Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting information for

Reshaping fish intestinal microbiota and facilitating barrier function by ZnO nanoparticles

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Table S1. Feed formulation and proximate composition analysis of diet

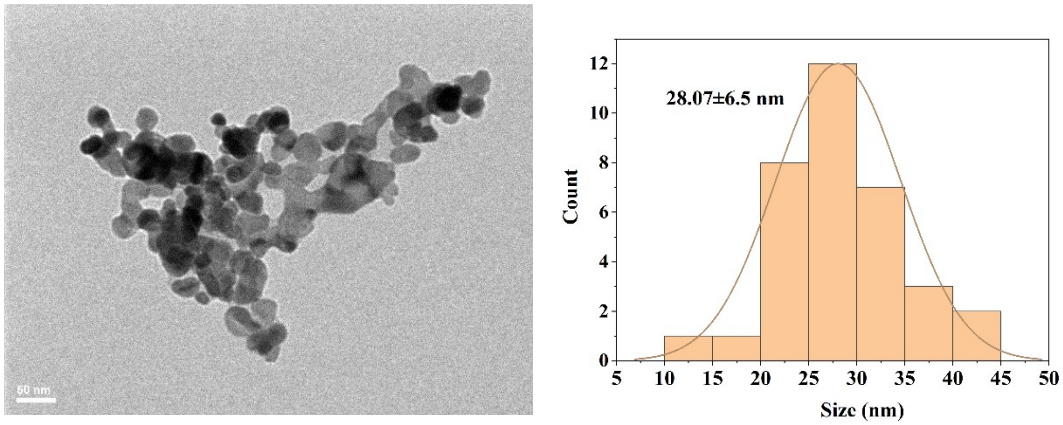
Ingredients (mg/kg)	Basic formula
Casein [1]	400
Fermented soybean meal [1]	180
Cassava starch [1]	160
α-Starch [1]	30
Soybean oil [1]	40
Soybean lecithin [1]	90
Choline chloride [1]	20
$Ca(H_2PO_3)_2$ [1]	20
Antioxidant [1]	10
Vitamin premix [1]	20
Mineral premix (Zn-free) [3]	20
Attractant [2]	10
Proximate composition (dry matter)	
Moisture (%)	8.40±0.02
Crude protein (%)	43.52±1.28
Crude lipid (%)	11.60±0.14
Ash (%)	5.87±0.00
Zn (mg/kg)	8.8 ± 1.41

[1] All these ingredients were purchased from Qingdao Fulin Biochemistry Co. Ltd.
[2] The attractant was purchased from Bayer (Sichuan) Animal health Co. Ltd.
[3] Mineral premix (Zn-free): mineral premix provided the following per kg of diet: NaF 4 mg; KI 1.6 mg; 1% $CoCl_2$❿$6H_2O$ 100 mg; $FeSO_4$❿H_2O 160 mg; $MgSO_4$❿$7H_2O$ 2.4 g; $Ca(H_2PO_4)_2$❿H_2O 6.0 g; $CuSO_4$❿$5H_2O$ 20 mg; $MnSO_4$❿H_2O 120 mg; NaCl 200 mg, Zeolite power 30.90 g.
[4] Ingredients were purchased from Aladdin Reagent (Shanghai) Co., Ltd.
Error bars represent the standard deviation of the replicates (n = 3)

Table S2. Forward and reverse primers for qPCR analysis

Genes	Forward primer (5'-3')	Reverse primer (3'-5')
Occludin A	ATCAGCGACCAGATGCACA	CCGAAGTTCTTTGCTGTGCC
Mucin	CGTACCTACCCGTCATGTCC	GACCCTCAAGCCAAGACTCG
β-Actin	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT

a



b

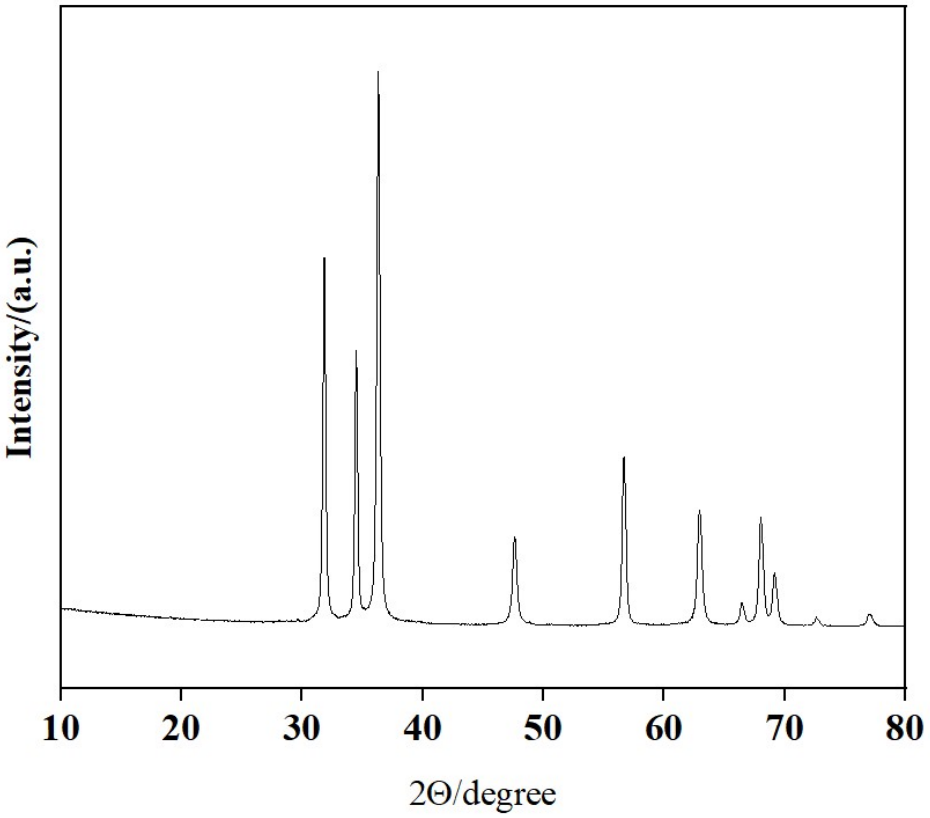


Fig S1. Particle characterization of ZnO NPs. (a) Representative TEM micrograph in absolute ethyl alcohol of the ZnO NP; (b) XRD spectrum pattern of the ZnO NPs.

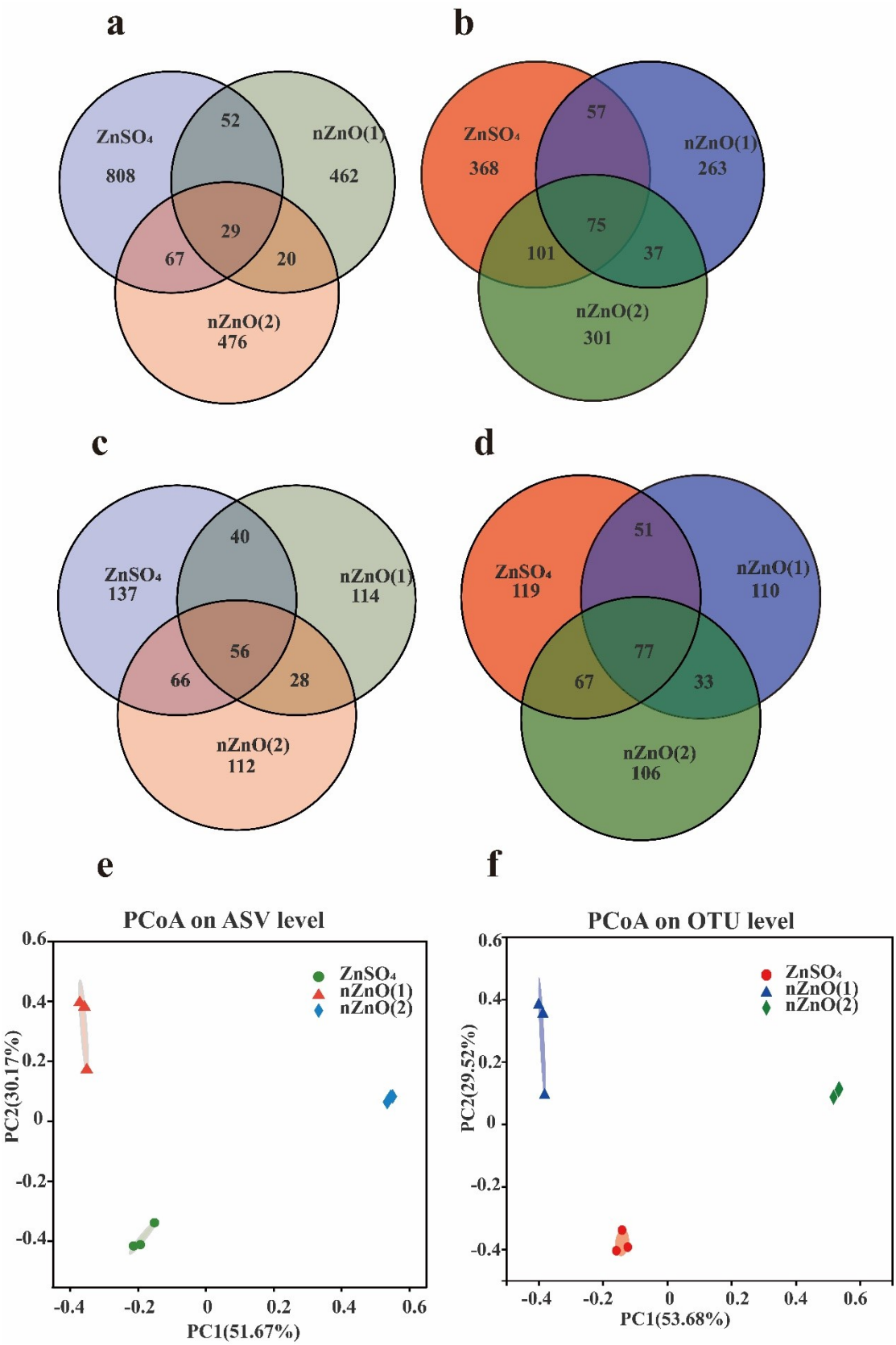


Figure S2 Quality analysis of microbial sequencing results. a, c, e: microorganisms at ASV level; b, d, f: microorganisms at OTU level.

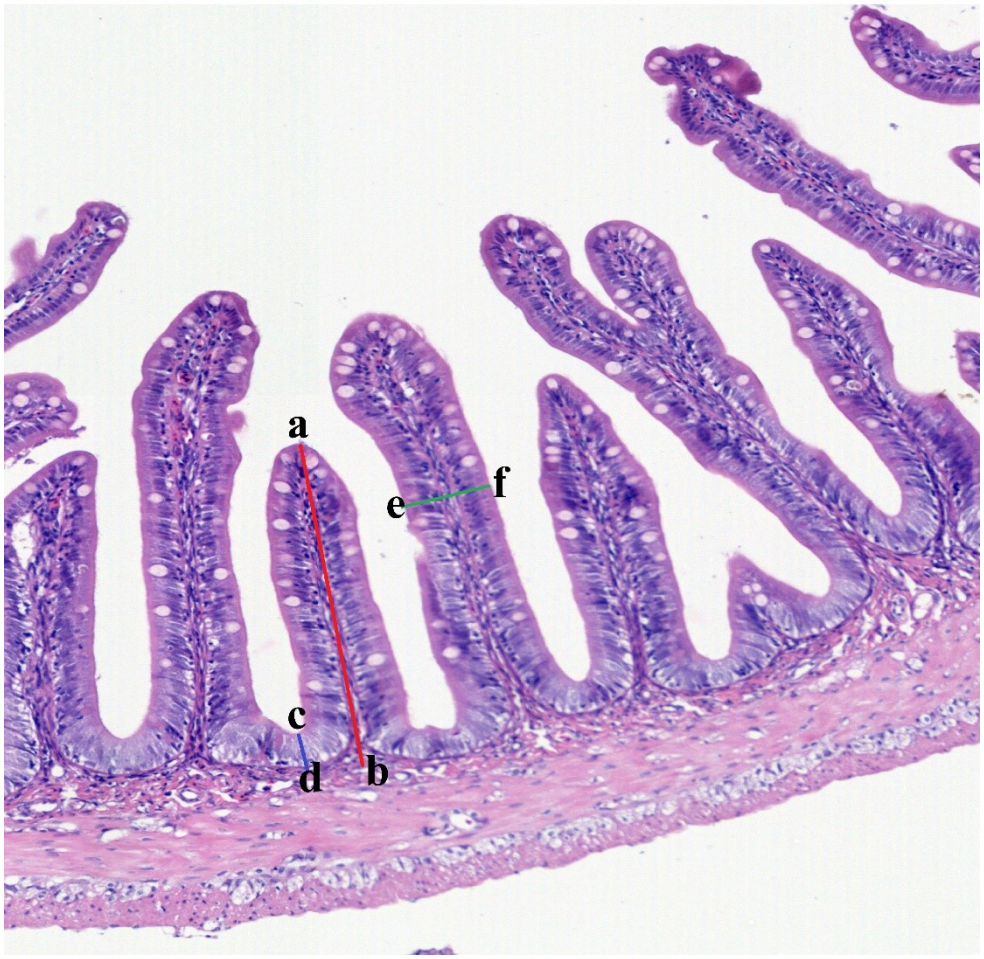


Fig S3. Schematic diagram of morphological measurement indexes of intestinal villi. The straight-line distances from a to b, c to d and e to f were the villus height, crypt height and villus width, respectively.

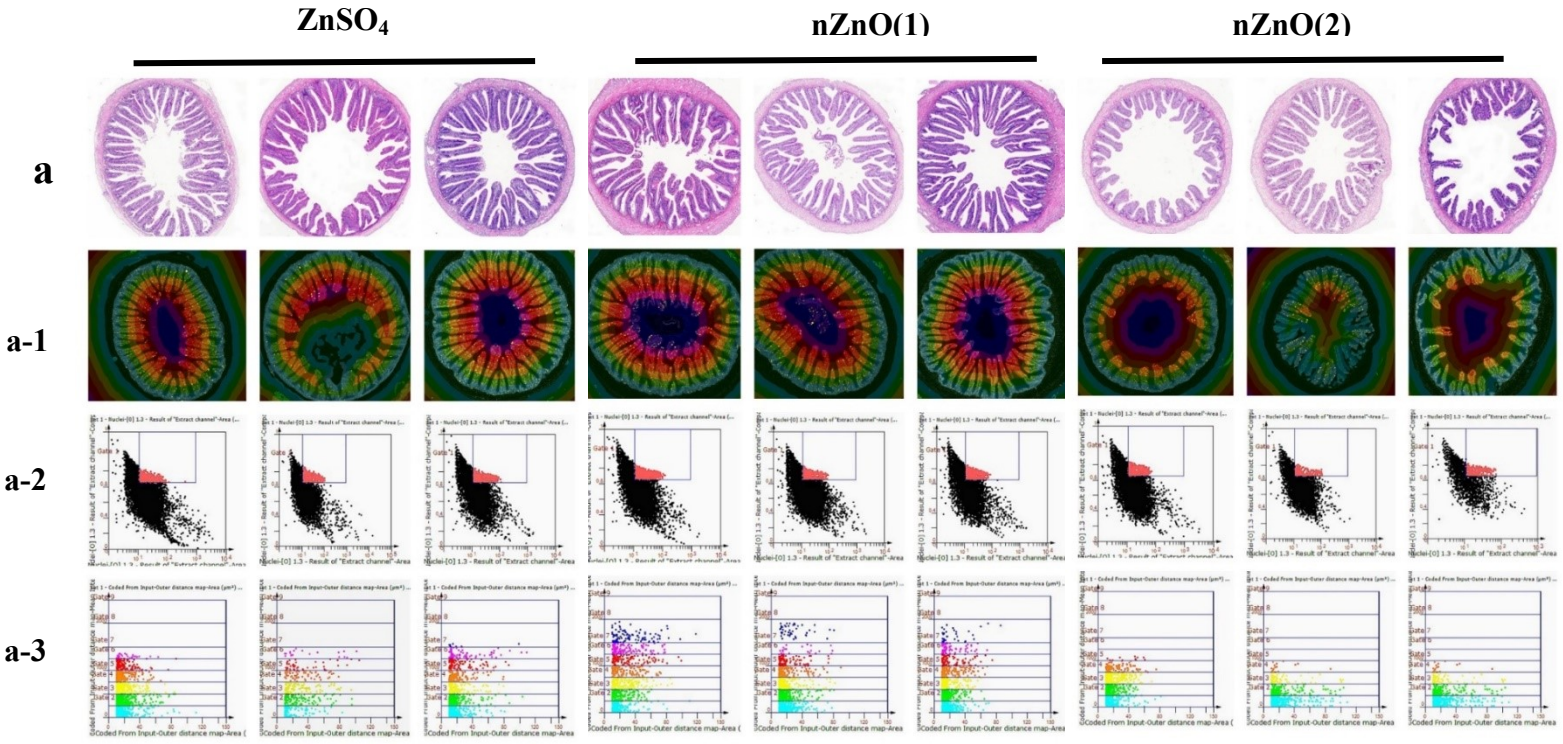


Fig S4. *In situ* spatial quantitative process of intestinal tissue of fish following 5-week dietary nZnO exposure.

(a) Histomorphological section results in different groups; (a-1) Localization of goblet cells; (a-2) Number of goblet cells in each section; (a-3) Distribution of goblet cells in different basal layers.

Text S1. The process of feed preparation

Step 1 Raw ingredients sieve.

This step aims to remove impurities from raw materials to ensure feed quality. We first sieved all ingredients through the sieve (60 mesh), respectively.

Step 2 Ingredients mix.

According to the feed formulation (Table S1), we measured the weight of all ingredients one by one. Then we mixed the ingredients using the blender mixer according to "the step by step" amplification principle. To blend all the ingredients, we made the different mixtures first.

For example, using 1 kg feed:

Mixture 1: 20 mg Choline chloride + 20 mg $Ca(H_2PO_3)_2$ + 10 mg antioxidant + 20 mg vitamin premix + 20 mg mineral premix (Zn-free) + 10 mg attractant.

Mixture 2: we mixed the *n*ZnO or $ZnSO_4$ with 30 mg α-Starch.

Mixture 3: mixed the 90 mg soybean lecithin, mixture 1 and mixture 2.

Mixture 4: mixed the mixture 3, fermented soybean meal (180 mg).

Mixture 5: mixed the 350 mg casein and 160 mg cassava starch.

Mixture 6: mix mixture 4 and mixture 5.

Step 3 Oil injection process.

Put the soybean oil into the automatic oil injector. The mixed ingredients in Step 2 were sprayed with oil in proportion. After sparing, the mixes which we got was called premix compound.

Step 4 Pelleting.

Different premix compounds were pelleted using a pellet-fodder expander through three processes: conditioning, curing and puffing. Finally, the expanded pellet diets were dried using the drying oven (50 ℃, 72 h).

Text S2 DNA extraction and PCR amplification

The quality of DNA extraction was determined using 1% agarose gel electrophoresis, and DNA concentration and purity were determined using NanoDrop2000. The DNA concentration and purity were determined using NanoDrop2000. The 16S rRNA gene V3-V4 variable region was amplified by PCR using 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplification procedure was as follows: 95°C pre-denaturation for 3 min, 27 cycles (95°C denaturation for 30 s, 55°C annealing for 30 s, 72°C extension for 30 s), followed by 72°C stable extension for 10 min and finally storage at 4°C (PCR instrument: ABI GeneAmp® Model 9700). FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, upstream primer (5uM) 0.8 μL, downstream primer (5uM) 0.8 μL, TransStart FastPfu DNA polymerase 0.4 μL, template DNA 10 ng, ddH2O make up to 20 μL. 3 replicates for each sample.

Text S3. Illumina Miseq sequencing

PCR products from the same sample were mixed and recovered using a 2% agarose gel. Fluorometer (Promega, USA) was used to quantify the recovered products. Library construction was performed using NEXTflexTM Rapid DNA-Seq Kit (Bioo Scientific, USA): (1) splice linkage; (2) removal of splice self-linked fragments using magnetic bead screening; (3) enrichment of library template using PCR amplification; (4) recovery of PCR products by magnetic beads to obtain the final library. Sequencing was performed using Illumina's Miseq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd.).

Text S4. Raw data stitching and optimization

Using fastp (https://github.com/OpenGene/fastp, version 0.20.0) software was used for quality control of the original sequencing sequence, and flash was used (http://www.cbcb.umd.edu/software/flash, version 1.2.7) software for splicing: (1) filter the bases with a mass value of less than 20 at the tail of reads and set a 50bp window. If the average mass value in the window is less than 20, cut off the back-end bases from the window, filter the reads with a mass value of less than 50bp after quality control and remove the reads containing N bases; (2) According to the overlap relationship between PE reads, paired reads are spliced into a sequence, and the minimum overlap length is 10 bp; (3) The maximum allowable mismatch ratio of overlap region of splicing sequence is 0.2, and the non-conforming sequences are screened; (4) The samples are distinguished according to the barcodes and primers at both ends of the sequence, and the sequence direction is adjusted. The allowable mismatch number of barcodes is 0 and the maximum primer mismatch number is 2.

Text S5. The quality of RNA test

The quality of RNA was assessed using agarose gel electrophoresis. The quality of RNA was determined by a Nano Drop® ND-2000 spectrophotometer and agarose gel electrophoresis to determine whether it was suitable for cDNA synthesis. The absorption ratios of all samples were between 1.9 and 2.0 (260:280 nm).