

## Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets

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### Abstract

The inclusion of fibre has been studied as an alternative to antimicrobials in weaning pig diets, while ZnO is used as an effective method to prevent post-weaning diarrhoea. However, it has not been investigated to what extent these two strategies interact with each other. The present study was designed to evaluate the effects of including wheat bran (WB) and ZnO alone or combined in the diet of early-weaning pigs on productive performance and microbial activity in the gastrointestinal tract (trial 1). A total of sixty-four piglets were distributed in a 2 × 2 factorial combination of two levels of WB (0 v. 40 g/kg) and ZnO (0 v. 3 g/kg) in the diet. The inclusion of ZnO in the diet improved the feed intake and growth of the animals and reduced the incidence of diarrhoea. The inclusion of WB increased SCFA concentrations and decreased *Escherichia coli* counts. However, simultaneous incorporation of WB and ZnO increased *E. coli* counts. Two *in vitro* trials were also designed to clarify hypotheses derived from the *in vivo* test: (1) the ability of WB and other fibre sources to bind *E. coli in vitro* (trial 2) and (2) the *in vitro* interactions between WB and ZnO with respect to *E. coli* growth (trial 3). We can conclude that incorporation of WB in the diet improved gut health by modulating the activity and composition of the microbial population. The negative interaction between WB and ZnO raises the interest of considering the inclusion of phytase enzymes to reduce the therapeutic levels of ZnO in post-weaning diets.

**Key words:** Colibacillosis; Microbiota; Piglets; Post-weaning diets

Post-weaning colibacillosis is a diarrhoeal disease of young pigs, strongly influenced by the drastic changes faced by the piglets immediately after weaning. Reduced feed intake, intestinal villous atrophy and reduced enzymatic and absorptive capacity of the gut may result in impaired digestion and absorption of nutrients and in the overgrowth of bacteria such as enterotoxigenic *Escherichia coli*<sup>(1)</sup>. The main aetiological agents are those enterotoxigenic *E. coli* expressing fimbrial antigens, which mediate adhesion to complementary carbohydrates located in the brush borders of villous enterocytes<sup>(2,3)</sup>.

So far, the most common strategy used to prevent enterotoxigenic *E. coli* growth in the intestine has been the addition of in-feed antimicrobial agents<sup>(4)</sup>. In those situations when the use of sub-therapeutic doses of antimicrobial agents has been suppressed (i.e. in the European Union in 2006<sup>(5)</sup>), the prescription of therapeutic antimicrobial agents has considerably increased<sup>(6)</sup>. In some European Union countries, pharmacological doses of ZnO (2500–3000 parts per million) are now extensively used in feed during the first 2 weeks after weaning

to reduce the incidence of post-weaning *E. coli* diarrhoea<sup>(7)</sup>. Besides having antimicrobial properties, some authors have proposed that high doses of Zn may increase feed intake by promoting an increase in the synthesis of ghrelin in the digestive tract<sup>(8)</sup> and may also reduce the inflammation of the intestinal mucosa<sup>(9)</sup>. Although the use of pharmacological doses of ZnO does not have the downside of selecting microbial resistances with its subsequent implications in human medicine (as opposed to the use of antibiotics), its use has raised environmental concerns about the low retention of Zn by pigs and soil toxicity.

Considerably, efforts are being made in the search of an efficient replacer of antimicrobial agents. One of the possible alternatives would be to modify the main ingredients of the feed formula to promote fermentation in the hindgut, which would protect animals from opportunistic bacterial proliferation. Several reports support that low-crude protein diets<sup>(10,11)</sup>, diets including protein of animal origin<sup>(7)</sup> or diets supplemented with fermentable carbohydrates (such as high lactose levels<sup>(12)</sup> or wheat bran (WB) and sugarbeet

**Abbreviations:** CT, control diet; OD, optical density; PA, phytic acid; TRF, terminal restriction fragments; WB, wheat bran.

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pulp<sup>(13,14)</sup>) may also help to maintain enteric health by lowering protein fermentation and promoting the proliferation of commensal microbiota<sup>(15)</sup>. In previous studies, inclusion of WB in the diet of early-weaned pigs increased intestinal fermentation<sup>(16,17)</sup> and diminished the incidence of diarrhoea and the attachment of *E. coli* K88 to the ileum mucosa<sup>(18)</sup>. The beneficial effects of fibrous ingredients could also be explained by additional mechanisms. Recent studies have shown, *in vitro*<sup>(19)</sup> and *in vivo*<sup>(20)</sup>, that dietary fibres from plants, because of their carbohydrate nature and low digestibility, may act as receptor analogues, which block the attachment of bacteria to the intestinal tract. However, no information is available regarding the ability of WB to block the attachment of *E. coli* to the intestinal tract.

Therefore, nutritionists have two main strategies available: either using an antimicrobial approach, which tends to delay microbiota maturation to later stages, or designing diets to promote and modulate the establishment of commensal microbiota in the intestinal tract immediately after weaning. This second option, however, requires a better knowledge of the effects of dietary components on digestive physiology and on the dynamic of the microbiota ecosystem. Both these strategies appear to be contradictory in the mode of action and are difficult to combine in a comprehensive way. However, in practice, they are frequently used together, although there is little information on the formulation of starter diets containing medicines. It would be of particular interest to know about the possible influence of dietary composition on the activity of pharmacological doses of ZnO in the diet.

In the present study, we evaluate the productive performance and gut microbiota responses associated with the incorporation of ZnO, WB or their combination in the diet of early-weaned piglets (trial 1). Two *in vitro* trials were also designed to clarify hypotheses derived from the *in vivo* test: (1) the ability of WB and other fibre sources to bind *E. coli* *in vitro* (trial 2) and (2) the *in vitro* interactions between WB and ZnO with respect to *E. coli* growth (trial 3).

## Materials and methods

### Trial 1: *in vivo* experiment

**Animals and diets.** This experiment was performed at the Animal Facilities of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review Committee of this institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines<sup>(21)</sup>. A total of sixty-four commercial crossbred piglets ((Large White × Landrace) × Pietrain), which had been excluded from receiving creep feed, were weaned at 21 d of age with an average body weight of 6.7 (SEM 0.37) kg. Pigs were transported from a commercial farm to the University facilities and were allocated into thirty-two pens (two animals/pen) based on the litter origin and body weight. Pens were allotted to four dietary treatments (eight replicates/treatment; Table 1) in a 2 × 2 factorial arrangement that included two levels of WB (0 *v.* 40 g/kg, control diet (CT) *v.* WB, respectively) and two

levels of ZnO (0 *v.* 3 g/kg, CT *v.* ZnO diet, respectively) in the diet. The diets were formulated to be isoenergetic (averaging 16.1 MJ/kg) and isoproteic (averaging 182 g crude protein/kg) based on ground maize, barley and soyabean protein concentrate.

**Experimental procedures and sampling.** Animals received the diets from day 1 to 12 of the experiment. Individual body weight and pen feed consumption were recorded on days 0, 3, 6, 9 and 12 after weaning. Physical and behavioural examination of the animals was done daily to evaluate their health status. Samples of fresh faeces were collected from the rectum of one animal/pen for microbial counts on days 3, 6, 9 and 12 after weaning. At the end of the experimental period, the faecal samples were kept in tubes and immediately frozen at -80 °C for analysing the microbial structure, quantifying the lactobacilli counts and determining the SCFA concentration.

**Analytical procedures.** Chemical analyses of the diets (Table 1) were performed according to the Association of Official Analytical Chemists<sup>(22)</sup> standard procedures.

Traditional culture methods were used to determine some bacterial groups. The faecal samples were diluted 1:10 in PBS (Sigma, St Louis, MO, USA) immediately after collection, and subsequently homogenised. Viable counts of enterococci

**Table 1.** Composition and chemical analysis of pre-starter diets\*

	Diets			
	CT	WB	ZnO	WB-ZnO
<b>Ingredients (g/kg DM)</b>				
Maize	414.0	367.0	414.0	364.0
Barley	200.0	200.0	200.0	200.0
Whey	112.0	102.0	112.0	102.0
High-fat whey	69.0	90.0	69.0	90.0
Soyabean protein concentrate	55.0	52.0	55.0	52.0
Spray-dried porcine plasma	50.0	50.0	50.0	50.0
Wheat gluten	30.0	30.0	30.0	30.0
Fishmeal LT†	40.0	40.0	40.0	40.0
Wheat bran	–	40.0	–	40.0
CaCO <sub>3</sub>	10.0	10.0	10.0	10.0
Dicalcium phosphate	7.0	7.0	7.0	7.0
Benzoic acid	5.0	5.0	5.0	5.0
Synthetic amino acids‡	11.2	11.2	11.2	11.2
Vitamin and mineral premix§	3.7	3.7	3.7	3.7
ZnO	–	–	3.0	3.0
<b>Chemical analysis (g/kg DM)</b>				
DM	902.0	903.0	902.0	903.0
Gross energy (MJ/kg)	17.8	17.9	17.8	17.9
Crude protein (N × 6.25)	203.0	200.0	203.0	200.0
Neutral-detergent fibre	79.0	92.0	79.0	92.0
Acid-detergent fibre	23.0	28.0	23.0	28.0
Diethyl ether extract	63.0	79.0	63.0	79.0
Ash	56.0	57.0	56.0	57.0

CT, control diet; WB, wheat bran diet.

\* Trial 1: *in vivo* experiment.

† Fishmeal LT, fishmeal low temperature: product obtained by removing most of the water and some or all of the oil from fish by heating at low temperature (<70 °C) and by pressing.

‡ Synthetic amino acids: L-Lys 0.99, DL-Met 0.99, L-Try 0.10, L-Thr 0.98.

§ Supplied per kg of feed: 13 000 IU (3900 µg) vitamin A, 1800 IU (45 µg) vitamin D<sub>3</sub>, 60.0 mg vitamin E, 3.0 mg vitamin K<sub>3</sub>, 2.0 mg vitamin B<sub>1</sub>, 6.0 mg vitamin B<sub>2</sub>, 3.0 mg vitamin B<sub>6</sub>, 0.02 mg vitamin B<sub>12</sub>, 35.0 mg niacin, 15.0 mg calcium pantothenate, 0.12 mg biotin, 1 mg folic acid, 20.0 mg Fe, 120.0 mg Cu, 110 mg Zn, 45.0 mg Mn, 0.30 mg Se, 0.10 mg Co, 1 mg I and 2.5 mg ethoxyquin as an antioxidant (Capsquin; Itpsa, Barcelona, Spain).

were done by plating serial tenfold dilutions onto Chromocult™ Enterococci-Agar (Merck K GaA, Darmstadt, Germany) and incubating the plates for 24 h at 37°C. For the enumeration of *E. coli* and coliforms, 1 ml of solution of the corresponding dilution was pipetted onto an *E. coli*-coliform count plate (3M Petrifilm; Europe Laboratories 3M Santé, Cergy-Pontoise, France) with Violet Red Bile gel as an indicator of glucuronidase activity. The plates were incubated for 48 h at 35°C, and the colonies were counted following the manufacturer's instructions. DNA from faeces was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), and the lactobacilli population was quantified by real-time PCR using SYBR Green dye, following Castillo *et al.*<sup>(23)</sup>. The terminal restriction fragment length polymorphism analysis of bacterial community was performed following the procedure described by Hojberg *et al.*<sup>(24)</sup> and adapted by Castillo *et al.*<sup>(25)</sup>. Finally, SCFA concentrations were determined by GC, after an acid-base treatment followed by diethyl ether extraction and derivatisation<sup>(26)</sup>.

### Trial 2: *in vitro* adhesion test

**Fibrous ingredients.** Seven different fibrous ingredients, WB, rice hulls, soyabean hulls, oat hulls, pea hulls, sugarbeet pulp and cereal straw, were selected as test products. Bovine serum albumin (Sigma) served as the reference (negative control), following the protocol described by Becker *et al.*<sup>(27)</sup>.

**Bacterial strains.** Two different *E. coli* strains were used in this experiment to elucidate the interaction between fibre substrates and bacterial fimbriae. The first one was an *E. coli* K88 (enterotoxigenic *E. coli*, strain FV12048) isolated from a colibacillosis outbreak in Spain<sup>(28)</sup>; the serotype (O149:K91:H10, F4+, LT1+, STb+) was provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The other strain was a non-fimbriated *E. coli* (F4-, F6-, F18-, LT1-, ST1-, ST2+, Stx2e-) isolated from the faeces of post-weaning piglets and kindly donated by the Departament de Sanitat i Anatomia Animal from the Universitat Autònoma de Barcelona.

Bacteria were cultured in unshaken Luria broth (Sigma) at 37°C and serially passaged every 48 h, at least five times. Bacterial cells from the culture were harvested and processed as described earlier<sup>(27)</sup>.

**Adhesion test methodology.** *E. coli* K88 and the non-fimbriated strain were allowed to adhere to different fibre components supplied as well coatings in microplates in a miniaturised adhesion test, following the protocol described by Becker *et al.*<sup>(27)</sup>. Briefly, fibre ingredients were suspended in PBS to a final concentration of 4% (w/v), and the soluble fraction was extracted for coating the flat-bottom wells of high-binding polystyrene microtitration plates. After blocking the non-specific sites with BSA, bacteria were added to a final concentration of  $1.20 \times 10^8$  colony-forming units/ml. Bacteria were allowed to adhere by incubation at room temperature for 30 min. Afterwards, the wells were washed to remove non-adherent bacteria, and bacteria were allowed to grow in Luria broth media by incubation in a microplate

reader (SPECTRAMax 384 Plus; Molecular Devices Corporation, Sunnyvale, CA, USA) at 37°C. Bacterial growth was monitored as optical density (OD) at 650 nm at intervals of 10 min. The test principle, as described by Becker *et al.*<sup>(27)</sup>, is based on an inverse relationship between initial cell densities and the appearance of growth defined as the duration (h) needed for the cultures to reach an OD of 0.05 at 650 nm ( $t_{OD} = 0.05$ ): the higher the adhering cell numbers, the shorter the detection times of growth.

### Trial 3: *in vitro* wheat bran and zinc oxide interaction test

**Sample preparation.** In order to elucidate the interaction between WB and ZnO and the likely role of phytates, eight different samples were prepared in a  $4 \times 2$  factorial design, which included four different buffered solutions (a negative control; 4% WB; 4% WB + 0.02% phytase enzyme (5000 IU/g; Ronozyme™ P500; DSM Nutritional Products Limited, Basel, Switzerland); and 4% WB + 0.02% xylanase and glucanase enzyme mixture (22 000 visco units/g xylanase and 2000 visco units/g glucanase/g; Rovabio™ Excel AP, Adisseo, France)), and two levels of ZnO (0 *v.* 0.3%, w/v). Samples of buffered solutions were adjusted to a pH of 5.1 with HCl and incubated for 4 h at room temperature. Then, the suspensions were sonicated three times for 30 s each and then centrifuged at 460 g for 5 min. The supernatant obtained was adjusted to a pH of 7.0 with NaOH and ZnO added appropriate to the specific treatment.

**Bacterial strains.** Two different *E. coli* strains (*E. coli* K88 and a non-fimbriated *E. coli* strain) were used in this experiment as described earlier.

***In vitro* test.** *E. coli* K88 and the non-fimbriated *E. coli* strains were centrifuged (1700 g) and adjusted to a final concentration of approximately  $3.5\text{--}3.9 \times 10^8$  colony-forming units/ml in Luria broth. Subsequently, 750 µl of each bacterial suspension were incubated with 750 µl of each experimental treatment. Thereafter, 300 µl of each suspension were added to polystyrene microtitration plates, and the growth of the bacteria was measured in a microplate reader at 37°C following the protocol described by Becker *et al.*<sup>(27)</sup>. Bacterial growth was monitored as OD at 650 nm at intervals of 10 min for 10 h. All readings were done in two independent assays and in triplicate per assay.

### Statistical analyses

The OD data from the *in vitro* experiments were processed by non-linear regression analysis using the non-linear P-NLIN (Gauss-Newton method) procedure<sup>(29)</sup> following the equations described by Becker *et al.*<sup>(27)</sup> in order to obtain  $t_{OD} = 0.05$  (h) for each treatment.

The  $t_{OD} = 0.05$  (h) results from the *in vitro* tests (trials 2 and 3), as well as all data from the *in vivo* trial (trial 1) were subjected to ANOVA using the generalised linear model procedure<sup>(29)</sup>. Classification factors included in each model were WB, ZnO level and their interaction for trial 1, fibrous ingredient, bacterial strain and their interaction for trial 2 and buffered solution, ZnO inclusion and their interaction

for trial 3. Means were calculated as least-squared means, and multiple mean comparisons were done using Tukey's correction. The  $\alpha$  level for the determination of significance was 0.05, and tendencies for  $0.05 < P < 0.15$  were also presented.

## Results

### Trial 1: *in vivo* experiment

**Animal performance and health status.** The effects of WB and ZnO on the average daily feed intake and average daily gain of the animals as well as the incidence of diarrhoea are shown in Table 2. The inclusion of ZnO in the diet increased the average daily feed intake of the animals from day 6 to 12 ( $P=0.006$ ) and from day 0 to 12 ( $P=0.035$ ). This resulted in an increased average daily gain of the animals for the same periods ( $P=0.008$  and  $0.036$ , respectively) and a higher body weight at the end of the experiment ( $P=0.044$ ) compared with the animals not receiving ZnO in the feed. The inclusion of ZnO in the diet also reduced the incidence of diarrhoea ( $P=0.009$ ).

**Metabolic activity and composition of faecal microbiota.** Concentrations of total and individual SCFA in the faecal samples and also the counts of major bacterial groups using culturing methods or quantitative PCR are given in Table 3. Significant differences were observed for the SCFA concentration associated with the incorporation of WB and ZnO in the diet. Moreover, the interaction between WB and ZnO was also significant for the total SCFA ( $P=0.048$ ), propionic acid ( $P=0.018$ ) and butyric acid concentrations ( $P=0.007$ ) and also tended to be significant ( $P=0.120$ ) for acetic acid. Thus, the WB diet increased the total SCFA, propionic acid and butyric acid concentrations in comparison with the CT, ZnO and WB-ZnO diets. The incorporation of WB (WB and WB-ZnO diets) increased the concentration of isoacids ( $P=0.001$ ), and the inclusion of ZnO (ZnO and WB-ZnO diets) diminished the concentrations of acetic acid ( $P=0.024$ ) and isoacids ( $P=0.001$ ).

A significant interaction was observed between the ZnO and WB supplementation on the counts of *E. coli* and coliform after weaning. The simultaneous incorporation of ZnO and WB in the diet increased the *E. coli* and coliform counts compared with the ZnO diet on day 6 after weaning ( $P=0.026$ ) and compared with the WB diet on day 9 after weaning ( $P=0.024$ ). On day 12, animals fed the WB diet showed lower counts of *E. coli* ( $P=0.034$ ) and coliforms ( $P=0.028$ ) than those fed the CT diet, but no significant differences were observed with the ZnO or WB-ZnO diet. On day 12, the incorporation of ZnO in the diets (ZnO and WB-ZnO) also tended to increase the enterococci population ( $P=0.064$ ) and to reduce the lactobacilli counts ( $P=0.084$ ) in the faeces of pigs compared with that in the animals that did not receive ZnO.

The terminal restriction fragment length polymorphism method was employed to evaluate global changes in the microbial ecosystem. Fig. 1 shows the analysis focused on two of the diets: the WB and ZnO diets. It shows the microbial profiles of all pens except one from which we were unable to take faecal samples. The effect of the diet on the composition of faeces was clearly observed as most of the animals were grouped into two separate clusters. Microbial profiles of pigs fed the WB diet were more similar (50–75%) than those of pigs fed the ZnO diet, which showed more heterogeneous microbial profiles (52–90%). *In silico* restriction, using Ribosomal Database Project II and MiCA software (version 3; Department of Biological Sciences, University of Idaho; <http://mica.ibest.uidaho.edu/>)<sup>(30)</sup>, was used to deduce potential ecological changes in the samples. Results are presented as potential compatible bacterial species. The WB dietary treatment showed higher diversity in compatible terminal restriction fragments (TRF) than the ZnO or the WB-ZnO treatment exposed by the following results: four animals of the WB treatment showed a TRF of 105–106 bp that was not found in any of the other three treatments (CT, ZnO and WB-ZnO). Possible bacteria compatible with this fragment size are *Bacteroides fragilis* (102 bp), *Prevotella ruminicola*

**Table 2.** Body weight (BW; g), average daily feed intake (ADFI, g/animal per d), average daily gain (ADG, g/animal per d) and diarrhoea incidence (no. of animals) in early-weaned pigs\* (Mean values with their standard errors,  $n$  8)

	Period (d)	Diets				SEM	<i>P</i>		
		CT	WB	ZnO	WB-ZnO		WB	ZnO	WB × ZnO
<b>Performance results</b>									
BW	0	6700	6725	6742	6698	53.7	0.735	0.783	0.217
	6	6859	7014	7130	6948	249.6	0.918	0.428	0.202
	12	7933	8120	8583	8350	390.2	0.908	0.044	0.303
ADFI	0–6	74.9	91.0	105.1	75.3	25.41	0.603	0.578	0.096
	6–12	278.8	279.9	358.9	321.6	39.91	0.333	0.006	0.331
	0–12	176.8	184.9	231.9	198.5	28.89	0.397	0.035	0.175
ADG	0–6	26.5	48.1	64.6	41.7	40.54	0.975	0.450	0.294
	6–12	179.1	184.3	242.3	233.7	35.34	0.927	0.008	0.702
	0–12	102.8	116.2	153.4	137.7	30.42	0.941	0.036	0.356
<b>Health status</b>									
Diarrhoea incidence	0–12	14/16	16/16	7/16	11/16	0.41	0.735	0.009	0.319

CT, control diet; WB, wheat bran diet; WB-ZnO, wheat bran and ZnO diet; WB × ZnO, effect of WB and ZnO inclusion in the diet.

\* Trial 1: *in vivo* experiment.

**Table 3.** SCFA concentration ( $\mu\text{mol/g}$  fresh matter (FM)) in day 12 after weaning, enterococci and *Escherichia coli* counts (log colony-forming units/g FM) and lactobacilli population (log copies gen 16S rDNA/g FM) in the faeces of piglets early after weaning\*(Mean values with their standard errors,  $n$  8)

	Diets				SEM	<i>P</i>		
	CT	WB	ZnO	WB–ZnO		WB	ZnO	WB $\times$ ZnO
<b>SCFA</b>								
Total	71.6 <sup>b</sup>	109.7 <sup>a</sup>	59.5 <sup>b</sup>	65.1 <sup>b</sup>	18.41	0.011	0.002	0.048
Acetic acid	42.2	56.9	37.8	35.7	12.93	0.241	0.024	0.120
Propionic acid	17.8 <sup>b</sup>	29.2 <sup>a</sup>	14.6 <sup>b</sup>	14.8 <sup>b</sup>	5.32	0.014	0.001	0.018
Butyric acid	9.5 <sup>b</sup>	18.3 <sup>a</sup>	10.2 <sup>b</sup>	9.2 <sup>b</sup>	3.98	0.027	0.019	0.007
Isoacids	4.3	5.1	1.9	3.2	0.91	0.001	0.001	0.494
<b>Microbial population</b>								
Day 3								
Enterococci	6.1	5.9	5.4	5.8	0.71	0.721	0.299	0.405
<i>E. coli</i>	6.6	6.2	6.1	6.5	0.89	0.988	0.923	0.418
Coliforms	6.9	6.6	6.2	6.8	0.95	0.720	0.573	0.434
Day 6								
Enterococci	5.8	5.8	6.9	5.9	0.73	0.166	0.158	0.186
<i>E. coli</i>	4.9 <sup>a,b</sup>	4.3 <sup>a,b</sup>	2.6 <sup>b</sup>	5.0 <sup>a</sup>	1.11	0.154	0.201	0.026
Coliforms	4.9	4.4	3.6	5.1	0.97	0.356	0.523	0.068
Day 9								
Enterococci	6.8	6.3	6.8	5.8	0.56	0.016	0.405	0.418
<i>E. coli</i>	5.0 <sup>a,b</sup>	3.9 <sup>b</sup>	4.2 <sup>a,b</sup>	5.3 <sup>a</sup>	0.87	0.986	0.515	0.024
Coliforms	5.1 <sup>a,b</sup>	4.1 <sup>b</sup>	4.7 <sup>a,b</sup>	5.4 <sup>a</sup>	0.72	0.580	0.229	0.033
Day 12								
Enterococci	6.3	5.9	6.8	6.7	0.65	0.357	0.064	0.589
<i>E. coli</i>	6.6 <sup>a</sup>	5.2 <sup>b</sup>	5.6 <sup>a,b</sup>	5.9 <sup>a,b</sup>	0.72	0.148	0.700	0.034
Coliforms	6.7 <sup>a</sup>	5.4 <sup>b</sup>	5.7 <sup>a,b</sup>	6.1 <sup>a,b</sup>	0.63	0.222	0.862	0.028
Lactobacilli	11.9	11.7	11.1	11.5	0.47	0.761	0.084	0.532

CT, control diet; WB, wheat bran diet; WB–ZnO, wheat bran and ZnO diet; WB  $\times$  ZnO, effect of WB and ZnO inclusion in the diet.<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).\* Trial 1: *in vivo* experiment.

(102 bp) and numerous uncultured rumen bacteria (102–104 bp). Analysing the final part of the electropherograms, animals that received the CT and WB diets showed eight and thirteen TRF of 522–582 bp, respectively, whereas only one TRF (575 bp) was found in the ZnO group, and three TRF (two of 558 bp and one of 565 bp) were found in the animals that received the WB–ZnO combination. This range of TRF is compatible with different *Bacillus* species (*B. cereus*, *B. thuringiensis* and *B. megaterium* (578–579 bp)), different *Streptococcus* species (*S. mitis*, *S. bovis* and *S. salivarius* (570–581 bp)), some *Lactococcus* species (*L. lactis* and *L. garvieae* (582–583 bp)) and numerous uncultured rumen bacteria.

### Trial 2: *in vitro* adhesion test

Table 4 presents the detection times of growth for *E. coli* K88 and for the non-fimbriated *E. coli*. In the present study, an interaction was found between the *E. coli* strain and the fibre source ( $P = 0.0001$ ). Significant differences between the fibre substrates were found related to the adhesion of the two *E. coli* strains. The *E. coli* K88 adhered more strongly ( $P = 0.0001$ ) to the WB substrate compared with the other fibre substrates and the negative control treatment. Similarly, the non-fimbriated *E. coli* showed a higher attachment ( $P = 0.0001$ ) to the WB substrate compared with soyabean hulls, sugarbeet pulp, oat hulls and the negative control treatment.

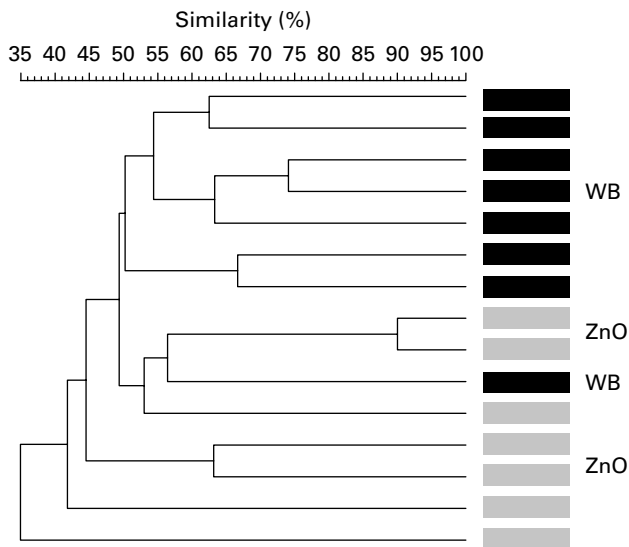
### Trial 3: *in vitro* analysis of interaction between wheat bran and zinc oxide

Table 5 presents the results related to trial 3 as detection times of growth for *E. coli* K88 and the non-fimbriated *E. coli*. A significant ( $P = 0.0001$ ) interaction between the ZnO and buffered solutions was found related to the growth of the two *E. coli* strains. The incorporation of ZnO in the buffered solution inhibited ( $P = 0.0001$ ) the bacterial growth for both *E. coli* strains in comparison with the negative control. Also, the ZnO supplementation showed antimicrobial effects when supplemented into the WB + phytase treatment. However, when it was added to the WB or WB + xylanase treatment, ZnO did not reduce the growth of *E. coli*.

## Discussion

### The influence of zinc oxide and wheat bran on the adaptation of piglets after weaning

The present results showed that a dietary supplementation with a high level of ZnO (3 g/kg) increased the feed intake and average daily gain of the animals and reduced the onset of diarrhoea in weaning piglets during the first days after weaning. These results are in accordance with observations from animal performance studies, in which a larger number of animals were used<sup>(31,32)</sup>. Since ZnO is known to possess antimicrobial properties, it has been usually assumed that it enhances growth by controlling pathogenic bacteria. In the



**Fig. 1.** Dendrogram illustrating the correlation between the experimental diets: 4% wheat bran (WB) diet and 0.3% ZnO diet, in terminal restriction fragment length polymorphism banding patterns of faeces of post-weaning piglets. The dendrogram distances are in percentage of similarity (trial 1: *in vivo* experiment).

present study, ZnO reduced the fermentation activity and the counts of lactobacilli and increased the counts of enterococci, as described by Hojberg *et al.*<sup>(24)</sup>. On the other hand, some studies have also demonstrated that high doses of ZnO are effective in increasing the feed intake related to modulations of gene expression<sup>(9,33)</sup>. Yin *et al.*<sup>(8)</sup> observed that ZnO supplementation increased plasma levels of ghrelin in early-weaned piglets. Ghrelin is a hormone released by the stomach, which is involved in the secretion of growth hormone and insulin-like growth factor 1 and in the stimulation of the feed intake and muscle growth.

The present results also revealed that ZnO supplementation decreased the incidence of diarrhoea and the counts of *E. coli* in faeces, as observed by Cardinal *et al.*<sup>(7)</sup>. Diarrhoea in piglets is an important problem, which is associated, in some cases, with an overproliferation of enteropathogenic *E. coli*. However, animals in the present study presented diarrhoea without a pathological picture of fever, dehydration or apathy. Ou *et al.*<sup>(9)</sup> demonstrated that Zn is also able to ameliorate intestinal inflammation due to inadequate feed intake after weaning by reducing the number of mast cells in the small-intestinal mucosa and submucosa and by inhibiting histamine release from mast cells.

In contrast to the results observed with the ZnO treatments, the incorporation of WB did not improve the productive performance, as was also shown in earlier studies<sup>(16,17)</sup>, but increased the concentration of fermentation products in the faeces, especially the concentration of butyrate. These results confirm that the early-weaned pigs were able to increase carbohydrate fermentation with the inclusion of WB in the diet. It is accepted that starch and bran from wheat or oats stimulate the formation of butyrate<sup>(26)</sup>, which is considered the principal oxidative fuel for colonocytes and may have beneficial trophic effects on the inflamed caeco-colonic

mucosa<sup>(34)</sup>. As observed previously with other insoluble fibre sources<sup>(17,35)</sup>, the addition of WB in the diet decreased the *E. coli* and coliform bacteria counts in faeces. Previous results from our group have also indicated that the incorporation of WB in the diet also decreased the enterobacteria counts in the caecal digesta<sup>(16)</sup> and the K88 *E. coli* attachment to the ileum mucosa after an experimental infection<sup>(18)</sup>. The main mechanism involved in these changes could be an increased fermentation of carbohydrates, which may reduce protein fermentation<sup>(14)</sup>, or likely changes in the physico-chemical properties of digesta, which can increase its water-binding capacity or the ability of some long-chain NSP to block the attachment of *E. coli* to the intestinal tract<sup>(20)</sup>.

However, a significant two-way interaction was observed between the WB and ZnO supplementation. The administration of medicated feed (ZnO) decreased the concentration of SCFA, increased coliform bacteria counts in faeces and reduced some of the microbial groups that were growth promoted when WB was included in the diet. It could be suggested that the antimicrobial effect of ZnO may have reduced the fermentation of fibre, and the main microbial changes were promoted by the WB supplementation. However, it is also intriguing that the combination of WB and ZnO also reduced the effect of therapeutic doses of ZnO in the counts of *E. coli* and coliform in faeces. The following two trials were designed to test hypotheses evidenced in the *in vivo* trial: (1) the ability of WB to bind *E. coli* in the intestinal digesta (trial 2) and (2) the likely mechanisms by which WB may affect the antimicrobial activity of the ZnO on the *E. coli* growth *in vitro* (trial 3).

**Potential of different fibrous substrates to bind Escherichia coli**

Different studies have shown the promising effects of glycoconjugates from different origins such as cranberry and

**Table 4.** Detection times of bacterial growth  $t_{OD} = 0.05$  (h) for *Escherichia coli* K88, non-fimbriated *E. coli*, as a measure for adhesion in different fibre ingredients\* (Least-squared means)

Product	$t_{OD} = 0.05$ <i>E. coli</i> K88	$t_{OD} = 0.05$ Non-fimbriated <i>E. coli</i>
Wheat bran (4%)	0.94 <sup>a</sup>	2.73 <sup>a</sup>
Rice hulls (4%)	2.74 <sup>b</sup>	2.88 <sup>a,b</sup>
Soyabean hulls (4%)	3.11 <sup>b</sup>	3.27 <sup>b,c</sup>
Cereal straw (4%)	3.12 <sup>b</sup>	3.01 <sup>a,b,c</sup>
Sugarbeet pulp (4%)	3.22 <sup>b</sup>	3.36 <sup>b,c</sup>
Pea hulls (4%)	3.00 <sup>b</sup>	3.11 <sup>a,b,c</sup>
Oat hulls (4%)	2.69 <sup>b</sup>	3.43 <sup>c</sup>
Negative control	2.92 <sup>b</sup>	3.34 <sup>b,c</sup>
SEM	0.193	
<i>P</i>		
Fibre product	0.0001	
Bacteria	0.0001	
Fibre product × bacteria	0.0001	

<sup>a,b,c</sup> Mean values within a column with unlike superscript letters were significantly different ( $P < 0.05$ ).  
\* Trial 2: *in vitro* adhesion test.

**Table 5.** Detection times of bacterial growth  $t_{OD} = 0.05$  (h) for *Escherichia coli* K88, non-fimbriated *E. coli*, as a measure of the ability of the *E. coli* strains to grow on different substrates\* (Least-squared means)

Items†	ZnO inclusion‡	$t_{OD} = 0.05$	
		<i>E. coli</i> K88	Non-fimbriated <i>E. coli</i>
Negative control	–	0.22 <sup>a</sup>	0.24 <sup>a</sup>
	+	– <sup>d§</sup>	– <sup>b§</sup>
WB	–	0.50 <sup>b</sup>	0.20 <sup>a</sup>
	+	0.42 <sup>b</sup>	0.35 <sup>a</sup>
WB + phytase	–	1.10 <sup>c</sup>	0.45 <sup>a</sup>
	+	1.53 <sup>d</sup>	– <sup>b§</sup>
WB + xylanase	–	0.33 <sup>a,b</sup>	0.17 <sup>a</sup>
	+	0.50 <sup>b</sup>	0.48 <sup>a</sup>
SEM		0.502	
P			
Buffered solutions		0.0046	
ZnO		0.0001	
Buffered solutions × ZnO		0.0001	

WB, wheat bran.

<sup>a,b,c,d</sup> Mean values within a column with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Trial 3: *in vitro* wheat bran and ZnO test.

† Negative control was based on PBS; the WB inclusion was at a level of 4%, and the phytase and xylanase inclusion was at a level of 0.02% (w/v).

‡ ZnO inclusion was at a level of 0.3% (w/v).

§ Total inhibition of the bacterial growth.

blueberry extracts<sup>(36)</sup>, mannan-oligosaccharides<sup>(37,38)</sup>, palm kernel extracts<sup>(39)</sup> or soya and fermented soyabean products<sup>(40)</sup> to inhibit the adhesion of different pathogens such as *E. coli* or *Salmonella* to the intestinal mucosa of different animal species. Dietary fibre from plants may provide an alternative adhesion matrix to enteropathogenic bacteria because of their carbohydrate nature similar to the intestinal receptors of such pathogens and low digestibility. Becker & Galletti<sup>(19)</sup> tested the binding capacity of different food and feed components for *E. coli* K88, *Salmonella enterica* sv. *typhimurium* and *Lactobacillus* spp. isolated from pigs, chickens, calves and humans. They reported positive scores for sesame seed extract and soyabean products against *E. coli* K88 *in vitro*. In recent studies, Kim *et al.*<sup>(35)</sup> and Becker *et al.*<sup>(20)</sup> also reported the blocking capacity of oat hulls or pea hulls against *E. coli* K88. In the present study, WB extracts showed the highest ability to bind *E. coli* K88 among the different fibre sources evaluated. The binding activity was higher in the presence of the F4-fimbriated *E. coli* K88 in comparison with the non-fimbriated *E. coli*. These results are in good agreement with those we found earlier regarding the reduction promoted by WB on enterobacteria and coliform counts in the digesta and attached *E. coli* K88 to the ileum mucosa<sup>(16,18)</sup>. WB is one of the more available fibre sources for human and animal feeding. It contains insoluble NSP<sup>(41)</sup> mainly as arabinoxylan, cellulose and  $\beta$ -glucan but also minute levels of glucomannans<sup>(42)</sup> and arabinogalactans<sup>(43)</sup> originating from the aleurone and endosperm cells. It might be speculated that the soluble fraction of WB may form a matrix in the gut in which fimbriated *E. coli* is captured. The adhesion of bacteria to the WB matrix may allow their

growth, as is observed in the *in vitro* system, but it also provides a mechanism by which the attachment and proliferation of *E. coli* K88 at the intestinal epithelium is inhibited or reduced.

### Possible mechanism involved in the interaction between wheat bran and zinc oxide

Negative interactions between WB and ZnO have been reported in the present study *in vivo* and *in vitro*. In the *in vivo* trial, the WB–ZnO combination did not reduce *E. coli* and the coliform counts as ZnO or WB did. In the *in vitro* trial, the WB–ZnO combination did not have the same antimicrobial effect on the *E. coli* strains as ZnO and the combination of WB–phytase and ZnO did. Therefore, it is suggested that a negative interaction between phytic acid (PA) and ZnO modifies the antimicrobial properties of therapeutic doses of ZnO *in vivo* and *in vitro*. Champagne & Fisher<sup>(44)</sup> suggested that PA, primarily found in the pericarp of cereal grains, may form a rather stable complex with bivalent cations, such as  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . These complexes are known not to affect Zn bioavailability in chicks<sup>(45)</sup> and also in humans<sup>(46)</sup>. Procedures that degrade phytate have been studied as a means to increase the bioavailability of Zn and other cations in the diet<sup>(47,48)</sup>. It is known that fermentation of feed may reduce the PA:Zn ratio, promoting a better Zn absorption<sup>(49)</sup>. Other authors, namely Gaetke *et al.*<sup>(50)</sup>, also have shown that yogurt (both active and heat-treated) protects against growth retardation in weanling rats fed high PA. In animals, feeding PA has been regarded as an anti-nutrient, which reduces P availability, and most research in this field has been aimed at eliminating PA from the animal feed by adding exogenous phytase to it. The term phytase is defined as a class of phosphatases with the *in vitro* capability to release at least one phosphate from PA<sup>(51)</sup>. Some authors<sup>(52,53)</sup> have suggested that phytase supplementation may increase the amount of Zn absorbed, even when pharmacological doses of Zn are included in the diet. Thus, Martinez *et al.*<sup>(52)</sup> suggested that present pharmacological doses of Zn (2000 mg/kg) fed to pigs could be reduced to 1000 mg/kg by adding phytase. The present study confirms a negative interaction between the WB and therapeutic doses of ZnO, which appears to be related to the high levels of PA in WB. Taking into account these results, phytase supplementation may be proposed as a good approach to increase the effectiveness or to reduce the levels of ZnO in post-weaning diets.

### Conclusion

Based on the results of the present study, we conclude that the incorporation of WB in the diet of early-weaning piglets may improve their gut health by modulating the activity of the intestinal microbiota, enhancing the fermentation and blocking the attachment of *E. coli* K88 to the intestinal mucosa. A negative interaction observed *in vivo* and *in vitro* between WB (rich in phytate) and ZnO raises the interest of considering the inclusion of phytase enzymes to reduce the required levels of ZnO in post-weaning diets.

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