commercial pig herd. They arrived at the experimental facility on the day of weaning (day 0), 28 days of age (initial weight 7.63 ± 0.98 kg). Upon arrival (d 0), pigs were randomly distributed to one of six diets (n=30/diet) after blocking according to body weight and gender. Pigs were housed individually in pens (1.5 × 2.4 m, 1/3 of the area slattered floor) with access to snout-contact to the neighbouring pig, *ad libitum* access to water and feed and provided a 12-hour light/dark cycle. The experiment was conducted in eight blocks repeated over time with

24 pigs/block, 4 pigs/treatment/block in block 1-7 and 12 pigs/block, 2 pigs/treatment/block in block 8.

The basic experimental diet was formulated to meet the Danish recommendations for nutrients for pigs between 6 and 15 kg (**Error! Reference source not found.**) but without Zn in the vitamin-mineral mixture. The grain ingredients were milled through a 3 mm screen before combined with the remaining ingredients. High purity (80%) ZnO (VetZink, Vepidan Aps, Løgstør, Denmark) was added separately to generate six diets with increasing Zn concentration (**Error! Reference source not found.**). Diets were pelleted at 60 ºC into 2 mm pellets and pigs received the experimental diet throughout the experiment.

Table 1. Ingredients and calculated composition of the basal diet.

Ingredients	**%**
Wheat	40.3
Barley	20.0
Soy protein concentrate, HP300	9.8
Soybean meal, 45.8% protein	8.0
Oats	5.9
Vegetable fat and oil	4.8
Lactose	4.1
Fishmeal	3.0
Vitamin/mineral premix[2]	2.2
Monocalcium phosphate	1.1
Salt	0.7
Aroma	0.1
Natuphos 10000 E[1]	0.02
Calculated composition (as-fed)	
Crude protein, %	18.3
Lysine, %	1.25
Ca, %	0.74
P, % (available)	0.40
Fe, mg/kg	180
Zn, mg/kg	29
Cu, mg/kg	120
Added dietary zinc (mg Zn/kg)[3]	**Analysed zinc concentration (as-fed), mg Zn/kg feed**
Basal diet (no added ZnO)	50
100	153
450	493
950	1,022
1,450	1,601
1,950	2,052
2,450	2,407

[1] 200 % phytase = 1000 FUT/kg, Natuphos 10000 E. [2] Zn-free vitamin-trace mineral mix providing the following per kilogram of diet: 5.989 IU vitamin A, 598 IU vitamin D3, 156 mg Vitamin E, 2.4 mg vitamin K3, 2.4 mg vitamin B1, 4.8 mg vitamin B2, 3.6 mg vitamin B6, 0.02 mg vitamin B12, 24 mg Niacin, 0.24 mg Biotin, 6.0 mg Ca, 6.0 mg P, 3.3 mg Na, 5.1 mg Kl, 180 mg Fe (FeSO4), 120 mg Cu (CuSO4), 48 mg Mn (MnO), 0.2 mg Se (Na2SeO3). [3] High purity ZnO (80%) VetZink, Vepidan Aps, Løgstør, Denmark.

2.2. Registrations

To calculate average daily gain (ADG) and ADFI, pigs and feed residues were weighed daily from day 0 to day 14 and again on day 21. The pigs were euthanized if they lost more than 15 % of the initial weight.

The faecal score was assessed daily throughout the experiment based on a four-category scale with faecal score 3 and 4 classified as diarrhoea [32]. Pigs were immediately

treated with antibiotic if their faeces was categorised as score 4. If the faeces was categorised as a score 3, the potential antibiotic treatment was postponed to the following day to await another faecal scoring.

2.3. Feed and blood sample collection

At the beginning of each block, representative sample of the experimental diets were obtained and at the end of the experiment, the samples were pooled for each diet and a representative sample of each diet was used for further analysis.

Blood samples from the jugular vein were obtained on day 0, 7, 14, and 21 PW. For mineral analysis, the blood samples were collected in vacutainers specifically for mineral analysis (Becton Dickinson AS, Kongens Lyngby, Denmark), and the serum was derived by centrifugation (1,300 *g* for 10 min at 4 °C) and stored in polyethene tubes at -20 °C. For analysis of diamine oxidase activity (DAO, only day 21) and D-lactate concentration, the blood samples were collected in Na/Hep vacutainers, centrifuged (1,300 *g* for 10 min at 4 °C) and stored in two 2 ml cryotubes at -20 °C.

2.4. Feed and blood mineral analyses

The dry matter content of diets was determined by drying the samples at 103 °C for 20 hours. The feed was analysed for Zn and copper (Cu) concentration, while serum was analysed for Zn concentration. The feed sample was ground to 1 mm and acidified with 5 mL HNO_3 (65 %), followed by destruction at 1,500 W at 230 °C for 35 minutes using a microwave system (Ultra wave, single reaction chamber, Milestone, Shelton, USA). Serum samples were prepared by adding 800 µL serum and 7,200 µL 2% HNO_3 to a 15 ml PP tube and afterward centrifuged (16,000 *g* for 10 min at 5 °C). Hereafter, 1-2 mL of the supernatant was filtered through a 0.20 µm filter into a PP microtube and further diluted 5 times with 0.1% HNO_3. The mineral content was measured on an iCAP TQ ICP-MS (Inductively Coupled Plasma-Mass Spectrometer) equipped with a MicroMist DC nebulizer, a Quartz cyclonic spray chamber operated at 2.7 °C (Thermo Scientific, Bremen, Germany) and a CETAC auto sampler model ASX 560. The instrument settings were forward power 1,550 W, plasma gas (Ar) 14 L/min, nebulizer gas (Ar) 0.96 L/min, auxiliary gas (Ar) 0.8 L/min. The sample uptake was approximately 0.4 mL/min. Data were collected using the QtegraTM version 2.10.9.3324.131 (Thermo Fisher Scientific, Bremen, Germany). Two isotopes of Zn and Cu were measured: ^{64}Zn, ^{66}Zn, ^{63}Cu, and ^{65}Cu. The ^{66}Zn and ^{63}Cu isotopes were used as quantifier and ^{64}Zn and ^{65}Cu as qualifier. Both isotopes were measured in KED mode. The standard curve contained Zn and Cu in a concentration ranging from 0.3125 to 250 ppb with ^{45}Sc, ^{71}Ga and ^{103}Rh as calibration standards (AQ0-053-841 bought from Labsupport).

The method was validated by spiking standards of Zn and Cu to in-house reference plasma and calculating the recovery and accuracy by using five replicates per spiked concentration. The mean recoveries for ^{66}Zn and ^{63}Cu were 87% and 94 %, respectively, with relative standard deviation between 2% and 8 %.

2.5. Analysis of DAO and D-lactate in plasma

D-lactate was analysed according to Larsen [33]. Diamine oxidase (DAO) was determined by a kinetic-fluorometric method where 1,5 diamino pentane (cadaverine, Sigma C8561) was the substrate, and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) was the profluophore oxidized by the developed hydrogen peroxide. Units were defined as d-emission per min at 590 nm after excitation at 544 nm.

2.6. Faecal microbial analysis

Faecal samples obtained at day 21 from 20 random selected pigs receiving 153, 1022, 1601 or 2407 mg Zn/kg diet were analysed for the number of total bacteria, total *Lactobacilli* and total *E. coli*. Extraction of DNA from 50mg of digesta samples was performed following the manufacturer's guidelines using the Nucleospin Fecal DNA extraction kit (Machery-Nagel, Düren, Germany). Thereafter, DNA concentration was quantified using a Qubit fluorometer 3.0 (Life Technologies). Target groups were quantified from DNA-extracted samples using qPCR and a set of primers as described in **Error! Reference source not found.** (Sigma-Aldrich). The annealing temperature, primer concentration and standard for each pair of primers are listed in **Error! Reference source not found.**. For each reaction, solution containing 5 μL of RealQ Plus 2x Master Mix, green (low ROX) (Amplicon III, Denmark), primers in concentrations as stated in **Error! Reference source not found.**, 2 μL of template DNA (template DNA was diluted x100 for total bacteria (All bacteria) primers), and nuclease-free water up to the final volume of 10 μL were used. The qPCR analysis was performed using a MicroAmp Optical 384-well reaction plate (Applied Biosystems) and an ABI ViiA7 real-time PCR system (Thermo Fisher Scientific) under the following run conditions; pretreatment of 2 min at 50°C, followed by initial denaturation (15 min at 95°C) and subsequently 40 cycles of denaturation for 15 s at 95°C, 30 s for primer annealing at different temperatures (**Error! Reference source not found.**), and 30 s at 72°C for base extension. Melting curves were derived by increasing the temperature from 60 to 95°C at a rate of 0.05°C/s, recording continuously. These curves were used to evaluate the quality of the PCR products. All analyses were performed in triplicate and a no-template control was included in every run. DNA from pure cultures were used to generate standard curves (see **Error! Reference source not found.**). Copy number in the standards was calculated from the genome size and the DNA concentration using the DNA to copy number calculator [34]. The concentrations of target DNA in the samples were estimated using PCR cycle threshold values, using QuantStudio real-time PCR software version 3.1 (Thermo Fisher Scientific).

Table 2. Primers and quantitative PCR conditions used for real-time PCR.

Primer name [1]	Target sequence	Sequence (5′–3′)	Conc. [2] (μM)	AT [3] (°C)	Size (bp)	Standard	Reference
Bank-lacto-F	All *Lactobacillus* (23S rRNA)	GCGGTGAAATTCCAAACG	0.30	60	216	*Lactobacillus reuteri* DSM 20016	[35]
Bank-lacto-R		GGGACCTTAACTGGTGAT	0.30				
E. coli 401 F	All *E. coli* (ybbW gene)	TGATTGGCAAAATCTGGCCG	0.50	65	211	*E. coli* K12	[36]
E. coli 611 R		GAAATCGCCCAAATCGCCAT	0.50				
16S_BAC-F (SRV3-1)	All bacteria (16S rRNA)	CGGYCCAGACTCCTACGG	0.30	65	200	*E. coli* K12	[37]
16S_BAC-R (SRV3-2)		TTACCGCGGCTGCTGGCAC	0.30				

[1] F = forward; R = reverse. [2] Primer concentration. [3] AT = Annealing temperature.

2.7. Statistical analyses

The statistical analyses of data were performed using R studio version 1.4 [38] as a randomized complete block design with the individual pig as the experimental unit. Shapiro-Wilk normality tests, Q-Q plots and residual plots verified the normality of the data. Dietary Zn level, gender and initial weight were included as fixed effect and block as random effect in all statistical analyses. Insignificant variables were omitted from the models. Linear mixed models tested the ADFI and ADG with linear and quadratic effects of dietary Zn concentration using the *lme4* package [39]. The DAO activity and D-lactate concentration was also tested with linear mixed model with dietary Zn level as categorical

variable, and the daily Zn intake included an interaction between dietary Zn concentration and day as fixed effect pig as random effects. Lastly, linear mixed models tested also the ADG with linear and quadratic effects of serum Zn concentration, the number of bacterial with the total Zn intake. A generalized additive mixed models (GAMMs) tested serum Zn concentration with a smooth term of dietary Zn concentration with six dimensions and the initial serum Zn at day 0 using the *mgcv* package [40]. The data were fitted using a gamma distribution and a log-transformation, and results is showed as transformed log-results. A cumulative logit mixed model with faecal score as categorical variable was tested using an ordinal logistic regression from the package *ordinal* [41]. The model included pig as a random effect, and the obtained values were square means of log odds, transformed to probabilities of transformed log odds. A cumulative logit mixed model tested also whether the daily feed intake was above or below 312 g, the model included an interaction of day and dietary Zn concentration as a fixed effect and pig as random effects. The relative risk of diarrhoea in relation to serum Zn status above or below the serum Zn level at weaning was evaluated with the package *fmsb* [42].Values are presented as LS-means with 95% confidence interval (95% CI), obtained with the *emmeans* package [43]. A threshold of $P \leq 0.05$ was considered as statistically significant while *p*-values between < 0.05 and ≤ 0.1 were considered as a statistical tendency.

3. Results

3.1. Feed, feed intake and weight gain

One hundred seventy-nine pigs completed the experiment. The basal diet had a higher Zn content than expected (50 vs. 29 mg/kg), but the experimental diets showed a Zn content close to the intended levels (Table 1). The deviation between calculated and analysed Zn concentration was 2-7% in the six dietary groups.

Dietary Zn concentration did not affect the ADFI during the first week PW ($p \geq 0.137$, **Error! Reference source not found.**A) and there was a very high probability (0.84-1.00) for pigs eating less than 312 g/day during day 2-7 PW (Figure S1). Even though dietary Zn level had no effect on the feed intake it had an effect on the Zn intake during the first week PW, as pigs fed a diet with 153 mg dietary Zn/kg showed the lowest Zn intake of less than 23 mg/day ($p<0.05$, Figure S2). ADFI had a strong tendency of being quadratic affected by dietary Zn concentration during the second week PW ($p=0.051$, **Error! Reference source not found.**B) and the first two weeks PW ($p=0.068$, **Error! Reference source not found.**C), while overall (week 1-3 PW) the effect was significant ($p=0.038$, data not shown). The estimated turning points occurred at dietary Zn levels of 1364 mg/kg during the second week PW, 1375 mg/kg during the first two weeks PW and 1344 mg/kg overall with ADFI of 405 g/day, 287 g/day and 433 g/day, respectively (Table S1). These dietary Zn levels were estimated to a Zn intake of 553 mg/day during the second week PW, 395 mg/day during the first two weeks PW and 583 mg/day during the three weeks PW.

Dietary Zn concentration had a tendency of a quadratically effect on the ADG during the first week PW ($p=0.102$, **Error! Reference source not found.**D), and a significant quadratically effect during the second week PW ($p=0.013$, **Error! Reference source not found.**E), the first two weeks PW ($p=0.032$, **Error! Reference source not found.**F) and overall (Week 1-3 PW, $p=0.003$, data not shown). The estimated turning points occurred at dietary Zn levels of 1394 mg/kg the first week PW, 1216 mg/kg the second week PW and 1408 mg/kg the first two weeks PW with ADG of 104 g/day, 401 g/day and 253 g/day, respectively (Table S2). These dietary Zn levels would lead to estimated Zn intake of 207 mg/day during the first week PW, 492 mg/day during the second week PW and 404 mg/day during the first two weeks PW. The overall ADG during the three weeks PW was estimated to have a turning point at a dietary Zn level of 1265 mg/kg with an ADFI of 367 g/day and this dietary Zn level would lead to a Zn intake of 547 mg/day.

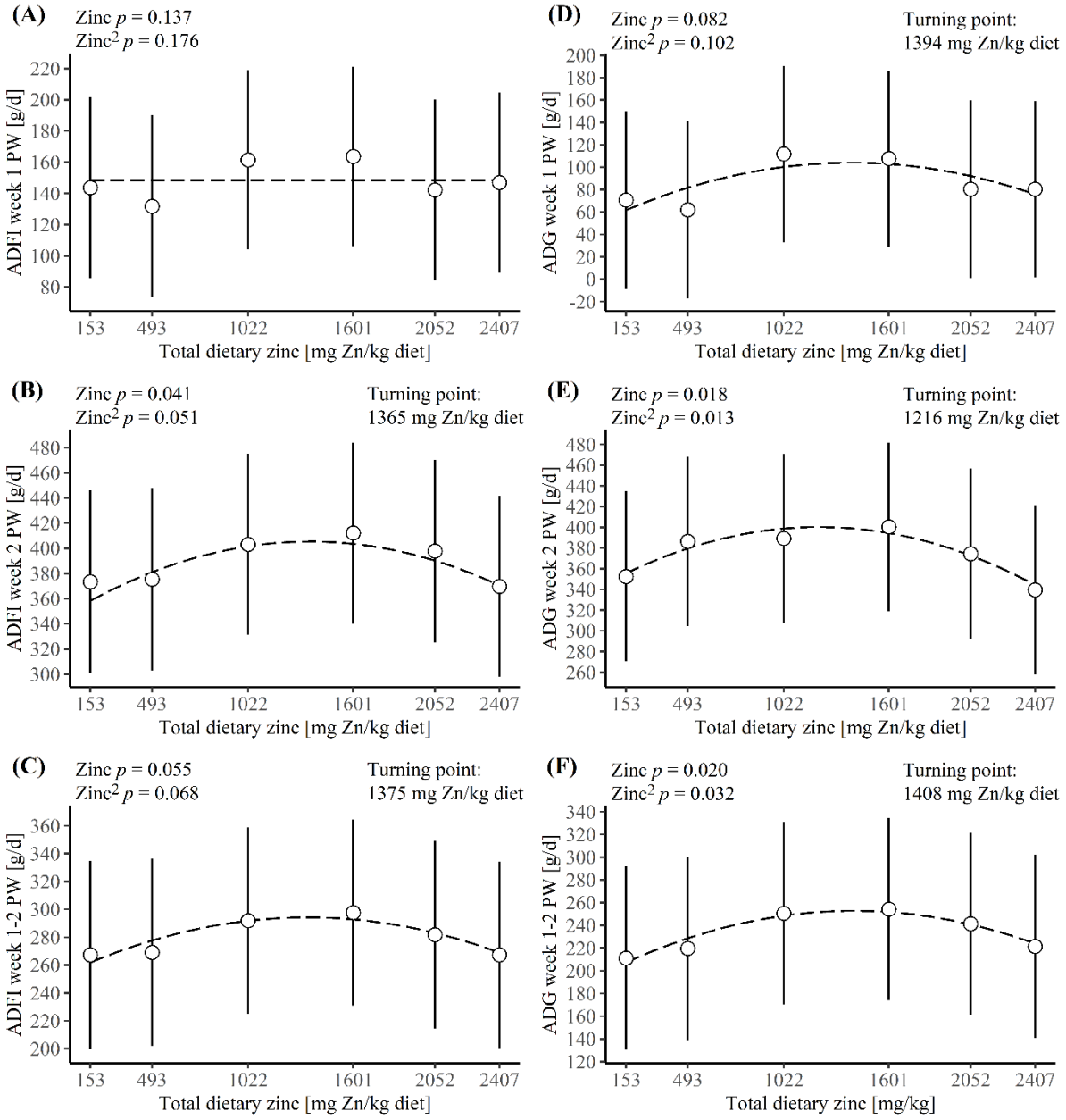

Figure 1. The average daily feed intake (ADFI) and average daily gain (ADG) obtained with six dietary zinc concentration during (**A**, **D**) the first week post-weaning (PW), (**B**, **E**) the second week PW and (**C**, **F**) the two first weeks PW. The dashed lines illustrate the effect of dietary zinc concentration on ADFI or ADG. The turning point and *p*-value of the linear and quadratic parameters are denoted at the top of each graph.

3.2. Diarrhoea probability

The probability of diarrhoea (faecal score ≥3 on a scale from 1 to 4) was higher with 153 mg Zn/kg diet compared to 2407 mg Zn/kg at any time point (**Error! Reference source not found.**). Moreover, from the second week 2052 mg Zn/kg diet had a lower diarrhoea probability than 153 mg Zn/kg diet. Diarrhoea probability was similar for the dietary Zn levels between 153 and 1601 mg/kg at any time point and 493 and 1601 mg Zn/kg diet had a similar diarrhoea probability as 2052 mg/kg at any time point. However, in the third week 153 mg Zn/kg diet tended to have higher probability than 1601 mg Zn/kg (p=0.087). The diarrhoea probability for week 1-2 had a tendency of being higher for 493 mg Zn/kg diet compared to 2407 mg Zn/kg diet (p=0.086).

Table 3. Probability (%) of diarrhoea in pigs in different intervals post-weaning dependent on total dietary zinc concentration [1].

	Total dietary zinc concentration [mg Zn/kg diet] [2]							
	153	**493**	**1022**	**1601**	**2052**	**2407**	**95% CI**	***p*-values**
Week 1	28.2 ^{a}	19.1 ^{ab}	13.2 ^{ab}	23.3 ^{a}	10.6 ^{ab}	6.6 ^{b}	11.7-19.9	<0.05
Week 2	22.7 ^{a}	13.6 ^{ab}	16.1 ^{ab}	16.0 ^{ab}	6.9 ^{b}	6.4 ^{b}	9.6-16.0	<0.05
Week 3	43.7 ^{a}	26.9 ^{ab}	27.7 ^{ab}	16.9 ^{abc}	12.4 ^{bc}	4.5 ^{c}	13.1-26.0	<0.05
Week 1-2	26.3 ^{a}	17.1 ^{abc}	15.7 ^{abc}	20.0 ^{ab}	9.7 ^{bc}	7.0 ^{c}	12.2-17.9	<0.05
Week 1-3	33.3 ^{a}	22.0 ^{ab}	21.5 ^{ab}	21.1 ^{ab}	13.1 ^{bc}	7.7 ^{c}	15.5-21.6	<0.05

[1] Probability is calculated as Prob = (odds/(1 + odds)) * 100, where odds = $e^{loge(odds)}$.
[2] Values are presented as least squares means in per cent.

3.3. Serum Zn status

The initial serum Zn concentration at weaning was similar for the six dietary treatment groups (767±19 µg/L, p>0.05, data not shown). Pigs with a serum Zn level lower than 767 µg/L at day 7 PW had 52% higher risk of a diarrheal episode during the second week PW compared to pigs with a higher serum Zn level than 767 µg/L. The risk of a diarrheal episode increased to 60% the third week PW for pigs with a serum Zn level day 14 lower than 767 µg/L (**Error! Reference source not found.**).

Table 4. Relative risk of diarrheal episode over the second and third week post-weaning by dividing the pigs into two groups depending on their serum zinc level was higher or lower than weaning level (767 µg/L).

	Serum zinc concentration		Relative risk (95% CI)	*p*-value
	≤767 µg/L	**>767 µg/L**		
Serum Zn concentration day 7				
Number of pigs	87	85		
Days with diarrhoea (d 7-13)*	126	81	1.52	<0.01
Total number of days	609	595	(1.18-1.96)	
Serum Zn concentration day 14				
Number of pigs	54	111		
Days with diarrhoea (d 14-20)*	142	182	1.60	<0.01
Total number of days	378	777	(1.34-1.92)	

* Events of diarrhoea: the number of observations of score 3 or 4.

Error! Reference source not found. show the relation between serum Zn concentration and the dietary Zn concentration at day 7, 14 and 21 PW (p<0.001). To maintain the serum Zn level at 767 µg/L day 7 PW required the dietary Zn concentration to be 1,121 mg/kg (**Error! Reference source not found.**A), which would lead to a Zn intake of 166 mg/day during the first week PW. The dietary Zn concentration required to maintain the serum Zn level at 767 µg/L at day 14 and 21 PW decreased to 778 and 461 mg/kg, respectively (**Error! Reference source not found.**B-C), which would lead to a Zn intake of 307 and 209 mg/day the second and third week PW.

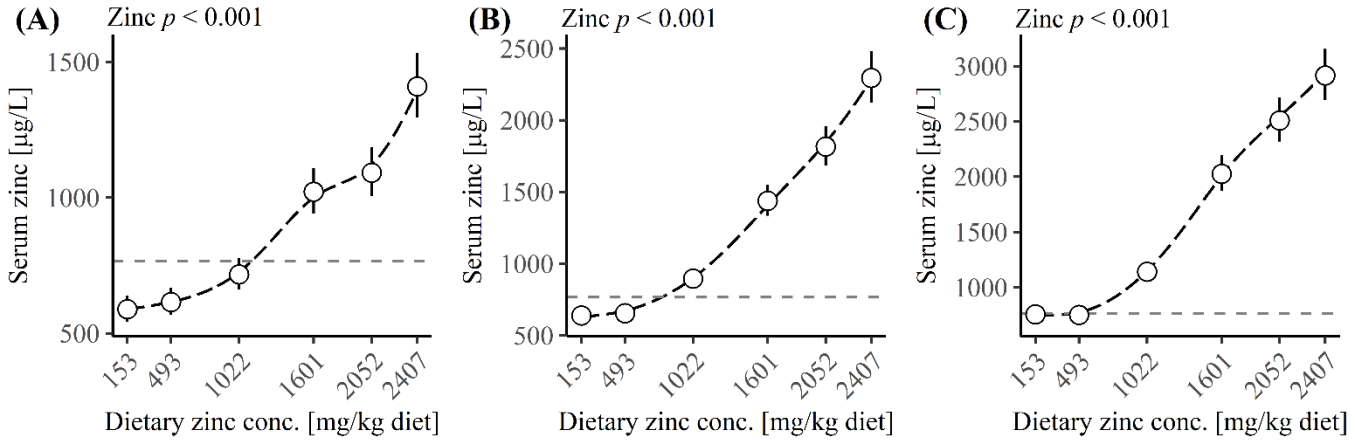

Figure 3. Average serum zinc concentration [µg/L] at (**A**) day 7 post-weaning (PW), (**B**) day 14 PW, (**C**) day 21 PW. The black dashed lines illustrate the effect of dietary zinc concentration, while the grey dashed line illustrates the serum zinc concentration at day 0.

3.4. Serum Zn status and weight gain

Error! Reference source not found. shows the quadratic correlation between serum Zn concentration and the ADG. The highest ADG during the first week PW was observed with a serum Zn level of 717 µg Zn/L (dietary Zn concentration of 1,022 mg Zn/kg, **Error! Reference source not found.**A), but the turning point was calculated to 1,011 µg Zn/L serum (Table S3). The highest ADG during the second week PW was obtained with 1,022-1,601 mg Zn/kg in the diet, which had a serum Zn concentration at day 14 PW of 902-1,406 µg/L (p<0.001, **Error! Reference source not found.**B). The maximal ADG during the second week PW was estimated to occur with a serum Zn level at day 14 PW of 1,279 µg/L (389 g/day, Table S3). To obtain this serum Zn level day 14 PW required 1,471 mg dietary Zn/kg during the second week PW (**Error! Reference source not found.**B). The highest ADG during the third week PW was obtained with a serum Zn concentration at day 21 PW between 1,142 and 2,527 µg/L (p<0.001, **Error! Reference source not found.**C). The maximal ADG during the third week PW was estimated to occur with a serum Zn concentration day 21 PW of 1,724 µg/L (585 g/day, Table S3). To obtain this serum Zn level at day 21 PW required 1,418 mg dietary Zn/kg during the third week PW (**Error! Reference source not found.**C).

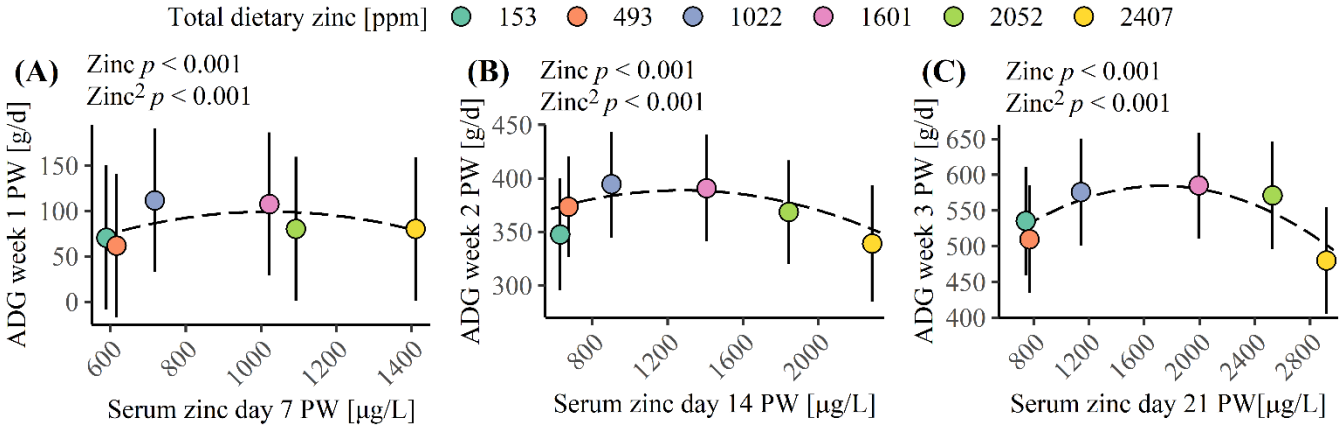

Figure 4. Average daily gain (ADG) as a function of serum zinc concentration. (a) The ADG the first week post-weaning (PW) and the mean serum zinc concentrations at day 7 PW. (b) The ADG the second week PW and the mean serum zinc concentrations at day 14 PW. (c) The ADG the third week PW and the mean serum zinc concentrations at day 21 PW (30 pigs/diet).

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3.5. Intestinal integrity and faecal bacteria

Dietary Zn level had no effect on the concentration of D-lactate at day 7, 14 and 21 PW, nor on the DAO activity at day 21 PW (p>0.05, **Error! Reference source not found.**).

Table 5. Effect of dietary Zn level on D-lactate concentration and diamine oxidase (DAO) activity in plasma.

	Dietary Zn [mg Zn/kg diet]							
	153	**493**	**1022**	**1601**	**2052**	**2407**	**95 % CI**	***p*-value**
				D-lactate [mg/L]				
Day 0	0.71	0.91	1.48	1.18	0.88	1.54	0.63-1.82	0.06
Day 7	2.27	3.01	2.74	2.23	1.61	1.87	1.54-3.25	0.6
Day 14	1.97	1.60	1.93	2.43	2.11	1.67	1.54-2.42	0.81
Day 21	2.10	1.11	1.43	4.14	1.48	1.03	0.92-1.96	0.47
				DAO [Units / min]				
Day 21	100	119	104	123	123	124	82-149	0.25

The dietary Zn concentration had a tendency of reducing the number of total faecal bacteria day 21 (p=0.08), while the number of total *E. coli* and *Lactobacilli* were reduced by the dietary Zn concentration (p=0.04 and 0.001, respectively; **Error! Reference source not found.**). A dietary Zn concentration of 2,407 mg/kg tended to have a lower number of total bacteria and total *E. coli* compared to 153 mg/kg (p=0.065 and 0.054, respectively; **Error! Reference source not found.**), while the number of total *Lactobacilli* were lower with 2,407 mg/kg than 153 mg/kg (p=0.001). Dietary Zn levels of 1022 and 1601mg/kg had a similar number as both 153 and 2,407 mg/kg (**Error! Reference source not found.**). **Error! Reference source not found.** shows that the number of total bacteria, *Lactobacilli*, and *E. coli* were negative correlated with the total Zn intake with R^2 values between 0.13 and 0.39 (p<0.05).

Table 6. The number of total bacteria, total *Lactobacilli* and total *E. coli* in faeces at day 21 post-weaning (log copies/g sample, N = 20 pigs/dietary group).

	Total dietary Zn [mg Zn/kg diet]					
	153	**1022**	**1601**	**2407**	**95 % CI**	***p*-value**
Total bacteria count (log copies/g sample)	10.4^{A}	10.3^{AB}	10.2^{AB}	10.0^{B}	10.0-10.4	0.08
Total *Lactobacilli* count (log copies/g sample)	9.80^{a}	9.44^{ab}	9.39^{ab}	8.92^{b}	8.98-9.82	0.001
Total *E. coli* count (log copies/g sample)	5.88^{A}	5.51^{AB}	5.25^{AB}	4.97^{B}	5.08-5.73	0.04

^{a,b} Different letters indicate significant difference (p<0.05). $^{A, B}$ Different letters indicate tendency of a difference (0.05<P<0.01).

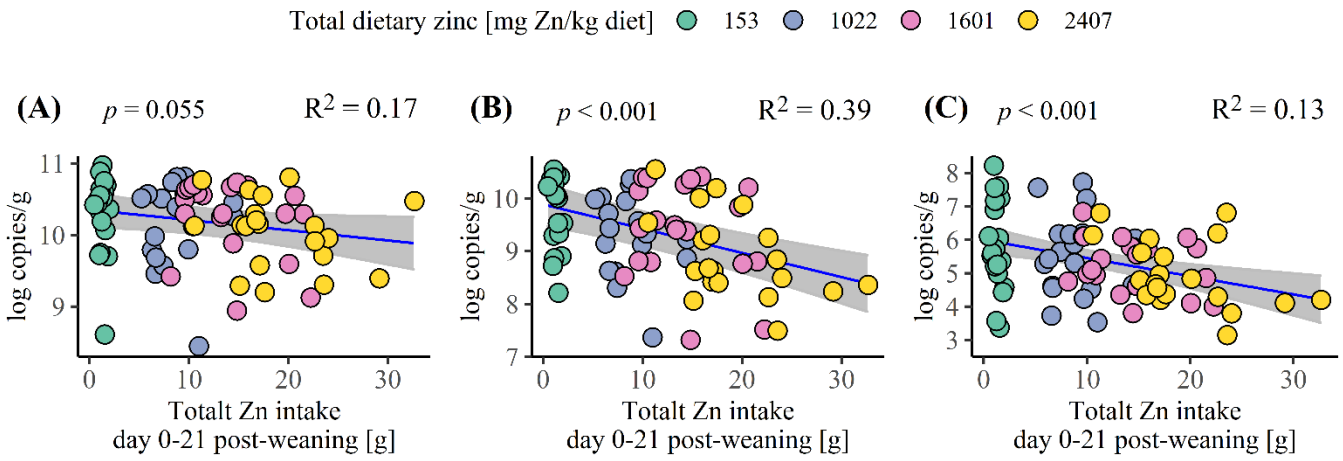

Figure 5. The correlation between the total zinc intake during the 21 days of experiment and the faecal number of (A) total bacteria, (B) total *Lactobacilli*, and (C) total *Escherichia coli* day 21 post-weaning. The correlation is assessed based on the R^2. The blue line illustrate the correlation and the grey area illustrate the 95% confidence interval (20 pigs/diet).

4. Discussion

The Zn requirement in weaned pigs has been investigated in several studies over the years [30,31,44-46]. However, the experimental setup in most of these studies included an adjustment period during the first 7-14 days PW [30,31,45] and thereby, the results are not applicable to estimate the Zn requirement in pigs the first week PW. In Poulsen [44], six dietary Zn supplementation levels (0, 100, 200, 1,000, 2,500 and 4,000 mg/kg diet) were provided from weaning at 28 days of age and the following five weeks. The ADG decreased when the dietary Zn supplementation was above 2,500 mg/kg diet. However, as there was no supplementation doses between 1,000 and 2,500 mg Zn/kg diet, it is unknown what the ADG would have been in this range. Hahn and Baker [45] included no adjustment period PW (weaning at day 28 of age), and showed similar growth performance following six Zn supplementation doses (0, 250, 500, 1000, 3000 or 5000 mg/kg diet). Antibiotics were added to all diets this study, and therefore, results cannot be compared with our results.

4.1. Feed intake and weight gain

The calculated turning point for maximal feed intake of approximately 1400 mg/kg week 2 and week 1-2 is in agreement with results of Hill et al. [46]. They reported a quadratic effect of dietary Zn content on ADFI and the highest ADFI was achieved when pigs were fed a diet with 1,500 mg Zn/kg diet supplementation compared to 0 or 3,000 mg Zn/kg diet. Since many studies apply only two dietary Zn concentrations: a low and a high (typically 100-250 and 2,000-3,000 mg Zn/kg diet), this may explain why some studies report no effect of dietary Zn content on the feed intake [47-49]. The quadratic effect of dietary Zn content on feed intake may relate to the influence of Zn on the palatability of feed. Reynolds et al. [50] found that when pigs weaned at day 28 of age were given the choice between a Zn unsupplemented diet and a diet supplemented with 3,100 mg Zn/kg diet (as ZnO), up to 91 % of the total feed consumption was of the unsupplemented diet. Possibly, the pigs chooses an unsupplemented diet because of an unpleasant taste in the Zn supplemented diet. Zhang and Guo [51] and Yin et al. [52] found a higher concentration of circulating ghrelin in pigs (24-28 days of age) fed 2,000 mg Zn/kg diet compared to 100-130 mg Zn/kg diet. Ghrelin secreted from enteroendocrine cells of the gastrointestinal tract is involved in appetite regulation, and increased ghrelin secretion stimulates appetite [53]. It can therefore be speculated, that the highest feed intake we calculated to coincide with approximately 1,400 mg Zn/kg diet represents a balance between increased ghrelin

secretion with higher levels of dietary Zn and reduced palatability of feed with high levels of ZnO.

Some studies have shown that 2,000-3,000 mg Zn/kg diet increase ADG of weaned pigs compared to 100-150 mg Zn/kg diet during the first four weeks PW [45,47,54]. However, the quadratic effect of dietary Zn on ADG shown in the present study corresponds to other studies [44,46,55]. A quadratic effect of dietary Zn level on ADG may explain why other studies observed no effect of dietary Zn content on ADG, as it has been most common to compare <250 mg Zn/kg diet to >2,000 mg Zn/kg diet [22,49,56]. The quadratic relationship between dietary Zn content and ADG is strongly related to the quadratic relationship between dietary Zn content and feed intake. Kjeldsen et al. [57] showed with a large Danish farm trial (750 pigs/diet) that a dietary Zn level of 2,500 mg/kg compared to 1,500 mg/kg resulted in a slightly higher feed intake (264 g/day vs. 247 g/day) and ADG (222 g/day vs. 207 g/day) during the first 11 days PW. From day 14 to 30 kg, all pigs were fed a diet with 100 mg added Zn/kg diet and over the entire experimental period (7-30 kg) there was no difference in production parameters (gain, feed intake, feed conversion ratio) between 1,500 and 2,500 mg Zn/kg diet. Pigs fed only 100 mg Zn/kg diet during the entire experimental period showed reduced production results, indicating that adding 100 mg Zn/kg diet is not enough to fulfil the Zn requirement. Another factor potentially influencing feed intake is housing type as individually housed pigs may show a lower feed intake compared with group housed animals, as Shirali et al. [58] found singled house pigs 60-90 kg had a 7% lower energy intake compared to grouped housed pigs. Therefore, the optimal dietary Zn concentration in newly weaned pigs of approximately 1400 mg Zn/kg estimated from our results on growth and feed intake may represent a maximum, since group housed pigs under normal production management practices are expected to eat somewhat more than our individually housed pigs.

The level of feed intake during the first week PW was similar to levels reported by Bruininx et al. [24,25]. After June 2022, only 150 mg Zn/kg diet will be allowed for weaned pigs in the EU and if NRC´s current recommendation of 48.6 mg Zn/day for 7-11 kg pigs is then to be achieved, they should eat 312 g/day. The probability of a feed intake less than 312 g/day was more than 0.84 the first seven days PW, and pigs fed 153 mg Zn/kg diet had a Zn intake of less than 23 mg/day the first seven days PW. Brugger et al. [30] estimated that pigs fed a Zn deficient diet would reach clinical Zn deficiency after approximately 10 days because of depletion of whole-body Zn storage. Clinical symptoms of Zn deficiency include depressed growth, reduced feed intake, diarrhoea and impaired immune function, while subclinical Zn deficiency is associated with reduced Zn status parameters such as serum Zn concentration [1]. Brugger et al. [30] mainly focus on the Zn status parameters to determine sufficient dietary Zn content, but King et al. [28] stated that increased growth (weight gain and linear growth) as a result of Zn supplementation reflects Zn deficiency and thus growth is a functional indicator of Zn requirement. Based on this, our data indicate that the daily dietary Zn requirement for the first two weeks PW might be up to 9 times higher than the NRC recommendation as we found the highest ADFI and ADG would lead to an average Zn intake of 395-436 mg/day compared to NRC recommendation of 48.6 mg/day.

4.2. Diarrhoea probability and faecal microbial composition

Dietary Zn level did not affect the probability of diarrhetic faecal scores in week 1 PW, which is in line with results from other studies [59,60]. It is possible that the various stressors piglets are exposed to around weaning, potentially leading to disruption of gut microbiota and inflammation may not manifest into PW diarrhoea until the second week PW [61]. However, in the second and third week PW, pigs receiving 153 mg Zn/kg diet had the highest probability of diarrhetic faecal scores. Human studies link Zn deficiency in children to diarrhoea [13,14,62] and therefore it can be speculated that a higher probability of diarrhoea for pigs receiving 153 compared to 2407 mg Zn/kg indicates Zn defi-

ciency. The two highest dietary Zn levels showed the lowest probability of diarrhetic faeces scores, whereas the intermediate Zn concentrations were not different from neither the 153 nor the 2,052 mg Zn/kg diet. A possible interpretation of this is that a diet containing 491 to 2407 mg Zn/kg is sufficient to prevent Zn deficiency manifested as increased probability of diarrhoea in PW week 2. Kjeldsen et al. [57] showed that there was no further beneficial effect of going from 1,500 to 2,500 mg Zn/kg diet, when evaluating the proportion of pigs receiving antibiotic treatment due to diarrhoea.

One of the arguments for no longer allowing the use of high doses of Zn to newly weaned piglets is that some studies indicate that it increases the risk of development of antimicrobial resistance [63,64] and general anti-microbial effects on the microbiota richness [49,65]. Our results showed similar numbers of total bacteria, Lactobacilli and total coliform bacteria in faeces at dietary Zn contents of 153, 1,022 and 1,601 mg/kg, which is similar as the results obtained by Pieper et al. [66]. Therefore, there are no indications, that 1,400 mg Zn/kg diet would modulate the faecal proportions of the three groups of bacteria investigated here or exert anti-microbial effects.

4.3. Serum Zn status

The risk of diarrhoea have been related the Zn status in blood in humans with Zn deficiency [13]. Bahl et al. [13] found children's risk of diarrhoea increased by 47% if the plasma Zn concentration was lower than 550 µg/L compared to a higher plasma Zn level. This corresponds to our result, that if the serum Zn level decreased below the average at weaning, the risk of diarrhoea increased to 52-60%. Therefore, it seems essential to maintain the weaning serum Zn level PW if increased risk of diarrhoea the following weeks should be avoided. Pigs receiving 153 to 493 mg Zn/kg diet showed reduced serum Zn status relative to the day of weaning on day 7 and 14 PW corresponding to findings of Carlson et al. [67] and Burrough et al. [68]. We calculated that to maintain the serum Zn level at weaning it required 1,121, 778 and 461 mg Zn/kg diet at day 7, 14 and 21 PW, respectively.

4.4. Serum Zn status and weight gain

Only Hahn and Baker [45] have previously studied the correlation between Zn status in the blood and ADG in weaned pigs. They reported highest ADG (day 0-21) with a plasma Zn status (day 21) of approximately 1,500 µg/L, which corresponds well with our results. Together, these results agree well with the assumed upper limit of the adequate Zn level stated by Puls [69].

4.5. Intestinal integrity

The activity of DAO and D-lactate concentration are well-established biomarkers of integrity of the mucosal barrier in the small and large intestine, respectively [70,71], as the plasma concentration will increase if the permeability of the intestinal wall is compromised [72,73]. Some studies have shown that high dietary Zn level (2,200-3,000 mg Zn/kg diet) reduce the DAO activity in plasma/serum compared to low dietary Zn levels (100 mg Zn/kg diet) [74,75]. This is in contrast to our results but it could be due to differences in when it was measured PW (7-14 vs. 21 days), as well as the methods used to determine the level of activity. However, the lack of effect of dietary Zn on D-lactate corresponds to findings from other studies [75-77]. Overall, these results indicate that the integrity of the intestines are unaffected by the level of dietary Zn, but it could be due to a high hygiene level in the experimental barn.

5. Conclusions

In conclusion, more than 80% of pigs were undersupplied with Zn during the first week PW according to current NRC recommendations, if they received a diet containing 153 mg Zn/kg. The optimal level of dietary Zn the first two weeks PW was approximately

1400 mg/kg corresponding to a daily intake of 400 mg Zn when ZnO is the source of added Zn and feed intake and growth are the primary endpoints. This is approximately 9 times higher current NRC recommendations. There were no indications that a dietary level of 1400 mg Zn/kg diet exceeded a nutritional level the first two weeks PW. Serum Zn is related to the risk of diarrhoea and if the Zn status drop below the weaning level, the risk of diarrhoea increases up to 60% the following two weeks.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: The probability of pigs eating less than 312 g/day the first 14 days post-weaning; Figure S2: The average daily zinc intake during day 2-7 post-weaning. Letters indicate a significant (P<0.05) difference between dietary groups within each day. Values are LS-means ± 95% CI; Table S1: Estimates of the parameters of the equation of the average daily feed intake fitted on the dietary zinc concentration.; Table S2: Overview of the average daily gain equations fitted on the dietary zinc concentration; Table S3: Overview of the average daily gain equations fitted on the serum zinc concentration.

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