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Effects of alkaline mineral complex water supplementation on growth performance, inflammatory response, and intestinal barrier function in weaned piglets

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Abstract

The purpose of the present study was to investigate the effects of drinking water alkaline mineral complex (AMC) supplementation on growth performance, intestinal morphology, inflammatory response, immunity, antioxidant defense system, and barrier functions in weaned piglets. In a 15-d trial, 240 weaned piglets (9.35 ± 0.86 kg) at 28 d of age (large white × landrace × Duroc) were randomly divided into two groups: the control (Con) group and the AMC group. Drinking water AMC supplementation improved ($P < 0.01$) final body weight (BW) and average daily gain (ADG) in weaned piglets compared to the Con group. Importantly, AMC reduced ($P < 0.01$) the feed-to-gain (F:G) ratio. AMC water improved the physical health conditions of piglets under weaning stress, as reflected by the decreased ($P < 0.05$) hair score and conjunctival score. Moreover, there was no significant ($P > 0.05$) difference in relatively small intestinal length, organ (liver, spleen, and kidney) indices, or gastrointestinal pH value in weaned piglets between the two groups. Of note, AMC significantly promoted the microvilli numbers in the small intestine and effectively ameliorated the gut morphology damage induced by weaning stress, as evidenced by the increased ($P < 0.05$) villous height (VH) and ratio of VH to crypt depth. Additionally, AMC lessened the levels of lipopolysaccharide (LPS, $P < 0.01$) and the contents of IL1β ($P<0.05$), and TNF-α ($P<0.05$) in the weaned piglet small intestine. Conversely, the gut immune barrier marker, secretory immunoglobulin A (sIgA) levels in serum and small intestine mucosa were elevated after AMC water treatment ($P < 0.01$). Furthermore, AMC elevated the antioxidant mRNA levels of ($P < 0.05$) SOD 1-2, ($P < 0.01$) CAT, and ($P < 0.01$) GPX 1-2 in the small intestine. Likewise, the mRNA levels of the small intestine tight junction factors Occludin ($P < 0.01$), ZO-1 ($P < 0.05$), Claudin 2 ($P < 0.01$), and Claudin 5 ($P<0.01$) in the AMC treatment group were notably higher than those in the Con group. In conclusion, drinking water AMC supplementation has an accelerative effect on growth performance by elevating gut health by improving intestinal morphology, the inflammatory response, the antioxidant defense system, and barrier function in weaned piglets.

Lay Summary

The piglet suffers vital physiological, environmental, and social challenges when it is weaned from the sow that can predispose the piglet to subsequent diseases and other production losses, and these challenges are responsible for serious economic losses to the swine industry. Weaning stress induces intestinal injury, decreased immunity, and digestive system dysfunction, which then reduces feed intake and inhibits the growth performance of piglets. It is well known that alternatives to antibiotics for preventing weaning stress in weaned farm animals are sorely needed. The biologically beneficial effects of alkaline mineral water are widely reported. Alkaline mineral complex (AMC), as an immunomodulator, is considered to have antistress effects in the swine industry. In addition, treatment through drinking water is considered to be an efficient and low-cost feasible disease control strategy. Drinking water AMC supplementation is expected to exert health benefits in pigs; however, the responses of weaned piglets to water supplemented with AMC have not been fully explored. Thus, this study explored the effects of drinking water AMC supplementation on growth performance and gut health in weaned piglets. Our results showed that AMC water supplementation conspicuously enhanced the growth performance by improving the gut health.

Key words: alkaline mineral complex water, growth performance, intestinal barrier function, intestinal inflammation, weaned piglets

Abbreviations: AMC, alkaline mineral complex; ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; CAT, catalase; Con, Control; CD, crypt depth; cDNA, complementary DNA; F: G, feed to gain ratio; GPX, glutathione peroxidase; H&E staining, Hematoxylin and Eosin staining; IL1β, interleukin1β; IL6, interleukin6; LPS, lipopolysaccharide; qRT-PCR, quantitative Real-Time polymerase chain reaction; ROS, reactive oxygen species; sIgA, secretory immunoglobulin A; SOD, superoxide dismutase; SEM, scanning electron microscopy; TNF-α, tumor necrosis factor-alpha; VH, villous height; ZO-1, zonula occludens-1

Introduction

Weaning is one of the most threatening but inevitable stressors for piglets (Upadhaya and Kim, 2021). The stress induced by weaning, especially within the first two weeks, can cause intestinal injury, immune system disorder, and digestive system dysfunction (Campbell et al., 2013), which then inhibit growth and decrease the feed intake of piglets. After weaning, piglets experience many stresses, such as transport stress

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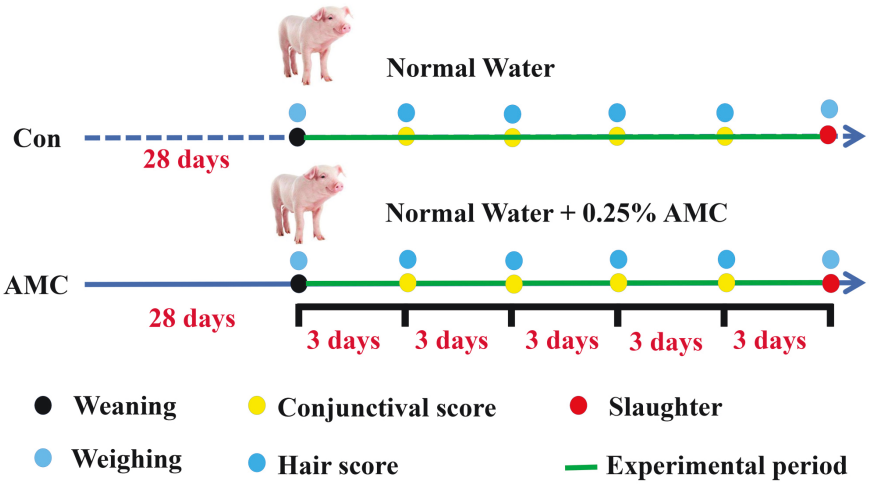

Figure 1. The time flow chart for treatment and data collection.

(Johnson and Lay, 2017), handling stress, feed change (from liquid to solid) stress (Campbell et al., 2013), and social stress (Corbett et al., 2021), together with a gradual reduction in maternal antibodies (Hao et al., 2021). Meanwhile, for piglets, changes in the physical environment (space, building, farm, water supply, etc.) increase exposure to pathogens as well as dietary and environmental antigens. Therefore, the weaning stage is also known as the "immune blank period," in which piglets have decreased disease resistance. They must adapt quickly to all these stressors to increase productivity and efficiency; however, when the stress exceeds a threshold that the piglet cannot overcome, it contributes to decreased performance and increased mortality (Xiong et al., 2019). It is well known that since the discovery of antibiotics, they have been extensively used in the pig industry as a promoter of growth at subtherapeutic doses, effectively alleviating the damage to piglets caused by weaning stress (Chen et al., 2020). However, a series of potential hazards caused by antibiotic application have also caused great concern in modern society, including bacterial resistance and residues (Chen et al., 2019). Therefore, finding an antibiotic alternative to alleviate the performance decline in weaned piglets is crucial for pork production and food security.

Water is an essential part of the diet and exerts a key role in nutrition (Dore et al., 2021). Alkaline minerals, such as Na, K, and Zn, are not only ample inorganic constituents in biota but are also the most studied elements because of their pivotal roles in biophysiological metabolism and catalytic processes (Nan et al., 2020). A previous study demonstrated that greater BW gain and lower morbidity were observed in animals treated with ionized alkaline mineral complex (AMC) water compared to untreated animals (Shin et al., 2014). The biologically beneficial and therapeutic effects of AMC water composed of various electrolytes have been demonstrated, such as controlling the proliferation of cancer cells (Pavelic et al., 2001), eliminating reactive oxygen species (ROS) in vivo (Zhu et al., 2021), and treating functional bowel disease and irritable bowel syndrome (Shin et al., 2018). The AMC water used in this study was an alkaline solution (pH 9.1) containing silicon, sodium, potassium, zinc, and germanium, and its properties are based on its mineral composition. Among these minerals, silicon has been linked to skin, hair, and nail health, bone mineralization, collagen production, Alzheimer's disease, atherosclerosis, immune system augmentation, and a variety of other diseases and pharmacological effects (Jurkic et al., 2013). Silicon is an essential trace element related to longevity, and the content of metasilicic acid is one of the indicators used to identify natural mineral water. Unfortunately, the biologically beneficial effects of silicon appear to be overlooked. On the other hand, minerals are involved in digestion, biosynthesis, and many physiological processes, including maintaining the normal function of bones, muscles, the heart, and the brain (Domingo and Marques, 2021). Meanwhile, they are also key components in the generation of enzymes and hormones (Wang et al., 2020a), thus exerting a vital role in sustaining metabolism and homeostasis. Moreover, as a nonspecific immunostimulator, drinking water AMC supplementation has been widely used to promote the growth and development of pigs as early as 2001 (Choi et al., 2001). However, the biologically beneficial effects of AMC water on weaned piglets have not been evaluated.

The gut is one of the major target organs of weaning stress (Hu et al., 2018; Lauridsen, 2020). Given its multiple functions (Campbell et al., 2013), including digestion and absorption of electrolytes and nutrients, regulation of fluid transport, and secretion of mucins, immunoglobulins, and digestive enzymes, it serves as a host barrier against harmful pathogens and antigens. Hence, our study aimed to investigate the effects of drinking water AMC supplementation on growth performance, intestinal morphology, intestinal inflammatory response, intestinal immune and antioxidant function, and intestinal barrier integrity in weaned piglets.

Materials and Methods

Animal and experimental design

All experimental methods and humane end points for decreasing pain in animals were performed after ratification from the Guide for the Care and Use of Laboratory Animals at Northeast Agricultural University, Harbin, China. Animal research was performed in conformity with the procedures and rules of the laboratory of animals defined by the state council (Decree No. 676).

The experimental design and basic data collection details are shown in Figure 1. In total, 240 weaned piglets at 28 d of age (large white × landrace × Duroc) were randomly allocated

into two groups (six replicate pens per group and 20 piglets per pen) based on body weight (BW; 9.35 ± 0.86 kg) and sex. All piglets were fed the same basal diet (NRC, 2012, Table 1). For ease of administration, and based on the ratio of the animal's demand for the element, we employed an AMC concentrate, which contains sodium metasilicate pentahydrate ($5H_2O{\cdot}Na_2SiO_3$, 200 g/L), potassium bicarbonate ($KHCO_3$, 100 g/L), zinc oxide (ZnO, 10 mg/L), and bis-(carboxyethyl-germanium) sesquioxide (Ge-132, 1 mg/L). Piglets obtained water ad libitum from two different tanks that were fitted with a water meter, agitator, and a reactor system: one tank contained basal water plus 0.25% AMC concentrate (AMC, pH 9.1), and the second contained basal water without AMC supplementation (Con, pH 7.0). The calculated content of ions in AMC water and the analyzed content in basal water are shown in Table 2. The mineral content of AMC water used in this study was assessed based on our preliminary test, which showed that piglets had the best growth performance and lowest diarrhea rates (data not presented here) at this dose. All piglets were weaned at 28 d and had free access to food and water during this 15-d trial. All raw materials for AMC concentrate were purchased from Nail Biotechnology Co., Ltd, Beijing, China.

Growth performance determination

The initial BW of all experimental piglets was determined on day 1 (weaning point), and the end of the trial (Day 15); four piglets were randomly selected from each pen to determine the final BW. Meanwhile, daily feed intakes were recorded every day. On the basis of these data, the average daily feed intake (ADFI), average daily gain (ADG), and the feed-to-gain (F:G) ratio were obtained.

Table 1. Composition of the basal diet (as-fed basis)

Item	Ingredient, %	Nutrient composition[2]	Content, g/kg
Corn	43.3	DE(Mcal/kg)	3.35
Soybean meal	25.4	Crude protein (%)	21.51
Soy protein isolate	3.00	Lysine (%)	1.41
Whey powder	7.50	Methionine + Cystine (%)	0.81
Soybean oil	1.50	Threonine (%)	0.94
Lactose	10.0	Calcium (%)	0.86
Stone powder	0.75	Total phosphorus (%)	0.70
Calcium hydrogen phosphate	1.05		
50% choline chloride	0.10		
Lysine	0.22		
L-Methionine	0.10		
L-Threonine	0.08		
Vitamin and mineral premix[1]	1.00		
Total	100		

[1]Vitamin and mineral premix supplied per kilogram diet: vitamin A, 18,000 IU; vitamin D, 4,000 IU; vitamin E, 50 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 4 mg; vitamin B_{12}, 30 μg; pantothenic acid, 30 mg; folic acid, 2 mg; biotin, 0.16 mg; Fe, 150 mg; Cu, 18 mg; Mn, 48 mg; Zn, 150 mg; I, 1.5 mg; and Se, 0.3 mg.
[2]Values were calculated according to NRC (2012).

Conjunctival score and hair score

The dorsal hair and conjunctiva were scored on two piglets randomly selected in each replicate (n = 12) every 3 d. The hair scores are defined as follows: 1 point, shiny and smooth hair; 2 points, slightly shiny and coarse hair; and 3 points, dull, rough, and dirty hair. The conjunctiva scoring of weaned piglets is as follows: 1 point, normal conjunctiva, and no tear stain; 2 points, normal conjunctiva with tear stain; 3 points, conjunctival flushing with tear stain.

Sample collection and gastrointestinal pH detection

On day 15, one piglet from each pen (n = 6) was randomly selected and slaughtered for sample collection. By jugular venipuncture, blood samples were obtained, and serum was obtained as described in a previous report (Chen et al., 2021). The piglets were euthanized, and the small intestine length was measured with a tape measure. The liver, spleen, and kidney were weighed with an electronic scale. Based on these data, the organ indices of the liver, spleen, and kidney and the relative length of the small intestine in piglets were calculated. The abdominal cavity was opened to directly collect the midsection of the duodenum, jejunum, and ileum. Meanwhile, the pH values of the stomach, midduodenum, midjejunum, midileum, midcecum, and midcolon contents were detected using a mobile pH detector (testo 205, Chunan Electronic Co., Ltd, Shanghai, China). Correspondingly sized sections were taken from the midduodenum, midjejunum, and midileum and fixed in 4% paraformaldehyde or 2.5% glutaraldehyde for paraffin section and scanning electron microscopy (SEM). A 10-cm section was snap-frozen in liquid nitrogen and then stored at –80 °C for various analyses. Mucosal samples from small intestines were collected by scraping with a glass slide.

Small intestine morphology analysis

Hematoxylin and eosin (H&E) staining and SEM were performed to analyze intestinal morphology as described previously (Yi et al., 2016; Dai et al., 2021). The SEM visualized using a Philips Model SU8010 FASEM (HITACHI, Japan). The villous height (VH) and crypt depth (CD) of each intestinal segment were measured with ImageJ software, and VH:CD ratio was calculated. A minimum of 10 villi from each sample were measured for each group.

Inflammatory marker detection

The levels of inflammatory markers (LPS/IL1β/IL6/TNF-α) in the small intestinal mucosa were measured using LPS (Beijing

Table 2. The calculated content of ions in AMC water and analyzed content in basal water

Ion ingredients	AMC water	Basal water
	calculated contents, mg/L	analyzed contents, mg/L
SiO_3^{2-}	179.25	ND[1]
Na^+	108.49	2.79
K^+	97.50	0.95
Zn^{2+}	0.02	ND
Ge^{4+}	0.0005	ND
HCO_3^-	152.50	13.20

[1]ND, not detected

Chenglin Biological Technology Co. Ltd, China, AD11746Po), IL1β (Beijing Chenglin Biological Technology Co. Ltd, China, AD0125Po), IL-6 (Beijing Chenglin Biological Technology Co. Ltd, China, AD0120Po), and TNF-α (Beijing Chenglin Biological Technology Co. Ltd, China, AD0070Po) ELISA kits according to the manufacturer's protocol.

Intestinal immune function evaluation

The levels of the gut immune marker sIgA in the small intestinal mucosa and serum were detected by a commercial kit (Beijing Chenglin Biological Technology Co. Ltd, China, AD12416Po).

Quantitative real-time PCR

Total RNA was extracted from midduodenum, midjejunum, and midileum tissues (100 mg) using RNAout reagent (Beijing Tiandi, Inc., Beijing, P.R. China) following the manufacturer's description. The quality and concentration of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand complementary DNA (cDNA) was produced from 4 μg total RNA using a commercial reagent kit (product category: AU311-02, TransGen Biotech, Beijing, China) following the manufacturer's description. The cDNA was stored at −80 °C before the quantitative real-time PCR (qRT-PCR). The primers used in this study for qRT-PCR (Table 3) were designed by Primer Premier software 6.

Table 3. Primers used in this study for qRT-PCR analysis

Gene name[1]	Accession number	Primer and probe sequences (5′ to 3′)[2]
GAPDH1	NM_001206359.1	F: TCGGAGTGAACGGATTTGGC R: TGACAAGCTTCCCGTTCTCC
GAPDH2	NM_001206359.1	F: CGGAGTGAACGGATTTGGC R: CACCCCATTTGATGTTGGCG
SOD-1	NM_001190422.1	F: AAGGCCGTGTGTGTGCTGAA R: AGTGGCCACACCATCTTTGC
SOD-2	NM_214127.2	F: GGCCTACGTGAACAACCTGA R: TGATTGATGTGGCCTCCACC
SOD-3	NM_001078688.1	F: TGACGCTGCTCTGTGCTTAC R: AACTCCTGCCAGATCTCCGT
CAT	NM_214301.2	F: CCTGCAACGTTCTGTAAGGC R: GCTTCATCTGGTCACTGGCT
GPX-1	NM_214201.1	F: CCTAGCAGTGCCTAGAGTGC R: CGCCCATCTCAGGGGATTTT
GPX-2	NM_001115136.1	F: CTGGACGGGGAGAAGGTAGA R: CGGACGTACTTGAGGCTGTT
ZO-1	XM_021098856.1	F: TCAAGGTCTGCCGAGACAAC R: ATCACAGTGTGGTAAGCGCA
Claudin-2	NM_001161638.1	F: AACGAGTTCTTACGTCGGGG R: CGAGGAGATGGCGCTAGATG
Claudin-5	NM_001161636.1	F: CCTGTCAAGTATTCGGCCCC R: CGACACCCTCAGACGTAGTT
Occludin	NM_001163647.2	F: CAGGTGCACCCTCCAGATTG R: ATGTCGTTGCTGGGTGCATA

[1]GAPDH, Housekeeping gene; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; ZO-1, zonula occludens-1.
[2]F, forward; R, reverse.

Statistical analysis

All statistical data were analyzed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) software. Statistical analysis was performed using Student's *t*-tests to compare differences between the two groups. A significant difference and extremely significant difference were considered if $P < 0.05$ and $P < 0.01$, respectively, and a trend was considered if the *P*-value was between 0.05 and 0.10.

Results

Growth performance

As shown in Table 4, under the precondition of no difference ($P > 0.05$) in initial BW, drinking water supplementation with AMC significantly improved the final BW ($P < 0.01$) and ADG ($P < 0.01$) in weaned piglets compared to the Con group. However, no changes in ADFI were observed ($P > 0.05$) between the Con and AMC groups. In the field of economic benefits, AMC treatment significantly reduced the F:G ($P < 0.01$) ratio. These results indicated that AMC water promoted growth performance in piglets under weaning stress. Importantly, although there was no significant change in the ADFI, the economic benefit was significantly improved by decreasing the F:G ratio.

Health status

To evaluate the health status of weaned piglets, we regularly scored dorsal hair and conjunctiva. As presented in Table 5, compared to the Con group, the hair score of the AMC treatment group was notably decreased on Days 6 ($P < 0.01$) and 12 ($P < 0.05$). Meanwhile, a decreasing trend of hair score was observed in the AMC group on Day 15 ($P = 0.080$). Likewise, the conjunctival score was apparently reduced ($P < 0.01$) on Day 6 after AMC water treatment, but no difference was recorded on the other experimental days ($P > 0.05$). These results suggested that treating piglets under weaning stress with AMC water has a positive effect on health.

Relative length of the small intestine and organ indices

As shown in Table 6, compared to the Con group, water supplementation with AMC did not significantly affect the relative length of the small intestine ($P > 0.05$) or the organ

Table 4. Effect of AMC water on the growth performance of weaned piglets[1]

Item[2]	Con	AMC	SD	*P*-value
BW, kg				
Initial BW	9.22	9.49	0.86	0.274
Final BW	12.29^{A}	14.37^{B}	1.00	<0.0001
ADG, kg/d	0.20^{A}	0.33^{B}	0.05	<0.0001
ADFI, g/d	335.7	379.7	200.65	0.554
F:G ratio	1.70^{A}	1.20^{B}	0.05	<0.0001

[1]Statistical significance was determined using Student's *t*-tests to compare differences between two groups ($n = 24$); Data are presented as the mean and SD.
[2]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F:G, feed to gain ratio.
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

Table 5. Effect of AMC water on the hair score and conjunctival score of weaned piglets[1]

Item	Con	AMC	SD	*P*-value
Hair score				
Day 3	1.80	1.70	0.56	0.696
Day 6	1.90[A]	1.20[B]	0.49	0.006
Day 9	1.90	1.50	0.64	0.180
Day12	1.70[a]	1.20[b]	0.45	0.024
Day15	1.70	1.30	0.48	0.080
Conjunctival score				
Day 3	1.20	1.20	0.42	>0.999
Day 6	1.70[A]	1.10[B]	0.40	0.004
Day 9	1.60	1.30	0.50	0.196
Day12	1.60	1.40	0.51	0.3979
Day15	1.50	1.20	0.47	0.177

[1]Statistical significance was determined using Student's *t*-tests to compare differences between two groups (n = 12); Data are presented as the mean and SD.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

Table 6. Effect of AMC water on the relative length of the small intestine and organ indices of weaned piglets[1]

Item	Con	AMC	SD	*P*-value
Relative length				
Small intestine	0.97	0.98	0.04	0.796
Organs indexes				
Liver	1.00	0.94	0.10	0.311
Spleen	1.00	0.85	0.18	0.176
Kidney	1.00	0.99	0.09	0.856

[1] Statistical significance was determined using Student's *t*-tests to compare differences between two groups (N = 6); Data are presented as the mean and SD.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

Table 7. Effect of AMC water on the gastrointestinal pH value of weaned piglets[1]

Item	Con	AMC	SD	*P*-value
Digestive tract pH				
Stomach	2.62	2.74	0.56	0.796
Duodenum	5.41	6.10	0.72	0.259
Jejunum	6.20	6.56	0.25	0.067
Ileum	6.86	6.92	0.20	0.625
Cecum	6.41	6.25	0.31	0.407
Colon	6.28	6.30	0.27	0.921

[1]Statistical significance was determined using Student's *t*-tests to compare differences between two groups (N = 6); Data are presented as the mean and SD.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

indices of the liver, spleen, or kidney in weaned piglets ($P > 0.05$).

Gastrointestinal pH value

The gastrointestinal pH value is an important parameter for intestinal homeostasis (Table 7). As an alkaline liquid, there was no obvious difference in the pH value of the stomach and small intestinal contents (including the duodenum, jejunum, and ileum) or the contents of the cecum and colon in piglets of the AMC treatment group compared to the Con group (P>0.05).

Intestinal morphology

SEM and H&E staining were used to evaluate the effect of AMC treatment on morphological injury of the small intestinal epithelium in weaned piglets. SEM at 200x magnification revealed that surface injury to villi in the duodenum, jejunum, and ileum was alleviated by AMC water (Figure 2). Interestingly, AMC also promoted the number of microvilli in the small intestine, manifesting as a more tidy and dense microvilli morphology at 30,000 times magnification. This result was also supported by H&E staining (Figure 3). As shown in Table 8, AMC treatment markedly increased VH in the duodenum ($P < 0.01$), jejunum ($P < 0.05$), and ileum ($P < 0.05$) compared to the Con group. However, there was no significant difference in CD in the small intestine between the Con and AMC groups ($P > 0.05$). Accordingly, the VH:CD ratio was obviously increased in the duodenum ($P < 0.01$), jejunum ($P < 0.05$), and ileum ($P < 0.01$) of weaned piglets after AMC treatment. These results demonstrated that AMC ameliorated the surface injury to villi in the small intestine and repaired the intestinal damage of piglets induced by weaning stress.

Intestinal inflammatory marker levels

Activation of the inflammatory response and LPS activity is a hallmark of weaning stress-induced intestinal damage in piglets. As shown in Table 9, AMC water significantly reduced the mucosal level of LPS in the duodenum, jejunum, and ileum ($P < 0.01$). Meanwhile, the levels of IL1β (P<0.05) and TNF-α (P<0.05) in the small intestinal mucosa were markedly decreased after AMC water treatment. Similarly, a significant decrease in IL6 levels ($P < 0.01$) was noticed in the duodenal and jejunal mucosa of piglets treated with AMC water, whereas only a decreasing trend was observed in the ileal mucosa ($P = 0.067$). These results showed that AMC water effectively reduced intestinal inflammation induced by weaning stress in piglets.

Intestinal immune function

sIgA is considered an immune barrier on the mucosal surface, and can effectively inhibit pathogen adhesion, colonization, and invasion. Surprisingly, the mucosa level of sIgA in the duodenum, jejunum, and ileum was prominently elevated after treatment with AMC water (Table 10). The results of sIgA levels obtained in this study indicated that AMC treatment could improve the mucosal immune barrier by promoting the secretion of sIgA in the small intestine of weaned piglets.

Intestinal expression of genes related to antioxidant enzymes

In this study, the effect of AMC on the antioxidant defense system was determined by analyzing the mRNA levels of

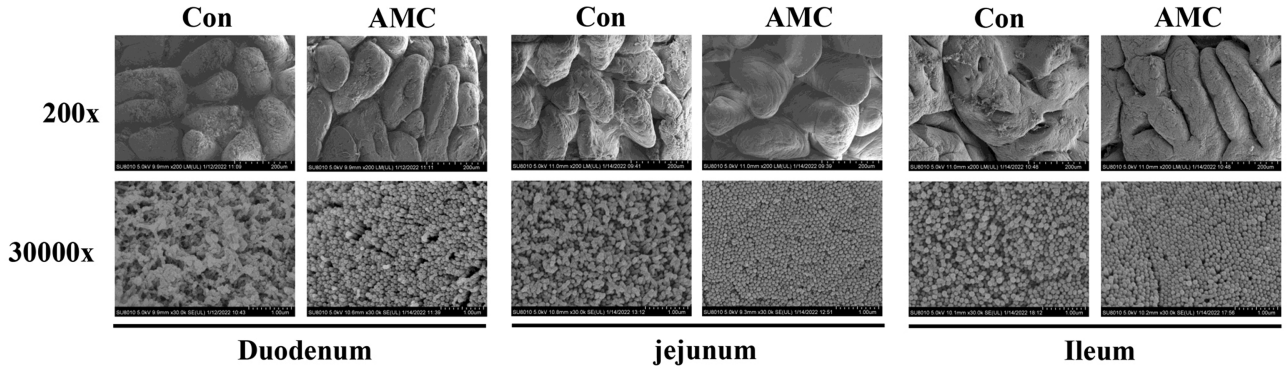

Figure 2. The effect of AMC water on small intestinal morphology shown by SEM. Upper, SEM at 200 times magnification; Nether, SEM at 30,000 times magnification.

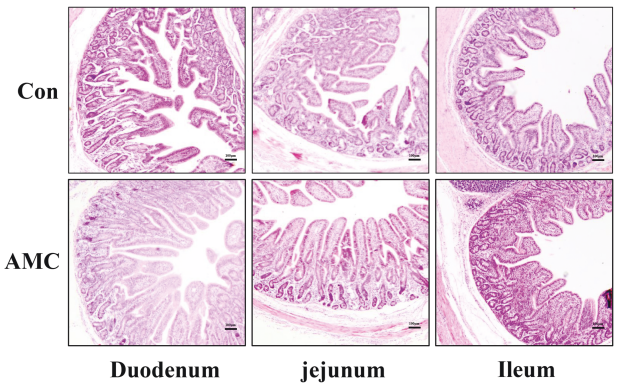

Figure 3. The effect of AMC water on small intestinal morphology shown by H&E staining.

key antioxidants, including SOD 1-3, CAT, and GPX 1-2 (Figure 4). Compared to the Con group, the relative expression levels of SOD-1 and SOD-2 in the duodenum ($P < 0.01$), jejunum ($P < 0.05$), and ileum ($P < 0.01$) were markedly increased by AMC treatment, while no change was noticed in the SOD-3 mRNA level ($P > 0.05$). Likewise, water supplementation with AMC significantly boosted the mRNA expression of peroxidases, including CAT, GPX-1, and GPX-2, in the small intestine ($P < 0.01$). These results suggested that the repair of weaning stress-induced piglet intestinal damage by AMC may be related to the enhancement of the antioxidant defense system.

Intestinal expression of genes related to barrier function

Small intestinal tight junction function was determined by measuring the Occludin, ZO-1, Claudin-2, and Claudin-5 expression in the duodenum, jejunum, and ileum. As shown in Figure 5, the mRNA levels of Occludin, Claudin-2, and Claudin-5 were extremely significantly elevated in the small intestine of weaned piglets after AMC treatment ($P < 0.01$). Additionally, the relative mRNA expression of ZO-1 was remarkably promoted in the duodenum ($P < 0.01$), jejunum ($P < 0.05$), and ileum ($P < 0.01$) when the piglets were treated with AMC water. These results revealed that AMC improved gut barrier function by increasing the expression of tight junction-related genes.

Discussion

ADG and ADFI are important parameters for evaluating the growth performance and health status of piglets, which are closely related to the economic benefits of the farm (Bai et al., 2021). Weaning stress induces reduced appetite, decreased feed intake, and inhibited growth in piglets and, in more severe cases, leads to secondary infection and even death of piglets (Sun et al., 2020). Since antibiotics have been banned from feed additives, veterinarians, and livestock practitioners have been looking for effective alternatives. AMC is considered one of the most promising alternatives to antibiotics (Koo et al., 2006; Shin et al., 2014). Previous studies have shown that drinking water supplemented with AMC improves growth performance in a variety of animal models, including piglets (Park et al., 2002), cattle (Kim et al., 2018), ostriches (Seyfori et al., 2018), and olive flounder (Shin et al., 2014). In agreement with these studies, our results also indicated that drinking water supplementation with AMC increased piglet growth performance, as evidenced by the promoted ADG and decreased F:G ratio. Of note, the improved performance of weaned piglets may be associated with improved overall piglet health.

After weaning, most piglets are in a subhealthy state, showing messy and rough hair, tear stains in the conjunctiva of the eye, and even color changes due to disease factors (Campbell et al., 2013). To determine whether AMC water treatment could improve piglet health, hair scoring and conjunctival scoring were performed in this study. Surprisingly, we observed that AMC water significantly decreased the hair score and conjunctival score of weaned piglets on day 6 after weaning, suggesting that AMC has the potential to ameliorate the subhealthy state of weaned piglets. On the other hand, the relative intestinal length (Wang et al., 2020b) and organ index (Yu et al., 2021) can partly reflect the growth and development of piglets. In this study, there was no significant difference in the relative length of the small intestine and indices of the liver, spleen, and kidney in weaned piglets. The period of this trial was 15 d, which may explain the abovementioned changes in the length of the small intestine and organ indices. We speculated that the beneficial effects of AMC in promoting intestinal and organ development may be amplified with prolonged treatment. It is worth emphasizing again that the main purpose of this study was to evaluate the effect of AMC water on the growth performance and intestinal health of weaning-stressed piglets. Thus, we may extend the experimental

Table 8. Effect of AMC water on the intestinal morphology of weaned piglets[1]

Item[2]	Con	AMC	SD	*P*-value
Duodenum				
VH, μm	321.10[A]	464.60[B]	62.17	<0.0001
CD, μm	161.30	141.70	47.28	0.369
VH:CD	1.99[A]	3.28[B]	0.42	<0.0001
Jejunum				
VH, μm	352.90[a]	416.10[b]	56.05	0.023
CD, μm	151.50	149.60	31.35	0.900
VH:CD	2.33[a]	2.78[b]	0.37	0.016
Ileum				
VH, μm	272.30[a]	327.80[b]	49.65	0.022
CD, μm	120.60	105.60	35.72	0.248
VH:CD	2.29[A]	3.02[B]	0.44	0.001

[1]Statistical significance was determined using Student's *t*-tests to compare differences between two groups *N* =10); Data are presented as the mean and SD.
[2]VH, villus height; CD, crypt depth; VH:CD, villus height to crypt depth ratio.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

Table 9. Effect of AMC water on small intestinal inflammatory markers of weaned piglets[1]

Item[2]	Con	AMC	SD	*P*-value
Duodenum				
LPS, ng/L	262.10[A]	188.80[B]	27.30	0.001
IL1β, ng/L	141.30[a]	104.40[b]	24.21	0.025
IL6, ng/L	57.83[A]	39.28[B]	5.94	0.001
TNF-α, ng/L	97.83[a]	84.82[b]	7.79	0.021
Jejunum				
LPS, ng/L	291.00[A]	166.80[B]	33.91	<0.0001
IL1β, ng/L	153.50[A]	106.80[B]	18.54	0.002
IL6, ng/L	56.00[A]	39.45[B]	7.46	0.007
TNF-α, ng/L	102.30[a]	84.82[b]	11.13	0.024
Ileum				
LPS, ng/L	326.90[A]	250.30[B]	26.86	0.0006
IL1β, ng/L	138.50[A]	98.43[B]	8.47	<0.0001
IL6, ng/L	49.33	41.11	6.88	0.067
TNF-α, ng/L	103.80[a]	88.16[b]	11.75	0.045

[1] Statistical significance was determined using Student's *t*-tests to compare differences between two groups (*N* = 6); data are presented as the mean and SD.
[2]LPS, lipopolysaccharide; IL1β, interleukin 1β; IL6, interleukin 6; TNF-α, tumor necrosis factor-alpha.
[a,b]Means in a row with different superscripts differ significantly ($P < 0.05$).
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

period in future studies to explore the effect of AMC water on the gut or organ development of piglets.

It has been recognized that an acidic gut environment may be more conducive to gut health (Williams, 2010), leading to the subconscious belief that alkaline substances may damage the gut. A previous study on AMC showed that AMC water has a protective effect on ethanol-induced hemorrhagic gastric injury in mice (Nassini et al., 2010). The mucus-bicarbonate-phospholipid "barrier," which is made up of mucus gel, bicarbonate, and surfactant phospholipids, is the first line of defense against gastrointestinal tissue injury (Kao and Lichtenberger, 1991). Thus, the statement that a more acidic internal environment is better for gastrointestinal health is not absolute. Our results indicated that there was no difference in the pH values of the stomach and gut lumen, which may be attributed to the strong humoral buffering system in mammals.

Table 10. Effect of AMC water on intestinal immune function markers of weaned piglets

Item[2]	Con	AMC	SD	*P*-value
sIgA, μg/mL				
Serum	9.17[A]	13.89[B]	0.74	<0.0001
Duodenum	7.43[A]	9.80[B]	0.79	0.0005
Jejunum	7.95[A]	9.29[B]	0.45	0.0004
Ileum	8.64[A]	10.34[B]	0.85	0.007

[1]Statistical significance was determined using Student's *t*-tests to compare differences between two groups (*N* = 6); Data are presented as the mean and SD.
[2]sIgA secretory immunoglobulin A.
[A,B]Means in a row with different superscripts differ significantly ($P < 0.01$).

Due to the immature digestive system and low nutrient digestibility, changes in structure and physiological function (enzymatic activity and absorption or secretion) in the gut occur in weaned piglets (Gu et al., 2017). The majority of studies showed that long-lasting structural and functional changes in the small intestine were induced after weaning, including villous atrophy and increased CD (Pluske et al., 1997; Boudry et al., 2004). The SEM result showed that weaning caused marked injury to the epithelium and a decrease in microvilli density in all small intestine segments of piglets, whereas these changes were reversed by AMC treatment. Meanwhile, intestinal VH and the VH/CD ratio are the gold standard for assessing intestinal morphology (Xie et al., 2021). It plays an essential role in nutrient absorption and provides a protective barrier. Moreover, the reduction in VH/CD caused by weaning can cause malabsorption, which in turn hinders intestinal functional repair (Song et al., 2018; Wang et al., 2019). The intestinal H&E staining results in this study proved that oral administration of AMC to weaned piglets had a better effect on VH and the VH:CD ratio in the duodenum, jejunum, and ileum, which are beneficial to intestinal function. Emerging evidence indicates that intestinal epithelial injury is usually accompanied by bacterial translocation, leading to a dramatic inflammatory response, oxidative stress, and metabolic endotoxemia (Ma et al., 2021). Our results indicated that the levels of LPS and proinflammatory factors, such as IL1β, IL6, and TNF-α, were significantly reduced in the weaned piglet small intestine after AMC treatment, which may be related to AMC promoting the secretion of sIgA in the small intestine (Noval Rivas et al., 2019). Moreover, AMC water alleviated the oxidative stress induced by weaning or inflammation by boosting the transcriptional levels of antioxidant enzymes, including SOD 1-2, CAT, and GPX 1-2, accordingly enhancing the gut antioxidant defense system of weaned piglets. The alleviation of the inflammatory response and oxidative stress

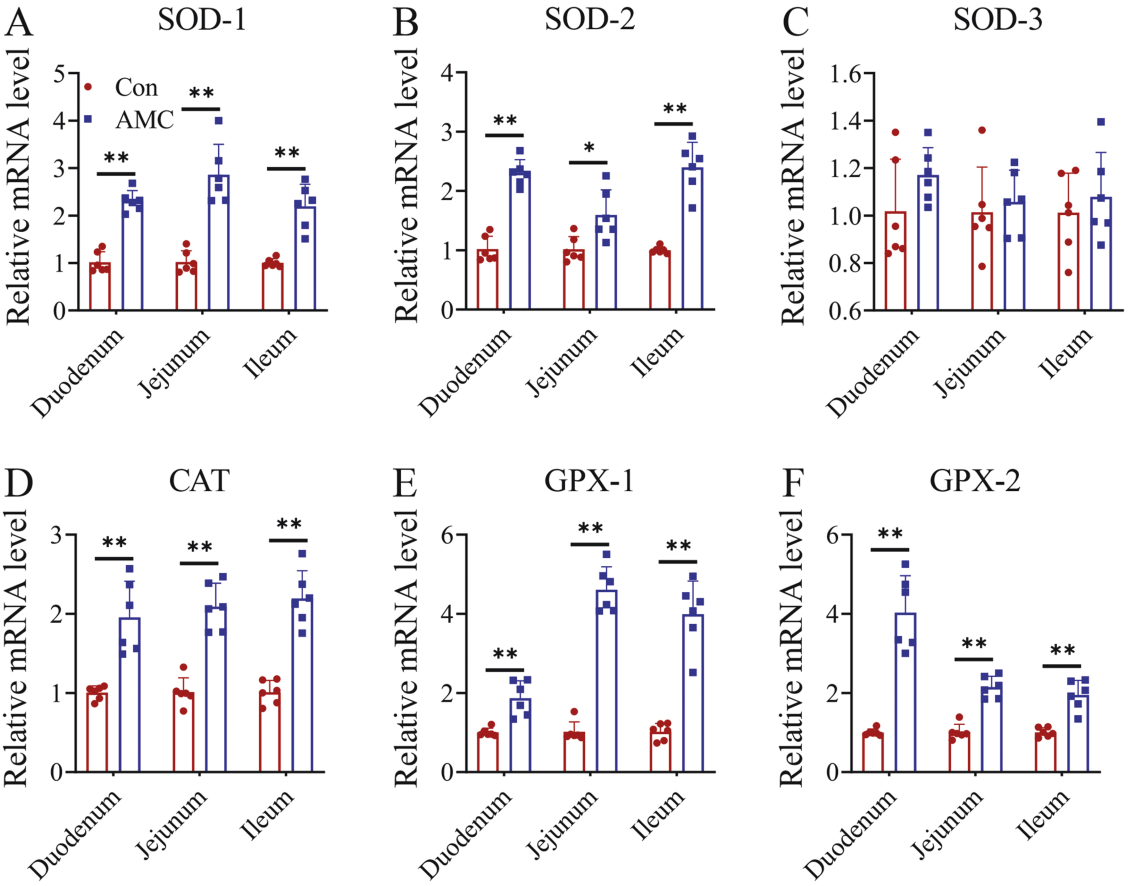

Figure 4. The relative mRNA expression levels of antioxidant-related genes in the small intestine of weaned piglets. (A) SOD-1, (B) SOD-2, (C) SOD-3, (D) CAT, (E) GPX-1, and (F) GPX-2. Data are presented as the means ± SD (n = 6 per group). Statistical significance was determined using Student's t-tests to compare differences between two groups. *P < 0.05, **P < 0.01.

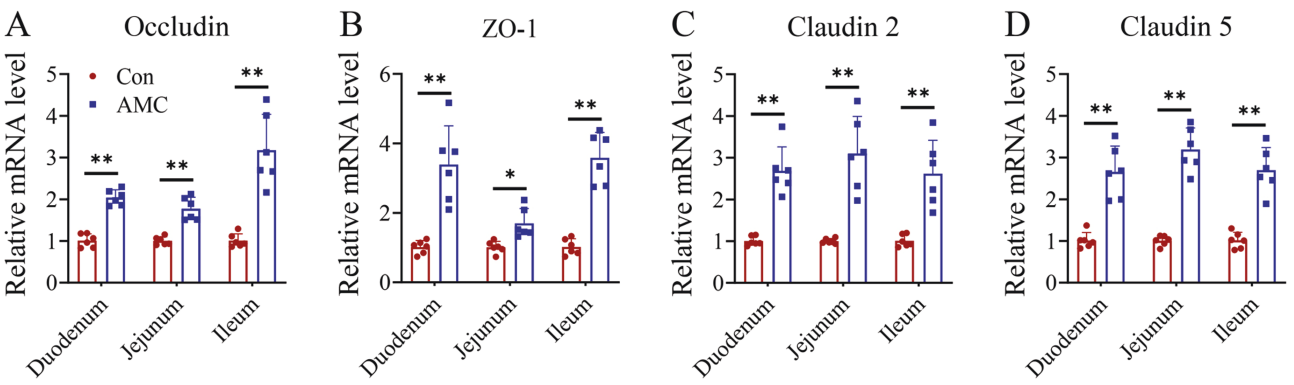

Figure 5. The relative mRNA expression levels of intestinal epithelium integrity-related genes in the small intestine of weaned piglets. (A) Occludin, (B) ZO-1, (C) Claudin 2, and (D) Claudin 5. Data are presented as the means ± SD (n = 6 per group). Statistical significance was determined using Student's t-tests to compare differences between two groups. *P < 0.05, **P < 0.01.

may be linked to the increase in intestinal goblet cell number by AMC treatment (Shin et al., 2014), whose main function is to synthesize and secrete mucins to form a mucosal barrier to protect epithelial cells.

On the other hand, the alteration of LPS levels and mRNA expression of proinflammatory factors and antioxidant enzymes also indirectly supported the improvement of the intestinal epithelial barrier. The promotion of small gut epithelial barrier function may be a consequence of elevated gut morphology, immunological function, and lower inflammation levels in weaned piglets (Barbara et al., 2021). The breakdown of the intestinal barrier provides favorable conditions for the proliferation and invasion of pathogens and exacerbates more severe injury (Xie et al., 2021). In this study, we found that the mRNA expression levels of tight junction proteins, including Occludin, ZO-1, Claudin2, and Claudin5, in the duodenum, jejunum and ileum were significantly increased by AMC water treatment. Tight junctions are structures that connect epithelial cells, giving them the function of sealing paracellular spaces between

cells, accordingly limiting bacterial toxins and pathogens. A variety of transmembrane and cytoplasmic proteins, including Occludins, Claudins, and ZOs, are important components of tight junctions, which interact and form complex closed structures with the cytoskeleton (Chelakkot et al., 2018). A study found that the increase in intestinal permeability in weaned piglets was negatively correlated with the mRNA expression of Occludin, Claudins, and ZO-1 in the intestinal epithelium (Hu et al., 2013). Therefore, our results demonstrated that AMC water plays a crucial role in maintaining intestinal permeability in weaned piglets, and the mechanism may involve promotion of the expression of tight junction proteins.

In conclusion, our study found that drinking water AMC supplementation could effectively enhance growth performance by alleviating the inflammatory response, promoting the immune barrier and antioxidant defense system, improving small intestinal morphology, and maintaining small intestinal barrier function in weaned piglets. Further studies should be performed to determine the optimal dose of AMC supplementation.

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Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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