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RESEARCH ARTICLE

Antibacterial Activity of Chlorocresol Nanoemulsion Disinfectant Against *E. coli* and Its Application Evaluation

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Abstract

This study investigated the antibacterial activity and disinfection efficacy of chlorocresol nanoemulsion disinfectant (CND) against *Escherichia coli*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, and alterations in bacterial ultrastructure were examined using scanning and transmission electron microscopy (SEM and TEM). The disinfection performance was further evaluated through simulated and field surface disinfection trials. The results showed that the MIC and MBC of CND against *E. coli* were 100 μg/mL and 200 μg/mL, respectively. After 0.06% CND treatment for 10 min, SEM revealed disrupted surface architecture with flagella loss and cell wall damage, while TEM showed compromised internal integrity including periplasmic narrowing and cytoplasmic aggregation. The disinfectant efficacy of CND increased with increasing contact time and concentration. In simulated surface trials, effective disinfection was achieved with 0.1% CND applied for ≥15 min or with CND concentrations ≥0.2%. In field trials, effective disinfection was achieved with 0.05% CND applied for ≥10 min or with CND concentrations ≥0.1%. These findings indicate that CND exhibits strong antibacterial activity against E. coli, causes significant ultrastructural alterations, and demonstrates effective disinfection performance.

Keywords: Antibacterial activity, Chlorocresol nanoemulsion disinfectant, Disinfection efficacy, *E. coli*, Ultrastructure

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Introduction

The global transition of animal husbandry toward intensification and large-scale operations has led to high-density farming models. These models significantly enhance pathogen transmission and accelerate the spread of drug-resistant microorganisms, posing severe challenges to animal disease prevention and control [1,2]. To block the transmission of pathogenic microorganisms, environmental disinfection has become one of the most fundamental and critical measures for establishing a biosecurity system on farms [3,4]. Extensive evidence indicates that disinfection is an essential measure for the effective prevention and control of infectious diseases [5-7]. Disinfectants play a leading role in reducing the spread of pathogens among animals and their transfer from animals to humans as well as limiting the infections in animals and humans [6]. Currently, the thorough and standardized application of disinfectants in livestock housing and the environment is a key preventive strategy. It interrupts transmission routes, eliminates sources of infection, and inactivates pathogens. This approach represents one of the most efficient and practical methods for implementing a preventive disease control strategy. However, some disinfectants exhibit limitations, including unstable efficacy, a propensity to induce pathogen resistance, and the generation of substantial by-products, which restrict their widespread application [8,9]. Therefore, the development and practical implementation of novel disinfectants with high efficacy, environmental safety, and low cost is a key task in veterinary sanitation [5].

Chlorocresol is a chemical disinfectant composed of chlorine and phenols [10,11]. It is primarily used for

disinfecting livestock housing, vehicles, equipment, and the environment [12-14]. However, its development and clinical application are limited by several drawbacks, notably poor water solubility and a characteristic phenolic odor [12]. According to the Veterinary Drug Quality Standards (2017 edition, Chemical Drugs Volume), the existing formulation is Chlorocresol Solution [15]. Chlorocresol nanoemulsion disinfectant (CND) is a novel environmental disinfectant developed by our research group, with chlorocresol as the active ingredient. It offers several advantages, including good water solubility, reduced phenolic odor, strong disinfection efficacy, and a simple production process [16,17]. Additionally, our group has previously investigated its bactericidal effects and mechanism of action against common microorganisms found in livestock and poultry environments, such as *Staphylococcus aureus* [18] and *Candida albicans* [19]. While the efficacy of CND against these microorganisms has been established, its activity against Gram-negative bacteria, particularly *Escherichia coli,* a key indicator organism in environmental hygiene, remains unexplored. *E. coli* is a Gram-negative bacterium widely present in the natural environment. Given its impact on animal and human health, developing effective management strategies is essential [20]. The purpose of this study was to evaluate the antibacterial activity of CND against *E. coli*, its effects on bacterial ultrastructure, and its field disinfection efficacy. The findings are intended to support its future clinical application.

Material and Methods

Ethical Statement

This study did not require ethical approval.

Bacterial Strain

E. coli 8099 was used in accordance with the guidelines of China's Technical Standard for Disinfection (Ministry of Health of the People's Republic of China, 2002) [21]. This bacterial strain was maintained and supplied by the Animal Pharmacology Laboratory at Henan Institute of Science and Technology, in Xinxiang, China.

Disinfectant Preparation

CND was prepared as previously described by Yang et al.[17]. CND was an O/W nanoemulsion and its mean droplet size was 27.43 nm with normal distribution. The conductivity, viscosity and pH values were 479.67 μs/cm, 204.33 cp and 2.52, respectively. CND has good stability and could be stored for 2 years. Before use, CND was diluted to the required concentration with distilled water.

Neutralizer Selection

Selecting an appropriate neutralizer is essential for accurately evaluating disinfectant efficacy [22]. To ensure precise quantification of surviving microorganisms, a suitable neutralizer must be applied immediately after the contact period to rapidly inactivate any residual disinfectant, thereby preventing its continued antimicrobial action. To ensure accurate efficacy assessment, a neutralizer validation test was conducted following the Technical Standard for Disinfection (2002) [21]. The results confirmed that a solution of 3% Tween 80 in phosphate-buffered saline (PBS) effectively neutralized CND without affecting *E. coli* viability, and was therefore used as the appropriate neutralizer for all subsequent antimicrobial evaluations [17].

Determination of *In Vitro* Antibacterial Activity

The minimum inhibitory concentration (MIC) of CND against *E. coli* was determined using the broth dilution method, following the Technical Standard for Disinfection (2002) [21]. First, CND was diluted with distilled water to obtain a series of test solutions at concentrations of 10, 30, 50, 100, 200, 300, 400, and 500 μg/mL. Then, 2.5 mL of each test solution was added to tubes containing 2.5 mL of Mueller-Hinton broth (MHB) and mixed thoroughly. Subsequently, 100 μL of an *E. coli* suspension (10^8 CFU/mL) was inoculated into each tube to form the test groups. For the positive control, the bacterial suspension was inoculated into tubes containing MHB without disinfectant, following the same procedure. The tube served as the positive control group. Another tube containing only MHB medium was used as the negative control. All tubes from the test, positive control, and negative control groups were incubated at 37°C for 24 h, after which the results were observed and recorded. The MIC was considered as the lowest concentration capable of inhibiting 100% of microbial growth [23]. Furthermore, 1 mL of broth from each tube showing no visible growth was subcultured onto disinfectant-free agar medium. After incubation at 37°C for 24 h, the minimum concentration at which no bacterial growth was observed on the subculture was defined as the minimum bactericidal concentration (MBC). The experiment was repeated five times, and the mode values were taken as the final MIC and MBC [24].

Effects of CND on the Ultrastructure of *E. coli*

Sample Preparation

A 5 mL aliquot of an *E. coli* suspension (1×10^8 to 5×10^8 CFU/mL) was added to 45 mL of 0.06% CND and mixed thoroughly. After 10 min of exposure, the mixture was rapidly neutralized by adding 450 mL of a neutralizer solution (3% Tween 80) and mixed for an additional 10 min. The sample was then centrifuged at 6000 rpm for 3 min at 4°C. The supernatant was discarded, and the resulting bacterial pellet was washed three times with PBS by centrifugation. The final pellet was retained for subsequent analysis. For the control group, PBS was used in place of CND, while all other steps remained identical. Both the test and control samples were each divided into

two portions: one for observation by scanning electron microscopy (SEM) and the other for transmission electron microscopy (TEM).

Preparation and Observation of SEM Samples

The bacterial pellet was resuspended and fixed in 2.5% glutaraldehyde, then stored overnight at 4°C. It was subsequently centrifuged at 6000 rpm for 3 min at 4°C. After discarding the supernatant, the pellet was dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100%), with each step lasting 8 min. The sample was then resuspended in 100% ethanol for an additional 8 min. A drop of the suspension was evenly spread on a glass slide and air-dried. Finally, the sample was sputter-coated with gold. Morphological changes in *E. coli* before and after disinfectant treatment were observed and photographed using a scanning electron microscope (SEM, Quanta 200, FEI Company, USA). Control group samples were processed identically.

Preparation and Observation of TEM Samples

The bacterial pellet was fixed with 2.5% glutaraldehyde and then post-fixed with 1% osmium tetroxide. Dehydration was performed sequentially in 50% ethanol for 20 min, 70% ethanol overnight, 90% ethanol for 20 min, followed by another overnight incubation in 90% ethanol. The sample was further dehydrated in a 1:1 (v/v) mixture of 90% acetone and the bacterial suspension for 20 min, followed by 90% acetone for an additional 20 min. It was then infiltrated with 100% acetone three times (15 min each) at room temperature. Subsequently, the sample was embedded in resin and sectioned into ultrathin slices. The sections were stained with uranyl acetate and lead citrate, then observed and photographed using a transmission electron microscope (TEM, H-7500, digital CCD camera system, Hitachi Company, Japan). Control group samples were processed identically.

Simulated Field Trial for Surface Disinfection

A simulated field trial was conducted following a standard method for evaluating disinfection efficacy on general surfaces [21]. A wooden desktop was used as the representative test surface. The test microbial suspension was *E. coli* at a concentration of 2.5×10^7 to 1.25×10^8 CFU per sample, which was used to evaluate the disinfectant efficacy of CND.

The trial included a negative control group, a positive control group, and CND test groups. For the test groups, CND was applied at concentrations of 0.1%, 0.2%, 0.5%, and 1.0%. The contact times tested for each concentration were 5, 10, and 15 min.

The experimental procedure was conducted as follows: ① Wooden desktop surfaces were disinfected by applying different concentrations of CND. After 5, 10, and 15 min of contact, a sterile cotton swab was moistened in a tube containing 10 mL of neutralizer solution, pressed against the tube wall to remove excess liquid, and then used to swab the disinfected area. The swab tip was aseptically cut into the original neutralizer tube, mixed thoroughly, and subjected to bacterial culture and counting as the test group sample. ② For the positive control group, a sterile cotton swab was moistened in a tube containing 10 mL of PBS dilution solution, pressed against the tube wall, and then used to swab the control area. The swab tip was aseptically cut into the original dilution tube, mixed thoroughly, appropriately diluted, and then cultured for bacterial counting. ③ Samples of the neutralizer solution, dilution solution, swabs, and culture media from the same batch were cultured and counted as the negative control. ④ The test was repeated three times. Killing log (KL) value was calculated as follows: KL value = l g (viable bacterial concentration in the positive control) - l g (viable bacterial concentration in the test group). According to the Chinese Technical Standard for Disinfection (2002), effective disinfection was defined as achieving a mean KL value ≥3 for the simulated field trial.

Field Trial for Surface Disinfection

A field trial was conducted on the concrete floor of the Animal Physiology Laboratory at Henan University of Science and Technology to evaluate the disinfection efficacy of CND. The procedure followed the standard method for field evaluation of disinfection efficacy on general surfaces [21], as described in reference [17]. The experimental groups and contact times were identical to those used in the simulated field trial. However, the disinfectant concentrations tested for the CND groups were 0.05%, 0.1%, 0.2%, and 0.5%. The test was repeated three times. According to the Chinese Technical Standard for Disinfection (2002), effective disinfection was defined as achieving a mean KL value ≥1 for the field trial, whereas a mean KL value ≥3 was required for the simulated field trial.

Results

In Vitro Antibacterial Activity

After incubation, no visible bacterial growth was observed in the negative control tube or in tubes containing CND at concentrations ranging from 100 to 250 μg/mL. In contrast, visible growth was observed in the positive control tube and in tubes with CND concentrations between 5 and 50 μg/mL. These results indicated that the MIC of CND against *E. coli* was 100 μg/mL. Subsequently, broth from tubes containing CND at concentrations of 100 to 250 μg/mL was subcultured onto agar medium. Bacterial growth was observed from subcultures of tubes with CND concentrations between 100 and 150 μg/mL,

while no growth occurred from those with concentrations between 200 and 250 µg/mL. These findings demonstrate that the MBC of CND against *E. coli* was 200 µg/mL.

Effects of CND on the Ultrastructure of *E. coli* Cells

SEM Observations

SEM observations of *E. coli* were shown in *Fig. 1*. Untreated *E. coli* cells exhibited a short, rod-shaped morphology with peritrichous flagella and rounded ends. The cell surfaces were smooth, without folds, protrusions, or depressions *(Fig. 1-A)*. After treatment with CND for 10 min, significant alterations in surface structure were observed. The peritrichous flagella were absent, and the cell surfaces became rough and uneven. Some bacteria exhibited distinct depressions or protrusions *(Fig. 1-B)*. In other cells, damage to the cell wall structure was observed, characterized by spiky projections *(Fig. 1-C)* or severe surface disruption leading to fragmentation, accompanied by wrinkling and spiky protrusions *(Fig. 1-D)*.

TEM Observations

TEM observations of *E. coli* were shown in *Fig. 2*. Untreated cells displayed a short, rod-shaped morphology with intact structure. A clear periplasmic space was observed between the thick, electron-dense outer cell wall and the inner cell membrane. The cytoplasm was uniformly distributed, and no gaps were observed between the cytoplasmic boundary and the cell membrane *(Fig. 2-A)*. After treatment with CND for 10 min *(Fig.2-B,C)*, the cellular architecture was altered. The periplasmic space appeared markedly narrower than that of untreated cells. In most bacteria, the cell wall appeared thinned, ruptured, or entirely absent. Gaps of varying sizes formed between the cytoplasmic boundary and the cell membrane. Furthermore, the intracellular contents exhibited an uneven distribution with aggregation.

Results of Simulated Field Trial for Surface Disinfection

The results of the simulated field trial evaluating the efficacy of CND in disinfecting general surfaces were presented in *Table 1*. The disinfectant efficacy of CND increased with longer contact time and higher concentrations. When surfaces were treated with 0.1% CND for 5 or 10 min, mean KL values were below 3, and thus did not meet the disinfection criterion. However, when the contact time was extended to ≥15 min, mean KL values exceeded 3, indicating effective disinfection. For CND concentrations ≥0.2%, mean KL values consistently exceeded 3 under all tested contact times, demonstrating effective disinfection. These results indicated that CND exhibited effective disinfection performance under simulated field conditions.

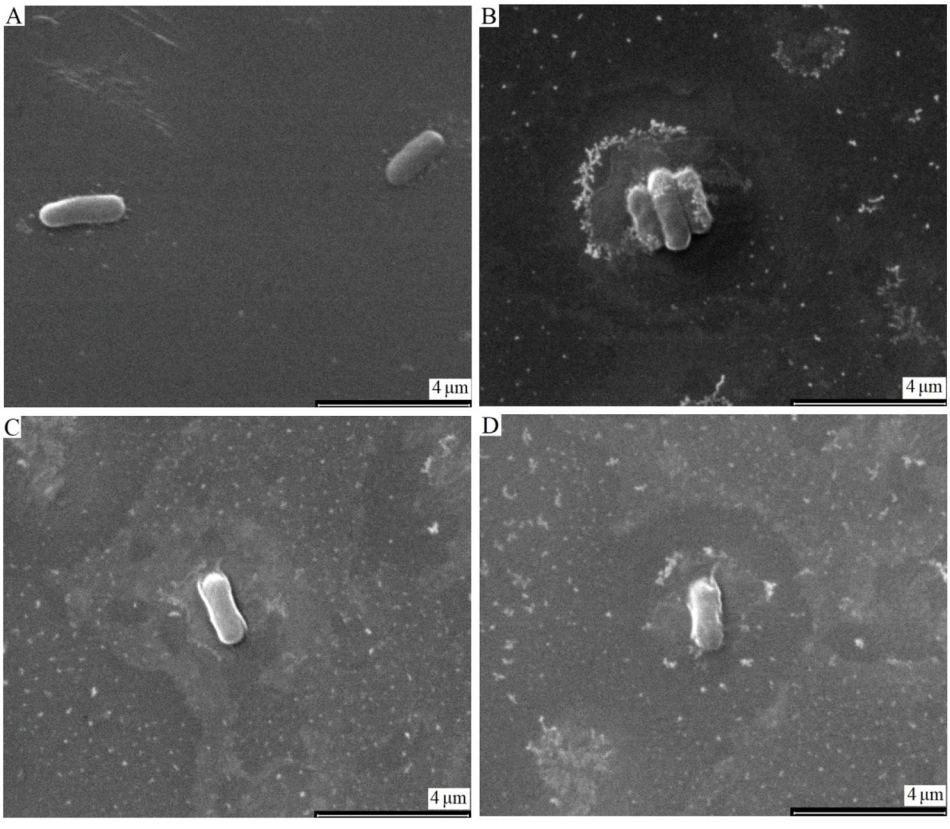

Fig 1. Scanning electron microscope observation about the effect of CND on the ultrastructure of *E. coli*. **A-** *E. coli* of the control group (×12 000); **B, C** and **D-** *E. coli* disinfected with CND for 10 min (× 12 000)

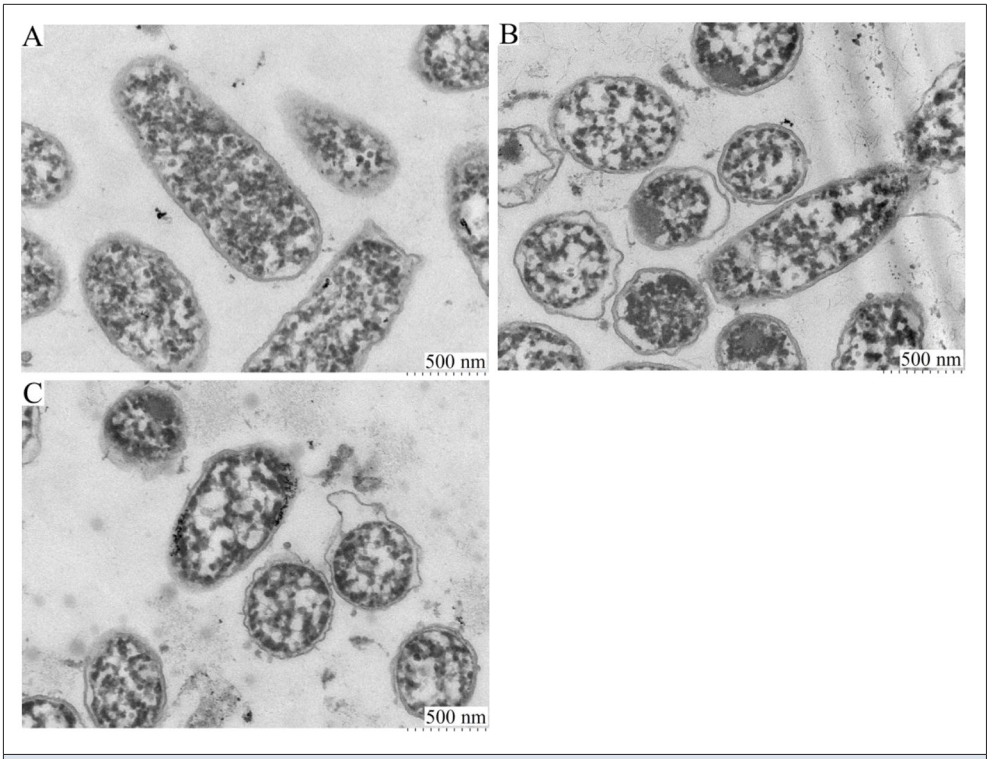

Fig 2. Transmission electron microscope observation about the effect of CND on the ultrastructure of *E. coli*. **A**- *E. coli* in the control group (×12 000); **B** and **C**- *E. coli* disinfected with CND for 10 min (×12 000)

Results of Field Trial for Surface Disinfection

The results of the field trial evaluating the surface disinfection efficacy of CND were presented in *Table 2*. The disinfectant efficacy increased gradually with longer contact time and higher concentrations. When 0.05% CND was applied for 5 min, the mean KL value was less than 1, and thus did not meet the disinfection standard. However, with contact time ≥10 min, mean KL values exceeded 1, indicating effective disinfection. For

Table 1. *Results of simulated field trial for surface disinfection*

Group	Log Values of Viable Bacteria Concentration After Disinfecting for Different Time			Mean KL Values After Disinfecting for Different Time		
	5 min	10 min	15 min	5 min	10 min	15 min
0.1% CND	4.78	5.05	4.46	2.97	2.70	3.29
0.2% CND	4.62	4.54	4.39	3.13	3.21	3.36
0.5% CND	4.58	4.30	3.07	3.17	3.45	4.68
1.0% CND	4.44	3.92	2.62	3.31	3.83	5.13

Log value of viable bacteria concentration was 7.75 in the positive control group, and no bacterial growth occurred in the negative control groups

Table 2. *Results of field trial for surface disinfection*

Group	Log Values of Viable Bacteria Concentration After Disinfecting for Different Time			Mean KL Values After Disinfecting for Different Time		
	5 min	10 min	15 min	5 min	10 min	15 min
0.05% CND	2.79	2.60	2.50	0.87	1.06	1.16
0.1% CND	2.62	2.18	1.60	1.04	1.48	2.06
0.2% CND	1.95	1.43	1.11	1.71	2.23	2.55
0.5% CND	1.41	-	-	2.25	3.66	3.66

Log value of viable bacteria concentration was 3.66 in the positive control group, and no bacterial growth occurred in the negative control groups

disinfectant concentrations ≥0.1%, all applications with contact time ≥5 min yielded mean KL values greater than 1, meeting the disinfection requirement. Notably, the 0.5% CND solution achieved complete inactivation of natural microorganisms on surfaces when applied for 10 min or longer. These results demonstrated that CND exhibited effective disinfection performance under field conditions.

Discussion

Disinfectants are effective in inhibiting or eliminating microorganisms on surfaces and within transmission media, and are extensively applied in various industries. Thorough and standardized disinfection of livestock housing and the environment serves as a crucial preventive measure to block transmission routes, eliminate sources of infection, and inactivate pathogens. It is also one of the most efficient and practical approaches for disease prevention. *E. coli* is an opportunistic pathogen widely present in nature. Although it can cause severe illnesses in humans and animals, it also plays a significant role in the commensal microbiota [20]. Therefore, *E. coli* serves as an ideal indicator organism for evaluating the efficacy of environmental disinfectants. Following the Technical Standard for Disinfection (2002) [21], this study used *E. coli* to evaluate the antibacterial activity of CND, a novel environmental disinfectant, as well as its effects on bacterial ultrastructure and its field disinfection efficacy.

Some nanomaterial-based disinfectants have been reported in recent years. Jamshidinia et al. demonstrated that nanomaterial-augmented formulations enhanced the antiviral activity of disinfectants and antiseptics against respiratory viruses, particularly SARS-CoV-2 [25]. These formulations are thought to enhance disinfectant efficacy by inhibiting viral penetration into cells, disrupting the lipid bilayer envelope, and increasing reactive oxygen species production. Similarly, sandalwood nanoemulsion had shown strong antibacterial efficacy and could be used as a disinfectant for various applications, including hand hygiene, surface disinfection, and surgical instrument sterilization [26]. Risso et al.[23] demonstrated that chlorhexidine nanoemulsion could decrease chlorhexidine concentrations. Both *in vitro* and *in vivo* experiments indicated its potential as an antiseptic for cutaneous microbiota. In another study, nano-emulsified cresol disinfectant had been reported for environmental disinfection in biosafety cabinets (BSCs). It is considered an ideal alternative to formaldehyde and chlorine dioxide for BSCs disinfection [27]. Collectively, these findings highlight the potential of nanostructured formulations as effective disinfectants [23], supporting the development of CND as a novel nanoemulsion-based disinfectant for environmental applications.

To evaluate the antibacterial activity of CND, its in *vitro* efficacy against *E. coli* was first examined. The results showed that the MIC and MBC of CND against *E. coli* were 100 μg/mL and 200 μg/mL, respectively. Our previous findings demonstrated that the MIC and MBC of CND against *S. aureus* were 100 μg/mL and 200 μg/mL, respectively [18]. In contrast, both the MIC and MBC for *Candida albicans* were 3 mg/mL [19]. In the present study, CND showed strong inhibitory activity against *E. coli* (Gram-negative). Taken together with our previous findings showing its efficacy against *S. aureus* (Gram-positive) and *Candida albicans* (fungus), these results indicate that CND possesses broad-spectrum antimicrobial activity. Notably, its bactericidal potency against both Gram-negative and Gram-positive bacteria appears to be greater than its fungicidal activity.

Previous studies from our research group indicated that exposure to 0.06% CND for 10 min led to varied outcomes in quantitative suspension tests [17]. Some bacterial cells died, some survived, and others showed partial loss of structural integrity. To investigate the antibacterial mechanism of CND against *E. coli*, bacterial samples were treated with 0.06% CND for 10 min and then processed into ultrathin sections for electron microscopic observation of ultrastructural changes. SEM observations showed that CND exerts its antibacterial effect by damaging the surface structure of bacterial cells. TEM further revealed that CND disrupted the internal structural integrity of *E. coli*, contributing to its antibacterial activity. The bacterial cell wall is a specialized structure that serves not only as the primary barrier against external threats but also plays essential roles in maintaining cell morphology, providing protection against damage, and mediating adhesion to host cells. These observations underscore the critical role of cell wall integrity in bacterial survival. Once the cell wall or membrane is compromised, bacteria lose their normal shape; the exchange of materials across the membrane is hindered; and membrane-associated enzymes fail to function properly. This reduces nutrient uptake and ultimately inhibits bacterial growth. Other studies have shown that upon contact with microorganisms, nanoemulsions fuse with the microbial cell membrane via their lipid bilayers, releasing the energy stored in the oil and disinfectants. This process destabilizes the bacterial lipid membrane, ultimately leading to cell death [26]. The present results suggest that one likely mechanism of CND against *E. coli* involves inducing ultrastructural alterations in bacterial cells. However, as this conclusion is primarily based on morphological observations, it remains partly hypothetical within the scope of the current study and requires further mechanistic validation.

Disinfection is a key measure for interrupting pathogen transmission, and evaluating its efficacy is a primary focus in environmental hygiene. To further evaluate the environmental disinfectant performance of CND

and support its future practical application, simulated and field trials for surface disinfection were conducted. The results demonstrated that the disinfectant efficacy of CND improved with longer contact time and higher concentrations. In the simulated surface disinfection trial, effective disinfection was achieved with 0.1% CND applied for ≥15 min or with CND concentrations ≥0.2%. In the field trial, effective disinfection was achieved with 0.05% CND applied for ≥10 min or with CND concentrations ≥0.1%. Notably, 0.5% CND applied for ≥10 min completely inactivated natural microorganisms on surfaces, resulting in a 100% bactericidal rate. These findings indicate that CND performs effectively under field conditions. Previous studies from our group have also shown that CND exhibits stronger disinfectant efficacy than conventional chlorocresol formulations [17-19]. Therefore, CND has great potential for use as a new-generation disinfectant. Extensive literature indicates that nanoemulsions serve as an ideal drug delivery system with unique advantages over other carriers, such as enhancing the solubility of poorly soluble drugs, improving bioavailability, and promoting transdermal absorption [28-30]. Nanoemulsions can be exploited in the formulation of sanitizers or related products with antibacterial activity [26,31]. In this study, CND was developed as a novel formulation using a nanoemulsion as the drug carrier. Its superior field disinfection efficacy is likely attributable to the distinctive properties of this nanoemulsion-based delivery system. CND is an oil-in-water nanoemulsion [17], in which chlorocresol is encapsulated within the nanoemulsion droplets. The resulting nanoscale particle size facilitates penetration through the bacterial cell wall, increasing the intracellular drug concentration and enhancing antibacterial activity [18], thereby contributing to its potent bactericidal effect. It has also been reported that the reduced particle size may enhance passive cellular absorption, facilitating drug entry into microbial cells and thereby increasing antimicrobial activity and improving the therapeutic index [23,32]. Furthermore, the nanoemulsion structure itself has been reported to possess broad-spectrum antimicrobial properties [33].

Several limitations of this study should be acknowledged. This study focused only on *E. coli* under controlled conditions; the efficacy of CND against other Gram-negative bacteria, drug-resistant strains, viruses, and spores remains unknown. Furthermore, the precise molecular mechanism of action has not been fully elucidated. Although our research group has systematically evaluated CND against multiple microorganisms, future studies should focus on elucidating the molecular mechanism using biochemical and omics approaches, evaluating its antiviral and sporicidal activity, and conducting long-term safety and ecotoxicological assessments.

In conclusion, this study evaluated the antibacterial efficacy of CND against *E. coli* by determining the MIC and MBC. The effects of CND on bacterial ultrastructure were examined using SEM and TEM. Furthermore, its disinfection performance was assessed through simulated and field trials on general surfaces. The results demonstrate that CND exhibits strong antibacterial activity against *E. coli*, induces significant alterations in cellular ultrastructure, and demonstrates effective disinfection under field conditions. Therefore, CND represents a promising environmental disinfectant, although further toxicity and *in vivo* safety assessments are needed before clinical application.

Declarations

Availability of Data and Materials: Data and materials for this research are available upon request.

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Competing Interests: The authors declare that there is no competing of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: Conceived of or designed study: XF Y, CZ L; Performed research: XF Y, YW S; Analyzed data: ZX A, DY L; Wrote the paper: XF Y. All authors critically reviewed and approved the final version of the manuscript.

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