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Organic acid-preserved grain improves growth and gut health in weanling pigs fed zinc oxide free diets

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The effects of organic acid (OA)-preserved grain and zinc oxide (ZnO) supplementation on post-weaning (PW) piglet performance and intestinal health were evaluated in a 2 × 2 factorial study. Ninety-six piglets (28 days old) were allocated to four diets: dried grain, OA-preserved grain, dried grain + ZnO, and OA-preserved grain + ZnO, for 35 days. Diets contained 600 g/kg grain (450 g/kg wheat, 150 g/kg barley). On day 10 PW, 28 piglets (n = 7/treatment) were euthanised for small intestinal morphology, gene expression, microbial, and volatile fatty acid (VFA) analyses. OA-preserved grain reduced dietary Ochratoxin A and Deoxynivalenol concentrations and increased average daily gain ($P < 0.05$), but provided no additional growth benefit when combined with ZnO. ZnO increased feed intake, body weight, colonic *Lactobacillus* abundance, and villus height-to-crypt depth ratio, while reducing faecal scores and colonic branched-chain fatty acids ($P < 0.05$). OA-preserved grain increased ileal *Faecalibacterium* and reduced *Escherichia* populations, and downregulated duodenal *IL17* and ileal *FOXP3* expression ($P < 0.05$). ZnO broadly suppressed pro-inflammatory cytokines and upregulated nutrient transporter genes (*SLC15A1*, *SLC16A1*). These findings indicate that OA-preserved grain improves growth and gut health but does not fully replace ZnO in mitigating PW diarrhoea.

Keywords Zinc oxide, Weaning, Organic acids, Microbiota, Immunity

In the commercial setting, piglets are typically weaned before their digestive and immune systems have fully matured[1]. The sudden transition from sow's milk to a grain-based diet leads to reduced feed intake, intestinal inflammation, nutrient malabsorption, and microbial imbalances, which together impair growth performance[2]. This intestinal dysfunction fosters the growth of *Escherichia coli*, a primary cause of post-weaning diarrhoea (PWD)[3]. Zinc oxide (ZnO) supplementation has been shown to mitigate PWD and support growth[4]. However, the European Union banned the use of in-feed antimicrobials, including ZnO, in 2022 due to concerns around the environment and antimicrobial resistance[5]. Consequently, identifying nutritional strategies to enhance growth and intestinal health post-weaning is critical.

While antimicrobials inhibit microbial proliferation, organic acid (OA) supplementation encourages the growth of beneficial bacteria that outcompete pathogenic bacteria in the gut[6]. Supplementing piglets with OA modulates the intestinal microbiome, reduces inflammation and diarrhoea, and improves growth performance[7]. However, the effects of OA depend on factors such as the type of acid used, inclusion levels, and interactions with other dietary elements[8]. Additionally, OAs, particularly propionic acid, serve as effective grain preservatives[9].

Cereal grains are essential components of pig diets[10]. In temperate, humid climates with significant rainfall, grain moisture content can reach 20–25% during harvest[11], promoting fungal growth and mycotoxin production[12]. Grain can be mechanically dried[13] to reduce moisture content, thereby limiting microbial load and preserving quality[14]. However, inconsistencies in mechanical drying can lead to uneven moisture content and elevated mycotoxin levels[15]. Moreover, mechanical drying is energy-intensive[16] and generates substantial greenhouse gas emissions[17].

Preserving grain with OA can reduce bacterial and fungal growth without affecting its chemical composition. Additionally, OA-preserving improves post-weaned piglet growth and gut health more effectively than direct OA supplementation[18]. Implementing post-harvest preservation techniques with OA to maintain grain quality may

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enhance piglet growth post-weaning through a linked mechanism: first, by reducing microbial contamination in the grain, thereby improving its quality and nutritional value; and second, by directly enhancing gastrointestinal function and health in piglets through lower levels of harmful pathogens and increased nutrient absorption. Previous research has reported that pigs offered OA-preserved grain exhibited increased growth, ileal and total tract nutrient digestibility, and beneficial gut microbial shifts at day 35 post-weaning compared to those fed dried grain[19]. Therefore, it is essential to compare the benefits and underlying mechanisms of OA-preserved grain with ZnO during the critical post-weaning period.

This study aimed to assess the intestinal health and growth of newly weaned piglets offered grain preserved with OA at harvest against those offered conventionally dried grain. Additionally, it sought to compare the mechanisms by which OA-preserved grain and ZnO improve piglet performance by assessing various intestinal health and growth parameters. The hypotheses were, firstly, that OA-preserved grain would enhance piglet intestinal health and growth compared to conventionally dried grain, and secondly, OA-preserved grain would have similar effects on piglet intestinal health and growth post-weaning as ZnO supplementation, presenting a viable alternative to in-feed antimicrobials.

Results

Grain quality

Prior to preservation, the initial quality assessment of wheat showed a moisture content of 180 g/kg, a hectolitre weight of 73 kg/hL, and a thousand grain weight (TGW) of 49.8 g. For barley, the corresponding values were 183 g/kg for moisture, 61 kg/hL for hectolitre weight, and a TGW of 48.2 g. Comprehensive chemical and microbial profiles of both grains at the point of diet formulation are detailed in Table 1. Grains preserved with OA exhibited lower DM content compared to those that were dried. Dried wheat and barley also had higher concentrations of deoxynivalenol (DON) than their OA-preserved counterparts. In barley, higher levels of HT-2 toxin and ochratoxin A (OTA) were observed in the dried samples. Across both preservation methods and grain types, levels of aflatoxins B1, B2, G1, and G2 (≤ 10 μg/kg), fumonisins B1 and B2 (< 1000 μg/kg), T-2 and HT-2 toxins (< 50 μg/kg), and zearalenone (ZEA; < 250 μg/kg) remained below the detectable threshold (Tables 2, 3 and 4).

Growth performance and faecal scores

Table 5 summarises the effects of the dietary treatments on growth metrics, body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), gain-to-feed ratio (G:F), and faecal consistency scores (FS) over the post-weaning (PW) periods: days 0–21, 21–35, and the overall 0–35 days. One piglet per treatment group died during the first week PW due to causes unrelated to dietary intake.

During days 0–21, significant interactions between the grain preservation method and ZnO inclusion were observed for ADG, G:F, and BW ($P < 0.05$). Piglets fed OA-preserved grains had improved ADG and G:F compared to those receiving dried grains; however, these differences were not present when ZnO was added. In contrast, ZnO supplementation enhanced BW in piglets on dried grain diets but had no measurable effect in the OA-preserved grain group. ADFI was higher in ZnO-supplemented diets relative to those without ZnO (386 vs. 337 g/day, SEM 0.007; $P < 0.001$).During days 21–35, significant grain preservation × ZnO interactions were also found for ADFI, ADG, and BW ($P < 0.05$). ZnO inclusion increased ADFI in pigs fed dried grain diets, but not in those fed OA-preserved grains. ZnO reduced ADG in the OA-preserved grain group but showed no effect in the dried grain group. BW was increased by ZnO in the dried grain group only. Pigs fed OA-preserved grains had significantly higher G:F compared to those receiving dried grains (747 vs. 680, SEM 0.026; $P < 0.05$).

Cereal crop type	Wheat		Barley	
Grain preservation method	**Dried**	**OA-preserved**	**Dried**	**OA-preserved**
Analysis post storage (g/kg)				
DM	860.0	820.8	859.3	818.8
Ash	13.4	13.2	14.1	13.6
GE (MJ/kg)	15.6	15.5	15.2	15.0
Crude protein	107.7	103.4	96.3	94.4
Crude fibre	23.5	21.5	50.5	45.5
Starch	618.5	593.0	521.3	510.7
Fat	13.7	13.0	15.3	14.7
Mycotoxin levels (μg/kg)[a]				
Deoxynivalenol	87.0	< 75.0	240.0	< 75.0
T-2 Toxin	< 4.00	< 4.00	< 4.00	< 4.00
HT-2 Toxin	< 4.00	< 4.00	5.77	< 4.00
Zearalenone	< 10.0	< 10.0	< 10.0	< 10.0
Ochratoxin A	< 1.00	< 1.00	15.76	< 1.00

Table 1. The chemical and microbiological analysis of experimental grain after storage (g/kg as fed) unless otherwise stated. DM, dry matter; GE, gross energy; NDF, neutral detergent fibre. [a] The following mycotoxins were below detectable levels: Aflatoxin B1, B2, G1 and G2.

	Treatments*			
Grain preservation method	**Dried**	**OA-preserved**	**Dried**	**OA-preserved**
Zinc oxide supplementation	**No**	**No**	**Yes**	**Yes**
Ingredients (g/kg)				
Wheat	452	452	448.7	448.7
Barley	150	150	150	150
Full fat soya	150	150	150	150
Soya bean meal	101.5	101.5	101.5	101.5
Whey powder	60	60	60	60
Soya oil	65	65	65	65
Vitamin and mineral concentrate[a]	2.5	2.5	2.5	2.5
Salt	2	2	2	2
Mono calcium phosphate	4	4	4	4
Calcium carbonate	6	6	6	6
Lysine HCl	4	4	4	4
DL-Methionine	1.5	1.5	1.5	1.5
L-Threonine	1.5	1.5	1.5	1.5
Zinc Oxide	0	0	3.3	3.3

Table 2. The ingredient composition of dietary treatments (g/kg). *Treatments: (1) dried grain diet; (2) OA-preserved grain diet; (3) dried grain diet + 3.3 g/kg of zinc oxide; (4) OA-grain + 3.3 g/kg of zinc oxide. [a] Vitamin and mineral concentrate (per kg diet): 250 mg choline chloride; 140 mg Fe; 120 mg Zn; 47 mg Mn; 25 mg Cu; 0.6 mg I; 0.3 mg S; 12 mg nicotinic acid; 10 mg pantothenic acid; 67 mg tocopherol; 4 mg menaquinone; 2 mg riboflavin; 2 mg thiamine; 1.8 mg retinol; 0.025 mg cholecalciferol; 0.015 mg pyridoxine; 0.01 mg cyanocobalamin; 500 mg phytase.

During the overall period (day 0–35), an interaction between grain preservation and ZnO supplementation influenced both ADFI and ADG ($P < 0.05$). ZnO enhanced ADFI and ADG in dried grain diets but had no such effect in OA-preserved diets. Overall, pigs fed OA-preserved grains achieved superior G:F ratios compared to dried grain-fed pigs (775 vs. 709, SEM 0.024; $P < 0.05$).

Small intestinal morphology

The effects of dietary treatments on small intestinal morphology are provided in Table 6. Neither grain preservation method, ZnO inclusion, nor their interaction significantly influenced villus height (VH), crypt depth (CD), or VH:CD ratio in the duodenum ($P > 0.05$).

In the jejunum, pigs receiving OA-preserved grain had shorter villi compared to those receiving dried grain (217.9 µm vs. 281.5 µm, SEM 15.03; $P < 0.05$). ZnO inclusion did not impact jejunal CD or VH:CD ratio ($P > 0.05$). In the ileum, a grain × ZnO interaction was evident for VH ($P < 0.05$); ZnO supplementation increased VH in the OA-preserved grain diet but not in the dried grain diet. ZnO also reduced CD (92.7 µm vs. 107.7 µm, SEM 4.71; $P < 0.05$) and improved VH:CD ratio (2.8 vs. 2.1, SEM 0.21; $P < 0.05$).

Gene expression in the gastrointestinal tract

Differential gene expression related to nutrient absorption, mucosal immunity, and barrier function is shown in Table 7.

In the duodenum, there was a significant interaction between grain preservation and ZnO on *MUC2* expression ($P < 0.05$); ZnO reduced *MUC2* expression in the dried grain group but had no effect in the OA-preserved group. *IL17* expression was reduced in pigs fed OA-preserved grains (1.03 vs. 1.74, SEM 0.213; $P < 0.05$). ZnO supplementation significantly decreased expression of *FABP2*, *IL6,* and *IL17* compared to non-supplemented diets ($P < 0.05$). In the jejunum, ZnO inclusion upregulated *SLC15A1* (1.98 vs. 0.99, SEM 0.280) and *OCCLN* (1.55 vs. 0.95, SEM 0.207), while reducing *IL1A, IL1B, CXCL8, IL17, IL22,* and *NOX1* ($P < 0.05$). Grain preservation had no effect ($P > 0.05$). In the ileum, a significant interaction was noted for *TNF* expression ($P < 0.05$); ZnO reduced *TNF* levels in OA-preserved diets only. The OA-preserved grain diets were associated with lower *FABP2* and *FOXP3* expression ($P < 0.05$). ZnO increased *SLC15A1* expression and reduced pro-inflammatory markers (*IL1A, IL1B, CXCL8, IL17, IL22, NOX1*) ($P < 0.05$).

In the colon, ZnO × grain preservation interactions were observed for *IL6* ($P < 0.05$); ZnO reduced *IL6* in the dried grain group only. ZnO supplementation increased *SLC16A1* and *MUC2* expression while decreasing *IL1A, IL1B, CXCL8, TLR4, IL17,* and *NOX1* ($P < 0.05$). Grain preservation had no significant effect on colonic gene expression.

Bacterial richness and diversity

Alpha diversity metrics are summarised in Table 8, with beta diversity visualised in Figs. 1 and 2. No significant effects of grain preservation, ZnO supplementation, or their interaction were observed on richness or diversity indices (Observed, Fisher, Shannon, Simpson) in the ileum ($P > 0.05$).

	Dietary treatments			
Grain preservation method	**Dried**	**OA-preserved**	**Dried**	**OA-preserved**
Zinc oxide supplementation	**No**	**No**	**Yes**	**Yes**
Chemical analysis (g/kg)				
DM	884.0	872.0	889.0	873.0
Ash	46.0	46.0	47.0	44.0
GE (MJ/kg)	16.67	16.07	16.48	16.49
Crude fat	55.0	51.0	53.0	55.0
Crude protein	187.0	186.0	185.0	183.0
Crude fibre	34.0	31.0	33.0	30.0
NDF	124.0	120.0	124.0	114.0
ADF	40.0	37.0	49.0	35.0
Starch	376.0	346.0	345.0	336.0
Mycotoxin levels (mg/kg)[b]				
Deoxynivalenol	< 75	< 75	< 75	< 75
T-2 Toxin	< 4.00	< 4.00	< 4.00	< 4.00
HT-2 toxin	< 20.00	< 20.00	< 20.00	< 20.00
Zearalenone	< 10	< 10	< 10	< 10
Ochratoxin A	24.01	< 1.00	10.72	2.15
Essential amino acids (g/kg)				
Arginine	9.6	9.9	9.7	10.2
Histidine	4.1	4.3	4.3	4.2
Isoleucine	6.9	7.0	7.0	6.7
Leucine	12.3	12.1	12.3	12.3
Lysine	13.3	13.2	13.7	13.7
Methionine + Cysteine	5.0	4.9	5.1	4.7
Phenylalanine	8.1	7.8	8.3	8
Threonine	8.8	8.8	8.9	8.9
Tryptophan	2.7	2.6	2.7	2.6
Valine	9.0	9.2	9.5	9.1

Table 3. The analysed composition of experimental diets offered to pigs (g/kg unless otherwise stated). DM, dry matter; GE, gross energy; NDF, neutral detergent fibre; ADF, acid detergent fibre. [a] The following mycotoxins were below detectable levels: Aflatoxin B1, B2, G1 and G2.

In the colon, OA-preserved grain diets led to a reduction in Shannon (3.31 vs. 3.55, SEM 0.060) and Simpson (0.94 vs. 0.96, SEM 0.004) diversity indices ($P < 0.05$). ZnO inclusion had no significant effect on colonic alpha diversity ($P > 0.05$). PERMANOVA analysis showed no significant differences in beta diversity for either intestinal region ($P > 0.05$).

Differential bacterial abundance

Phylum level

Table 9 presents the effects of grain preservation method and ZnO supplementation on the relative abundance of bacterial phyla. In the ileum, the predominant phyla were Firmicutes (~ 85.25%), Proteobacteria (~ 8.98%), Actinobacteria (~ 1.76%), and Bacteroidetes (~ 1.67%). Grain × ZnO interactions were detected for Proteobacteria ($P < 0.05$); OA-preserved grains reduced Proteobacteria abundance compared to dried grains, with no effect under ZnO supplementation. ZnO lowered Bacteroidetes abundance (1.03 vs. 2.24, SEM 0.421; $P < 0.05$). In the colon, Firmicutes (~ 91.60%) dominated, followed by Proteobacteria (~ 4.10%), and Bacteroidetes (~ 1.58%). Interactions were observed for Firmicutes and Proteobacteria ($P < 0.05$).

Family level

Table 10 presents the effects of grain preservation method and ZnO supplementation on the relative abundance of bacterial families. In the ileum, ZnO × preservation interactions affected *Lactobacillaceae, Lachnospiraceae,* and *Enterobacteriaceae* ($P < 0.05$). *Ruminococcaceae* were more abundant in OA-preserved diets, while ZnO increased *Erysipelotrichaceae* and decreased *Oscillospiraceae* ($P < 0.05$). In the colon, interactions were seen for *Lachnospiraceae* and *Enterobacteriaceae. Lactobacillaceae* abundance was reduced in OA-preserved grain diets but increased by ZnO supplementation ($P < 0.01$).

Genus level

Table 11 presents the effects of grain preservation method and ZnO supplementation on the relative abundance of bacterial genera. The OA-preserved grains increased *Holdemanella* and *Faecalibacterium* while reducing

Target gene	Gene name	Accession no	Forward primer (5′-3′) Reverse primer (5′-3′)
Nutrient transporters			
FABP2	Fatty Acid Binding Protein 2	NM_001031780.1	F: CAGCCTCGCAGACGGAACTGAA R: GTGTTCTGGGCTGTGCTCCAAGA
SLC2A1	Solute Carrier family 2 Member 1	XM_003482115.1	F: TGCTCATCAACCGCAATGA R: GTTCCGCGCAGCTTCTTC
SLC2A5	Solute Carrier family 2 Member 5	EU012359	F: CCCAGGAGCCGGTCAAG R: TCAGCGTCGCCAAAGCA
SLC15A1	Solute Carrier Family 15 Member 1	NM_214347.1	F: GGATAGCCTGTACCCCAAGCT R: CATCCTCCACGTGCTTCTTGA
SLC2A7	Solute Carrier Family 2 Member 7	XM_003127552.3	F: ACATCGCCGGACATTCCATA R: GCGAGGACTGCAGGAAGATC
SLC5A1	Solute Carrier Family 5 Member 7	NM_001164021	F: GGCTGGACGAAGTATGGTG R: ACAACCACCCAAATCAGAGC
Short chain fatty acids transporters			
SLC16A1	Monocarboxylate Transporter 1	NM_001128445.1	F: GCAGCCCTGTGTTCCTCTCT R: CCAGCCGTAGATACCGAAGAAA
Response to acid secretion			
SCT	Secretin	NM_001009583.1	F: CCCTGGAGCCTGAAGATTTG R: GCCGGGTTTGAGTTAGCTGAT
AQP10	Aquaporin 10	NM_001128454.1	F: CTCCGGATCCGCAACCT R: GAGCATGAGCACAAACACACCTA
GIP	Gastric Inhibitory Polypeptide	NM_001287408.1	F: AAGAGCGACTGGAAACACAACA R: TTAGACTGATGGGCCAGCTCTAG
HRH2	Histamine receptor H2	XM_003354192.4	F: CCAGCCTGGATGTCATGCCT R: CCGGTCGAGGCTGATCAT
APOA4	Apolipoprotein A-1	NM_214388.1	F: GGCTGTGGAACATCTCCAGAA R: GTGTTCACTTCCCCAAGTTTGTC
Inflammatory markers			
IL1A	Interleukin 1A	NM_214029.1	F: CAGCCAACGGGAAGATTCTG R: ATGGCTTCCAGGTCGTCAT
IL1B	Interleukin 1B	NM_001005149.1	F: TTGAATTCGAGTCTGCCCTGT R: CCCAGGAAGACGGGCTTT
IL6	Interleukin 6	NM_214399.1	F: GACAAAGCCACCACCCCTAA R: CTCGTTCTGTGACTGCAGCTTATC
CXCL8	C-X-C motif chemokine ligand 8	NM_213867.1	F: TGCACTTACTCTTGCCAGAACTG R: CAAACTGGCTGTTGCCTTCTT
IL10	Interleukin 10	NM_214041.1	F: GCCTTCGGCCCAGTGAA R: AGAGACCCGGTCAGCAACAA
IL17	Interleukin 17	NM_001005729.1	F: CCCTGTCACTGCTGCTTCTG R: TCATGATTCCCGCCTTCAC
IL22	Interleukin 22	XM_001926156.1	F: GATGAGAGAGCGCTGCTACCTGG R: GAAGGACGCCACCTCCTGCATGT
IFNG	Interferon gamma	NM_213948.1	F:TCTAACCTAAGAAAGCGGAAGAGAA R: TTGCAGGCAGGATGACAATTA
TNF	Tumour Necrosis Factor	NM_214022.1	F: TGGCCCCTTGAGCATCA R: CGGGCTTATCTGAGGTTTGAGA
FOXP3	Forkhead box P3	NM_001128438.1	F: GTGGTGCAGTCTCTGGAACAAC R: AGGTGGGCCTGCATAGCA
MMP1	Matrix Metalloproteinase-histone H3	NM_001166229.1	F: GGACCGTGCCATTGAGAAA R: CCTCGGAGACCTTGGTGAAG
Oxidative stress			
NOX1	NADPH Oxidase 1	XM_003484140.3	F: AGCCATGCTGAGATCCCAAT R: TGCTTTATGGCAGGCTTTCA
Tight junctions			
TJP1	Tight Junction Protein 1	XM_021098827.1	F: TGAGAGCCAACCATGTCTTGAA R: CTCAGACCCGGCTCTCTGTCT
CLDN1	Claudin 1	NM 001244539.1	F: CTGGGAGGTGCCCTACTTTG R: TGGATAGGGCCTTGGTGTTG
Toll like receptors			
TLR2	Toll-like Receptor 2	NM_213761.1	F:CATCTTCGTGCTTTCCGAGAAC R:AAAGAGACGGAAGTGGGAGAAGT
TLR4	Toll-like Receptor 4	NM_001293317.1	F: TGCATGGAGCTGAATTTCTACAA R: GATAAATCCAGCACCTGCAGTTC
Mucins			
MUC2	Mucin 2	AK231524	F: CAACGGCCTCTCCTTCTCTGT R: GCCACACTGGCCCTTTGT
Continued			

Target gene	Gene name	Accession no	Forward primer (5′-3′) Reverse primer (5′-3′)
Reference genes			
HMBS	Hydroxymethylbilane Synthase	NM_001097412.1	F: CTGAACAAAGGTGCCAAGAACA R: GCCCCGCAGACCAGTTAGT
H3F3A	Histone H3.3	NM_213930.1	F: CATGGCTCGTACAAAGCAGA R: ACCAGGCCTGTAACGATGAG
YWHAZ	Tyrosine 3-Monooxygenase/tyrtophan 5-monooxygenase Activation Protein Zeta	NM_001315726.1	F: GGACATCGGATACCCAAGGA R: AAGTTGGAAGGCCGGTTAATTT
ACTB	Actin Beta	XM_001927228.1	F:GGACATCGGATACCCAAGGA R:AAGTTGGAAGGCCGGTTAATTT
B2M	Beta-2-Microglobulin	NM_213978.1	F:CGGAAAGCCAAATTACCTGAAC R:TCTCCCCGTTTTTCAGCAAAT

Table 4. Primer sequences for QPCR analysis of genes involved gastrointestinal health and function.

	Treatment*					*P* value		
Grain preservation method	**Dried**	**OA-preserved**	**Dried**	**OA-preserved**				
Zinc Oxide supplementation	**No**	**No**	**Yes**	**Yes**	**SEM**	**Grain**	**ZnO**	**Grain × ZnO**
D 0–21								
ADFI (g/DM/day)	325	349	388	384	0.011	0.357	**< 0.001**	0.209
ADG (g/d)	235[a]	295[b]	313[b]	287[ab]	0.020	0.396	0.080	**0.030**
G:F	707[a]	844[b]	808[ab]	742[ab]	0.049	0.465	0.996	**0.039**
BW (kg)	11.99[a]	13.25[b]	13.63[b]	13.07[ab]	0.417	0.396	0.080	**0.030**
FS	3.03	3.00	2.51	2.40	0.206	0.399	**<0.001**	0.524
D 21–35								
ADFI (g/DM/day)	833[ab]	860[ab]	903[a]	781[b]	0.034	0.168	0.889	**0.030**
ADG (g/d)	573[ab]	647[a]	593[ab]	559[b]	0.029	0.483	0.240	**0.049**
G:F	691	753	670	742	0.036	**0.049**	0.664	0.886
BW (kg)	20.01[a]	22.71[b]	21.93[b]	20.90[ab]	0.718	0.242	0.936	**0.011**
D 0–35								
ADFI (g/DM/day)	528[a]	553[ab]	594[b]	543[ab]	0.018	0.473	0.133	**0.038**
ADG (g/d)	370[a]	447[b]	425[b]	396[ab]	0.021	0.242	0.936	**0.011**
G:F	696	810	721	742 739	0.034	**0.047**	0.494	0.162

Table 5. The effect of dietary treatment on growth performance and faecal scores (Least-square means with their standard errors). BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio; FS, faecal score. [a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).*A total of eight replicates were used per treatment group for the first 10 days (replicate = pen, 3 pigs/pen), following the collection of samples on day 10 PW, (replicate = pen, 2 pigs/pen).

Escherichia in the ileum. ZnO also raised *Faecalibacterium* levels. In the colon, interactions affected *Frisingicoccus, Gemmiger,* and *Faecalibacterium. Mediterraneibacter* was higher and *Lactobacillus* lower in OA-preserved grain-fed pigs; ZnO reversed this trend.

Volatile fatty acids (VFAs)

Table 12 details the volatile fatty acid (VFA) profile. In the ileum, ZnO × grain preservation interactions influenced butyrate ($P<0.05$); ZnO reduced butyrate in OA-preserved grain diets only. The OA-preserved grains raised acetate and lowered propionate proportions ($P<0.05$). In the colon, OA-preserved diets increased propionate (0.267 vs. 0.237, SEM 0.0094; $P<0.05$), while ZnO decreased branched-chain fatty acids (BCFA) (0.036 vs. 0.051, SEM 0.0046; $P<0.05$). No significant interaction effects were found in the colon.

Discussion

The hypothesis of this study was that OA-preserved grain would improve piglet growth performance compared to conventionally dried grain by reducing the microorganism load in the grain, thereby increasing grain quality and directly influencing the gastrointestinal health and function of the pig. It was also hypothesised that OA-preserved grain would have similar effects on piglet growth and intestinal health compared to ZnO and could serve as an alternative to in-feed antimicrobials. The OA-preserved grain diet had the best BW, ADG, and G:F among all dietary treatments, while the beneficial effects of ZnO on growth performance were reduced after the initial 21 days PW. However, OA-preserved grain did not reduce FS to levels comparable with ZnO. These results

	Treatment*					*P* value		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc Oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Duodenum								
VH µm	271.3	269.6	260.7	233.4	23.56	0.538	0.335	0.587
CD µm	104.9	112.0	110.1	103.2	7.62	0.986	0.815	0.363
VH:CD	2.7	2.4	2.4	2.4	0.26	0.609	0.545	0.749
Jejunum								
VH µm	273.9	187.1	289.0	248.7	22.69	**0.006**	0.089	0.281
CD µm	106.7	87.0	84.9	108.5	11.60	0.858	0.988	0.070
VH:CD	2.7	2.3	3.7	2.5	0.44	0.078	0.162	0.371
Ileum								
VH µm	251.9[a]	189.3[b]	236.4[a]	257.8[a]	21.18	0.332	0.226	**0.057**
CD µm	108.8	106.6	90.7	94.7	6.70	0.898	**0.036**	0.648
VH:CD	2.4	1.8	2.8	2.8	0.30	0.416	**0.027**	0.280

Table 6. The effect of dietary treatment on small intestinal morphology (Least-square means with their standard errors). VH, villus height; CD, crypt depth; VH:CD, villus height to crypt depth ratio. * a total of seven replicates were used per treatment group.

indicate that OA-preserved grain could support piglet growth throughout the PW period. However, further dietary manipulation of OA-preserved grain diets is necessary to reduce FS to levels reported with ZnO.

In this study, OA-preserved grain had a lower DM and reduced levels of OTA and DON compared to dried grain, indicating improved grain quality. Post-weaned piglets are particularly vulnerable to mycotoxin contamination due to their physiological immaturity[20]. Mycotoxins impair immune function and increase pathogen susceptibility[21], ultimately reducing voluntary feed intake and growth[22]. Specifically, OTA promotes oxidative stress and an inflammatory state[23], while DON increases inflammation and digestive disorders[24]. Propionic acid and ammonium propionate are well-established grain preservatives[26,27]. Previous research suggests they provide greater benefits for gut health and growth when used as grain preservatives rather than as feed additives[18]. Therefore, improved grain quality could be partially responsible for the increased ADFI, growth performance, and improved gut microbiome observed in the OA-preserved grain diet. To confirm these protective effects, future studies could employ non-invasive mycotoxin biomarkers in piglet urine, faeces, or blood[25].

Feed intake typically declines immediately PW[28], leading to villous atrophy, crypt hyperplasia, elevated T-cell production[29], and impaired intestinal development[30]. Reports on the effects of OA supplementation on piglet feed intake remain inconsistent, with studies showing no effect[31–34], negative effects[35], or positive effects[36–38]. Additionally, supplementation with glycerol polyethylene ricinoleate has been shown to increase ADG and improve feed efficiency in broiler chickens[39,40]. In the present study, OA-preserved grain increased ADFI compared to dried grain, aligning with previous findings[18,19]. Therefore, the observed increase in ADFI may contribute to the gut microbial shifts and improved growth performance seen in piglets fed the OA-preserved grain diet.

Interestingly, ZnO supplementation improved BW and ADFI in dried grain but did not provide any additional benefit on BW and ADFI in OA-preserved grain, consistent with previous research[19]. Although research examining ZnO supplementation in OA-preserved grain is limited, ZnO supplementation in non-OA-preserved diets has consistently increased growth performance[41–43]. However, the beneficial effect of ZnO on growth performance in pigs fed dried grain was diminished during the second phase of the experiment (D21–35). This finding supports the notion that ZnO efficacy declines after the initial 21 days PW[44]. In contrast, OA-preserved grain maintained its positive effects on growth throughout the experimental period, likely due to its influence on feed intake and modulation of the gut microbiome. Although both OA-preserved grain and ZnO improved growth performance, their effects on FS differed. OA-preserved grain did not improve FS compared with dried grain, whereas ZnO supplementation led to higher FS values relative to non-ZnO diets, consistent with previous findings[45,46]. The growth and FS improvements associated with ZnO may stem from enhanced gut morphology, reduced immune activation, improved nutrient absorption, and alterations in intestinal microbial populations.

The intestinal microbiome is vital in maintaining the physiological, immunological, and nutritional state of the pig. Thus, manipulating the gut microbiota could reduce reliance on antimicrobials in pig production[47]. In this study, piglets offered OA-preserved grain had reduced colonic Shannon and Simpson diversity compared to those offered dried grain. Reduced microbial diversity has been associated with diarrhetic piglets PW[48], however, other studies have reported no correlation between reduced diversity and piglet gut health[49]. Pigs offered the OA-preserved grain diet had the highest abundance of ileal and colonic *Faecalibacterium* among all treatments. *Faecalibacterium,* specifically *Faecalibacterium prausnitzii* are indicators of increased intestinal health due to their immunomodulatory[50] and butyrate-producing abilities[51]. Supplementing *Faecalibacterium prausnitzii* to mice increased tight-junction protein expression, reduced pro-inflammatory cytokine expression, and decreased the abundance of faecal *Staphylococcus* and *Klebsiella*[52]. Additionally, the OA-preserved grain was associated with reduced abundance of ileal *Escherichia* compared to dried grain. *Escherichia coli* is one of the

	Treatment*					*P* value		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc Oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Duodenum								
FABP2	1.25	1.26	1.11	0.71	0.171	0.266	**0.050**	0.240
MUC2	1.64[a]	1.13[ab]	0.83[b]	1.28[ab]	0.204	0.860	0.116	**0.027**
IL6	1.05	1.26	0.88	0.90	0.112	0.326	**0.027**	0.425
IL17	2.56	1.35	0.93	0.71	0.302	**0.027**	**0.001**	0.114
SCT	1.27	0.98	1.13	0.94	0.130	0.079	0.498	0.707
Jejunum								
SLC15A1	1.30	0.69	2.07	1.89	0.397	0.330	**0.020**	0.589
OCCLN	0.97	0.93	1.66	1.44	0.292	0.659	**0.050**	0.752
IL1A	1.46	1.31	0.82	0.67	0.249	0.550	**0.018**	0.996
IL1B	2.57	1.98	0.98	0.58	0.661	0.464	**0.033**	0.887
CXCL8	1.99	1.51	0.82	0.54	0.396	0.346	**0.013**	0.799
lL17	3.76	2.95	0.66	0.21	0.844	0.461	**0.002**	0.834
IL22	2.58	3.21	0.72	0.58	0.525	0.640	**<0.001**	0.467
NOX1	1.43	1.53	0.96	0.66	0.218	0.639	**0.005**	0.380
Ileum								
FABP2	1.46	0.96	1.25	0.88	0.219	**0.045**	0.494	0.748
SLC15A1	1.03	0.86	1.89	1.98	0.340	0.906	**0.008**	0.704
IL1A	1.16	1.29	0.79	0.68	0.154	0.938	**0.004**	0.452
IL1B	1.11	2.17	0.78	0.68	0.361	0.174	**0.014**	0.104
CXCL8	1.73	1.75	0.79	0.56	0.299	0.722	**0.002**	0.673
TNF	1.16[a]	1.22[a]	1.51[a]	0.70[b]	0.157	0.025	0.593	**0.011**
IL17	2.96	1.90	0.92	0.27	0.681	0.200	**0.009**	0.753
IL22	2.96	4.35	0.77	0.43	0.816	0.529	**0.001**	0.300
FOXP3	1.27	0.98	1.71	0.87	0.238	**0.026**	0.491	0.260
NOX1	1.31	1.51	1.21	0.62	0.239	0.425	**0.047**	0.111
Colon								
SLC16A1	1.05	0.74	1.73	1.30	0.246	0.134	**0.016**	0.801
MUC2	0.97	0.84	1.45	1.29	0.177	0.410	**0.013**	0.924
IL1A	1.83	1.32	0.74	0.70	0.232	0.236	**<0.001**	0.308
IL1B	2.11	1.94	0.41	1.30	0.479	0.442	**0.019**	0.263
IL6	1.35[a]	1.13[ab]	0.69[b]	1.32[a]	0.191	0.277	0.215	**0.030**
CXCL8	2.16	1.36	0.61	0.86	0.278	0.323	**<0.001**	0.064
TLR4	1.11	1.29	0.91	0.89	0.154	0.576	**0.050**	0.499
IL17	1.97	1.65	0.63	0.74	0.367	0.768	**0.004**	0.542
NOX1	1.56	1.47	0.56	0.95	0.231	0.511	**0.003**	0.292

Table 7. The effect of dietary treatment on the expression of genes involved in stomach functionality, nutrient transportation, inflammation and barrier function in the small intestine and colon (Least-square means with their standard errors). FABP2, fatty acid binding protein 2; MUC2, mucin 2; IL6, interleukin 6; IL17, interleukin 17; SCT, secretin; SLC2A1, glucose transporter 1; SLC15A1, peptide transporter 1; OCCLN, occludin; IL1A, interleukin 1A; IL1B, interleukin 1B; CXCL8, C-X-C motif chemokine ligand 8; IL22, interleukin 22; TNF, tumour necrosis factor; FOXP3, forkhead box P3; NOX1, NADPH Oxidase 1; SLC16A7, solute carrier family 16 member 7, TLR4, toll-like receptor 4.

main causative agents of PWD[53]. Previous research has demonstrated OA supplementation can reduce *E. coli*[54,55] and increase faecal *Faecalibacterium* in weaned piglets[56]. Despite the reduction in colonic bacterial diversity, the microbial shifts associated with OA-preserved grain could contribute to enhanced growth performance and intestinal health.

Supplementing ZnO can beneficially modulate the PW gut microbiota by promoting the growth of beneficial bacteria, enhancing short-chain fatty acid (SCFA) production, and inhibiting the proliferation of pathogens[57], thereby supporting a more stable and mature gut ecosystem. ZnO supplementation has been reported to reduce the abundance of *Enterobacteriaceae*, *Escherichia*, and *Lactobacillus* spp. in the small intestine[58]. In contrast, the present study found that ZnO supplementation increased colonic *Lactobacillus* abundance compared with non-ZnO diets, differing from previous reports that observed no effect of ZnO on intestinal *Lactobacillus* populations[4,45]. Notably, *Lactobacillus* species have been associated with improved nutrient digestion and barrier integrity,

	Treatment*					P value		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc Oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Ileum								
Observed	53.29	48.33	52.00	50.29	3.039	0.267	0.926	0.578
Fisher	8.30	7.41	8.07	7.75	0.554	0.272	0.926	0.596
Shannon	3.39	3.34	3.36	3.35	0.084	0.711	0.860	0.843
Simpson	0.95	0.95	0.94	0.95	0.009	0.810	0.649	0.743
Colon								
Observed	54.43	47.71	58.14	50.71	3.644	0.064	0.366	0.923
Fisher	8.52	7.33	9.20	7.84	0.657	0.064	0.375	0.897
Shannon	3.51	3.25	3.59	3.38	0.084	**0.011**	0.238	0.778
Simpson	0.96	0.94	0.96	0.95	0.006	**0.023**	0.109	0.458

Table 8. The effect of dietary treatment on measures of alpha diversity in ileal and colonic digesta (least square means with their standard errors). * A total of seven replicates were used per treatment group.

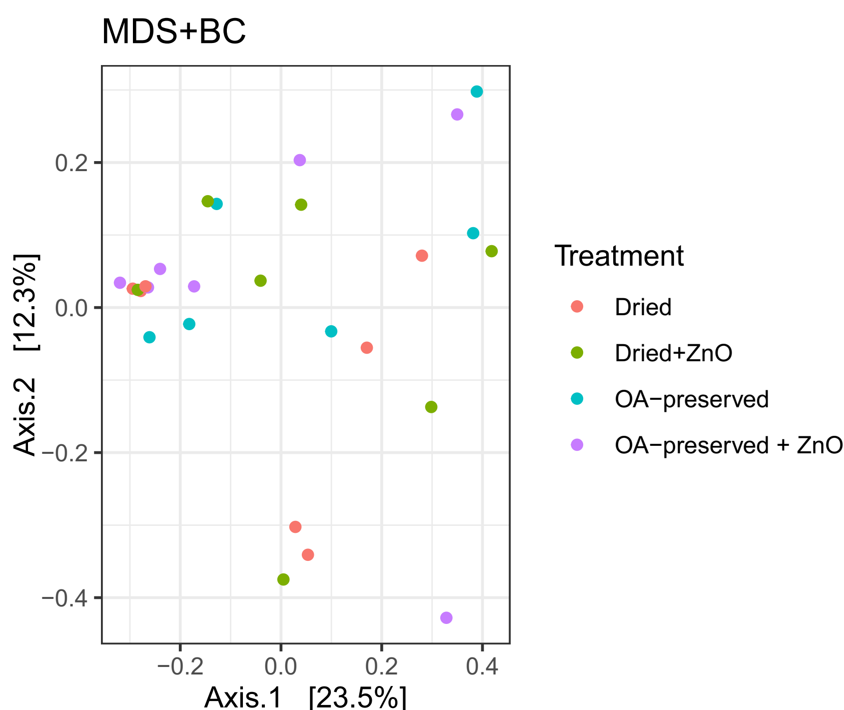

Fig. 1. Bacterial beta diversity of ileal digesta based on Permanova analysis and through visualisation using the Bray Curtis distance matrix and multi-dimensional scaling.

reduced diarrhoea[59], and decreased expression of pro-inflammatory cytokines[60], which could partially explain the positive effects observed here. The beneficial influence of ZnO on the gut microbiota was further reflected in the VFA profile. ZnO supplementation reduced the molar proportions of colonic BCFAs compared with non-ZnO diets, indicating decreased protein fermentation[61]. Excessive protein fermentation generates harmful metabolites[62], disrupts intestinal barrier function, promotes inflammation[63], and facilitates the proliferation of pathogenic bacteria[40]. Together, the microbial and VFA shifts observed with ZnO supplementation may underlie the initial improvements in growth performance in pigs fed the dried grain diet with ZnO, as well as the reductions in FS and intestinal inflammatory markers.

Post-weaning inflammation reduces intestinal barrier function, alters the microbiome[64] and affects intestinal morphology[65]. Supplementing OAs has been reported to reduce the expression of pro-inflammatory cytokines[66] and increase the expression of genes related to intestinal barrier function[67]. In the current experiment, OA-preserved grain reduced the relative expression of duodenal *IL17* and ileal *FOXP3* compared to dried grain. The supplementation of ZnO decreased the relative expression of multiple pro-inflammatory cytokines, including *IL6* and *IL17* in the duodenum, *IL1A, IL1B, CXCL8, IL17*, and *IL22* in the jejunum and ileum, and *IL1A, IL1B*,

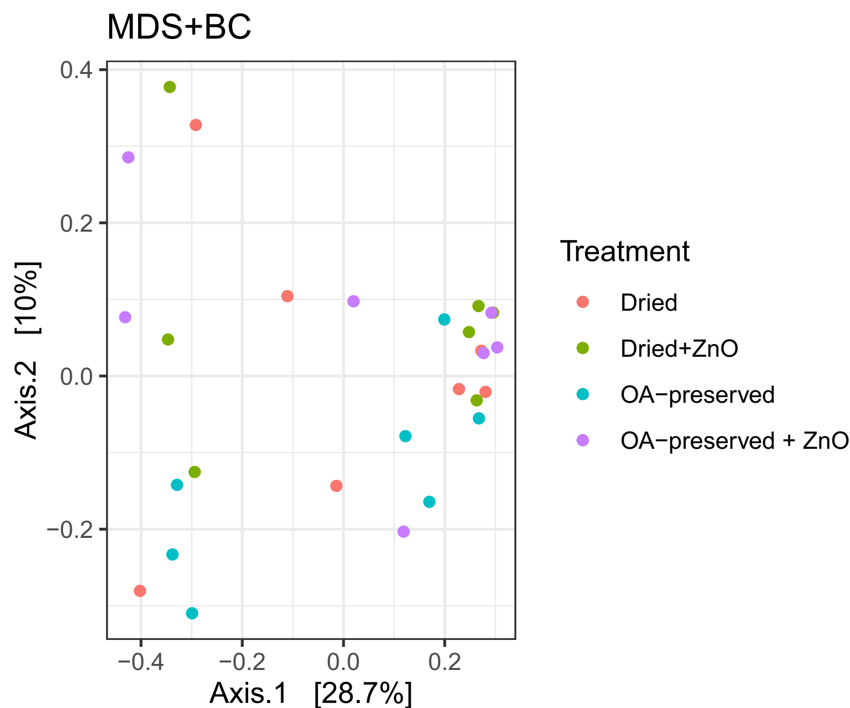

Fig. 2. Bacterial beta diversity of colonic digesta based on Permanova analysis and through visualisation using the Bray Curtis distance matrix and multi-dimensional scaling.

Phylum	Treatment*				SEM	*P* value		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Ileum								
Firmicutes	81.68	89.85	83.64	86.48	3.870	0.137	0.864	0.466
Bacteroidetes	2.61	1.92	1.48	0.72	0.610	0.137	**0.028**	0.540
Proteobacteria	13.02^{a}	4.38^{bc}	2.07^{b}	7.56^{c}	1.364	0.599	0.003	**< 0.001**
Actinobacteria	0.52	1.09	1.49	1.70	0.532	0.281	0.071	0.445
Colon								
Firmicutes	95.02^{a}	83.39^{b}	92.61^{ab}	95.36^{a}	3.691	0.213	0.183	**0.050**
Bacteroidetes	1.27	1.39	1.64	0.44	0.485	0.140	0.280	0.094
Proteobacteria	0.26^{a}	6.44^{c}	2.92^{b}	1.32^{ab}	1.036	0.010	0.344	**< 0.001**
Actinobacteria	0.50	0.99	2.82	2.70	0.635	0.408	**0.002**	0.348

Table 9. The effect of dietary treatment the relative abundance of bacterial phyla in ileal and colonic digesta (mean % relative abundance with their standard errors). * A total of seven replicates were used per treatment group. ^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

IL6, *CXCL8*, and *IL17* in the colon on day 10 PW. Additionally, *NOX1*, an indicator of oxidative stress, was reduced in the jejunum, ileum, and colon of ZnO-supplemented pigs. The expression of genes involved in barrier integrity, such as *OCCLN* in the jejunum and *MUC2* in the colon, were increased in ZnO-supplemented pigs. These results suggest that the reduction in pro-inflammatory cytokine expression and increased expression of genes associated with the integrity of the intestine in ZnO-supplemented diets could contribute to reduced FS. Moreover, physiological changes during an immune response create a homeorhetic state, redirecting nutrients from growth to the immune system[68]. Thus, the reduced expression of pro-inflammatory cytokines may increase the energy available for growth, partially explaining the improved growth performance of the dried grain diet with ZnO in the initial 21 days.

The relationship between intestinal morphology and growth performance is well established, with morphological disruptions associated with reduced growth performance[69]. Interestingly, although there was no negative effect on growth, OA-preserved grain reduced jejunal VH compared to dried grain. Furthermore, OA-preserved grain reduced the relative expression of ileal *FABP2* compared to dried grain. The ability of OA-

Family	Treatment*				SEM	*P* value		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Ileum								
Lactobacillaceae	60.78[ab]	56.91[ab]	54.53[a]	62.68[b]	3.080	0.473	0.907	**0.050**
Lachnospiraceae	9.29[a]	2.19[b]	5.38[ab]	5.30[ab]	1.152	0.001	0.393	**0.001**
Erysipelotrichaceae	0.06	0.51	2.27	1.83	0.569	0.272	**0.007**	0.179
Eubacteriaceae	0.62	0.46	0.42	0.38	0.299	0.722	0.619	0.872
Oscillospiraceae	0.70	1.72	0.41	0.30	0.536	0.603	**0.049**	0.282
Ruminococcaceae	7.58	10.99	9.72	12.20	1.483	0.027	0.174	0.571
Enterobacteriaceae	12.53[a]	2.08[bc]	0.90[b]	4.20[c]	0.774	0.650	0.002	**< 0.001**
Clostridiaceae	1.16	2.64	0.89	0.55	0.664	0.663	0.663	0.108
Acidaminococcaceae	0.06	0.09	0.11	0.10	0.131	0.864	0.786	0.840
Hungateiclostridiaceae	1.54	1.34	0.63	0.79	0.473	0.907	0.084	0.647
Colon								
Lactobacillaceae	58.96	45.63	65.62	60.03	3.062	**0.002**	**0.001**	0.109
Lachnospiraceae	12.80[ab]	9.07[a]	8.63[a]	15.43[b]	1.485	0.311	0.555	**0.001**
Erysipelotrichaceae	0.52	0.20	0.27	1.03	0.383	0.784	0.456	0.092
Eubacteriaceae	1.05	0.47	1.37	1.73	0.498	0.481	0.063	0.207
Ruminococcaceae	13.86	13.55	14.87	14.38	1.503	0.790	0.534	0.959
Clostridiaceae	0.34	0.55	1.01	0.79	0.380	0.815	0.171	0.475
Enterobacteriaceae	0.29[a]	5.73[b]	1.23[a]	1.34[a]	0.977	0.002	0.993	**0.003**

Table 10. The effect of dietary treatment the relative abundance of selected bacterial family in ileal and colonic digesta (mean % relative abundance with their standard errors). * A total of seven replicates were used per treatment group. [a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

preserved grain to sustain superior growth performance, despite evidence of reduced absorptive capacity, may be attributed to its previously reported enhancement of nutrient digestibility in weaned pigs[19,70], possibly mediated by increased digestive enzyme activity. Conversely, ZnO supplementation improved intestinal morphology by reducing ileal CD and increasing the ileal VH:CD ratio compared to non-ZnO supplemented diets. Supplementing ZnO also increased the relative expression of jejunal and ileal *SLC15A1* and colonic *SLC16A1* compared to non-ZnO supplemented diets, indicating increased protein and SCFA absorption. The increased digestive and absorptive capacity of ZnO-supplemented pigs may be linked to reduced FS and improved growth performance in the dried grain diet in the initial 21 days PW.

The ability of OA-preserved grain to enhance piglet growth performance throughout the PW period highlights its potential as an alternative to ZnO following the EU's restrictions on in-feed antimicrobials. However, the findings of this study may not be fully generalisable. The experiment used specific wheat (JB Diego) and barley (SY Errigal) varieties from Ireland, each with unique nutritional compositions that could influence how OA preservation affects grain quality, piglet growth, and intestinal health. Grains differing in variety, origin, or initial moisture content, as well as other cereals such as maize, oats, rye, triticale, or sorghum, may not respond identically to OA preservation. Variations in starch characteristics and fiber content can alter fermentation patterns and nutrient digestibility. Despite these potential differences, the core benefits of OA preservation, reducing microbial spoilage, stabilising nutrients, and supporting gut health, are likely to be broadly applicable across cereal types. Nonetheless, the magnitude and consistency of these effects are expected to depend on the specific properties of each grain and should be validated experimentally under different conditions. Finally, ZnO's strong capacity to reduce FS and suppress pro-inflammatory cytokine expression suggests that future research should aim to further optimise OA-preserved grain diets to achieve comparable improvements in intestinal health. Approaches such as lowering dietary crude protein or incorporating complementary feed additives may help reduce FS and enhance gut integrity to levels similar to those observed with ZnO supplementation.

Material and methods

All of the experimental procedures detailed in this study were approved by the University College Dublin Animal Research Ethics Committee (AREC-20-21-ODoherty) and performed in accordance with Irish legislation (*SI* no.543/2012) and the EU directive 2010/63/EU for animal experimentation and in compliance with the ARRIVE guidelines. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed the EU standards for the protection of animals used for scientific purposes and the ARRIVE guidelines.

Genus	Treatment*				SEM	*P* valu		
Grain preservation method	Dried	OA-preserved	Dried	OA-preserved				
Zinc oxide supplementation	No	No	Yes	Yes	SEM	Grain	ZnO	Grain x ZnO
Ileum								
Lactobacillus	66.06	58.36	60.64	63.51	3.119	0.437	0.993	0.096
Eubacterium	0.69	0.46	1.12	0.38	0.399	0.166	0.782	0.516
Oscillibacter	0.72	0.38	0.41	0.30	0.320	0.463	0.554	0.794
Butyricicoccus	0.33	0.25	0.34	0.55	0.281	0.860	0.565	0.596
Gemmiger	3.47	4.13	3.02	2.69	0.830	0.888	0.198	0.503
Escherichia	1.75	0.36	2.45	0.73	0.592	**0.005**	0.260	0.684
Anaerobacterium	0.93	0.08	0.55	0.79	0.394	0.219	0.297	0.099
Holdemanella	0.07	0.30	0.16	2.03	0.539	**0.049**	0.172	0.599
Faecalibacterium	3.05	6.02	6.50	8.66	1.112	**0.010**	**0.004**	0.265
Colon								
Coprococcus	5.34	4.24	4.51	3.34	0.874	0.172	0.289	0.856
Lactobacillus	59.04	52.15	66.22	60.92	3.076	**0.045**	**0.011**	0.683
Sporobacter	0.65^{a}	2.00^{b}	1.70^{ab}	1.33^{ab}	2.003	0.215	0.435	0.060
Holdemanella	0.16	0.06	0.21	0.12	0.175	0.507	0.665	0.838
Frisingicoccus	0.83^{ab}	1.68^{a}	1.13^{ab}	0.26^{b}	0.530	0.435	0.122	**0.035**
Eubacterium	0.77	0.49	1.39	1.55	0.471	0.672	0.048	0.502
Butyricicoccus	0.12	0.46	0.40	0.19	0.276	0.732	0.851	0.213
Gemmiger	7.27^{a}	1.66^{c}	6.47^{ab}	3.86^{bc}	1.019	< 0.001	0.087	**0.027**
Ruminococcus	0.90	2.37	1.35	1.68	0.581	0.088	0.927	0.269
Faecalibacterium	1.76^{a}	15.61^{c}	4.07^{b}	5.41^{b}	1.613	< 0.001	0.576	**< 0.001**
Mediterraneibacter	0.09	2.27	0.65	1.94	0.569	**0.005**	0.203	0.140
Clostridium	0.14	0.21	0.26	0.36	0.227	0.652	0.496	0.955

Table 11. The effect of dietary treatment the relative abundance of selected bacterial genera in ileal and colonic digesta (mean % relative abundance with their standard errors). * A total of seven replicates were used per treatment group. [a,b,c]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Grain processing and quality evaluation

Winter wheat (cv. *JB Diego*) and spring barley (cv. *SY Errigal*) grains sourced from McAuley Feeds (Burtonstown, Co. Meath, Ireland) were utilised in this study, following appropriate management and preservation practises[19]. The wheat was sown in October 2019, following standard agronomic practices [3-spray fungicide program and a 3-split nitrogen (N) application rate of 180 kg N/ha] and harvested in August 2020 at 180.0 g/kg moisture content. The barley was sown in March 2020, and received a 2-spray fungicide program and a 2-split N application rate of 140 kg/ha, before being harvested in August 2020, at 182.0 g/kg moisture content.

Prior to storage, both the wheat and barley grains were separated into two groups and preserved[19]. Briefly, one group was dried using a continuous flow dryer (Cimbria, Thisted, Denmark) at 65 °C for 3 h before a 2 h cooling period, reducing the moisture content of the wheat and barley to 140 g/kg and 140.7 g/kg, respectively. The second group was preserved with an organic acid liquid surfactant mould inhibitor [MycoCURB ES Liquid; containing propionic acid (650 g/kg), ammonium propionate (70 g/kg), glycerol polyethyleneglycol ricinoleate (17.5 g/kg) and water]. It was applied to the grain by spray action at the inclusion rate of 4 g/kg. Even distribution of the acid throughout the grain was ensured by using a mixing auger. This product was manufactured by Kemin Industries (Des Moines, IA) and sourced from Adesco Nutricines, (Dungarvan, Co. Waterford, Ireland).

At the time of harvest, various grain quality parameters was evaluated including moisture content, density (hectolitre weight), and thousand-grain weight (TGW). Moisture content was measured with a DICKEY-john GAC 2500-UGMA electronic moisture meter (Illinois, USA), while density was assessed using a Pfeuffer Chondrometer along with a bulk density calibration chart. The TGW was determined by weighing 1000 grains and recording their weights using a Pfeuffer Contador seed counter (Kitzingen, Germany). Prior to diet manufacture, samples were collected from both the wheat and the barley using the grab sample technique. These samples were analysed for dry matter (DM), ash, gross energy (GE) crude protein, crude fibre, starch, fat and mycotoxin levels. Mycotoxins including Aflatoxin B1, B2, G1 and G2, and trichothecenes [T-2 toxin, HT-2 toxin and DON], Fumonisin B1 and B2, OTA and ZEA was detected using liquid chromatography-mass spectrometry[71].

Experimental design and diet

Ninety-six newly piglets, which were weaned at 28 days (progeny of Meatline Hermitage boar (Sion Road, Kilkenny, Ireland) × (Large White × Landrace sow)) were selected from a commercial swine unit and assigned to 1 of 4 dietary treatments in a 2 × 2 factorial design across a 35-day experimental period (n = 8). The average

	Treatment*					*P* value		
Grain preservation method	**Dried**	**OA-preserved**	**Dried**	**OA-preserved**				
Zinc oxide supplementation	**No**	**No**	**Yes**	**Yes**	**SEM**	**Grain**	**ZnO**	**Grain x ZnO**
Ileum mmol/gram of digesta								
Acetate	0.733	0.787	0.615	0.792	0.0578	**0.025**	0.244	0.206
Propionate	0.166	0.101	0.255	0.107	0.0447	**0.010**	0.205	0.268
Butyrate	0.070[ab]	0.098[a]	0.087[a]	0.052[b]	0.0129	0.760	0.190	**0.009**
Valerate	0.008	0.003	0.014	0.014	0.0056	0.604	0.081	0.513
Isobutyrate	0.009	0.003	0.013	0.014	0.0061	0.632	0.152	0.472
Isovalerate	0.014	0.007	0.016	0.019	0.0067	0.787	0.204	0.391
BCFA	0.029	0.022	0.022	0.048	0.0180	0.522	0.538	0.267
Total	12.97	16.67	35.58	20.34	10.099	0.491	0.128	0.264
Colon mmol/gram of digesta								
Acetate	0.616	0.588	0.626	0.599	0.0202	0.177	0.602	0.965
Propionate	0.253	0.262	0.221	0.273	0.0133	**0.026**	0.434	0.101
Butyrate	0.084	0.095	0.093	0.093	0.0108	0.603	0.737	0.633
Valerate	0.016	0.020	0.028	0.013	0.0052	0.270	0.647	0.067
Isobutyrate	0.014	0.016	0.014	0.014	0.0026	0.778	0.742	0.786
Isovalerate	0.017	0.020	0.019	0.018	0.0034	0.674	0.946	0.559
BCFA	0.047	0.056	0.033	0.039	0.0068	0.272	**0.029**	0.849
Total	151.90	131.22	158.41	144.10	20.346	0.382	0.626	0.872

Table 12. The effect of dietary treatment on the molar proportions and total concentrations of VFA in mmol/gram in pig ileum and colon (Least-square means with their standard errors). VFA, volatile fatty acids; BCFA, branched-chain fatty acids. * A total of seven replicates were used per treatment group.

weaning weight of the piglets was 7.00 ± 1.2 kg (SD), and they were blocked by litter of origin, sex and body weight. The dietary treatments were as follows: (T1) dried grain diet; (T2) OA-preserved grain diet; [3] dried grain diet with zinc oxide (ZnO) and[4] OA-preserved grain diet with ZnO. The diets consisted of 600 g/kg of grain, with 450 g/kg being either dried or OA-preserved wheat and 150 g/kg being dried or OA-preserved barley. The remaining composition (400 g/kg) consisted of a concentrate obtained from Cargill (Naas, Co. Kildare, Ireland), as outlined in Table 2. The ZnO used in this study was obtained from Cargill (Naas, Co Kildare, Ireland) and contained 80% zinc. The ZnO was included at a concentration of 3.3 g ZnO/kg of feed, resulting in an inclusion level of 2.65 g Zn/kg of feed. After 15 days, the ZnO inclusion level was lowered to 1.5 g/ZnO/kg feed. The diets were formulated to have comparable levels of digestible energy (14.95 MJ/kg), net energy (10.95 MJ/kg) and standardised ileal digestible lysine (12.0 g/kg)[72]. The piglets' amino acid requirements were met relative to lysine[73]. All diets were milled on the research facility. The ingredient composition of the dietary treatments is presented in Table 2, while the chemical and microbial composition of the dietary treatments are presented in Table 3.

Animal housing and management

Piglets were housed in fully slatted pens (1.7 × 1.2 m) with three animals per pen. Environmental conditions were controlled, with the room temperature set at 30 °C for the first week and reduced by 2 °C each subsequent week. Humidity was maintained at 65%. Feed in the form of mash and water were provided ad libitum through two-space feeders and nipple drinkers.

Body weight was recorded at baseline (day 0) and subsequently at 7-day intervals. These data were used to calculate ADG. Feed intake was recorded weekly to calculate ADFI and G:F. Faecal consistency was scored twice daily using a 1–5 scale: 1 = firm; 2 = slightly soft; 3 = soft, partially formed; 4 = loose, semi-liquid; 5 = watery, mucous-like[46].

Sample collection

On day 10 PW, one piglet per pen (7 per treatment group) was selected for the collection of intestinal tissue and digesta samples, based on average body weight, as gut disturbances are greatest during the first 14 days PW[69,74,75]. Selected piglets were humanely euthanised. Euthanasia was performed by a trained technician in a separate area, using a lethal injection of pentobarbitone sodium (Euthanal, 200 mg/ml; Chanelle Pharma, Galway, Ireland) administered into the cranial vena cava at a dosage of 0.71 ml/kg body weight[18].

Immediately following euthanasia, the gastrointestinal tract was excised. Tissue samples were taken from the duodenum (10 cm distal to the stomach), jejunum (60 cm distal), and ileum (15 cm proximal to the cecum), and fixed in 10% neutral buffered formalin. These samples were analysed via quantitative PCR (QPCR) to assess expression of nutrient transporters, mucins, tight junction proteins, and cytokines. Additionally, colonic tissue was harvested for analysis of SCFA transporters, mucin, and cytokine expression via QPCR. Digesta from the ileum and colon was collected into sterile containers (Sarstedt, Wexford, Ireland) and immediately frozen at – 20 °C for subsequent VFA profiling and 16S rRNA gene sequencing.

Feed analysis

During diet preparation, representative feed samples were collected and ground using a 1 mm screen (Christy and Norris Hammer Mill, Chelmsford, UK). GE was determined via an adiabatic bomb calorimeter (Parr Instruments, St. Moline, IL, USA). DM content was measured by drying at 55 °C for 72 h. Crude ash was determined by combusting the samples in a muffle furnace (Nabertherm) at 550 °C for 6 h.

Nitrogen concentration was analysed using a LECO FP 528 (Leco Instruments, Stockport, UK Ltd.). Amino acid profiles were determined using high-performance liquid chromatography (HPLC)[76]. Crude fibre was assessed following AOAC (1990; method 978.10), and neutral detergent fibre (NDF) was analysed using an Ankom 220 Fibre Analyzer (Ankom Technology, USA)[77]. Starch content was measured with the Megazyme total starch assay (Megazyme, Bray, Co. Wicklow, Ireland). Crude fat was quantified using Soxtec extraction with light petroleum ether (Tecator, Hillerod, Sweden). Table 3 summarises the complete chemical composition of the dietary treatments.

Gut morphological analysis

Histological processing of intestinal tissues (duodenum, jejunum, ileum) was performed following the paraffin-embedding method[46]. Tissue sections were cut to 5 µm long and stained with haematoxylin and eosin. Morphometric measurements were conducted using a light microscope fitted with an image analysis system (Image-Pro Plus; Media Cybernetics, Oxon, UK). For each tissue per animal, a minimum of 15 measurements of VH and CD were recorded. VH was measured from the villus tip to the crypt-villus junction, while CD was measured from the base of the crypt to the same junction.

Gene expression in the gastrointestinal tract

Tissue sample preparation and RNA Analysis

Tissue samples were collected from the mesenteric aspect of the duodenum, jejunum, ileum, and colon. Following collection, samples were rinsed with sterile phosphate-buffered saline (PBS; Oxoid), and the external smooth muscle layers were carefully removed. The mucosal tissues were then sectioned into smaller fragments using a sterile scalpel and placed in 15 mL of RNAlater™ (Applied Biosystems) for overnight preservation. Subsequently, the samples were stored at − 20 °C until RNA extraction.

Total RNA was isolated from the collected tissues using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), adhering to the manufacturer's instructions[78]. For reverse transcription, 2 µg of total RNA was converted to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with oligo (dT) primers, achieving a final reaction volume of 40 µL. This cDNA was then diluted with nuclease-free water to a final volume of 360 µL for downstream applications.

QPCR was carried out in 20 µL reaction volumes, comprising 10 µL GoTaq Master Mix (Promega, Madison, WI, USA), 1.2 µL of 5 µM forward and reverse primers, 3.8 µL nuclease-free water, and 5 µL of cDNA template. Each sample was analysed in duplicate using the 7500 ABI Prism Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermocycling program included an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min.

Primers were designed using Primer Express Software (Applied Biosystems) and synthesised by MWG Biotech UK Ltd. (Milton Keynes, UK). Melting curve analysis was performed to confirm amplicon specificity. PCR efficiency was assessed via standard curves generated from four-fold serial dilutions of cDNA, and only assays with efficiencies between 90–100% and single specific products were considered.

Normalised relative expression levels were calculated using qbase PLUS software (Biogazelle, Ghent, Belgium), based on stable reference genes. For duodenum, jejunum, and ileum tissues, *HMBS, H3F3A*, and *YWHAZ* were used, while *ACTB* and *B2M* were chosen for colonic tissues. These genes were selected based on GeNorm-calculated M values (< 1.5). Genes assessed included *SLC15A1, FABP2, SLC2A1, SLC2A5, SLC2A7, SLC5A1, SLC16A1, CLDN1, TJP, OCCLN, IL1A, IL1B, IL10, IL6, IL17, IL22, INFG, TNF, CXCL8, MUC2, TLR2, TLR4, NOX1, GIP, SCT, HRH2, FOXP3, MMP1*, and *AQP10*. Differentially expressed genes are presented in Table 4.

Volatile fatty acid (VFA) quantification

VFA levels in ileal and colonic digesta were determined using gas–liquid chromatography[79]. For each sample, 1 g of digesta was homogenised with distilled water (2.5 × the sample weight) and centrifuged at 1400 × g for 10 min using a Sorvall GLC-2 B centrifuge (DuPont). A 1 mL aliquot of the supernatant was mixed with 1 mL of an internal standard (0.05% 3-methyl-n-valeric acid in 0.15 M oxalic acid dihydrate) and 3 mL of distilled water. This mixture was then centrifuged at 500 × g for 10 min.

The resulting supernatant was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter and transferred to chromatographic vials. One microlitre of each sample was analysed using a Varian 3800 GC fitted with an EC™ 1000 Grace column (15 m × 0.53 mm I.D., 1.20 µm film thickness). The temperature program ramped from 75 to 90 °C at 3 °C/min, then from 95 to 200 °C at 20 °C/min, with a final hold of 0.5 min. Injector and detector temperatures were set at 240 °C and 280 °C, respectively. Each run lasted 12.42 min.

Microbial DNA extraction and illumina sequencing

Genomic DNA was extracted from the ileal and colonic digesta using the QIAamp PowerFecal Pro DNA Kit (Qiagen, West Sussex, UK), following the supplier's protocol. DNA yield and purity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Sequencing of the V3–V5 hypervariable regions of the 16S rRNA gene was performed on the Illumina MiSeq platform at Eurofins Genomics (Ebersberg, Germany). Amplification used universal primers with adapter overhangs compatible with Nextera XT indexing. Amplicons were purified using AMPure XP magnetic beads

(Beckman Coulter, Indianapolis, IN), followed by indexing PCR. Indexed products were again purified and quantified using a fragment analyser (Agilent, Santa Clara, CA), pooled in equimolar ratios, and quantified using the Bioanalyser 7500 DNA kit (Agilent). Sequencing employed v3 chemistry for 2 × 300 bp paired-end reads.

Bioinformatic analysis
Sequence processing and analysis were carried out by Eurofins Genomics using QIIME (v1.9.1)[80]. Reads passing Illumina's chastity filter and with Phred scores > 30 were demultiplexed. Primer sequences were trimmed, and reads with mismatches were excluded. Paired-end reads were merged using FLASH v2.2.00[81], requiring a minimum 10 bp overlap. When merging was not possible, only forward reads were retained.

Reads were filtered to match the expected V3–V5 length and truncated to 300 bp to improve quality. Chimeric reads were identified using UCHIME in the VSEARCH tool[82] and removed. Operational taxonomic units (OTU) were formed using Minimum Entropy Decomposition (MED)[83]. Representative OTU were classified taxonomically via DC-MEGABLAST against the NCBO nucleotide database, requiring ≥ 70% similarity over ≥ 80% of the sequence.

Copy number correction was applied using gene-specific linear copy numbers[84]. The resulting dataset, comprising the normalised OTU table, metadata, and phylogenetic tree, was imported into the R package *phyloseq* (v3.5.0) for differential abundance analysis at phylum, family, and genus levels.

Statistical analysis
All statistical analyses were conducted using SAS® software (v9.4, SAS Institute Inc.). The Shapiro–Wilk test (PROC UNIVARIATE) assessed data normality for growth performance, FS, gene expression, intestinal morphology, and VFA profiles. Where needed, data were transformed to meet normality assumptions. Growth metrics (ADFI, ADG, G:F, BW) were analysed via PROC GLM for days 0–21, days 22–35 and days 0–35. The model included fixed effects of grain preservation, ZnO supplementation, and their interactions. The pen was used as the experimental unit. Initial body weight was used as a covariate. The FS were averaged every three days and analysed via PROC MIX, using repeated measures. The model included fixed effects of grain preservation, ZnO supplementation, time, and their associated interactions. The pen was used as the experimental unit. PROC GLM was used to analyse gut morphology, gene expression, VFA concentrations, and alpha diversity. Bonferroni's correction ($P < 0.05$) adjusted for multiple comparisons in gene expression. The Non-parametric microbial data were evaluated using PROC GLIMMIX, with P values corrected via the Benjamini–Hochberg method. Results are reported as least-square means ± standard error of the mean (SEM). Significance was set at $P < 0.05$, with $0.05 < P \leq 0.10$ considered a trend.

Alpha diversity (richness and evenness) was assessed using observed richness, Fisher, Shannon, and Simpson indices[85,86]. Beta diversity was calculated using the Bray–Curtis dissimilarity metric in *phyloseq*[84,87], with data normalised prior to comparison.

Conclusion
In summary, OA-preserved grain improved piglet growth performance, feed intake, and beneficially altered the intestinal microbiota, offering a promising alternative to ZnO following regulatory restrictions. However, it did not match ZnO's effectiveness in reducing FS or inflammatory responses. The growth-enhancing effects of OA-preserved grain may be linked to improved grain hygiene, increased feed intake, and favourable microbial profiles. Zinc oxide's superior impact on FS and gut health markers highlights the need for future strategies, such as adjusting dietary protein or incorporating complementary additives, to optimise OA-preserved grain diets. These approaches could provide a sustainable alternative to in-feed antimicrobials, aligning with animal welfare and regulatory goals.

Data availability
All data generated and/or analysed during this study are available upon reasonable request from the corresponding author.

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Author contributions

Conceptualisation and Methodology, K.R.C. and J.V.O.; Investigation, K.R.C.; Resources, J.V.O. and T.S.; Data curation, K.R.C., S.M., M.R. and V.G.; Formal analysis, K.R.C., J.V.O. and S.V.; Writing—original draft, K.R.C.; Writing—review and editing, K.R.C., J.V.O., T.S., and S.V.; Funding acquisition and Supervision, J.V.O. All authors approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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