EVALUATING THE REPLACEMENT OF ZINC OXIDE WITH AN ENCAPSULATED ZINC OXIDE PRODUCT AS A MEANS OF CONTROLLING POST-WEANING DIARRHOEA IN PIGLETS

Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

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By

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Executive Summary

As the use of in-feed antimicrobial growth promoters to control post-weaning diarrhoea is banned or limited, such has been the case in much of Europe due primarily to consumer concerns about the transference of resistant pathogens to humans, numerous dietary and management strategies have been introduced and implemented. Despite the efforts of many research groups around the world, poor gut health of pigs after weaning when fed an antibiotic-free diet compromises the potential growth of pigs. Gut health of weaned piglets is known to be influenced by many factors such as nutritional, physiological and psychological stressors, immune functions, hygienic conditions, intestinal barrier functions and diet composition. The most available strategies are summarised in recent reviews (Halas et al., 2007; Moran, 2007; Pluske et al., 2007). Among those strategies, pharmacological use of zinc oxide (2,500-3000 ppm ZnO) is widely accepted as the means of controlling post-weaning diarrhoea and is being used worldwide as an alternative for antibiotics. However, the strategy is criticized because high levels of zinc are excreted into the environment through the effluent system. Recently, a microencapsulated zinc oxide product was released on the market and the lipid-coated ZnO has been claimed to dramatically decrease inclusion of ZnO from 2,500-3,000 ppm to 100 ppm to achieve the same effect on PWD. Therefore, the microencapsulated zinc oxide was evaluated as a solution for the environmental issue as well as controlling PWD.

The results showed that inclusion of 100 ppm microencapsulated ZnO suppressed the expression of Post-weaning diarrhoea (PWD) in both enterotoxigenic *E. coli* (ETEC) challenged and non-challenged pigs, and kept the plasma and faecal zinc levels to the levels of that found in the pigs fed a control diet without additional ZnO supplementation. The results from this experiment suggest that expression of PWD can be reduced by supplementing 100 ppm microencapsulated ZnO in the diets for weaner pigs without compromising faecal zinc excretion levels.

Table of Contents

1. Introduction

In commercial piggeries, piglets are commonly weaned at 20 to 24 days of age to maximise the number of litters per sow per year. However, piglets at this age are particularly vulnerable to diarrhoea as their passive immunity from the sow is low and the piglet has only just started to build up its own immunity. Furthermore, weaning is also a time when the piglet is under extreme stress due to a change in diet, a new environment, and mixing with unfamiliar pigs (Pluske et al., 1997). As a consequence, post-weaning diarrhoea (PWD) caused by enterotoxigenic *E. coli* (ETEC) is a major problem in weaner piglets. Post-weaning diarrhoea is associated with an increase in morbidity and mortality rates, a high cost of treatment, and a severe decrease in growth rate during the weaner period (Hampson et al., 2001). Importantly, the growth check associated with PWD decreases lifetime performance of the pig (Pluske et al., 1997).

To minimise the post-weaning growth lag and the potential effects of *ETEC,* weaner piglets are treated with in-feed antibiotics and/or high concentrations (3kg/tonne) of zinc oxide in the diet. However, neither option is a viable long-term solution to the problem of PWD. There is considerable concern about the development of antibiotic resistance in bacteria and the toxic effect of faeces containing high concentrations of zinc on the environment (Case and Carlson, 2002; Poulsen, 1995). Therefore there is a need for an effective environmentally safe alternative.

Numerous studies have shown the production and/or anti-diarrhoeal benefits of including ZnO at high levels in weaner diets (Pluske et al, 2007). Reported effects include the increased gene expression of antimicrobial peptides in the small intestine, positive effects on the stability and diversity of the microbiota, bactericidal functions and reductions in electrolyte secretion *in vitro* from enterocytes. However, some studies have reported no benefit of feeding ZnO (Broom et al, 2006), whereas Hedermann et al (2006) concluded that while it was an effective treatment for PWD there were no definite answers as to how excess dietary Zn exerted its effects.

A recent study demonstrated that high levels of Zn in a weaner diet increases tight junction protein expression and hence decreases intestinal permeability (Zhang and Guo, 2009). The authors speculated that decreasing intestinal permeability due to dietary Zn supplementation may have prevented translocation of pathogenic bacteria through the intestinal barrier. Despite the ambiguity related to the exact mechanism(s) of action of ZnO, it is likely that it will continue to be used and studied because it is a cost effective nutritional tool. However, the high level of Zn excreted in the faeces is an environmental concern and in Europe high levels of ZnO can now only be used under veterinary prescription. It is possible that a similar ruling may apply in Australia at some stage in the future.

Shield Zn is a microencapsulated inorganic zinc oxide product, containing 100g/kg of ZnO. According to the Korean manufacturers of this product, CTCBio Inc, it is designed to deliver Zn^{++} ions to the ideal location in the gastrointestinal tract for maximum effectiveness in the control and treatment of PWD. The lipid matrix of Shield Zn prevents the absorption or chemical change of ZnO in the stomach, allowing it to enter the upper intestine where the lipid coating is broken down by lipase enzymes. This releases the zinc oxide in the critical area of the

gastrointestinal tract for maximum effectiveness. Limited company trials with Shield Zn have indicated similar control of PWD to that of ZnO, and in some case an improvement in the feed conversion ratio (FCR).

The incidence of PWD is difficult to predict for reasons that we do not understand. For this reason it is necessary to inoculate piglets at weaning with ETEC to ensure that we have an experiment in which PWD will occur. Similarly, it is also important to study the impact that these additives have on pigs when not challenged with ETEC. There is also anecdotal evidence that the inclusion of high levels of ZnO might actually decrease feed intake because of its bitter taste but we are not aware of any data to support this claim.

A high proportion of the Zn contained in ZnO is excreted from the body in faeces, and as such has the potential to be an environmental pollutant. If we can control PWD with a lower level of ZnO, such as in the form of Shield Zn, then this has benefits to the environment

2. Methodology

Experimental design
An experiment with a split-plot design for which the whole plots were arranged in randomised blocks was conducted. Challenge versus no-challenge with ETEC (*E. coli* O149:K91:K88) were the factors in the whole plot, and the three dietary treatments (control, ZnO and encapsulated Zn) were used as subplots (n=12). A total of 72 weaner pigs (castrate and female, 1:1) were used in a 3-week feeding experiment (housed individually, providing 12 replicates per treatment). The experiment assessed the incidence of PWD, monitored production indices (feed intake, daily gain, feed conversion ratio), and measured faecal zinc excretion levels and plasma zinc concentrations.

Detailed methods
Pigs (72 female and castrate, 1:1) were acquired at weaning (day 21 ± 1 days) from a commercial farm (Craig Mostyn Farm, Nambeelup, WA) and were transported to the Medina Research Station. Upon arrival, pigs were weighed, ear tagged, housed individually (space allowance 0.4 m^2 per pig) and were randomly stratified by live weight to one of following treatments.

1. Control - no ETEC challenge
2. ZnO 3,000 ppm- no ETEC challenge
3. Shield Zn 100 ppm - no ETEC challenge
4. Control + ETEC challenge
5. ZnO 3,000 ppm + ETEC challenge
6. Shield Zn 100 ppm + ETEC challenge

At approximately 72 hours after weaning, the pigs in the ETEC challenge treatment received an oral enterotoxigenic *E. coli* challenge (approximately 10 mL of 3 x 10^8 colony forming units of ETEC (*E. coli* O149:K90:K88) per ml of broth administered) to reproduce post-weaning diarrhoea. At the same occasion, piglets on the non- ETEC challenge treatments received the same amount of physiological saline solution orally to equilibrate the level of stress associated with the oral ETEC challenge. Piglets were fed their respective diets on an *ad libitum* basis for 3 weeks post-weaning. Fresh water was available throughout the experiment. The

composition of experimental diets is presented in Table 1. Pigs were weighed weekly and feed intake was measured on a weekly basis.

Table 1. Composition of experimental diets (g/kg, as-fed basis).

	Control	ZnO	Shield Zn
Wheat	656	650	654
Soybean meal	150	150	150
Blood meal 85%	20	20	20
Meat meal 50%	43	45	44
Fishmeal 60%	39	39	39
Skim milk powder	50	50	50
Canola oil	29	31	29
Lysine	3.00	3.00	3.00
Methionine	1.59	1.60	1.59
Threonine	1.12	1.12	1.12
Mineral vitamin premix	1.00	1.00	1.00
Limestone	2.31	2.10	2.24
Dical Phos	0.94	0.60	0.83
Salt	1.00	1.00	1.00
ZnO		3.00	
Shield Zn[2]			1.00
Titanium	2.00	2.00	2.00
Total	1000	1000	1000
Calculated composition, g/kg			
DE, MJ/kg	15	15	15
CP	230	230	230
Available Phosphorus	4.5	4.5	4.5
Calcium	9	9	9
NDF	98	97	98
ADF	30	29	30
SID[1] Lysine/MJ DE	0.88	0.88	0.88
SID Lysine	13.2	13.2	13.2
SID Met+Cys	7.7	7.7	7.7
SIDThr	8.3	8.3	8.3
SID Trp	2.4	2.4	2.4
SDI Ile	7.8	7.8	7.8
SID Leu	15.4	15.4	15.4
SID Val	1.0	1.0	1.0
Analysed composition			
Zn, mg/kg	198	3,043	315

[1]SID: Standardised ileal digestible

[2]Microencapsulated zinc oxide- included 0.1 g/kg of the product supplying 100 ppm zn. Zamira Life Sciences Pty. Ltd., Knoxfield, Vic.

Pigs having diarrhoea were treated with either Trisoprim-480 (trimethropin 80 mg/ml, sulfadiazine, 400 mg/ml, 0.05 ml/kg body weight, Troy Laboratories, Smithfield, NSW, Australia) or Betamox (150 mg/mL amoxicillin, Norbrook Lab Ltd, Vic, Australia), until considered healthy and the number of antibiotic treatments were recorded. The treatment was initiated when the faecal score exceeded 4 and ceased at 3 (Kim et al., 2008).

Faecal score and incidence of diarrhoea were visually assessed daily for the first 2 weeks. Faecal consistency was recorded daily for the first 14 days of the experiment using a subjective score on a four-point scale ranging from 1 to 4, where 1 = firm well formed, 2 = soft, 3 = loose and 4=diarrhoea. Pigs with faecal score 4 was considered as pigs with diarrhoea and used for calculation of diarrhoea index. Diarrhoea index was expressed as a proportion of days with diarrhoea with respect to total number of days (14 d) (after Mateos et al. 2006).

Blood samples were collected in a Lithium Heparin coated vacutainer via the anterior vena cava at 14 days post-weaning and were centrifuged for 5 min at x 2000 g to harvest plasma samples. Plasma zinc and plasma urea nitrogen contents were determined using Atomic Absorbance Spectrophotometry and an enzymatic (urease) kinetic method (Randox, Crumlin, Co., Antrim, UK), respectively. Faecal samples were collected for 3 consecutive days at the end of week 2 to determine faecal Zn excretion levels using Atomic Absorbance Spectrophotometry.

Statistical analysis

Statistical analyses were performed using SAS for Windows, Version 9.13 (SAS Inst. Inc., Cay, NC) with each animal as the experimental unit. Three pigs from the shield zinc + ETEC challenge treatment and 2 pigs from the control + ETEC challenge treatment died a day after the experimental ETEC challenge. The post-mortem report suggests that dehydration, diarrhoea and some entry of E coli through airway were the likely cause of the death. The number of observations for shield zinc + ETEC challenge treatment and control + ETEC challenge treatment were 9 and 10, respectively, while all other treatments were 12. Data were analysed with the procedure MIXED using a split-plot model where challenge with E. coli was the factor in the main plot, and the three dietary treatments were used as subplots. As there were no gender effects for any of the measurements it was removed from the model. Faecal consistency score was considered as repeated measurements on the same animal and analysed accordingly. If an interaction was not significant ($P > 0.05$), it was removed from the model. All presented results are expressed as least square means and standard error of the means. Treatment effects were considered significant at $P < 0.05$, whereas $P < 0.10$ was considered a trend.

3. Outcomes

Performance of pigs

Pig weights and average daily gain (ADG) were not affected by the dietary treatments or ETEC challenge. Feed intake tended (P = 0.092) to be lower in the first week after weaning and overall (P = 0.109) for the pigs challenged with ETEC. The FCR was not calculated for the first week after weaning due to the high number of negative values. Overall FCR was affected by diet as the pigs fed Shield-Zn had an improved FCR compared with the pigs receiving ZnO (Table 2).

Table 2. Effects of feeding ZnO and Shield-Zn (S-Zn) and challenge with E. coli (ETEC) on pig weight, average daily gain, daily feed intake and feed conversion ratio.

	No ETEC challenge			ETEC challenge				P-value		
	Control	ZnO	S-Zn	Control	ZnO	S-Zn	SEM	Diet	ETEC	DxE
Pig weights, kg										
Weaning	5.6	5.6	5.6	5.7	5.6	5.6	0.28	-	-	-
Day 7	6.6	7.0	6.6	6.4	6.5	6.7	0.20	0.493	0.154	0.295
Day 14	9.1	9.6	9.0	8.8	8.7	8.9	0.41	0.819	0.210	0.639
Day 21	12.0	11.6	12.2	11.6	11.8	11.9	0.51	0.986	0.440	0.757
Average daily gain, g/d										
Week 1	147	200	137	112	123	150	28.2	0.493	0.154	0.295
Week 2	373	360	359	314	317	334	35.8	0.976	0.244	0.872
Week 3	413	411	406	409	407	427	35.5	0.983	0.884	0.923
Overall	313	314	303	275	282	304	23.0	0.964	0.236	0.649
Feed intake, g/d										
Week 1	247	281	219	201	219	218	25.3	0.361	0.092	0.478
Week 2	451	519	423	431	408	424	37.4	0.490	0.154	0.281
Week 3	676	700	664	647	652	652	45.2	0.908	0.419	0.927
Overall	453	490	430	409	416	423	30.9	0.593	0.109	0.562
Feed conversion ratio, g/g										
Week 1	-	-	-	-	-	-				
Week 2	1.17	1.47	1.20	1.34	1.27	1.23	0.099	0.191	0.914	0.099
Week 3	1.66	1.90	1.78	1.61	1.67	1.54	0.202	0.705	0.335	0.856
Overall	1.45	1.55	1.41	1.53	1.55	1.37	0.066	0.039	0.844	0.612

Post-weaning diarrhoea and mean antibiotic treatment days

Diet significantly affected the incidence of PWD expressed as the mean percentage of days with diarrhoea during 14 days post-weaning (P < 0.001) whereas challenge with ETEC in this experiment failed to increase the incidence of diarrhoea (Figure 1). Pigs fed the control diet had a higher incidence of PWD compared with the pigs fed ZnO and Shield-Zn supplemented diets. As a consequence pigs fed a control diet required more antibiotic treatments during the first 2 weeks after weaning (P<0.001, Table 3) compared with pigs fed either ZnO or Shield Zn supplemented diets. On average the control pigs had 2.1 antibiotic treatments, whilst the pigs fed ZnO and Shield-Zn had only 0.3 and 0.8 antibiotic treatments, respectively, during the first 14 days post-weaning. The development in faecal consistency score as a function of time after weaning is shown in Figure 2.

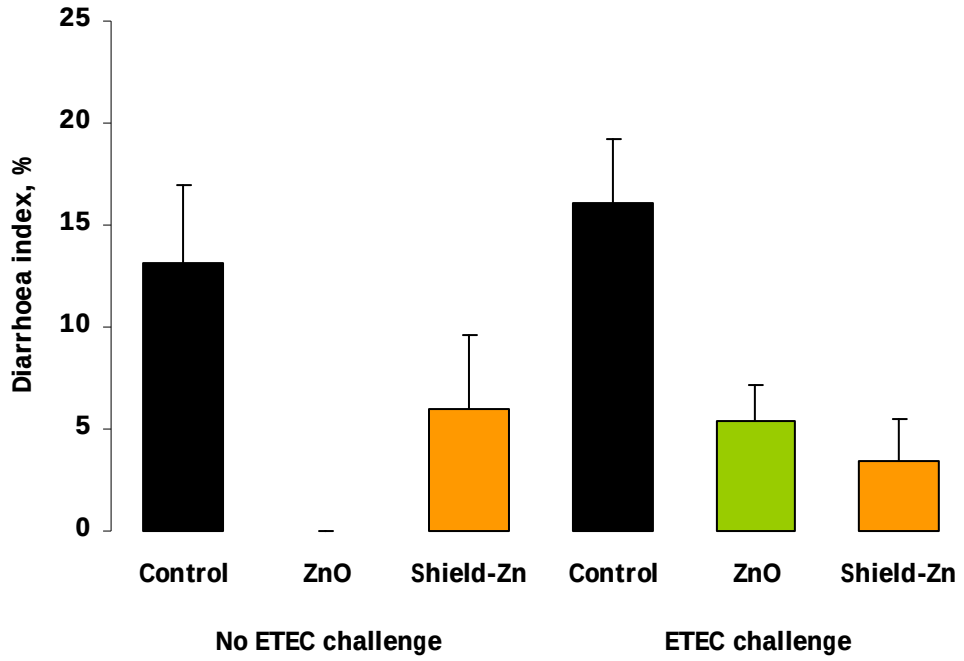

Figure 1. Effect of ZnO and Shield zinc supplementation in ETEC-challenged or non-challenged pigs on the incidence of PWD expressed as percentage of days with diarrhoea during the 14 days after weaning. Diet effect was significant (P<0.001), while ETEC challenge and interaction between diet and ETEC challenge were not significant.

Table 3. Effects of feeding ZnO and Shield-Zn, and challenge with ETEC on the percentage of pigs with diarrhoea, and the mean number of antibiotic treatments during the first 14 days post-weaning.

	No ETEC challenge			ETEC challenge				P-value		
	Control	ZnO	S-Zn	Control	ZnO	S-Zn	SEM	Diet	ETEC	DxE
Number of pigs with diarrhoea	8/12	0/12	3/12	10/10	6/12	4/12				
% pigs with diarrhoea	67	0	25	100	50	44				
Number of antibiotic treatments	1.8	0.0	0.8	2.3	0.7	0.5	0.41	<0.001	0.285	0.436

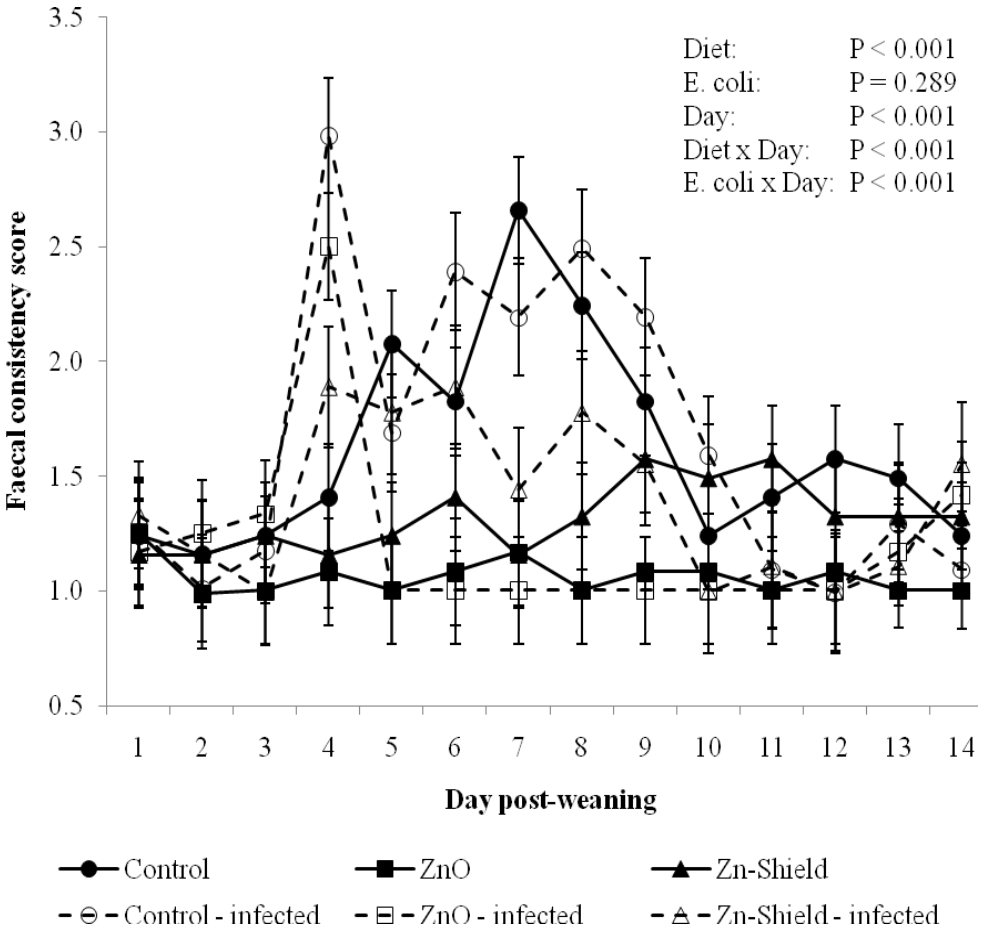

Figure 2. Effects of feeding ZnO and Shield-Zn, and experimental ETEC challenge, on faecal consistency score. 1: Firm, well formed; 2: Soft; 3: Loose; 4: Diarrhoea.

Plasma Urea Nitrogen

Plasma urea nitrogen (PUN) levels were affected (P = 0.046) by the dietary treatment, but not by ETEC challenge (Figure 3). PUN levels were lower in the pigs fed ZnO (4.7 mmol/L) and Shield-Zn (4.8 mmol/L) compared with the pigs receiving the control diet (5.5 mmol/L).

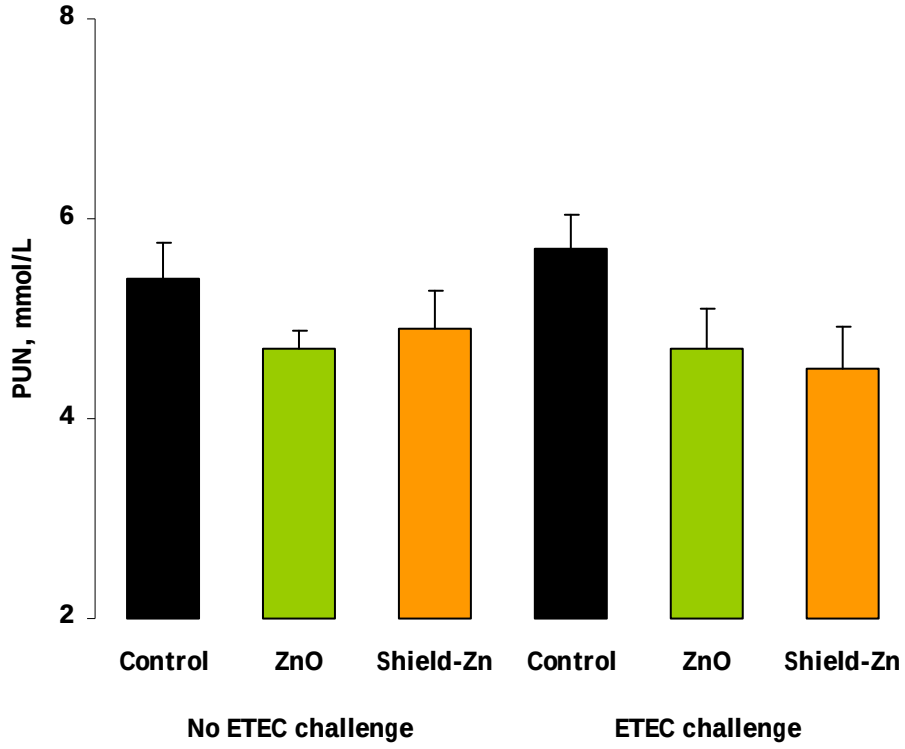

Figure 3. Effect of ZnO and Shield zinc supplementation in either ETEC-challenged or non-challenged pigs on plasma urea nitrogen (PUN) content determined on day 14 after weaning. Diet effect was significant (P=0.046), while ETEC challenge and interaction between diet and ETEC challenge were not significant.

Plasma and faecal zinc concentration

Plasma Zn concentration was significantly greater (P < 0.001) in the pigs fed the 3,000 ppm ZnO supplemented diet (2.38 mg/L) compared with that in the pigs that received the control diet (0.69 mg/L) and 100 ppm Shield-Zn diet (0.70 mg/L). Challenge with ETEC did not influence plasma Zn concentration.

Similarly, faecal Zn concentration was significantly greater (P < 0.001) in the pigs fed a diet supplemented 3,000 ppm ZnO (14,145 mg/kg) compared with that of the pigs that received the control (1,732 mg/kg) or Shield-Zn (2,504 mg/kg) diets. However, it is noteworthy that the faecal Zn concentration tended (P = 0.074) to be lower in the control pigs compared with the pigs fed Shield-Zn.

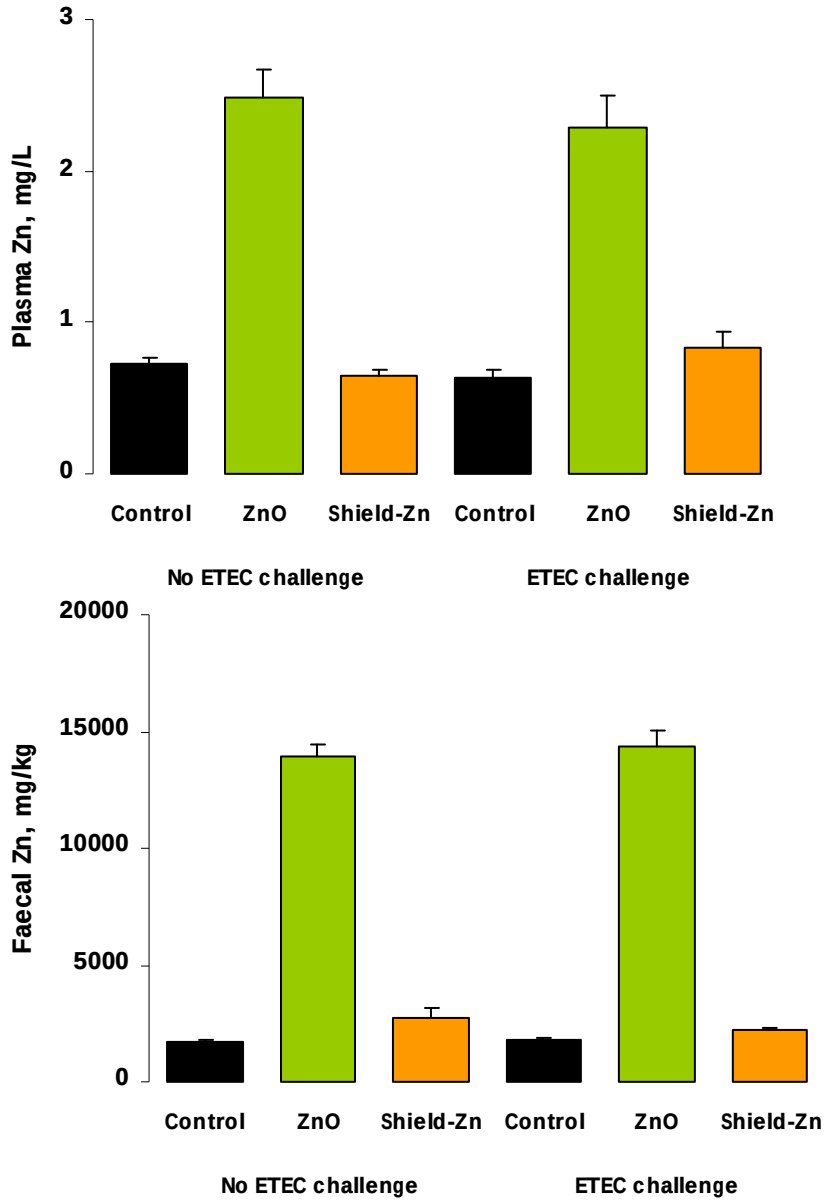

Figure 4. Effect of ZnO and Shield zinc supplementation on either ETEC-challenged or non-challenged pigs on plasma and faecal zinc concentrations determined on day 14 after weaning. Diet effect was significant (P<0.001), while ETEC challenge and interaction between diet and ETEC challenge were not significant.

4. Application of Research

Use of pharmaceutical levels of ZnO in diets for weaner pigs is widely accepted in the pig industry worldwide as a first choice for replacement for or in combination with in-feed antibiotics due to its proven effects on performance and PWD and its cost-effectiveness compared with other feed additives and dietary strategies. Exact mechanisms for how pharmaceutical levels of ZnO can reduce PWD is unclear but recent research suggests that suppression of PWD and growth promotion effects seen with high levels of ZnO supplementation in diets for weaner pigs are not associated with ETEC elimination in the GIT. For example, Hojberg et al. (2005) used a 16S rRNA gene sequencing technique and found that 2,500 ppm of ZnO significantly suppressed gram positive commensal microbes such as *Lactobacillus amylovorous, Lactobacillus reuteri,* and *Streptococcus alactolyticus* throughout the GIT but did not inhibit growth of potentially pathogenic gram negative microbes. Also, a recent *in vitro* study which examined *E. coli* K88 growth in tryptic soy broth dilute showed that addition of 250, 2,000, 3,000 and 5,000 ppm of ZnO did not suppress growth of *E. coli* K88 while zinc sulphate and copper sulphate did (Hardy et al., 2003). Rather, several molecular technique-based studies suggest that high levels of dietary ZnO reduces expression of PWD through reducing ETEC adhesion and intestinal permeability rather than manipulating pathogen population in the GIT (Li et al., 2001; Roseli et al., 2003; Zhang and Guo., 2009).

Although the beneficial effects of ZnO were proven and widely accepted, supplementation of pharmaceutical levels of ZnO in diets for weaner pigs has been scrutinised because increased Zn excretion in the effluent system can cause environmental pollution. Accordingly, some European countries banned the use of high levels of ZnO in diets for pigs and many other countries in the world are moving to limit use of ZnO in the diet for weaner pigs. Recently, microencapsulated zinc oxide was released on the market and the lipid-coated ZnO has been claimed to dramatically decrease inclusion of ZnO from 2,500-3,000 ppm to 100 ppm to achieve the same effect on PWD.

The results in this experiment showed that inclusion of 100 ppm microencapsulated ZnO suppressed the incidence of PWD in both ETEC-challenged and non-challenged pigs, and the suppressing effect on PWD was comparable to the pigs fed a diet supplemented with 3,000 ppm ZnO. Consequently, the number of antibiotic treatments that were required to control PWD was significantly decreased by supplementation of either 3,000 ppm ZnO or 100 ppm microencapsulated Zn. The results suggest that supplementation of 100 ppm microencapsulated Zn can replace conventional pharmaceutical use of ZnO for reduction of the number of pigs with diarrhoea and total incidence of PWD under both ETEC-challenged and non-challenged conditions.

In terms of performance responses, pigs fed a diet supplemented with microencapsulated ZnO had better feed conversion efficiency than pigs fed the control or 3000 ppm ZnO-supplemented diets. However the improved FCR in pigs fed 100 ppm microencapsulated zinc was evident only under conditions of ETEC challenge, especially in week 2 (Diet x ETEC challenge interaction, $P<0.1$). This finding may suggests that the microencapsulated ZnO can be beneficial under commercial production systems where a continual bacterial pathogenic challenge

exists. However, the performance results need to be interpreted with caution because only a small number of pigs (n=12) were used in this small scale study and therefore different results could be observed from a larger scale study.

Plasma urea nitrogen content was significantly lower in pigs fed diets supplemented with 3,000 ppm ZnO and 100 ppm microencapsulated Zn than pigs fed a control diet. As both Zn products reduced PWD possibly by better maintaining intestinal barrier functions, dietary amino acids could be more efficiently used in the pigs fed diets with both Zn products. However, in pigs fed a control diet, PWD was increased possibly due to compromised intestinal barrier functions, and some absorbed amino acids may have been partitioned for immune response, and hence increased plasma urea nitrogen. Therefore, this result indicates that both 3,000 ppm ZnO and 100 ppm microencapsulated ZnO supplementation improved amino acid utilization efficiency, although further experimentation is required to confirm this notion.

As expected, feeding a diet supplemented with 100 ppm microencapsulated ZnO kept the plasma and faecal zinc levels to the levels of that found in the pigs fed a control diet, whilst pigs fed 3,000 ppm ZnO dramatically increased their plasma Zn concentration and faecal Zn excretion. This result confirms that use of low levels of microencapsulated Zn can minimise Zn excretion levels to the effluent system.

Although further research is required to elucidate whether the use of low levels of microencapsulated Zn affects intestinal barrier functions *in vivo*, these data suggest that this feed additive reduces the incidence of PWD to a level similar to feeding ZnO whilst significantly reducing Zn excretion.

5. Conclusion

Results of this study clearly showed that supplementation of 100 ppm microencapsulated ZnO was as effective as supplementation of 3,000 ppm ZnO for suppression of PWD in the first 2 weeks after weaning. Also, supplementation of 100 ppm microencapsulated ZnO significantly reduced plasma zinc concentration and faecal zinc excretion levels. Therefore it is concluded that under the experimental conditions, expression of PWD can be reduced by supplementing 100 ppm microencapsulated ZnO in the diets for weaner pigs without compromising faecal zinc excretion levels.

6. Limitations/Risks

The experiment was conducted with a relatively small number of pigs and under hygienic condition using only 1 ETEC strain. Therefore, data should be interpreted with caution. The response might be different when pigs are exposed to less hygienic conditions and where multi-strains of pathogen might be present in the environment. A large-scale commercial validation study is required for confirmation of current findings.

7. Recommendations

Supplementation of 100 ppm microencapsulated ZnO for 2 weeks immediately after weaning is recommended to counteract pathogen-originated PWD and reduce Zn excretion.

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