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Antibacterial effects of the Cu(II)-exchanged montmorillonite on *Escherichia coli* K88 and *Salmonella choleraesuis*

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Abstract

The aim of this research was to determine the antibacterial properties and mechanisms of Cu(II)-exchanged montmorillonite (MMT-Cu) in vitro. *Escherichia coli* ATCC K88 and *Salmonella choleraesuis* ATCC 50020 were chosen as indicators of intestinal tract pathogenic bacteria in weanling pigs. The antibacterial activity of MMT-Cu and MMT were evaluated by determining the minimum inhibitory concentrations (MICs) using two-fold serial dilutions in MH broth, and the amount of Cu^{2+} released into the broth was measured by an atomic absorption technique. The rate of oxygen consumption was measured using a SP-II-type oxygen electrode analyzer; the structural integrity of cell walls of bacteria was observed by transmission electron microscope (TEM); enzymatic activity of bacteria was examined with a semi-automatic biochemical analyzer. The results showed that MMT-Cu inhibited the growth of *E. coli* K88 and *S. choleraesuis*, and the MICs were 1024 and 2048 μg/ml, respectively. The amount of Cu^{2+} released into the broth was in the range 6.51–45.65 μg/ml. Nevertheless, both tested bacteria still grew in broth containing 32,768 μg/ml of MMT. Treatment with MMT-Cu could lead to significant release of intracellular enzymes from the tested bacteria. Data from oxygen consumption of bacteria showed that MMT-Cu could inhibit the TCA pathway of the bacterial respiration metabolism. These results show that MMT-Cu has an antibacterial activity.

Keywords: Cu(II)-exchanged montmorillonite; *Escherichia coli* K88; *Salmonella choleraesuis*; Antibacterial activity; Oxygen consumption

1. Introduction

Diarrheal disease, which is mainly caused by enterotoxigenic *Escherichia coli* K88 and *Salmonella choleraesuis*, is by far the most common disease in neonatal and early-weaned pigs (Yokoyama et al., 1992; Alexander, 1994; Hampson, 1994). These bacteria are usually found in those hoggeries where the morbidity and mortality are relatively high (Fairbrother, 1999). Thus, it is very important to inhibit the growth and propagation of these pathogenic bacteria.

Antibiotics are the most commonly used drugs in modern animal production. Abuse or overuse of

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antibiotics causes various side-effects and also results in the emergence and increase of bacteria resistant to antibiotics (Levy, 1991; Kunin, 1993). Over the past 10 years, researchers have demonstrated a rise in antibiotic-resistant genes in the majority of bacterial species, found in humans, animals and the environment, including, not only pathogens and opportunistic species, but also normal flora species (Roberts, 1989; Levy, 1992). This has led to a decline in the effectiveness of antibiotics. Therefore, it is necessary to take action to develop new types of antibacterial agents in order to reduce the unnecessary usage of antibiotics against pathogenic bacteria in animal production.

Special attention has been paid to natural non-metallic minerals (zeolite, montmorillonite, kaolinite etc.), which were used as carriers of antibacterial agent (Ohashi and Oya, 1992). It is well known that a number of clay minerals have a layer structure which can load inorganic antibacterial cations (Ag^+, Cu^{2+}, Zn^{2+} etc.) within their interlayer space by an ion-exchange reaction.

In the present study, montmorillonite (MMT) was chosen as a carrier. There are a number of reasons for this. (1) It is an expandable 2:1-type clay mineral that has both interlayer sites and ionizable hydroxyl sites on its external surface for metal cation adsorption (Borchardt, 1989), and the permanent negative layer charge originating from the isomorphous substitution of Mg(II) or Fe(II) for octahedral Al(III) is the origin of the binding of exchangeable cations to the interlayer sites (Grim, 1998). These interlayer cations can be exchanged with inorganic cations under aqueous conditions. (2) It has a great adsorption capacity, which is attributed to its large specific surface area and high cation exchange capacity (CEC) (Ramos and Hernández, 1996). (3) MMT, as an additive of non-nutritive adsorptive materials to feedstuffs, can effectively improve gastrointestinal mucus resistance to various pathogenic bacterial aggressions by interacting closely with the mucous glucoproteins (Droy-Lefaix et al., 1985). (4) It occurs widely and also is very cheap.

It is well known that some metal ions, such as Ag^+, Cu^{2+}, Zn^{2+}, have antibacterial activity. Ag^+ has the highest antibacterial activity among metal ions (Grier, 1983; Uchida, 1995). It also has many disadvantages: (1) it is unstable under light and heat conditions; (2) it can interact with Cl^- ion in aqueous medium, which makes Ag^+ lose antibacterial activity; (3) Ag is a noble metal and is very expensive. Therefore, copper was chosen to prepare the cation-loaded inorganic antimicrobial material in this study. Domek et al. (1984) reported that in a very wide range of bacterial species, Cu^{2+} ion exhibited very high bactericidal activity in vitro. In recent years, various Cu(II)-organcomplexes displaying remarkable antimicrobial activity towards bacteria, yeasts or moulds have been synthesized (Carcelli et al., 1995; Jantova et al., 1997). Nevertheless, the use of inorganic antimicrobial agents has attracted more interest for the control of microbes (Okouchi et al., 1995; Wilczynski, 2000). The key advantages of inorganic antimicrobial agents are safety and stability, compared with organic antimicrobial agents.

The purpose of this study is to prepare an inorganic antibacterial agent—Cu(II)-exchanged montmorillonite (MMT-Cu) by cation exchange and to evaluate its antibacterial properties in vitro. The data from this study may be used as the foundation for the future development of new types of antibacterial agents to treat infectious disease in animal production.

2. Materials and methods

2.1. Materials

The montmorillonite sample used in this study was purchased from Inner Mongolia, China. After purification, its structural formula, as determined by chemical analysis, was $[Na_{0.158}K_{0.082}Ca_{0.256}Mg_{0.063}]$ $[Mg_{0.376}Fe^{2+}{}_{0.014}Fe^{3+}{}_{0.136}Al_{1.474}]$ $[Si_{3.87}Al_{0.13}]O_{10}$-$(OH)_2{\cdot}nH_2O$. The CEC of the montmorillonite was 127.4 mmol/100 g, determined by leaching with 1 M ammonium acetate at pH 7, washing with 90% ethanol, displacing the NH_4^+ with 1 M NaCl and measuring the amount displaced with an autoanalyzer (Theng et al., 1997). The specific surface area was 102.3 m^2/g, measured on NOVA ver. 3.70n by N_2 adsorption at 77 K and application of the BET equation (Stadler and Schindler, 1993).

2.2. Preparation of Cu(II)-exchanged montmorillonite

Methods used in the production of cation-exchanged clays are modifications of techniques