Original Article

Effects of copper-bearing montmorillonite on *Salmonella* spp. in vitro study

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Abstract

Clay is an important material available in nature. Montmorillonite is considered viable for producing enhanced performance in a variety of materials and products. Farmed animals such as pigs and poultry receive additional Zn and Cu in their diets as supplements in their compound feed and these elements are also used for medical remedies. This study aimed to observe the bactericidal activity of copper-bearing montmorillonite (Cu-MMT) against *Salmonella* spp. in vitro. The experiments were divided into two methods that effect *Salmonella* spp.. First, Cu-MMT was used to precipitate the Cu^{2+} ion supernatant mixed with bacteria. The second, Cu-MMT was directly mixed with the bacteria. The *results indicate* that the optimal bactericidal concentration of the Cu^{2+} ion supernatant precipitated by Cu-MMT was 0.1 mg/mL for 24 hours, and the optimal sterilisation concentration of Cu-MMT was directly mixed with *Salmonella* spp. at 0.05 mg/mL for 24 hours. When *Salmonella* spp. was mixed with Cu^{2+} ion or Cu-MMT, the effects on the cell types could be clearly discerned with an electron microscope. Scanning electron microscope (SEM) or Transmission electron microscope (TEM) showed that many bacteria were adsorbed by Cu-MMT. The appearance of the bacteria exhibited an uneven morphology, the cell wall was ruptured, the cell membrane was broken, the cells appeared as bubbles and the osmotic pressure of the Cu^{2+} ion liquid caused the cell wall to separate from the cytoplasm. Here was an important reason for the antibacterial effect and bactericidal ability of Cu-MMT.

Keywords: bacteria, Cu-MMT, electron microscope, Montmorillonite, *Salmonella*

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Introduction

Copper-bearing montmorillonite (Cu-MMT) is a feed additive that contains Cu^{2+} ion coatings with an antibacterial role. Farmed animals such as pigs and poultry receive additional Zn and Cu in their diets due to supplementation of elements into their compound feed as well as medical use (Yazdankhah *et al*., 2014; Poole, 2017). Cu^{2+} ion feed additives have demonstrated significant growth-promoting effects on pigs. The mechanism is through the use of high concentrations of Cu^{2+} ions, which can elicit a favourable antibacterial effect in animals, increasing the permeability of the intestinal wall cells and promoting the nutrition of absorption and utilisation (Zhou Yuhang *et al*., 2004; Sohrabnezhad and Sadeghi, 2015). Montmorillonite (MMT) is a natural silicate with a layered structure. Chemically, it can be described as hydrated sodium calcium aluminium magnesium silicate hydroxide (Uddin, 2018).

The antibacterial ability of Cu-MMT is related to two factors. Firstly, MMT produces a positive charge on the surface and most bacterial cell wall surfaces have a negative charge; thus, the static electricity between the MMT and the bacteria causes a number of bacteria to be adsorbed on the mineral surface. The copper ions (Cu^{2+}) cause changes in the morphology and permeability of the bacterial cell membranes, resulting in the loss of a large number of enzymes that create a bactericidal effect (Xia *et al*., 2006).

Salmonella is Gram-negative and can survive cold, dry and salty environments; however, it is easily destroyed by high temperatures, sunlight, phenols and halogen disinfectants (Minor L Le, 1984; Bean *et al*., 1990; Tindall *et al*., 2005; WHO, 2017). This bacteria is a common pathogen in pig farms and can cause enteric diseases, watery diarrhoea, sepsis and intestinal necrosis, which may be circumferential or scattered and may be superficial or deep (YAO *et al*., 2017). This paper describes an experimental study in a test-tube which isolated *Salmonella* spp. via the total number of bacteria by monitoring the selective culture medium (Bismuth sulphite agar) to observe the inhibitory effects of Cu-MMT on *Salmonella* spp. at different times and concentrations.

Materials and Methods

Bacterial culture preparation: Bacterial strains *S. typhimurium* were obtained from the Clinical Avian Medicine & Molecular Medicine Laboratory (Department of Veterinary Medicine, NPUST). A single colony was selected from the LB agar (SIGMA, Merck, USA) plate to culture in 10mL LB Broth (SIGMA, Merck, USA) for the initial bacterial culture. The culture was incubated at 37°C for 10 hours with a rotary shaker (LM-570 incubator, Yihder, Taiwan), and then transferred aseptically to a 50 mL LB broth culture at 37°C for 9 hours. The optical density (OD) (CT-2200 spectrophotometer, Chrom, USA) value had to be not more than 1.23, and the bacterial concentration was about 1×10^8 CFU/mL.

Testing for the bacterial content of *Cu-MMT***:** In order to ensure that the Cu-MMT was sterile, Cu-MMT 0.8g mix and 1.5mL sterile water were used to incubate the culture at 37°C for 20 hours; colony-forming units per mL (CFU/mL) were then counted.

The effect of copper ions in the supernatant and Salmonella: Four sterilised 50mL tubes were used, and 20 mL of LB broth was loaded into each tube, then 0 g, 4 g, 8 g and 16 g Cu-MMT were added respectively. Then they were completely mixed in a rotary shaker incubator for 2 hours. The tubes were centrifuged at 3000 r/min for 5 mins, the supernatant from each tube was extracted and divided equally into five 15 mL tubes. Then, 4 mL of the bacterial solution was added to each tube, so that each tube contained *S. typhimurium* 4×10^8 CFU/mL, and then mixed. LB broth was added to 8 mL, so that the final concentration of each tube was 0 g/mL, 0.1 g/mL, 0.2 g/mL, 0.4 g/mL respectively; the mixing times used were 0 hr, 6 hrs, 12 hrs, 24 hrs and 36 hrs, respectively. At the end of the mixing time, each tube was diluted to 10^{-6} using a ten-fold dilution method. Briefly, 0.1 mL of the bacteria solution was spread onto LB agar plates. These plates were incubated for 20 hours at 37° C. Finally, the CFU/ml was counted. This test was repeated 6 times and then averaged.

***The effect of direct contact of the Cu-MMT with salmonella*:** Four mL of the bacterial solution was added into 15 mL tubes so that each tube contained 4×10^8 CFU/mL *Salmonella* spp., was then added 0 g, 0.4 g, 0.8 g and 1.6g Cu-MMT and prepared in 5 tubes per each concentration. They were each mixed, and then LB broth was added to each tube of 8 mL, so that the final concentration of each tube was 0 g/mL, 0.05 g/mL, 0.1 g/mL and 0.2 g/mL, respectively; the mixing times were 0 hr, 6 hrs, 12 hrs, 24 hrs and 36 hrs, respectively. Furthermore, at the end of the mixing time, dilute, culture and counted CFU/ml were repeated 6 times and then averaged in the same way as in the above section.

***Sample preparation for electron microscopy*:** 1 mL of the bacterial culture was sampled and centrifuged at 3000 r/min for 5 mins, supernatant was discarded and then 1mL of glutaraldehyde was used to fix for at least two hours, then soaked in phosphate-buffered saline for 10 mins, repeated twice and centrifuged. Alcohol was then added. 50% alcohol was added for 10 mins, centrifugation; the 2nd, 70% alcohol for 10 mins, centrifugation; the 3rd, 80% alcohol for 10 min, centrifugation; the 4th, 90% alcohol for 10min, centrifugation; the 5th 100% alcohol for 15 mins, centrifugation, twice; soaked in acetone for 15 mins, twice. The gold coating was performed after critical point drying of the carbon dioxide. Specimens were observed at the Electron Microscopy Center at National Pingtung University of Science and Technology by Scanning electron microscope (SEM, Hitachi S-3000N, Japan) and Transmission electron microscope (TEM, Hitachi H-7500, Japan).

Results

Testing for the bacterial content of Cu-MMT**:** Zero CFU/mL was represented.

The effect of Cu^{2+}ion in the supernatant with Salmonella: The colony was numbered at five-time points, when the concentration was 0.1 g/mL, the colony number decreased compared with the control group (Fig. 1). Therefore, the concentration of 0.1 g/mL was the concentration with the best bactericidal effect. The concentration of 0.2 g/mL and the concentration of 0.4 g/mL also exhibited a bactericidal effect; however, it occasionally climbed, which meant that the higher-concentration bactericidal effect might not be directly proportional. Cu^{2+} ions in the supernatant showed the best antibacterial effects for *Salmonella* spp. when the concentration was 0.1 g/mL (Fig. 1).

***The effect of the direct contact of Cu-MMT with Salmonella*:** When the concentration was 0.05 g/mL at five-time points, the number of colonies decreased compared with the control group (Fig. 2). Therefore, the concentration of 0.05 g/mL was the best for sterilisation. The concentration of 0.1 g/mL and the concentration of 0.2 g/mL also had bactericidal effects; however, occasionally the climbing phenomenon occurred, which meant that at a higher concentrations the bactericidal effects might not be directly proportional, to the best time for sterilisation, because each group of time had fallen, so it worked every time.

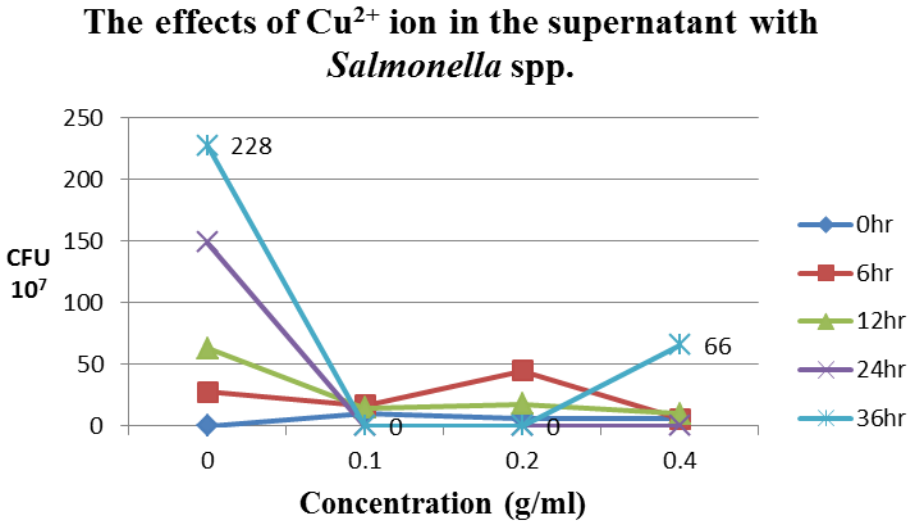


Figure 1 The effects of Cu^{2+} ions in the *supernatant* with *Salmonella* spp. Cu^{2+} ion in the supernatant; each tube was 0, 0.1, 0.2, 0.4 g/mL respectively; the mixing times used were 0, 6, 12, 24 and 36 hrs, respectively. The most favourable antibacterial effects on *Salmonella* spp. were shown when the concentration was 0.1 g/mL.

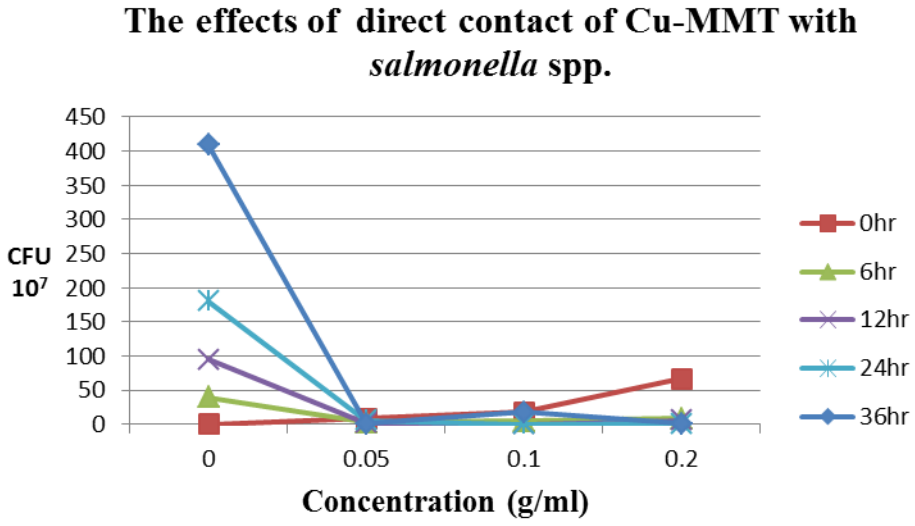
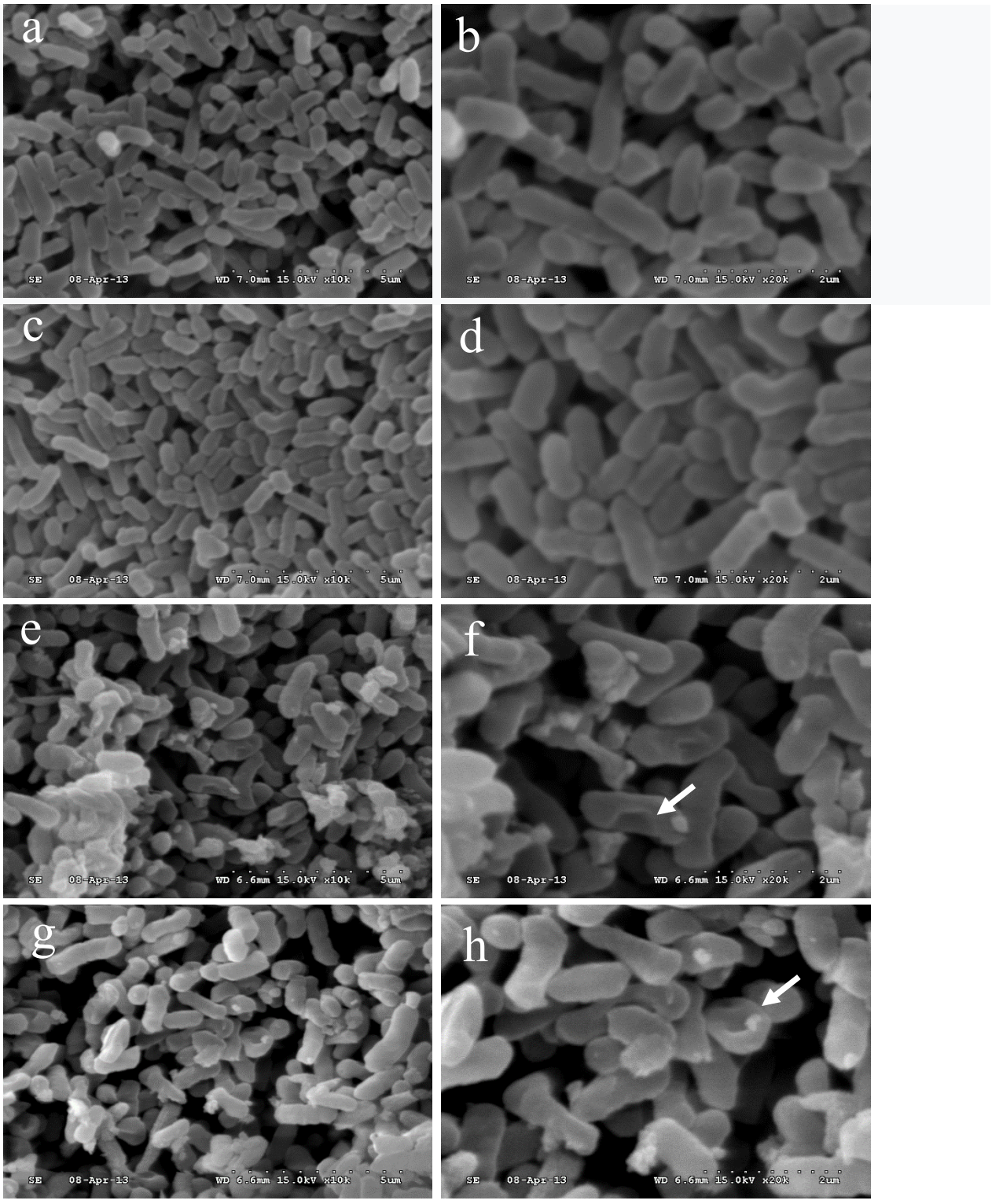


Figure 2 The effects of direct contact of Cu-MMT with *Salmonella* spp.; each tube was 0, 0.05, 0.1, 0.2 g/mL respectively; the mixing times used were 0, 6, 12, 24 and 36 hrs, respectively. The concentration of 0.05 g/mL was the best for sterilisation.

***Electron microscopy analysis*:** The SEM images are shown in Fig. 3-a to k. The TEM images are shown in Fig. 3-l to q showing that only Cu^{2+}ions in the supernatant have mixed with the bacteria. They all controlled the affections for 12 hours, with a magnification of 3,000× (Fig. 3-k), 10,000× (Fig. 3-a, c, e, g, i), 20,000× (Fig. 3-b, d, f, h,), 30,000× (Fig. 3-j), 60,000× (Fig.3-q, n), 100,000× (Fig.3-m, o, p) respectively. The stack of bacteria was found (Fig. 3-a). The appearance of the cells can be seen more clearly in (Fig. 3-b). A slight change in the bacterial cell type was observed (Fig. 3-e). The appearance of the bacteria was uneven

(Fig. 3-f, h, i, j). Slight changes were observed for the bacterial cell type (Fig. 3-g). Fig. 3-i~Fig. 3-k shows the effect of Cu-MMT mixed directly with the bacteria. Many bacteria overlapped and the appearance of the bacteria was slightly uneven (Fig. 3-i). A Cu-MMT particle adsorbs bacteria (Fig. 3-k). Bacterial cell membrane breaks occur (Fig. 3-l and m). The bacterial cell membrane ruptures (Fig. 3-n). When the cell membrane of the bacteria is broken the membrane, and cytoplasm separate via osmotic pressure (Fig. 3-o), revealing a substance in the cell that resembles bubbles (Fig. 3-p).



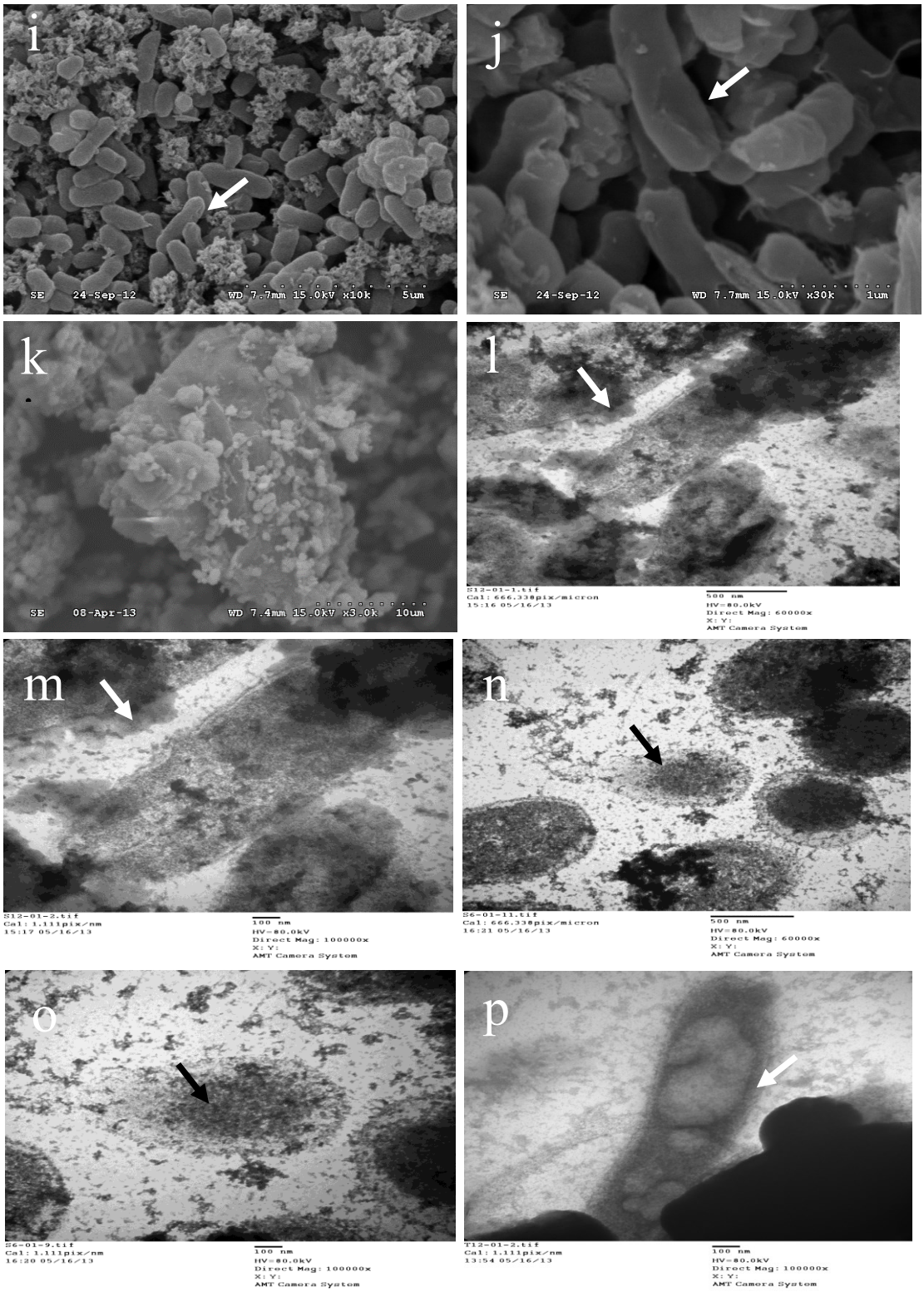


Figure 3 The effects of *Salmonella* spp. mixed with Cu-MMT under electron microscopy Fig. a~k are performed with a scanning electron microscope (SEM), and Fig. l~p are performed witha transmission electron microscope (TEM). Control experiments are performed in a~d. A slight change in the bacterial cell type was observed (Fig. 3-e, g). The bacteria appear uneven in f, h, i, j (arrow pointing). Fig. 3-k, shows the bacteria are adsorbed on the surface of Cu-MMT. The bacterial cell membrane ruptures and cytoplasm separated via osmotic pressure [(Fig. 3-l, m; 3-n (6,000×), 3-o (10,000×) arrow pointing)]. Fig. p, the cells appear as bubbles (arrow pointing).

Discussion

The results show that Cu^{2+} ions in the supernatant or Cu-MMT that directly contacted the *Salmonella* spp. could effectively reduce the number of colonies. The optimal antibacterial concentration of the Cu^{2+}ions in the supernatant was 0.1 g/mL. Compared with the number of colonies in the control group, the optimal antibacterial concentration decreased significantly. The optimal concentration of Cu-MMT for direct mixing was 0.05 g/mL.

The mechanism of MMT-Cu action works like activated carbon. MMT can adsorb a large number of bacteria, such as harmful bacteria in the digestive tract, and then they are excreted with the faeces. The disadvantage of MMT is that it can adsorb other additive feedstuff such as vitamins and minerals; thus, a greater concentration of vitamins and minerals must be added.

Copper ions are also an effective bactericide. Copper sulphate is usually used in feedstuff. However, when a high content of copper ion is added to pig feed, the feed is easily oxidised and quickly deteriorates due to the promotion of the copper ion. Following the addition of an appropriate amount of chelating agent such as Cu-MMT to the feed, the performance of the antioxidants in the high copper feed is significantly improved. Chelating agents effectively bind copper ions to form complex ring-like structures called chelates so that the copper ions lose their ability to promote oxidation.

The results of the SEM and TEM pictures show that the bacteria were destroyed, the cell wall was broken and the cytoplasm and cell wall were separated. In the future, we might be able to design more detailed experiments on the mechanism for destroying the cell wall, such as injecting jellyfish fluorescent substances into the bacteria. If the fluorescent substance leaks out, it will confirm the destruction of the cell wall. Furthermore, more extensive testing of the other bacterial, viruses or mould pulps requires further investigation in order to better understand their effects.

This experiment produced favourable results through testing on pig farms. We conducted field trials on pig farms and tested the total bacterial count, the effect of Salmonella on the Cu-MMT feed additives, plus the environment, feed, drinking water and faeces. As a result, the total bacterial count in the environment and the faeces decreased significantly. However, there was no obvious bacteriostatic effect on the amount of Salmonella in the feed and drinking water.

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