Effects of antibiotic substitutes on growth, intestinal microvillus morphology and M cells in pigs - electron microscopy study

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Research

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Abstract

Background: Misuse of antibiotics in feed seriously affects intestinal tract health of pigs. The feed strategy of alternative feed additives is essential for the pig industry. A single-factor test was conducted to study the effects of antibiotics in feed, glucose oxidase (GOD), and bacterial direct-fed microbials on pig growth and the morphology of M cells the small intestine.

Methods: A total of 160 40-d-old weaning piglets of the DLY (Duroc × Large White × Yorkshire) breed were randomly divided into 4 treatments with 4 replicates of 10 pigs each. Dietary treatments were control (commercial basal diet with no additive) and quantitative antibiotics (0.5 kg/t), bacterial direct-fed microbials, GOD (0.5 kg/t), and microbials + GOD added to the basal diet. The preliminary trial period was 7 days, and the formal test period was 35 days.

Results: The results showed that the combination of microbials and GOD significantly increased the average daily gain of pigs (ADG) and reduced the ratio of feed intake to gain (F/G) of pigs. The results of intestinal histology by electron microscopy suggested that antibiotics in feed seriously destroyed the morphology of intestinal microvilli and M cells, perturbed the intestinal microflora in nursery pigs. The bacterial direct-fed microbials were most beneficial to small intestine health, which is characterized by complete microvilli, compact and orderly arrangement of villi, and rich intestinal microbes. GOD changed the morphology of M cells in the small intestine, enhanced phagocytosis, but damaged the intestinal mucosa in a certain degree. The combined use of bacterial direct-fed microbials and GOD repaired the intestinal mucosa injury caused by GOD.

Conclusions: In this experiment, the microbials and GOD as feed additives could replace antibiotics and improved the growth and immune performance of nursery pigs. The bacterial direct-fed microbials were beneficial to intestinal health. The excessive use of antibiotics seriously affected the development and health of the small intestine. Our results provide a theoretical basis for the application of bacterial direct-fed microbials and GOD in pig production.

Background

Antibiotics are widely used in human health and veterinary practices, and the use of antibiotics benefits the rate and efficiency of body weight gain, reduces mortality and morbidity, and improves health and economic benefits in pigs [1]. Antibiotics have been administered to agricultural animals for disease prevention and growth promotion for decades. However, by altering the composition and functions of the microbiota, they can also produce long-lasting deleterious effects in the host. The emergence of multidrug-resistant pathogens raises concerns about the common, and at times inappropriate, use of antimicrobial agents [2]. In-feed antibiotics have been forbidden for use in swine and livestock production in many countries around the world. Modulation by natural feed supplements as alternatives to in-feed antibiotics has been successfully practiced, the most important being probiotics, prebiotics, bacteriocins, organic acids, enzymes, bioactive phytochemicals, and antimicrobial peptides [3-5]. The role of these

additives and their potential use in managing gut health and function in newly weaned pigs has been reviewed extensively [6,7]. GOD has been widely used in the feed production industry and has been reported to have the best antagonistic effect against different food-borne pathogens, such as *Salmonella infantis*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, *Campylobacter jejuni* and *Listeria monocytogenes* [8]. Supplementation of GOD in the diet promoted the growth of nursery pigs [9]. Lysozyme improved the growth performance and altered the small intestinal villus morphology in nursery pigs [4]. The dietary supplementation of microbials (a mixture of several beneficial microorganisms) increased the growth performance of birds at an early age, stimulated the immune response, and improved the ileal morphology of broiler chickens [5]. Using both prebiotics and probiotics in the diet of pigs from weaning to finishing improved the feed-conversion ratio [10]. So far, few results on the effect of feed additive on the ultra-microstructure of the intestinal villi of pigs have been reported.

Microfold cells (M cells) are enigmatic intestinal epithelial cells residing in the follicle-associated epithelium covering gut-associated lymphoid tissue follicles [11]. They have unique morphological characteristics, including irregular brush borders, reduced microvilli, and basolateral pockets containing mononuclear phagocytes and lymphocytes [12]. M cells initiate mucosal immune responses through the uptake and transcytosis of luminal microbial antigens, which are important for maintaining intestinal homeostasis [13]. It is generally known that antibiotics and probiotics can affect microbials in the intestinal tract. Do feed additives such as antibiotics and probiotics affect the morphology of M cells? This question remains unclear.

Therefore, the present study aims to investigate the influence of antibiotic substitutes on growth, intestinal microvillus morphology and M cells in pigs.

Methods

Animals and management

A total of 160 healthy weaned piglets (Duroc×Landrace×Large White) with similar weights (9.67 +0.13) kg were selected as test animals, which were provided by the Pig Breeding Farm of Shandong Huayu Group. The experiment was conducted at Huayu breeding farm in Guangrao County, Dongying City, Shandong Province, and the experimental pigs were fed at the same stationary pig house. Preparations for cleaning and disinfection of the pigsty were made before the start of the experiment. After the test started, the feed was given at 8 a.m. daily, and the pigs were given ad libitum access to feed and water. Piggery hygiene, pig immunization, insect repellent and other feeding management practices strictly followed the routine procedures of the pig farm.

Experimental design

A single-factor design was applied to study the effects of antibiotic substitutes on growth, intestinal microvillus morphology and M cells in pigs. There was no significant difference in starting body weight between treatments ($P > 0.05$). A total of 160 weaning piglets (Duroc × Large White × Yorkshire) aged 40 days were randomly divided into 4 treatments with 4 replicates of 10 pigs each. Dietary treatments included 1, control (commercial basal diet with no additive; diet composition and nutrition level are illustrated in Table 1); 2, basal diet plus antibiotics (main ingredients: 50% gentamicin, 0.19 kg/t; 10% clomiphene, 0.42 kg/t; 10% bacitracin zinc, 0.44 kg/t); 3, basal diet plus GOD (0.5 kg/t, 3 000 U/g, from KRVAB Bio-tech group of Beijing); and 4, basal diet plus bacterial direct-fed microbials (Lactobacillus, 3.0×10^9 cfu/g, *Bacillus subtilis* 1.4×10^{10} cfu/g, *Bacillus licheniformis* 1.3×10^{10} cfu/g; commercial name: Kofulai, from KRVAB Bio-tech group of Beijing) and GOD. The preliminary trial period was 7 days, and the formal test period was 35 days.

Table 1 Basic diet composition and nutrition levels.

Item	Content	Nutrient level	Content
Ingredients（%）		DE/(MJ/kg)	13.86
Corn	68.55	Crude Protein	19.68
46%Soybean	12.92	Ca	1.02
Fermented soybean	3.15	P	1.89
full fat soybean	6.77	Lys	0.57
Peru fishmeal	3.15	Met	0.16
Plasma protein	0.47	Thr	0.13
APC blood corpuscle	0.63		
$Ca(H_2PO_4)_2$	0.91		
Limestone	0.79		
NaCl	0.47		
Premix[1]	2.00		
Total	100.00		

Note: 1. The premix per kg of dietp rovided the following: VA, 8 000 IU; VD_3, 3 000 IU; VK_3, 2.00 mg; VB_1, 1.50 mg; VB_2, 6.00 mg; VB_6, 2.20 mg; VB_{12}, 0.04 mg; Pantothenic acid, 14.00 mg; Niacin, 45.00 mg; Biotin, 0.15 mg; Folic acid, 1.20 mg; Mn, 40.00 mg; Fe, 120 mg; Cu, 10.00 mg; Zn, 130 mg; Se, 0.30 mg; I, 0.50 mg.

Growth indicators

The quantities of feed and residues in the pig house were recorded every week to calculate the average daily feed intake (ADFI) of each pig. The lean weight of every pig was individually measured every two weeks to calculate the average daily gain (ADG). The feed to gain (F/G) ratio was calculated.

Electron-microscopic observation of intestinal villi

The approximately 0.5×0.5×0.2 cm epithelium samples from the middle portion of the ileum and jejunum were washed with PBS and fixed in 3% glutaraldehyde fixing solution (pH 7.2) at 4°C for 3 days. Then, the solution was fixed in 1% OsO_4 at 4°C for 3 hours. The samples were sent to the electron microscopy center of the State Key Laboratory of Crop Biology at Shandong Agricultural University for preparation of electron microscope slides, which were observed and photographed under a JEM-1400Plus transmission electron microscope and a JSM-6610LV scanning electron microscope.

Statistics and Analysis

All data were represented as the mean ± SEM. Statistical analysis was performed by the one-way ANOVA procedure of SAS 9.1. Differences among treatments were compared using Duncan's test and considered statistically significant at $P < 0.05$.

Results

Effects of different antibiotic substitutes on the growth performance of nursery pigs

The statistical results of the growth performance of the nursery pigs are shown in Table 2. The results showed that there were significant differences in ADG, ADFI and F/G among treatments ($P < 0.01$). Compared with the control, the addition of antibiotics and the antibiotic substitutes in the diet significantly increased the ADG and ADFI ($P < 0.05$) of the nursing pigs. In comparison with the ADG (0.43 kg/d) and F/G (2.19) of antibiotic treatment, the ADG of the GOD and the microbial + GOD treatments was significantly increased, with increases of 23% and 30%, respectively ($P < 0.05$), but F/G was significantly decreased (by 17% in both groups). Based on the analysis of each index of growth performance, the growth performance of pigs in the group of microbials + GOD was the best in the nursery stage by ADG (0.56 kg/d), ADFI (1.02 kg/d) and F/G (1.82).

Table 2 Effects of different antibiotic substitutes on the growth performance of nursery pigs

Items	ADG（kg/d）	ADFI（kg/d）	F/G
Control	0.38 ± 0.01^{d}	0.86 ± 0.01^{c}	2.29 ± 0.09^{a}
Antibiotic	0.43 ± 0.01^{c}	0.95 ± 0.08^{b}	2.19 ± 0.23^{ab}
GOD	0.53 ± 0.01^{a}	0.96 ± 0.04^{b}	1.82 ± 0.10^{c}
Microbials+GOD	0.56 ± 0.03^{a}	1.02 ± 0.04^{a}	1.82 ± 0.06^{c}
P-value	< 0.001	< 0.001	0.009

Note: ADG, average daily gain; ADFI, average daily feed intake; F/G, the ratio of feed to gain, indicating feeding efficiency. Feed was calculated based on dry weight. The same letters to the right of data in the same row denote no significant difference ($P > 0.05$), while different letters indicate significant differences ($P < 0.05$). The same holds in the following table.

Effects of different antibiotic substitutes on the intestinal microvilli of nursery pigs

Representative microvillus morphology of the ileum and jejunum is illustrated in Figure 1 and Figure 4. The results from scanning and transmission electron microscopy revealed that the addition of antibiotics to feed resulted in a large area of microvillus shedding in the jejunum and ileum (Figure 2A) and structural destruction of epithelial cells (Figure 2D), especially in the ileum, compared with the control group. Epithelial cells on the surface of the intestinal villus were not intact and exhibited histological lesions. There were many hollow holes on the surface of intestinal epithelial cells (Figure 1B). Transmission electron microscopy revealed that intestinal villus epithelial cells had lateral junctions with adjacent cells (Figure 4A, D). Antibiotics caused microvillus degradation (Figure 4B, C, G, H). Supplementing with GOD also damaged the intestinal mucosa microvilli to a certain degree (Figure 2B). In contrast, the damage to the villus barriers of the intestine from diets containing antibiotics were relieved significantly after adding microbials to the diet. The combined use of microbials and GOD made intestinal microvilli denser (Figure 1D) and longer (Figure 4J).

Figure 1. Effects of antibiotics and substitutes on microvilli in the intestinal epithelium of nursery pigs under scanning electron microscopy.

Figure 2. Effects of antibiotics and substitutes on villus development in the intestine of nursery pigs.

Note: Top three rows are scanning electron micrographs, and the last row is transmission electron micrographs. The arrows indicate lesions of microvilli. Bar: for scanning electron micrographs, 10 μm for the left two columns, 1 μm for the right column; for row D, 5 μm for the left, 2 μm for the right.

Intestinal microflora was also observed in the fold and surface of villi. Only a small number of cocci adsorbed among the villous folds, much fewer than in other treatments (Figure 3). This indicates that only a small number of drug-resistant cocci remained in the intestinal tract. Interestingly, under all treatments, including the basal diet, bacilli were rarely found on the surface of intestinal microvilli. Only after microbial treatment there were a small number of brevibacteria observed between the villus folds. The combination treatment with microbials and GOD significantly increased the number and richness of microorganisms between the microvilli of the jejunum (Figure 3). This showed that the abuse of antibiotics and additives destroyed the normal intestinal tract flora of pigs.

Figure 3. Effects of feed additives on the number of bacteria in the intestine of nursery pigs.

Note: A, control; B, antibiotics; C, GOD; D, microbials +GOD. The arrows indicate *Bacillus brevis*. Bar: 10 μm.

Figure 4. Effects of antibiotics and substitutes on microvilli in the intestinal epithelium of nursery pigs under transmission electron microscopy.

Note: The white arrow indicates the gap junction between cells. Bar: 1 μm.

Effects of different antibiotic substitutes on M cells in the intestinal epithelium of nursery pigs

Scanning electron microscopy revealed that M cells in antibiotics adding group had fewer microvilli or lacked normal microvilli on their apical plasma membrane and instead had short fold-like structures, showing a concave shape compared with normal villus epithelial cells (Figure 5A). Adding antibiotics destroyed the microvilli of intestinal epithelial cells, so it was difficult to identify which cells were M cells; we only observed M-like cells (Figure 5B). Treatment with GOD stimulated the development of short fold-like structures of M cells, resulting in papillary uplift structures on the apical surface of M cells, enhanced phagocytosis, and even bacterial-like particles adsorbed on the protrusion of M cells (Figure 5C). Combination treatment with GOD and microbials also promoted the growth of fold-like structures of M cells compared with that of the normal diet. The phenomenon that particles were captured on the protrusion of M cells was also observed.

Figure 5. Effects of antibiotics and substitutes on M cells in the intestinal epithelium of nursery pigs under scanning electron microscopy.

Note: The triangle indicates M cells. Bar: 10 μm for the left 1st column, 5 μm for the left 2nd column, 1 μm for the right columns.

Transmission electron microscopy observations showed that normal M cells lacked microvilli or had fewer and untidy, unhealthy microvilli (Figure 6A, C, E). The basal plasma membrane was deeply invaginated to form a large sac-like structure, the so-called M-cell pocket, where dendritic cells and/or lymphocytes could move in and reside. M cells possessed tight junctions and desmosomes that contacted adjacent columnar cells and lymphocytes (Figure 6D, Figure. 7E, F, Figure 8B, G, white arrow). In the cytoplasm, phagocytic endosomes and secondary lysosomes were observed (Figure 6E, F). Treatment with antibiotics resulted in organelle vacuolation and microvillus degradation and fragmentation that made the subcellular structure unrecognizable (Figure 6G, H).

Figure 6. Ultramicrostructure of M cells in the intestinal epithelium of nursery pigs under transmission electron microscopy.

Note: M indicates M cell; Ly, lysosome; mit, mitochondria; ER, endoplasmic reticulum; L, lymphocyte. The white arrow indicates a desmosome between lymphocytes and M cells, and the black arrow indicates an endocytosed cell fragment. Bar: 5 μm for the left column, 1 μm for the right columns beside 7-H (2 μm).

The addition of GOD changed the morphology of M cells in the small intestine, causing irregular microvillus uplifting on the apical surface of M cells and a stronger capture and phagocytosis capacity (Figure 7C, D), and cell fragments appeared in the deep cytoplasm (Figure 7G). In superficial cytoplasm, more phagocytic endosomes and secondary lysosomes were observed (Figure 6E, F). Multiple adjacent M cells had gap junctions between them (Figure 7E, F).

Figure 7. Effects of GOD on M cells in the intestinal epithelial cells of nursery pigs under transmission electron microscopy.

Note: M indicates M cell; Ly, lysosome; E, endosome; L, lymphocyte; the white arrow indicates a gap junction between M cells, the black arrow indicates an endocytosed cell fragment, and the arrow head indicates a particle to be phagocytosed; Bar: 5 μm for the left column, 1 μm for the right columns.

Interestingly, the combined use of GOD and microbials strengthened lipid mobilization and resulted in many lipid droplets in M cells and adjacent epithelial cells (Figure 8). There were large phagocytic fusion vesicles in the superficial cytoplasm of M cells (Figure 8F, H) and endosomes in the deep cytoplasm (Figure 8D).

Figure 8. Effects of the combination of microbials and GOD on M cells in the intestinal epithelial cells of nursery pigs under transmission electron microscopy.

Note: M indicates M cell; Ly, lysosome; E, endosome; L, lymphocyte; the white arrow indicates gap junction; the black arrow indicates a lipid droplet. Bar: 5 μm for the left column, 1 μm for the right columns beside part B (2 μm).

Discussion

The use of antibiotics in feed is being forbidden in the animal farm industry, and the development of new feeding strategies to substitute antibiotics and stimulate gut development and health in weaned pigs is essential for the long-term sustainability of the pig industry. This article thoroughly studied the ultra-microstructure of intestinal villi and M cells using electron microscopy and found that antibiotics in feed seriously impacted the morphology of both, while a substitute of antibiotics, bacterial direct-fed microbials, effectively improved the side effects of antibiotic additives on the microvillus microflora in nursery pigs.

It is well known that antibiotics in feed improve health and economic benefits and disrupt the gastrointestinal microbiota [2]. This paper provides visual ultra-microstructural/morphologic evidence of the influence of antibiotics on intestinal microvilli and microflora by electron microscopy. Earlier research has shown that feed additives affect intestinal villus morphology at the optical microscope level, comprising the length and width of the villi and the depth of the crypt [14-16]. In our experiment, feeding antibiotics obviously induced damage to microvilli and obviously decreased the count of microbes in the intestinal tract. The damage to microvilli induced by antibiotics is likely associated with mucosal inflammation induced by antibiotics [17,18]. It was interesting that, even in the normal control, bacilli were rare on the folds and the surface of intestinal villi. Adding microbials directly significantly increased the microflora and bacterial counts in the small intestine. This suggests that the long use of antibiotics in feed on pig farms has had an effect on the microbiota in the intestinal tract of pigs. This problem deserves attention. Our results also show that microbials could be an alternative to antibiotics in feed.

In this paper, treatment with GOD improved the growth performance of pigs. This is consistent with the reports in broiler chickens [19,20]. Although treatment with GOD promoted the growth of pigs, intestinal microvillus injury can also be induced by the product of GOD, hydrogen peroxide. Because GOD specifically catalyzes the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide [21], the latter has been evidenced to induce intestinal mucosal damage in a closed circulating intestinal loop [22]. This indicates that the additive dosage is noteworthy. Combination usage of bacterial direct-fed microbials and GOD effectively improved microvillus damage repair. This suggests that feeding direct microbials was beneficial to the health of the intestinal tract in pigs. In support of this hypothesis, *B. subtilis*-based probiotic supplementation influenced gut barrier integrity through increased tight junction gene expression in broilers [23]. It is worth noting that directly fed GOD changed the microfold morphology of M cells in the intestinal tract, showing a long papillary uplift structure in the apical membrane and gap junction between M cells in the side face. As far as we know, this has rarely been reported. Only Wang et al. [20] reported that dietary treatment with GOD had beneficial effects on the expression of intestinal

tight junction genes in broilers. This result indicates that directly fed GOD can intensify the phagocytic function of M cells. The relevant mechanisms need to be further identified.

Interestingly, many lipid droplets were observed in M cells and intestinal epithelial cells in the microbial group. Why did many lipid droplets appear in M cells and intestinal epithelial cells after treatment with microbials? What role do they play? These questions are not yet clear. Data have shown that lipid droplets are organelles involved in lipid metabolism and the production of inflammatory mediators [24]. We speculate that they are involved in the mobilization of lipids or in immunomodulation. Because research suggests that probiotic yeast has the ability to prevent inflammation by promoting proinflammatory immune function and increasing the production of short-chain fatty acids [25].

Conclusions

In summary, our results show that excessive use of antibiotics seriously affected the development and health of the small intestine. The microbials and GOD could replace antibiotics and improve the growth and immune performance of nursery pigs. The bacterial direct-fed microbials were beneficial to intestinal health.

List Of Abbreviations

GOD: Glucose oxidase; M cells: Microfold cells; ADG: average daily gain; ADFI average daily feed intake; F/G: the ratio of feed to gain;

Declarations

Funding

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

YWR and HLB designed the study. YXJ and HLB collected samples, YXJ participated preparation of electron microscope slides and photographed micrograph. XYU collected and analyzed the data. YXJ wrote the manuscript and HLB revised it. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Gilts used in all experiments were cared for in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The protocol of this study was approved by the animal Ethics Committee of Shandong Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

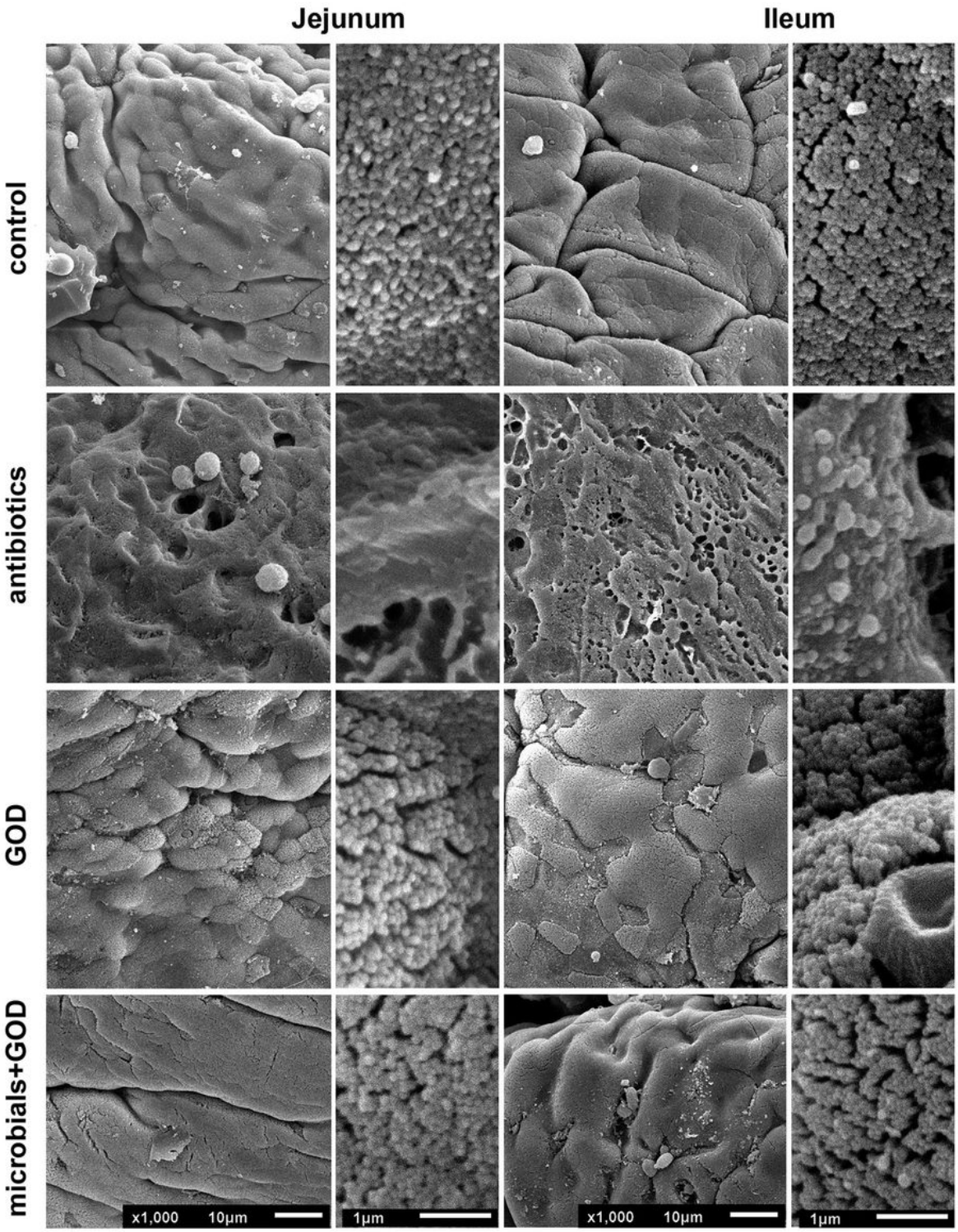

Figure 1

Effects of antibiotics and substitutes on microvilli in the intestinal epithelium of nursery pigs under scanning electron microscopy.

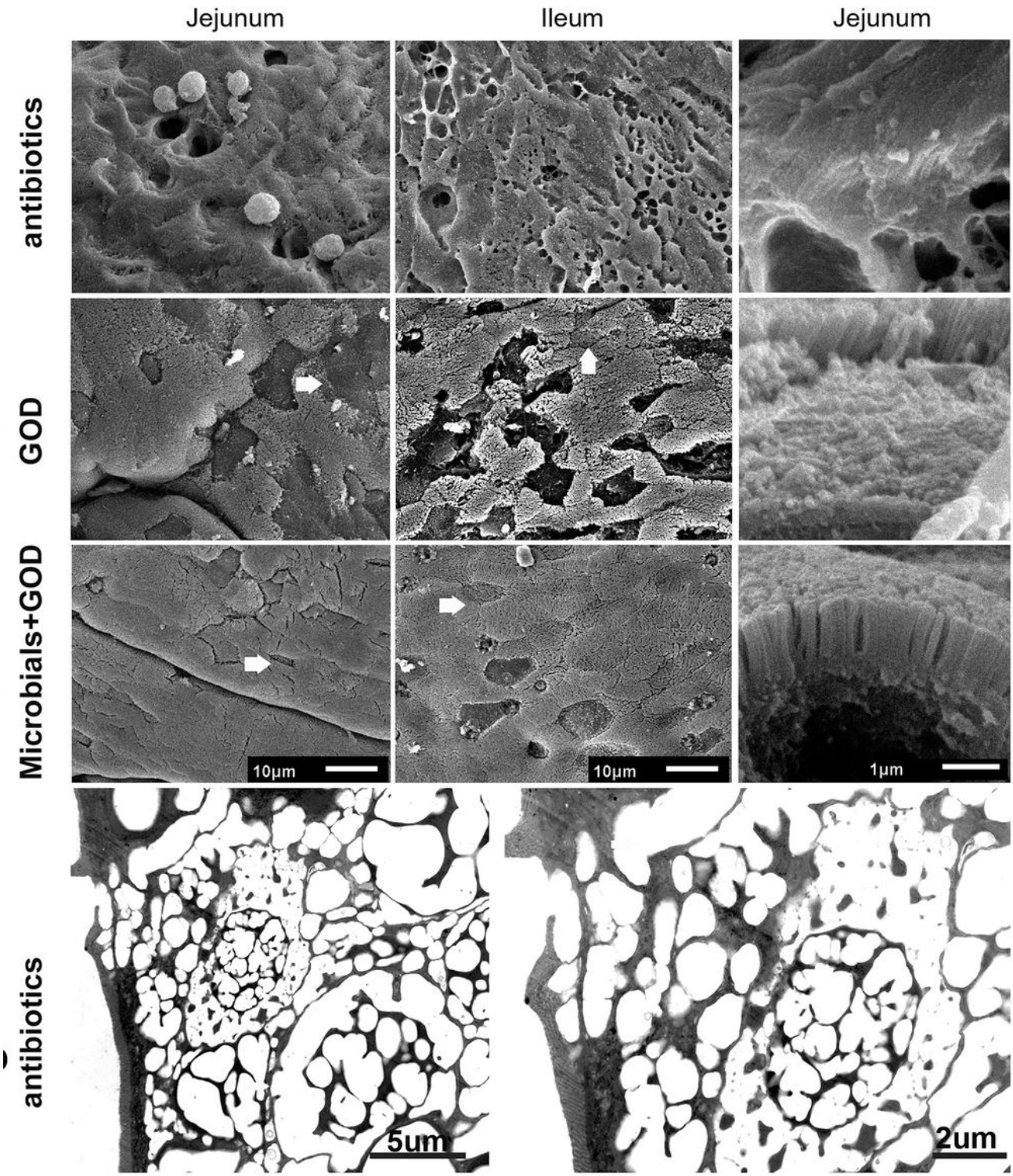

Figure 2

Effects of antibiotics and substitutes on villus development in the intestine of nursery pigs. Note: Top three rows are scanning electron micrographs, and the last row is transmission electron micrographs. The arrows indicate lesions of microvilli. Bar: for scanning electron micrographs, 10 μm for the left two columns, 1 μm for the right column; for row D, 5 μm for the left, 2 μm for the right.

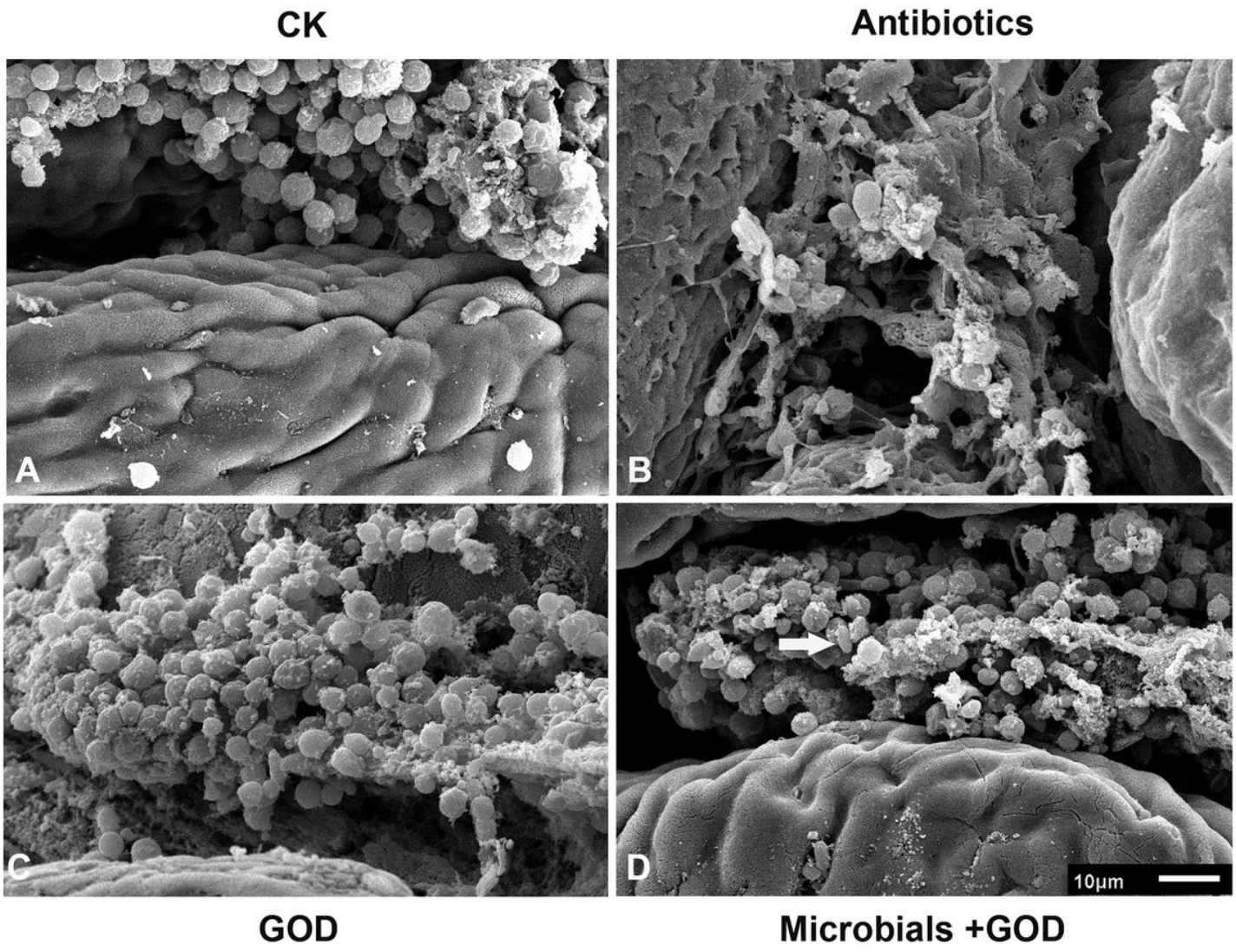

Figure 3

Effects of feed additives on the number of bacteria in the intestine of nursery pigs. Note: A, control; B, antibiotics; C, GOD; D, microbials +GOD. The arrows indicate Bacillus brevis. Bar: 10 μm.

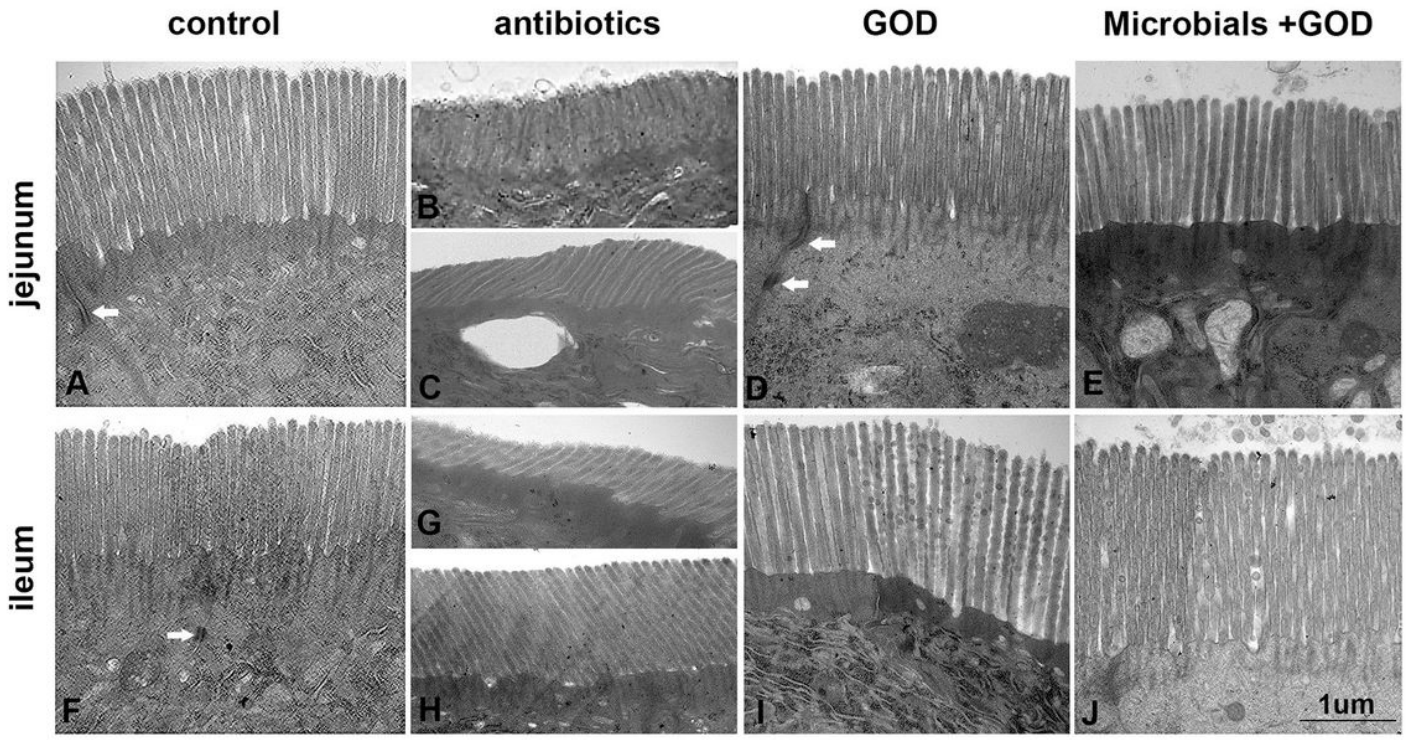

Figure 4

Effects of antibiotics and substitutes on microvilli in the intestinal epithelium of nursery pigs under transmission electron microscopy. Note: The white arrow indicates the gap junction between cells. Bar: 1 μm.

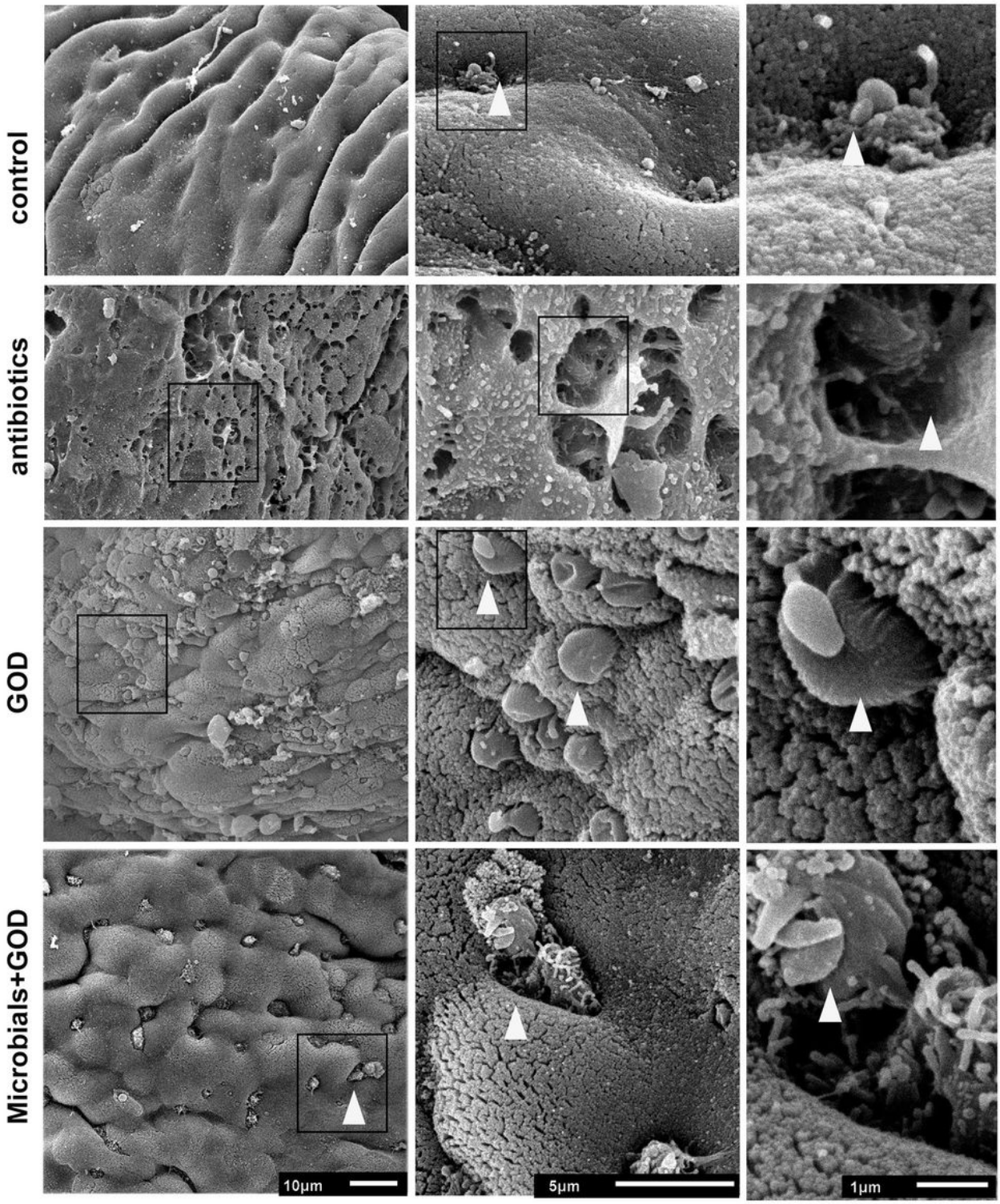

Figure 5

Effects of antibiotics and substitutes on M cells in the intestinal epithelium of nursery pigs under scanning electron microscopy. Note: The triangle indicates M cells. Bar: 10 μm for the left 1st column, 5 μm for the left 2nd column, 1 μm for the right columns.

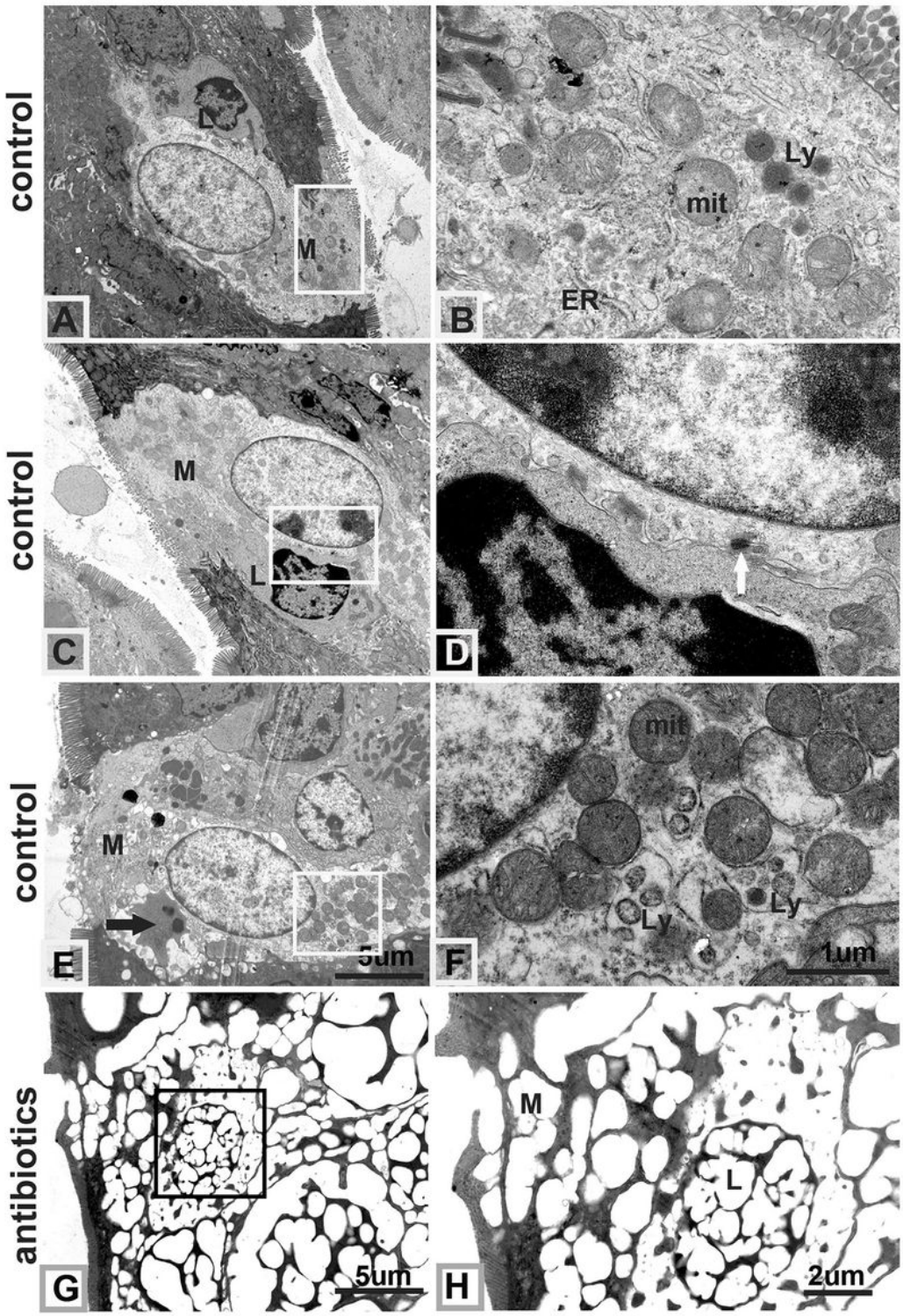

Figure 6

Ultramicrostructure of M cells in the intestinal epithelium of nursery pigs under transmission electron microscopy. Note: M indicates M cell; Ly, lysosome; mit, mitochondria; ER, endoplasmic reticulum; L, lymphocyte. The white arrow indicates a desmosome between lymphocytes and M cells, and the black arrow indicates an endocytosed cell fragment. Bar: 5 μm for the left column, 1 μm for the right columns beside 7-H (2 μm).

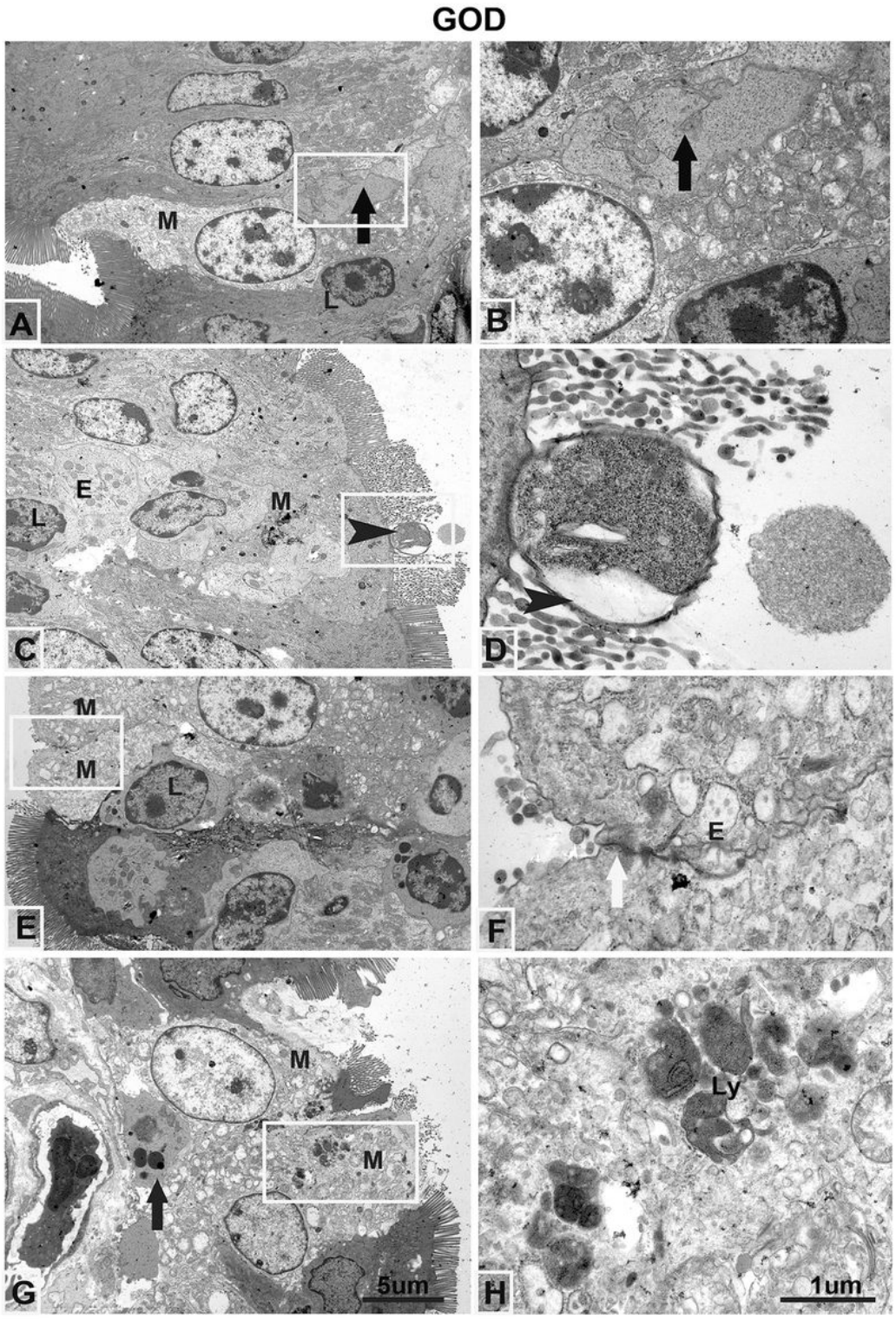

Figure 7

Effects of GOD on M cells in the intestinal epithelial cells of nursery pigs under transmission electron microscopy. Note: M indicates M cell; Ly, lysosome; E, endosome; L, lymphocyte; the white arrow indicates a gap junction between M cells, the black arrow indicates an endocytosed cell fragment, and the arrow head indicates a particle to be phagocytosed; Bar: 5 μm for the left column, 1 μm for the right columns.

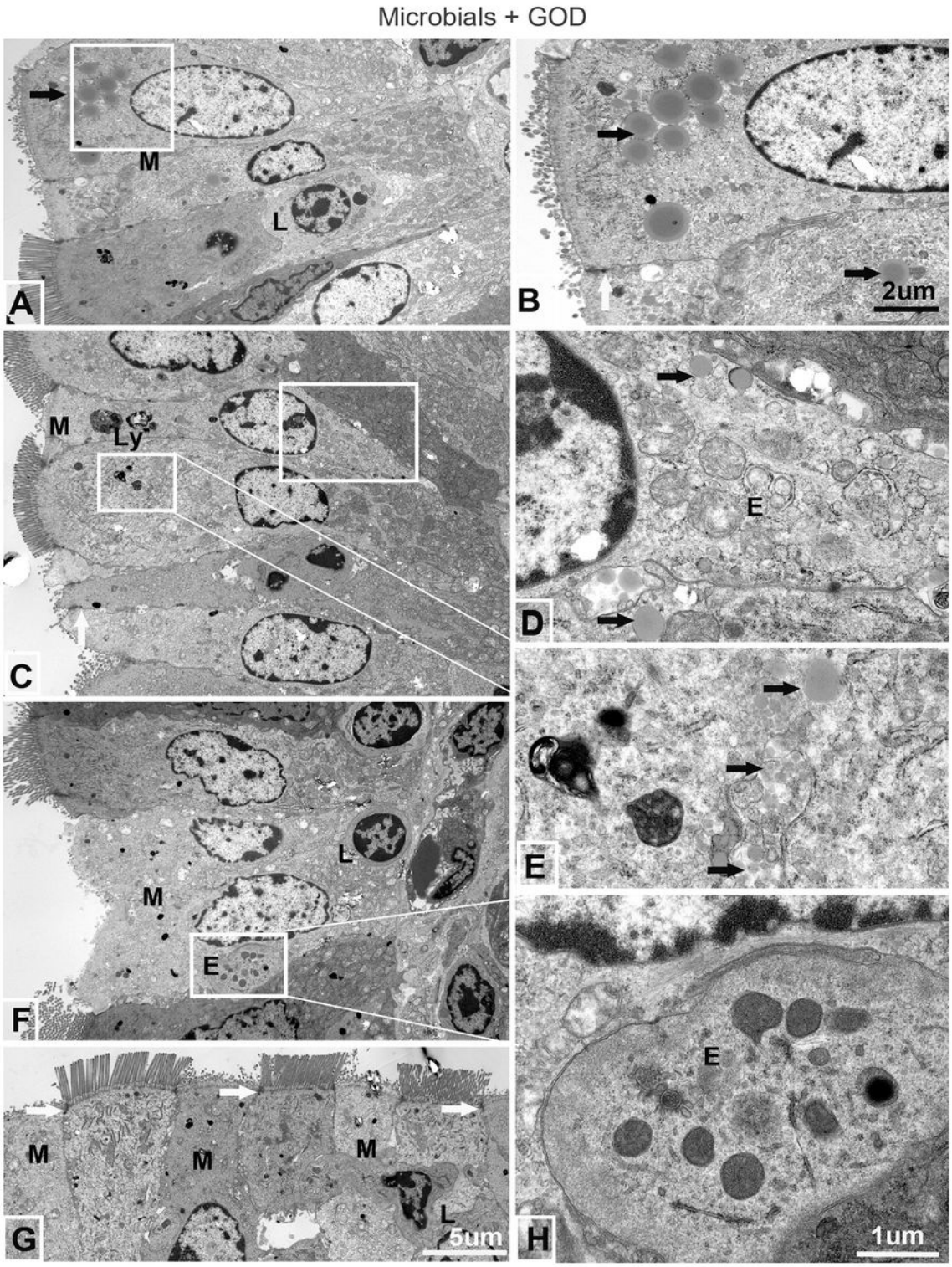

Figure 8

Effects of the combination of microbials and GOD on M cells in the intestinal epithelial cells of nursery pigs under transmission electron microscopy. Note: M indicates M cell; Ly, lysosome; E, endosome; L, lymphocyte; the white arrow indicates gap junction; the black arrow indicates a lipid droplet. Bar: 5 μm for the left column, 1 μm for the right columns beside part B (2 μm).