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Linalool as a potential agent for inhibiting *Escherichia coli* biofilm formation and exopolysaccharide production

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Abstract

Escherichia coli (*E. coli*) is one of the most common pathogens causing endometritis in dairy cows. The presence of genes encoding extended-spectrum β-lactamase (ESBL) and biofilm formation are important factors contributing to bacterial resistance, which poses a significant challenge to the treatment of endometritis in dairy cows. Essential oils containing linalool have been shown to improve the cure rate of bovine endometritis, but whether linalool can inhibit *E. coli* biofilm has not yet been reported. We proposed to ascertain the linalool implications on the development of *E. coli* biofilm and its extracellular polysaccharides, as well as to assess the impacts of linalool on *E. coli* in both planktonic and biofilm states. We discovered that the minimum biofilm inhibitory concentrations (MBICs) of linalool against *E. coli* were twice as high as the minimum inhibitory concentrations. Linalool exhibited a strong bactericidal effect on clinical *E. coli* strain producing ESBL and forming strong biofilm, regardless of whether they were in a planktonic or biofilm condition. Linalool suppressed the biofilm development in a way that was dependent on the dosage, with an MBIC 4 μL/mL. This was verified by the use of crystal violet test and scanning electron microscopy. Moreover, the CCK-8 assay and confocal laser scanning microscopy (CLSM) manifested significant reductions in live bacteria within the biofilm. The concentrations of extracellular polymeric compounds in the *E. coli* biofilm were also reduced. Furthermore, CLSM and RT-qPCR analysis confirmed that linalool (2 μL/mL) significantly suppressed exopolysaccharide (EPS) and the *pgaABCD* gene expression, regulating an essential exopolysaccharide expression in biofilm formation. These findings revealed that linalool effectively suppressed viable bacteria, EPS production, and *E. coli* biofilm formation, providing a theoretical foundation for alternative antibiotic therapy in endometritis in dairy cows and as a potential agent for preventing *E. coli* biofilm-related infections.

Keywords Linalool, *Escherichia coli*, Biofilm, Exopolysaccharide

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Introduction

Endometritis is an important disease damaging the reproductive performance of dairy cows, primarily caused by pathogenic bacteria. *Escherichia coli* (*E. coli*) is one of the most common pathogens causing endometritis in dairy cows, and the multidrug resistance strains are increasing [4, 54]. The presence of genes encoding extended-spectrum β-lactamase (ESBL) and biofilm formation are important factors contributing to bacterial resistance, often leading to failure in antibiotic treatment [22, 28]. However, it still mainly relies on antibiotic in the treatment for endometritis in dairy cows at present. Therefore, it is crucial to discover new alternatives to antibiotics for the prevention and treatment of endometritis in dairy cows.

Linalool, 3, 7-dimethyl-1, 6-octadien-3-ol, is a major component identified in multiple plant essential oils encompassing camphor oil (*Cinnamomum camphora*), basil oil (*Ocimum basilicum*), and lavender oil (*Lavandula angustifolia*) [6, 20, 50, 51]. Linalool possesses a range of pharmacologically beneficial properties, including anti-bacterial [21, 46], anti-inflammatory [32], analgesic [27], antioxidant [3], tissue-protective [15], and anti-cancer biological effects [44, 55]. Linalool demonstrates broad-spectrum anti-bacterial activity with its mechanism of action against various bacterial strains encompassing *Salmonella Senftenberg*, *E. coli*, and *Listeria monocytogenes*, which involves disrupting cell membranes and causing leakage of cytoplasmic contents [13, 23, 53]. Additionally, biofilm formation of *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Candida albicans* is inhibited by linalool [10, 31, 38]. Previous study reported that addition of 20% camphor oil improved the cure rate of the disease, when chloramphenicol and furacillin were used to treat persistent infective endometrium in cows [40]. In our laboratory, medicine containing 4% camphor oil was used to treat endometritis in dairy cows, and the cure rate was 72.9% (The data have not been published). And we found that camphor oil significantly inhibited *E. coli* biofilm [50]. As the main component of camphor oil, we speculated that linalool might play an important role in the process. However, there are no systematic studies on the effect of linalool on *E. coli* biofilm.

Bacterial biofilm is a complex and organized population of bacteria that attach to surfaces, and create a protective layer made of complex extracellular polymeric substances, making them harder to eliminate than plankton. This layer protects bacteria from evading antibiotics and host defense and cause persistent and recurring bacterial infections [7]. The extracellular polymeric substance of biofilm mainly contains exopolysaccharides (EPS), proteins, DNA, and other molecules [11, 26]. EPS participated in the development and growth of bacterial biofilms [8, 43]. Targeting the creation of EPS has been shown to effectively limit the development of biofilms. Disrupting the genes responsible for EPS production leads to the bacteria's inability to build a mature biofilm [1, 16, 35]. This investigation assessed linalool's capacity to hinder *E. coli* biofilm formation by evaluating the biofilm's biomass, morphology, and extracellular polymeric substances. Further investigations were conducted into its anti-bacterial properties and impact on EPS, a crucial biofilm component. This study aims to reveal the inhibitory effect of linalool on *E. coli* biofilm and related mechanisms, and provide a theoretical basis for the alternative antibiotic therapy to treat endometritis in dairy cows and a potential agent for infections associated with *E. coli* biofilm.

Materials and methods

Bacterial strains and growth conditions

In the present study, the strain *E. coli* D5, isolated from the uterine mucus of Holstein cows with clinical endometritis and characterized by its production of ESBL and its ability to form robust biofilms, was preserved at the Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences. The control strain, *E. coli* ATCC 25,922, was obtained from the American Type Culture Collection. Both strains were cultured in Nutrient Broth Medium at 37 °C for 24 h before the experiment.

Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The MICs and MBCs of linalool (Sigma-Aldrich, USA) against *E. coli* D5 and ATCC 25,922 were ascertained using the broth micro-dilution technique [29] with some changes. Linalool was subjected to serial two-fold dilutions, with concentrations ranging from 0.25 to 128 μL/mL in Mueller-Hinton (MH) broth containing 1% dimethyl sulfoxide (DMSO) (v/v) and *E. coli* was inoculated with 1×10^5 CFU/mL. In addition, various controls were established, such as a solvent control consisting of test bacteria and MH broth with 1% DMSO, a bacterial control consisting of test bacteria and MH broth, a blank control consisting of MH broth with 1% DMSO and corresponding linalool concentrations, a blank solvent control consisting of MH broth with 1% DMSO, and a blank medium consisting of MH broth. Bacterial cultures were incubated at 37 °C for 24 h and then $OD_{600\ nm}$ values were detected by using a BioTek Synergy LX multi-mode reader (Agilent, USA). The MIC was defined as the lowest concentration of linalool that inhibits ≥ 80% of growth as compared to the control without linalool. To determine the MBC, 100 μL aliquots from the MIC and three subsequent higher concentrations were aseptically transferred to MH agar plates, followed by incubation at 37 °C for

24 h. The number of colonies on the agar was counted, and the MBC was identified as the lowest linalool concentration resulting in no colony being observed. Three replicates were employed to test the MIC and MBC.

Estimation of minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs)

The MBICs of linalool against *E. coli* D5 and ATCC 25,922 were measured by the micro-dilution technique with minor adjustments [2]. Linalool was subjected to serial two-fold dilutions, with concentrations ranging from 0.25 to 128 µL/mL in Luria-Bertani (LB) broth containing 1% DMSO and *E. coli* was inoculated with 1×10^7 CFU/mL. Furthermore, the experimental configuration included multiple controls: a solvent control, which comprised LB broth containing test bacteria and 1% DMSO; a bacterial control, which comprised LB broth and test bacteria; a blank control, which comprised LB broth containing 1% DMSO and varying doses of linalool; a blank solvent control which comprised LB broth containing 1% DMSO; and a blank medium, which comprised LB broth. The bacteria were cultivated for 24 h at 26 °C. Following that time, the medium was withdrawn. Biofilm was stained with a 0.3% (w/v) crystal violet solution. The examination of absorbance was performed at 600 nm utilizing a BioTek Synergy LX multi-mode reader (Agilent, USA). MBIC of linalool was found to be the lowest concentration at which it produced a minimum of 90% decrease in biofilm formation, compared to the control group without linalool.

The MBECs of linalool against *E. coli* D5 and ATCC 25,922 were measured employing the micro-dilution technique, with minor adjustments [42]. The bacteria (1×10^7 CFU/mL) were cultivated at 26 °C for 24 h and then the samples underwent three rounds of washing with PBS. Subsequently, biofilm was treated with linalool in serial two-fold dilutions from 0.25 µL/mL progressing up to 128 µL/mL. Furthermore, several controls were implemented, including the solvent control (consisting of LB broth with biofilm and 1% DMSO), the biofilm control (consisting of LB broth with biofilm), the blank control (consisting of LB broth with 1% DMSO and the matching linalool concentrations), the blank solvent control (consisting of LB broth with 1% DMSO), and the blank medium (consisting of LB broth). After incubating for another 24 h at 26 °C, Finally, the specimen was stained with a 0.3% (w/v) solution of crystal violet, and the detections were carried out using the previously indicated procedure. The MBEC of linalool was defined as the smallest concentration at which at least 80% of biofilms were eradicated, compared to the control group lacking linalool.

Planktonic Time-dependent killing assay

E. coli D5, with an initial concentration of 1×10^5 CFU/mL, was subjected to incubation at 37 °C. During this process, linalool was introduced at final concentrations: 0, 1, 2, and 4 µL/mL in MH broth including 1% DMSO. At time intervals of 5, 15, and 30 min, as well as 1, 2, 4, 8, 12, and 24 h, a volume of 100 µL solution was extracted from each sample and then diluted in a series. Afterward, they were inoculated onto MH agar and placed in an incubator at 37 °C for 24 h. Ultimately, the quantity of functional *E. coli* cells was ascertained by the enumeration of the colonies that were produced. The minimum detectable concentration was 10 CFU/mL. Readings were acquired at time 0 before the addition of linalool. Measurements were carried out in three separate experiments. Time-kill curves were constructed by plotting the average colony counts (log10 CFU/mL) versus time.

Determination of biofilm Inhibition

The linalool impact on the suppression of biofilm formation by *E. coli* D5 was evaluated by employing the micro-dilution approach with minor modifications [2]. Bacterial suspensions were prepared in LB broth at a concentration of 1×10^7 CFU/mL and treated with linalool at concentrations of 0, 1, 2, and 4 µL/mL in 1% DMSO in LB broth at 26 °C. The specimens were collected at certain time intervals of 5, 15, and 30 min, as well as 1, 2, 4, 8, 12, 24, 48, 72, and 96 h. The biofilm was then determined using the crystal violet technique described earlier.

Biofilm Time-dependent killing assay

Firstly, 100 µL of *E. coli* D5 suspension (2×10^7 CFU/mL) was added to each individual well in a 96-well plate. The solution of 0, 2, 4, and 8 µL/mL linalool was prepared by adding linalool to an LB medium containing 1% DMSO (v/v). Each well received 100 µL of the linalool solution. The culture plates were incubated at 26 °C for varying periods of time, for 5, 15, and 30 min, and 1, 2, 4, 8, 12, 24, 48, 72, and 96 h. The culture media was removed and then washed three times with PBS. Following the addition of 200 µL of LB broth to each well, 40 µL of CCK-8 solutions (CCK-8, Beyotime Biotechnology, China) was then added to the wells containing the biofilm. The measurement of absorbance was performed at a specific wavelength of 450 nm after a period of incubation for 2 h at 37 °C. The assessments were conducted in three separate trials.

Analysis with scanning Electron microscopy (SEM)

The impact of linalool on the development of biofilms was investigated using SEM, following a previously established protocol with minor modifications [25]. *E. coli* D5 was grown in LB broth containing 1% DMSO (v/v) with linalool added at doses of 0, 1, 2, and 4 µL/mL. The

bacterial concentration was 1×10^7 CFU/mL. The specimens were cultured to generate biofilms on an 8 mm glass coverslip placed in a 24-well polystyrene plate. The culture was left undisturbed for 24 h at 26 °C. In addition, there was a control for antibiotics at a dose of 2 mg/mL of ampicillin. The specimens were treated with 2.5% glutaraldehyde solution for 4 h, rinsed with PBS, and then subjected to a stepwise dehydration process employing various levels of ethanol (30, 50, 70, 85, 90, and 100%) for 15 min at each level. Once the specimens were dried, they underwent a gold coating process using a sputter coater. After that, they were imaged utilizing an SEM (JSM-5600, JEOL, Japan).

Confocal laser scanning microscopy (CLSM) analysis

The linalool impact on living bacteria throughout the process of biofilm development was investigated by employing CLSM. *E. coli* D5 (1×10^7 CFU/mL) were cultured with linalool at final concentrations of 0, 1, and 2 μL/mL for 24 h without agitation at 26 °C. The biofilms were stained for duration of 15 min using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen, France). Subsequently, the specimens were cleansed using PBS and observed using a CLSM (LSM 700, Zeiss, Germany). The excitation/emission peaks for PI were at 305/617 nm, whereas for SYTO 9, they were roughly 483/500 nm. Each sample was photographed in six fields, and all samples were examined in three separate tests. The CLSM pictures were analyzed using COMSTAT to measure the biomass of both living and dead cells [19, 48].

The CLSM was also used to observe the changes in EPS throughout the process of biofilm development. *E. coli* D5 (1×10^7 CFU/mL) were exposed to linalool at final doses of 0, 1, and 2 μL/mL for 24 h at a constant temperature of 26 °C without agitation. The biofilms were stained using FITC-ConA/PI double staining. The specimens were treated with a 4% solution of paraformaldehyde for duration of 10 min. Then, they were stained in a light-restricted environment using FITC-ConA for 15 min. Finally, they were treated with PI for duration of 10 min.

Table 1 Specific primers for *PgaABCD* used for quantitative RT-PCR

Gene	Primer	Sequence (5′-3′)
pgaA	*pgaA*-F	AGGCTTATGTTCGCTGGTATC
	pgaA-R	TAGTATGGGGTATCGTGTTCTG
pgaB	*pgaB*-F	AAACATCCCTCAGGCTAAAGAC
	pgaB-R	CATTCAGTTGTAATAGGCTCATCC
pgaC	*pgaC*-F	GGCGTCTATTTCTGGGTCTATC
	pgaC-R	GCGGCGTGTATGGTTTCC
pgaD	*pgaD*-F	TCTGCTGACGGGTTATTACTG
	pgaD-R	TTGCGGCGTATATTGGTAGG
16 S	*16 S*-F	CTGGAACTGAGACACGGTCC
	16 S-R	GGTGCTTCTTCTGCGGGTAA

The excitation/emission peaks for FITC-ConA stain were at 495/519 nm, whereas for PI, they were roughly 305/617 nm. Following the staining process, the specimens were rinsed with PBS and visualized using a CLSM. Each sample consisted of six fields, and all samples were conducted in three separate trials. The CLSM pictures were further analyzed using COMSTAT to quantify the biomass of EPS and bacteria.

Quantification of EPS, protein, and DNA in biofilm

Each well of 96-well sterile polystyrene plates was inoculated with about 10^7 CFU/mL of *E. coli* D5, along with linalool solutions at concentrations of 0, 1, 2, and 4 μL/mL. The plates were then placed in an incubator set at 26 °C for 24 h to promote the biofilm growth. The wells were subsequently rinsed with PBS in a gentle manner to remove any planktonic microorganisms. After the samples were allowed to dry naturally, 200 μL of physiological saline solution was introduced to each well. Ultrasonication was used to disturb the biofilm. The identical samples were combined and subjected to centrifugation at a speed of 5000 g for duration of 30 min. The liquid portion was employed to quantify the concentration of EPS, proteins, and DNA. The EPS content in the biofilm was quantified using the phenol-H_2SO_4 reagent method. The protein content in the biofilm was estimated using a BCA protein assay kit (Beijing Solarbio Science & Technology Co., China). The DNA was isolated by deploying a bacterial DNA kit (Omega, USA). The $OD_{260\ nm}$ was measured using a UV-visible spectrophotometer (Liu et al., 2017).

RNA extraction and RT-qPCR detection of *PgaABCD* expression

The bacterial cells in the *E. coli* D5 biofilm, cultivated on 96-well polystyrene plates, were collected following the procedure described above, both with and without the presence of linalool. The RT-qPCR method was used to examine the transcription levels of *pgaABCD* in *E. coli* that were either treated with linalool or left untreated while undergoing biofilm formation. The gene-specific primers for RT-qPCR may be found in Table 1. The RNA isolation process was carried out utilizing a Bacterial RNA Kit (OMEGA, USA). The same amount of RNA in each group was used for reverse transcription, and cDNA was synthesized employing a PrimeScript™ reagent Kit in conjunction with a gDNA Eraser (Takara, Japan). Using TB Green™ Premix Ex Taq™ II (Takara, Japan) and the Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific Inc., USA), the RT-qPCR amplifications were conducted. Each sample underwent three separate tests. The determination of relative gene expression was conducted utilizing the 2−ΔΔCt method.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics 25 (SPSS Inc., USA). A one-way ANOVA was used to find significant variations among the data. Variations were deemed significant at $p < 0.05$.

Results

Anti-bacterial activity of Linalool and its implications on planktonic growth of *E. coli*

The antimicrobial effectiveness of linalool was evaluated by determining MIC, MBC, MBIC, and MBEC values. MIC and MBC values of linalool against *E. coli* D5 were 2 and 4 µL/mL, respectively. The MBIC and MBEC values were both 4 µL/mL (Fig. 1A). The MIC, MBC, MBIC, and MBEC values of linalool against *E. coli* ATCC25922 were 1, 2, 2, and 4 µL/mL. The growth curves were used to estimate the linalool impact on the *E. coli* growth (Fig. 1B). Following a 24-hour exposure to a concentration of 1 µL/mL linalool, the quantity of viable *E. coli* cells was reduced by 6 $\log_{10}$ in CFU/mL, resulting in a 99.9999% reduction compared to the control. The quantity of viable cells was significantly reduced after treatment with a concentration of 2 µL/mL linalool. No live *E. coli* was found between 5 min and 12 h. Nevertheless, detectable *E. coli* cells (about 360 CFU/mL) were present until 24 h. No viable *E. coli* cells were observed throughout the observation period after treatment with 4 µL/mL linalool. The rate of death varied based on the dose of linalool within the studied concentration range.

Anti-Biofilm activity of Linalool and SEM observation of biofilm formation

The development of *E. coli* biofilms was inhibited by linalool using quantitative crystal violet assay, as presented in Fig. 2. In the untreated control, biofilm growth began to increase at 4 h, surged rapidly from 12 to 48 h, peaked from 48 to 72 h, and then declined at 96 h. Compared to the control, linalool at a concentration of 1 µL/mL significantly inhibited biofilm formation from 4 h ($p < 0.01$). Linalool at 2 µL/mL inhibited more than 90% of biofilm formation from 4 h to 24 h, and then the inhibition ratios decreased over time. Complete inhibition of biofilm formation was achieved with 4 µL/mL linalool. There were significant variations in the effectiveness of biofilm inhibition between 1 and 2 µL/mL linalool after 4 h and between 2 and 4 µL/mL linalool after 12 h. SEM analysis revealed the implications of linalool on the structure of *E. coli* biofilms at 24 h, as depicted in Fig. 3. In the untreated control, the *E. coli* biofilms appeared densely arranged. With increasing concentrations of linalool, the biofilms became thinner and sparser compared to the control. The biofilm was reduced at 1 µL/mL linalool, and significant cellular disruption and loss of morphological features were observed at 2 µL/mL linalool. At 4 µL/mL linalool, *E. coli* was barely visible. Additionally, *E. coli* biofilm decreased with the use of 2 mg/mL ampicillin sodium.

Linalool implications on viable Bacteria growth throughout biofilm formation

The bacteria growth of *E. coli* during biofilm formation was estimated using the CCK-8 method. In the control group, the growth trend of bacteria in the biofilm was similar to that of the biofilm formation above. However, it reached the highest at 48 h and then decreased (Fig. 4). Linalool at 1 and 2 µL/mL significantly inhibited bacterial proliferation in the formation of *E. coli* biofilm ($p < 0.05$). The bacterial inhibition rate for 1 µL/mL linalool was highest at 24 h. And it was even higher for 2 µL/mL

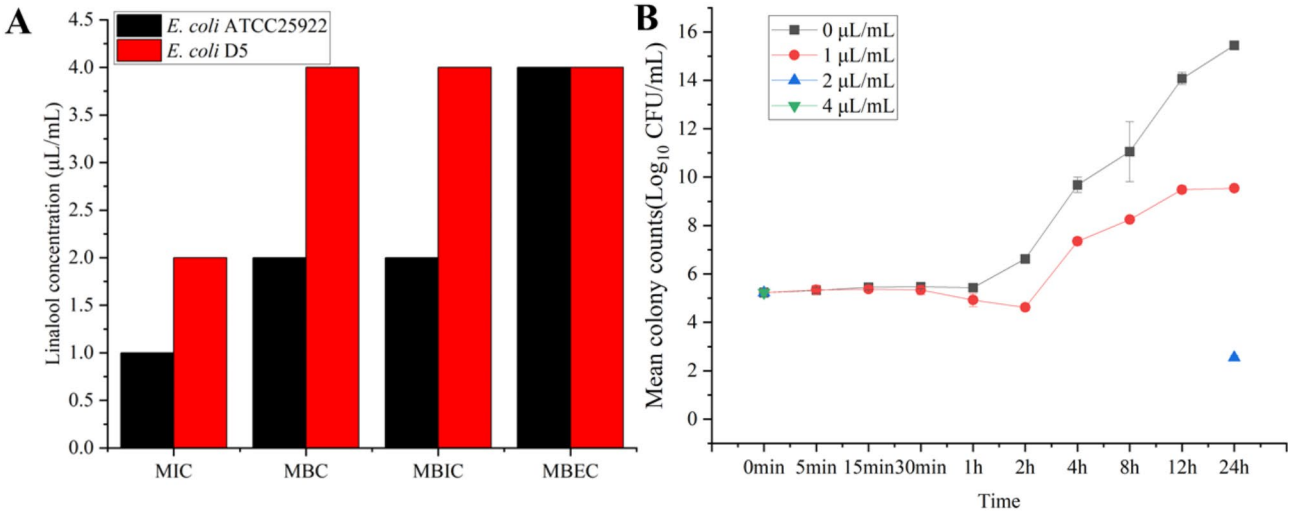

Fig. 1 Anti-bacterial activity of linalool and its implications on planktonic growth of *E. coli*: (**A**) Antimicrobial activity of linalool against *E. coli* in planktonic growth and biofilm; (**B**) Time-kill curves of linalool at 0, 1, 2, and 4 µL/mL against *E. coli* D5 in planktonic growth. A specimen of 0 µL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments

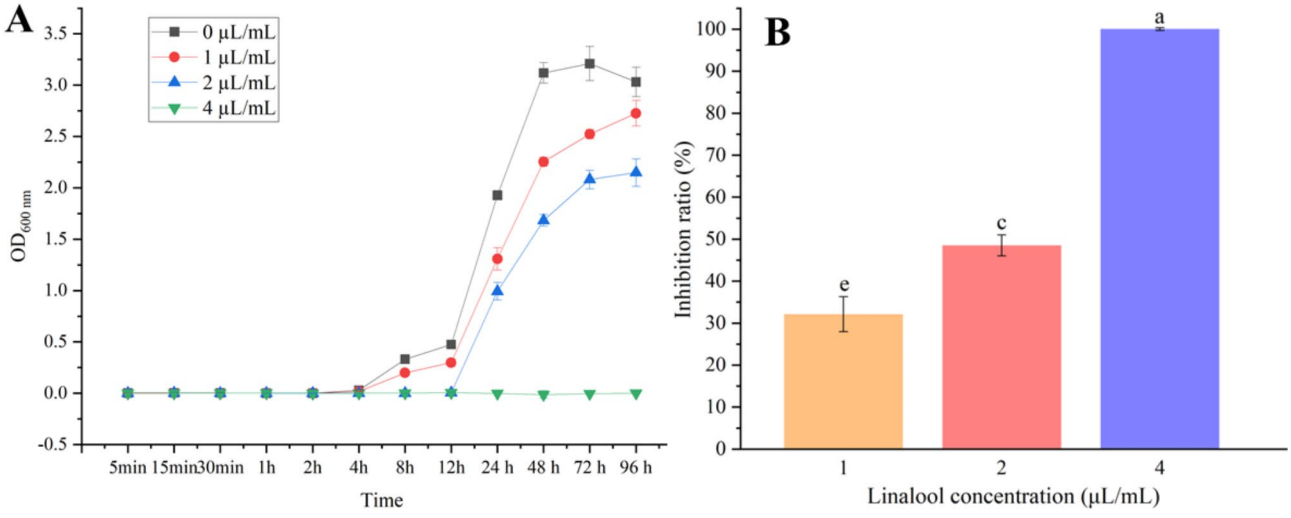

Fig. 2 The linalool implications (0, 1, 2, and 4 μL/mL) on *E. coli* D5 biofilms were ascertained by crystal violet assays: (**A**) Inhibition biofilm curve; (**B**) Inhibition biofilm ratio of linalool treated for 24 h. A specimen of 0 μL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments. Different lower cases indicate significant differences, and indirect letters manifest greatly significant variations ($p < 0.01$)

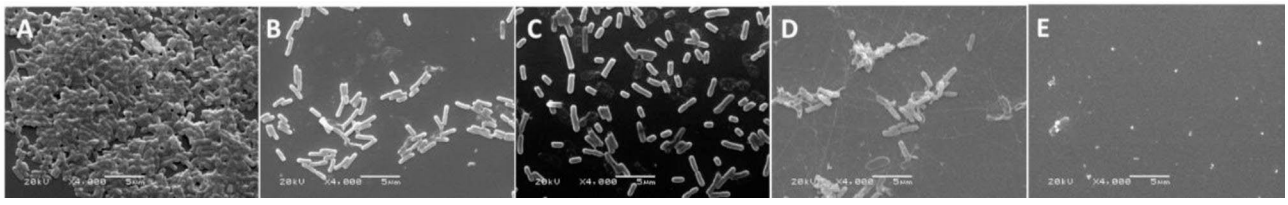

Fig. 3 Scanning electron microscopy (SEM) images of *E. coli* D5 biofilms treated with linalool for 24 h: (**A**) 0 μL/mL linalool; (**B**) 2 mg/mL Ampicillin; (**C**) 1 μL/mL linalool; (**D**) 2 μL/mL linalool; (**E**) 4 μL/mL linalool. A specimen of 0 μL/mL linalool was employed as a control. The images were acquired at 4000× magnification, with a scale bar representing 5 μm

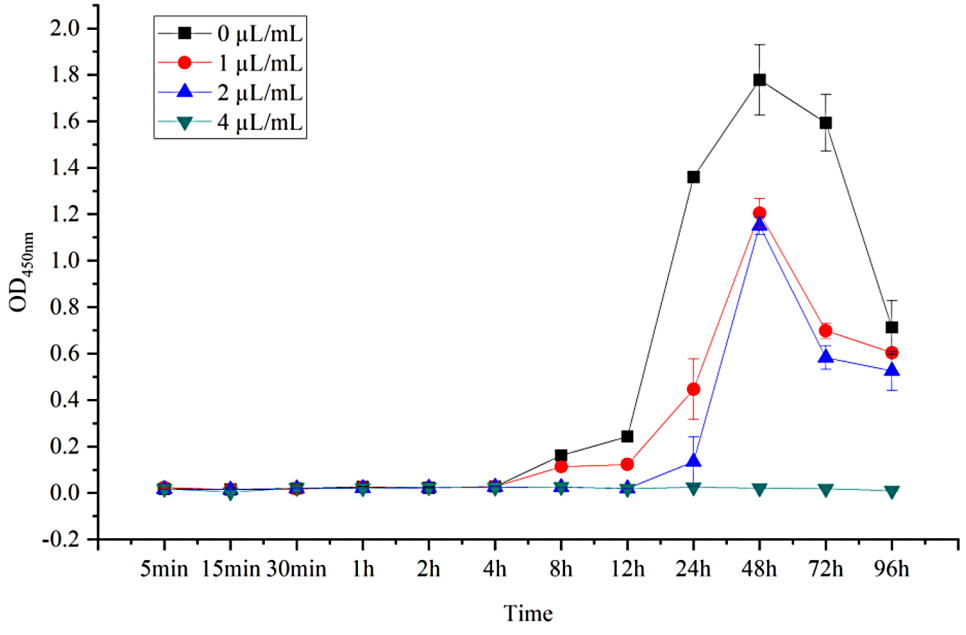

Fig. 4 Time-kill curves of linalool at 0, 1, 2, and 4 μL /mL against *E. coli* D 5 in the biofilm. A specimen of 0 μL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments

linalool at both 12 and 24 h, surpassing 90% effectiveness. Subsequently, the inhibition rate decreased, though it surged again at 72 h. There were significant variations in the bacteria viability at 8–72 h contrasted with the control for both 1 and 2 μL/mL linalool. Linalool at 4 μL/mL effectively eradicated bacteria, with an inhibition rate that exceeded 95% from 24 h onward in the development of *E. coli* biofilm. Significant differences in the ability to inhibit bacterial proliferation were observed: between 1 and 2 μL/mL linalool at 12 and 24 h, between 1 and 4 μL/mL linalool after 8 h, and between 2 and 4 μL/mL linalool following 48 h. Additionally, the viability of *E. coli* D5 in biofilm was assessed using CLSM after collective staining with SYTO 9 and PI. The SYTO 9 dye is used to color living bacteria green, whereas the PI dye is used to color dead bacteria red. The research showed that the presence of linalool in the biofilm treatment significantly decreased the survival of *E. coli* compared to the control group (Fig. 5A). The linalool treatment caused a significant hindrance in the biomass of live bacteria contrasted with the control ($p < 0.01$) (Fig. 5B). In contrast, the biomass of dead bacteria significantly increased following linalool treatment contrasted with the control ($p < 0.01$) (Fig. 5C). Consequently, the ratio of dead to live bacteria significantly increased after linalool treatment ($p < 0.01$) (Fig. 5D).

Linalool implications on extracellular polymeric substance in *E. coli* biofilms

We analyzed the alterations in extracellular polymeric substances in the biofilm, including EPS, proteins, and DNA in *E. coli* D5 biofilms (Fig. 6). When biofilm was treated with 1 μL/mL linalool, EPS was significantly reduced in the biofilm compared to the control ($p < 0.01$). The content of EPS in biofilm reduced with the rise of linalool concentration (Fig. 6A). The trends in protein and DNA changes were similar to those observed in EPS. When the concentration of linalool was greater than 1 μL/mL, the protein and DNA levels in the *E. coli* biofilm were significantly reduced compared to the control group ($p < 0.01$) (Figs. 6B-C). The outcomes manifest that

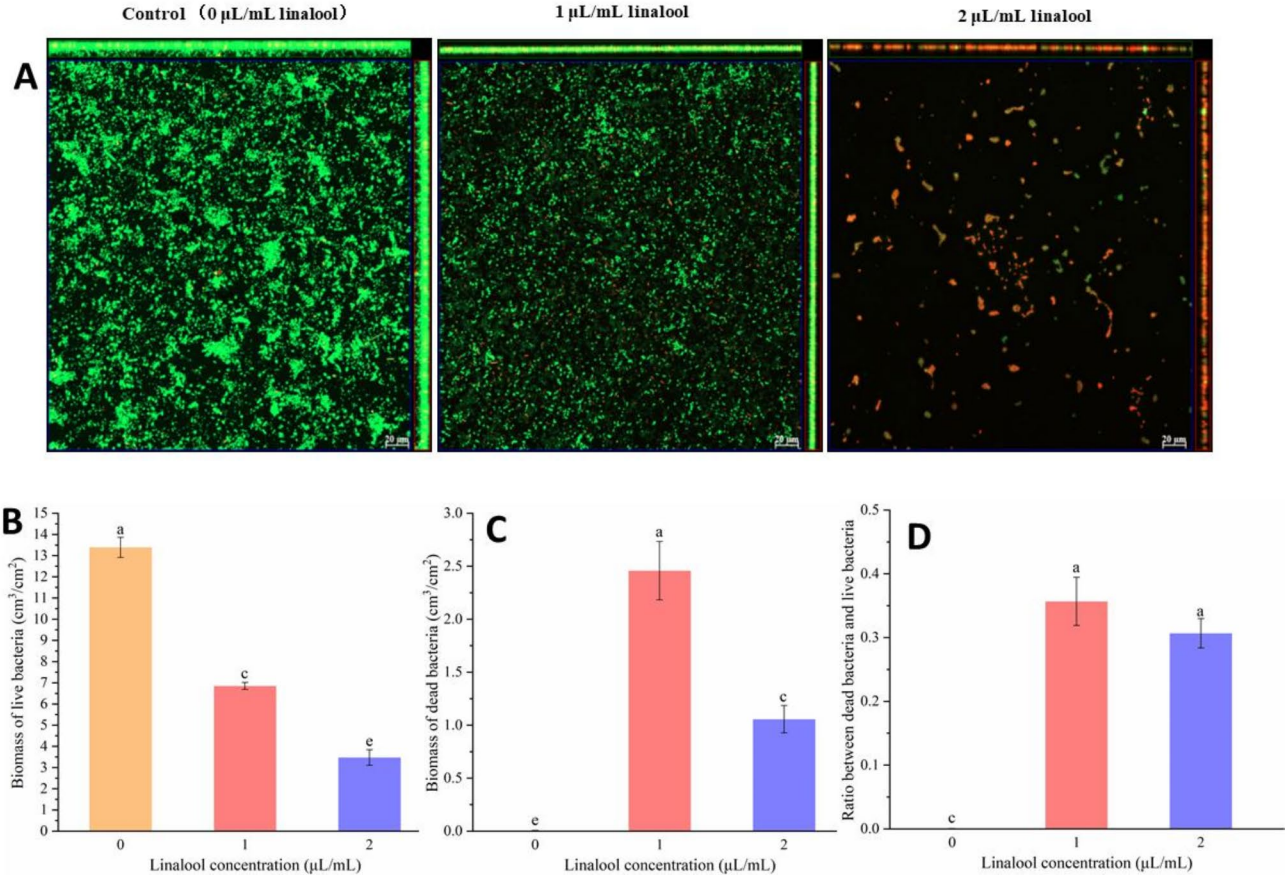

Fig. 5 Analyses of dead/live bacteria in *E. coli* D5 biofilms treated with linalool for 24 h. (**A**) Confocal laser scanning microscopy (CLSM) images of linalool at 0, 1, and 2 μL/mL linalool; green, live bacteria, red, dead bacteria; images from three independent replicates with 20 μm bars are representative. (**B**) Biomass of live cells in the biofilm. (**C**) Biomass of dead cells in the biofilm. (**D**) The proportion of dead to viable cells within a biofilm. A specimen of 0 μL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments. Different lower cases indicate significant differences, and adjacent letters indicate significant variations compared to previous data ($p < 0.05$). Indirect letters signify highly significant variations ($p < 0.01$)

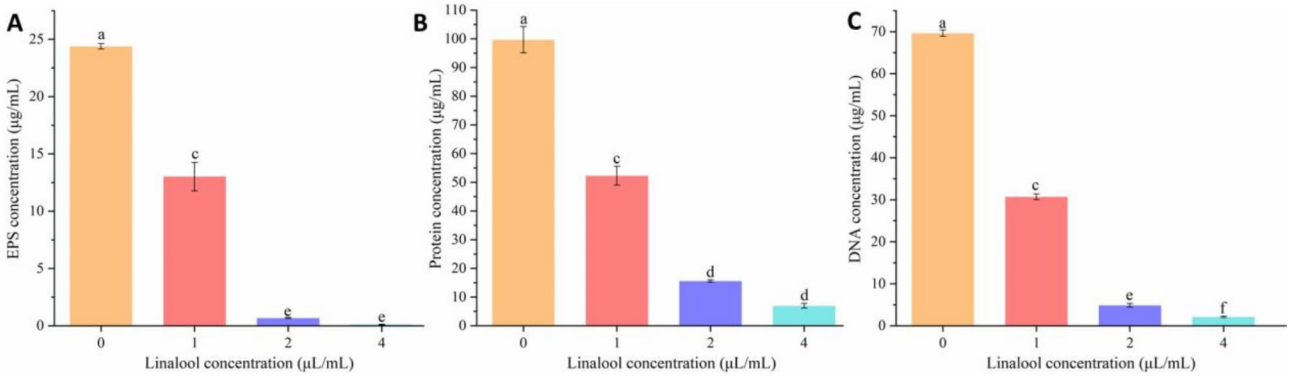

Fig. 6 The concentration of EPS (**A**), protein (**B**), and DNA (**C**) in *E. coli* D5 biofilms treated with linalool for 24 h. A specimen of 0 μL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments. Different lower cases indicate significant differences, and adjacent letters manifest significant variations compared to previous data ($p < 0.05$). Indirect letters signify greatly significant variations ($p < 0.01$)

linalool hindered the production of extracellular polymeric compounds in *E. coli* biofilm.

Linalool implications on EPS in *E. coli* biofilms

We also examined EPS in *E. coli* biofilms using CLSM. The FITC-ConA stains EPS green, whereas PI stains bacteria red. We observed a decrease in both EPS and bacterial signal intensities as the concentration of linalool increased (Fig. 7A). The EPS was significantly reduced in the biofilm with 1 μL/mL linalool compared to the control ($p < 0.01$). The EPS in biofilm was reduced with the elevation in linalool concentration (Fig. 7B). The trend in bacterial changes mirrored that of the EPS (Fig. 7C). Linalool can suppress the EPS production and the bacterial cells in *E. coli* D5 biofilms.

Figure 8 illustrates the alterations in the levels of gene expression in the *pga* family in *E. coli* D5 biofilm after being exposed to linalool. The expression levels of all these genes were significantly inhibited at a dose of 2 μL/mL linalool compared to the control ($p < 0.01$). At a concentration of 1 μL/mL, only the genes *pgaA* and *pgaB* were shown to be down-regulated contrasted with the control. This data is consistent with the findings obtained using the CLSM and phenol-H_2SO_4 test, thereby indicating that linalool could inhibit *E. coli* D5 biofilms by suppressing EPS production.

Discussion

E. coli is one of the main pathogens of endometritis in dairy cows and *E. coli* producing ESBL poses the greatest threat for the treatment. Because β-lactam antibiotics are the most commonly used antibiotics in dairy farms, such as penicillins, cephalosporins. This underscores the urgent need for novel alternative antibiotic therapeutic strategies. In this study, linalool demonstrated significant anti-bacterial efficacy against both the *E. coli* D5 and the ATCC25922 strain, as indicated by their respective MICs and MBCs. The strain *E. coli* D5 isolated from the uterine mucus of clinical endometritis in dairy cows, which can produce ESBL and form strong biofilms, is resistant to many drugs. For example, our results demonstrated that the MIC/MBC of ampicillin for *E. coli* D5 was 500-fold higher than for ATCC25922 strain in our previous studies. And the MIC and MBC of linalool for *E. coli* D5 were only twice for *E. coli* ATCC25922. This indicates that linalool may evade common resistance mechanisms associated with β-lactamase production. Our results are in agreement with earlier studies on the antimicrobial effect of linalool on *E. coli* [12, 17]. In addition, linalool has been reported to exhibit antimicrobial activity with the MIC and MBC being 5 μL/mL and 10 μL/mL against *Listeria monocytogenes* [13], the MIC being 5 μL/mL against *Candida albicans* [38], and the MIC being 1.5 μL/mL against *Pseudomonas aeruginosa* [18]. Collectively, these results demonstrate that linalool exhibits broad-spectrum antimicrobial activity, with greater efficacy against Gram-negative bacteria compared to Gram-positive bacteria and fungal species [37]. This finding indicates linalool's potential as a promising natural antimicrobial agent in preventing and treating endometritis in dairy cows.

The formation of *E. coli* biofilm is the leading cause of persistent infection in clinical practice [30]. MBEC and MBIC values have been proposed as a therapeutic reference for managing biofilm-related infections [47]. It has been reported that bacteria in biofilms are far more resistant to antibiotics compared to planktonic bacteria, with a concentration of around 1,000 times [39]. Notably, our findings revealed that the MBIC, MBEC, and MBC of linalool are all 4 μL/mL being twice the MIC against *E. coli* D5. *E. coli* D5 was capable of forming strong biofilm. $OD_{600\ nm}$ of *E. coli* D5 biofilm reached more than 3 using the crystal violet assay, while ATCC25922 is only 0.8. The change of linalool on MIC, MBC, MBIC and MBEC against *E. coli* was similar to those of natural product gallic acid against *Salmonella pullorum*, with the MIC, MBC, MBIC and MBEC were 4 mg/mL, 8 mg/mL,

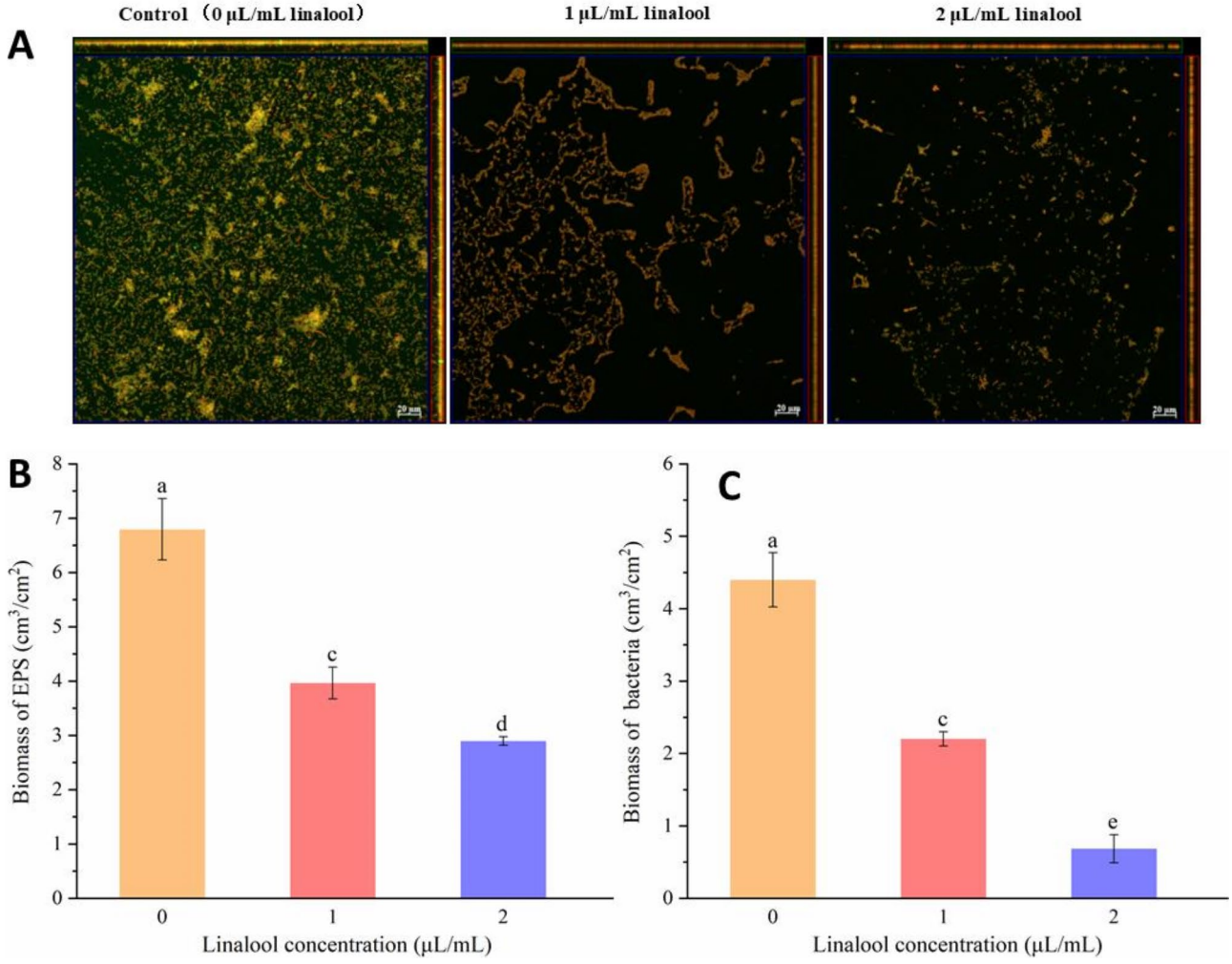

Fig. 7 Analyses of EPS and bacteria in *E. coli* D5 biofilms treated with linalool for 24 h. (**A**) Confocal laser scanning microscopy (CLSM) images of linalool at 0, 1, and 2 μL/mL linalool; green, EPS, and red, bacteria; Representative are the 20 μm bars on images derived from three independent replicates. (**B**) Biomass of EPS in the biofilm. (**C**) Biomass of bacteria in the biofilm. A specimen of 0 μL/mL linalool was employed as a control. Data represent the mean ± SD of three separate experiments. Different lower cases indicate significant differences, and adjacent letters indicate significant variations compared to previous data ($p < 0.05$). Indirect letters manifest greatly significant variations ($p < 0.01$)

8 mg/mL and 16 mg/mL [33]. In addition, Durgadevi et al. [10] reported that the MBIC of linalool against *Proteus mirabilis* was 0.2 mg/mL (about 0.25 μL/mL). In the study reported by Shen et al. [45], the MBICs of linalool against *Bacillus amyloliquefaciens* DY1a and DY1b were determined as 4 μL/mL and 8 μL/mL. Manoharan et al. [38] reported that 0.05 μL/mL linalool treatment with α-longipinene inhibited approximately 90% of the biofilm of *Candida albicans*. In the study reported by Ghazal et al. [14], 100 μM (about 0.02 μL/mL) linalool inhibited 34.44% of the biofilm of *S. aureus*, the effects of higher concentrations of linalool on biofilm were not studied. At present, most of the reports on the inhibitory effect of linalool on bacterial biofilm were at specific concentration of linalool, and the dose-effect relationship of linalool on bacterial biofilms is relatively rare. Furthermore, we found MBEC values were the same for resistant strain *E. coli* D5 and sensitive strain *E. coli* ATCC25922. This finding means that linalool can disrupt pre-formed biofilm, which is conducive to the radical treatment of persistent infection caused by biofilm [36]. The above results indicate that linalool has the capacity to be an antimicrobial agent for treating *E. coli* biofilm-related infections.

Research on linalool has mainly focused on its antibacterial effects and mechanisms, with few studies examining the anti-bacterial kinetics, especially its impact on *E. coli* within biofilms. This study demonstrated the anti-bacterial kinetic curves of linalool against *E. coli* in planktonic (Fig. 1B) and biofilm states (Fig. 4). The bacterial growth cycle includes the lag, exponential, stationary, and decline phases. At a concentration of 1 μL/mL, linalool significantly reduced the amount of planktonic *E. coli* after 1 h, while 2 μL/mL linalool eradicated over 99.999% of the *E. coli* within 5 min. These results suggest

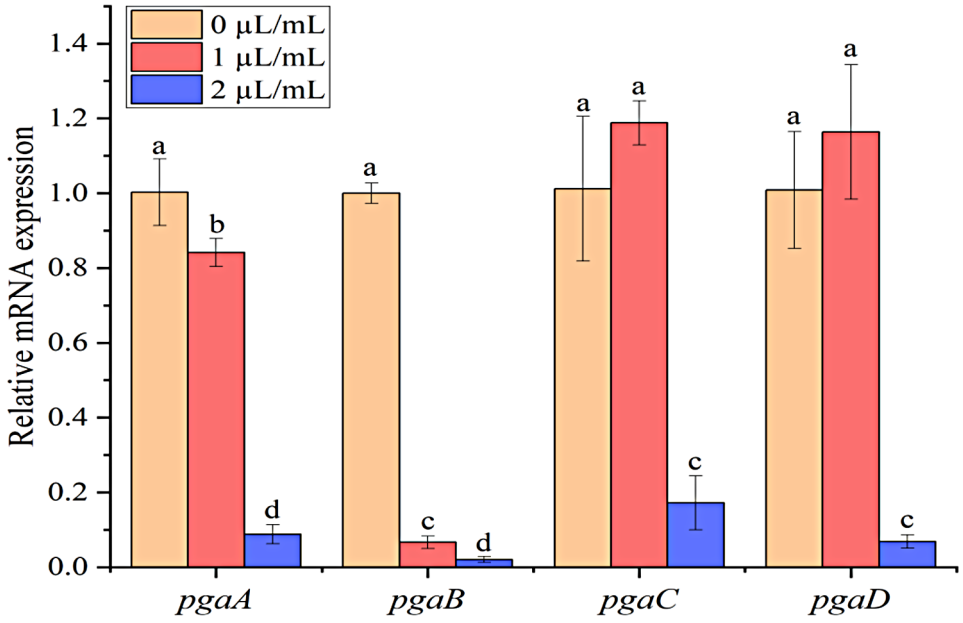

Fig. 8 RT-qPCR results of *pga* family genes of *E. coli* D5 treated with linalool (0, 1, and 2 μL/mL) for 24 h. Data manifest the mean ± SD of three separate experiments. Different lower cases indicate significant differences. The adjacent letters manifest significant variations compared to the previous data ($p < 0.05$). Indirect letters indicate greatly significant variation ($p < 0.01$)

that linalool possesses bactericidal properties. In contrast, lower concentrations (1/2 MIC) exhibit bacteriostatic effects by impeding bacterial proliferation during the exponential phase while not affecting the number of bacteria in the lag phase. This is consistent with the results reported by D'Auria et al. (2005), who found that 5 μL/mL linalool killed 100% of *Candida albicans* within 30 s. The development of bacterial biofilms progresses through stages of attachment, growth, maturation, and dispersal. This study employed the crystal violet assay and CCK-8 method to quantify biofilm mass and bacterial viability, respectively. Although the CCK-8 method is less damaging to bacteria in biofilms than the plate counting method and demonstrates higher repeatability, it indirectly reflects cell proliferation and survival status by measuring metabolic activity. Therefore, it cannot directly indicate changes in the number of viable bacteria. The suppressive effects of 1 and 2 μL/mL linalool on *E. coli* within biofilms were significant at 12 and 24 h but not at other time points, indicating that the inhibitory actions of low-concentration linalool is primarily evident during the biofilm growth phase and diminishes as biofilm mass increases. This pattern is consistent with the inhibition observed in planktonic bacteria. After 48 h, the difference in inhibitory effects between 2 and 4 μL/mL linalool on biofilm-associated *E. coli* was significant, suggesting that the MBIC can reduce bacterial quantities in the mature and dispersal stages of biofilm development, whereas 1/2 MBIC of linalool exhibits a weaker effect during these stages. SEM images revealed that low concentrations of linalool predominantly inhibit bacterial proliferation, while higher concentrations cause cellular damage, leading to cell rupture and death. The CLSM results confirmed that linalool reduces both the number and activity of bacteria. Collectively, these findings confirm a dose-dependent relationship between the suppressive effects of linalool on bacteria and biofilm formation and the concentration of linalool. Concentrations of 1 and 2 μL/mL linalool can suppress rapid *E. coli* proliferation, but the concentration of 4 μL/mL linalool is required for effective sterilization and biofilm inhibition of *E. coli*.

The experimental results of this investigation indicated no significant difference in the inhibitory effects of 1 and 2 μL/mL concentrations of linalool on the viability of *E. coli* in biofilm except for 12 and 24 h. However, the inhibition of linalool on biofilm formation was markedly significantly, suggesting that linalool also inhibits the production of extracellular polymeric substances. And our study explored the effects of linalool on various extracellular polymers, including EPS, proteins, and DNA. The data revealed that linalool significantly diminished EPS, DNA, and protein levels. These results are consistent with previous studies. Shen et al. [45] reported that linalool inhibited the production of extracellular polysaccharides and proteins of the biofilm matrix of *Bacillus amyloliquefaciens*. Linalool destroyed the biofilm framework of *Pseudomonas aeruginosa* by reducing protein and carbohydrate content of extracellular polymeric substance [31]. In addition, Peng et al. [41] reported that *Tankan peel* essential oil, which contains linalool, significantly inhibited *Listeria monocytogenes*

from forming biofilm by reducing the extracellular polymeric substances, including protein, polysaccharide, and eDNA.

The production of EPS was critical for the development of *E. coli* biofilm architecture [8]. It played the role of structural support and adhesion, providing an adhesion matrix for the formation of biofilm, and enhancing the adhesion between bacteria and surfaces and bacteria [49]. Poly-beta-1, 6-N-acetyl-D-glucosamine (PGA) is an important EPS found in *E. coli* biofilms [5]. The pgaABCD operon, which encodes four proteins of PGA synthase, regulates the synthesis of PGA [52]. Our results demonstrated that linalool significantly reduces EPS production by phenol-H_2SO_4 method and CLSM. This effect is likely mediated by two interdependent mechanisms: (1) its primary bactericidal activity, which reduces bacterial biomass and thereby indirectly diminishes EPS accumulation (Figs. 1, 4 and 5, and 7), and (2) a direct inhibitory effect on PGA biosynthesis via down-regulation of the pgaABCD operon (Fig. 8). Notably, the suppression of *pgaABCD* gene expression at 2 μL/mL linalool aligns with previous studies showing that natural compounds like gallic acid and (R)-(+)-pulegone similarly disrupt PGA synthesis [16, 24]. Such dual effects mirror observations in clinical isolates where *pgaABCD*-deficient strains exhibit biofilm vulnerabilities [34], yet the therapeutic priority lies in eliminating viable pathogens. These results demonstrated that linalool inhibits bacterial growth and EPS production, thereby reducing biofilm formation of *E. coli*, indicating the potential of linalool in the treatment of *E. coli* biofilm-related infections. However, its applicability and therapeutic efficacy in the treatment of biofilm-related infections remain to be further investigated through comprehensive experimental investigations in vivo.

Conclusion

These investigations demonstrated the significant antibacterial effects of linalool on *E. coli* in both planktonic and biofilm forms. Linalool was found to kill *E. coli* rapidly and efficiently, and destroyed *E. coli* biofilms at a dose of 4 μL/mL. Additionally, it reduced the production of EPS and the expression of the *pgaABCD* genes. Our findings demonstrated that linalool prevents the formation of *E. coli* biofilms by inhibiting bacterial growth and decreasing EPS production, establishing linalool as a promising natural anti-bacterial agent. The effects of linalool on bacterial biofilms in vivo require further investigation.

Author contributions

J.M. Wang, K. Zhang and J.Y. Zhang: Methodology, Investigation, Experiments and Writing-review & editing. D.A. Cui and J.Y. Wang: Data curation and Writing-review & editing. P. Ji and Y.M. Wei: Writing-review & editing. L. Wang and J.X. Li: Conceptualization, Project administration, Writing-review & editing, and Funding acquisition. All authors have reviewed and approved the final article.

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Data availability

The datasets generated for this study are available on request to the corresponding author.

Declarations

Ethical approval

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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