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RESEARCH ARTICLE

Evaluation of antimicrobial effect of lemon grass essential oil on biofilm forming pathogens in broth medium and on stainless steel chip

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Abstract: The objectives of this study was to check effect of lemon grass essential oil on biofilm forming selected pathogens in broth medium and on stainless steel chip. The antimicrobial effect of lemon grass essential oil (LMO) by well agar assay showed all four pathogenic strains were susceptible to LMO. 0.9% MIC was observed in strain *B. cereus* ATCC 10876 and it was 0.8% for both *S. aureus* ATCC 700698 and *Proteus mirabilis* ATCC 12453 strains. *E. coli* ATCC 10536 was found to be the species bearing the least MIC (0.6%). Time kill analysis with all the four planktonic pathogens in broth medium by using 0.8% lemongrass essential oil (selected based on MIC) was checked at their stationary growth phase. *E. coli* was found most sensitive to LMO treatment and gave complete reduction within 20 min contact time, while *S. aureus* gave 71% inhibition, *Proteus mirabilis* gave 53% inhibition after 80 min of treatment with LMO. *Bacillus cereus* was the most resistant among all against LMO and gave only 27% inhibition after 80 min of treatment. However all the pathogens inhibited completely after 24 h treatment with 0.8% LMO. On stainless steel chip against biofilm of *S. aureus* and *E. coli,* LMO (0.8%) treatment gave complete reduction within 24 h. This study demonstrated that LMO could be a reliable foodborne pathogen biofilm disrupter.

Keywords: Biofilm, Pathogens, Stainless steel chip, Lemon grass essential oil, Antimicrobial effect

Introduction

A food industry, particularly those in the dairy sector, always strives to satisfy customers and avoid microbial contamination, which can adversely affect product quality and consumer health (Mnif et al. 2020; Mishra et al. 2011). Internationally, both developed and developing countries see foodborne illnesses as a rising public health issue. The dairy industry's equipment are highly susceptible to microorganisms, especially in milk storage tanks and milk processing lines, and is the leading cause of milk contamination (Vishwakarma, 2020). The dairy industry often struggles with biofilm problems due to the presence of foodborne pathogens or spoilage microorganisms. Due to this major spoilage, dairy industry connected to both economic and public health consequences. Biofilms of pathogenic bacteria may also raise the risk of foodborne illness in milking equipment and milk storage tanks (Panebianco et al. 2022; Manju & Grover 2023). Due to their difficulty in eliminating, bacteria within biofilms act as a source of recurrent contamination of plants, products, and personnel after they have been established (Makwana et al. 2018)

To date, a wide range of commercially available disinfectants have been broadly used for lowering microbial contamination in order to produce safer and longer-lasting products. In order to eradicate biofilm, several factors need to be considered, such as concentration, pH, temperature, exposure time, type of surface, and relative humidity.

Traditionally, plants essential oils have been sought out as safe and natural alternatives to synthetic antiseptics and antibacterial drugs (Burt, 2004). Research has shown that essential oils and their component have antimicrobial properties against some bacteria and fungi. Several studies have implicated lemongrass in antidepressants, antioxidants, antiseptics, astringents, bactericidal, fungicidal, nervine, and sedative activities. LMO is having GRAS (Generally Recognised As Safe) status (Faheem et al. 2022). Reports state that LMO (probably referring to a specific chemical) has been discovered to have higher FRAC (ferric reducing antioxidant capacity) but lower scavenging activity. In comparison to compounds like ascorbic acid and butylated

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hydroxyl toluene, LMO is hence more efficient at chelating iron (II) ions (Viuda-Martos et al 2010). Lemongrass essential oil (LMO) is rich in terpenes like citral (85%) and geraniol (1.5%) (Ortega-Ramirez et al. 2020).

The objectives of this study was to check effect of lemon grass essential oil on biofilm forming selected pathogens in broth medium and on stainless steel chip.

Materials and Methods

Pathogenic cultures

Four pathogenic cultures for screening of biofilm formation were obtained from the Department of Dairy Microbiology, College of Dairy Science and Technology, Ludhiana, Punjab (Table 1). Stock cultures were preserved at -80ºC. Before any assay, strains were revived by transferring stock cultures into Brain Heart Infusion (BHI, Hi media) broth and further placed at 37ºC for 24 hours. Purity of every culture was ascertained by doing Gram staining and catalase test. The storage of cultures was done below 5ºC between transfers.

Procurement of Lemongrass essential oil (LMO)

LMO (extracted from *Cymbopogon citratus*) was obtained from Satuguru Trading firm located in Tarntaran, Punjab, India. The manufacturer confirms that the lemongrass oil was isolated through distillation from lemongrass leaves cultivated in Punjab. Furthermore, the oil's purity has been verified to be 100% using a chiral method.

Preparation of LMO concentrations

Various concentrations, specifically 5%, 10%, 15%, 20%, 25%, and 30%, (v/v) of lemongrass oil were prepared in sterile 0.6% tween-80 (v/v) in aseptic environment.

Evaluation of antimicrobial effect of LMO by disc diffusion assay

Using disc diffusion method of Adukwu et al. (2016), the antimicrobial effect of LMO was tested on bacterial cultures at different concentrations (5 %, 10 %, 15%, 20%, 25% and 30%). BHI agar having 0.6% of tween 80 was prepared and 15 mL agar was added to specific petri plates and allowed to solidify. A total of 100 µL of pathogenic suspension was spread on to agar aseptically. At the same time, 6 mm diameter sterile discs were soaked with 20 µL of various concentrations of LMO (5 %, 10 %, 15%, 20%, 25%, 30%) and then placed on the agar plates. For negative control only disc with tween 80 was placed. Inhibition Zone in mm was checked after the incubation period was over.

Evaluation of antimicrobial effect of LMO by well agar assay

Using agar well diffusion method, the inhibitory effect of LMO was tested on bacterial cultures at different concentrations (5 %, 10 %, 15%, 20%, 25% and 30%) as described by Adukwu et al. (2016). BHI broth, having 0.6% of tween 80 was prepared and 15 mL of agar was added to specific petri plates and allowed to solidify. A total of 100 µL of pathogenic suspension was spread on to agar aseptically. Wells of 6 mm diameter were made in solidified agar. A total of 50 µL of each LMO concentration was pipetted out and filled into wells. For negative control only tween 80 was added to the well. All the petri dishes were incubated at 37! for 24-36 hrs. Inhibition zone in mm was checked after the incubation period was over.

Evaluation of minimum inhibitory concentration (MIC) of LMO

The determination of the MIC for LMO on the pathogenic strain was conducted through the broth dilution method, following the protocol outlined by Hammer et al. (1999). To prepare the test strain cultures, the pathogenic strain was inoculated into sterilized test tubes containing 5 mL of nutrient broth. Subsequently, the tubes were placed in an incubator and allowed to incubate overnight at a temperature of 37±1ºC. The MIC was defined as the minimum amount of any compound capable of inhibiting the growth of pathogen. A total of 10 mL of tryptic soy broth (TSB, Hi media) supplemented with 0.6% (v/v) tween-80 was spiked with varying concentrations of LMO, spanning from 1% to 0.015% (v/v) in different test tubes. As a positive growth control, TSB with 0.6% (v/v) tween-80 and without LMO was employed. Uniformly, 25 µL of pathogenic suspension was added to individual tubes. Subsequently, the tubes were placed in an incubator at a temperature of 37±1ºC for incubation periods of both 24 and 48 hours. After the designated incubation periods, the tubes were assessed for turbidity. Additionally, to evaluate bacterial growth, the tubes that exhibited no increase in turbidity during each time interval of 24 to 36 h were streaked onto nutrient agar. This step aimed to confirm the absence of pathogenic growth. To ensure accuracy, each trial was repeated three times.

Time kill analysis of planktonic pathogens by LMO in broth medium at stationary phase of their growth

Method of Mitic-Culafic et al. (2005) was subjected to further modifications. 100 µL of each pathogenic culture was inoculated into 10 mL BHI (pH 6.80) tubes in duplicate and incubated at 37! for 16 hrs. The pathogenic cultures were centrifuged at 3000 rpm for 6 minutes and pellet were washed and diluted using sterile phosphate buffer to the final OD of 0.5 at 600 nm wavelength to obtain cell number about $8.7x10^8$ colony forming unit (CFU) per mL. The tubes containing test strains were inoculated with 0.8 % concentration of lemongrass oil (above the calculated MIC for each pathogen) along with tubes without any lemon grass oil as control. All the test tubes were incubated at 37! for a period 20, 40, 60, 80 min and 24 h. After every designated incubation period the dilutions were made for each tube and were plated on BHI agar. Plates were incubated at 37! for 24-48 hrs. Percent inhibition was calculated at each time interval in comparison with control.

Development of biofilm on stainless steel

The method initially proposed by Moltz and Martin (2005) was subjected to further modifications. A volume of 0.5 mL from cultures of selected pathogens (*E. coli* ATCC 10536 and *S. aureus* ATCC 700698) was inoculated into 500 mL of BHI broth at a pH of 6.80. This process was carried out in duplicate and the mixtures were then incubated at a temperature of 37°C for a period of 24 hours. These cultures were supplemented with 304 stainless steel chips measuring 2.54 x 2.54 cm^2 each. Before utilization, all stainless steel (SS) chips were immersed in a 70% ethanol solution for a period of 10 minutes, following which they were rinsed three times using sterile deionized water. Subsequently, the chips underwent autoclaving at a temperature of 121°C for 15 minutes and were then dried under a biosafety hood for 30 minutes. The incubation of the broth was executed on a shaker to replicate the bacterial growth on the SS chips. After the incubation, the chips were subjected to washing using a sterile phosphate buffer in order to eliminate any detached cells present on the surface of the SS chips.

Evaluation of antimicrobial effect of LMO on selected pathogenic biofilm developed on stainless steel

Stainless steel chips with biofilm were dipped in 0.8 % lemon grass solution and placed in a shaker incubator at 37! for 24 hrs for further incubation. After 24 hrs incubation stainless steel chip was taken out. The surface of the stainless steel chip was scraped using sterile swab. The swab was then used to make dilutions, and the diluted samples were plated on BHI agar and were incubated at 37! for 24 hrs.

Statistical analysis

Under the supervision of a statistician, data gathered from numerous experiments during the screening and comparative analysis process were analyzed for two-way analysis of variance (ANOVA) and t-test using SAS 9.3 version. Microsoft excel was used to calculate the mean, standard error of data, when needed.

Results and Discussion

Assessing purity of commercial pathogenic strains

During this study four pathogenic cultures for screening of biofilm formation were obtained from the Department of Dairy Microbiology, College of Dairy Science and Technology, Ludhiana, Punjab. All the strains were found to be pure under microscope, shown characteristic appearance on their selective growth medium and were catalase positive (Table 1 & Figure 1).

Evaluation of antimicrobial activity of lemongrass essential oil by Disc diffusion assay

The antimicrobial activity of lemongrass essential oil gradually increased with increased in concentration (Figure 2). Both Gram positive and Gram negative pathogens showed sensitivity towards LMO. Highest inhibition zone was observed at concentration 30%.

Evaluation of antimicrobial activity of LMO by well agar assay

As given in Figure 3 and Table 2, for *B. cereus* ATCC 10876, *S. aureus* ATCC 700698, *E. coli* ATCC 10536 and *Proteus mirabilis* ATCC 12453 highest inhibition zone was observed at 30% LMO concentration. The findings of the current investigation unequivocally demonstrate the considerable antibacterial potential of lemongrass oil against the examined microorganisms. The outcomes derived from agar diffusion assay align with the prevailing notion that Gram-positive organisms exhibit more susceptibility to the LMO compared to Gram-negative bacteria. Comparable conclusions were also drawn by Cimanga et al. (2002).

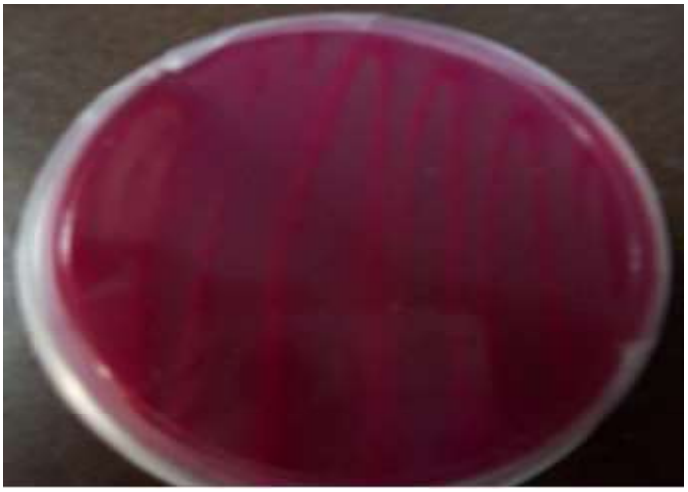
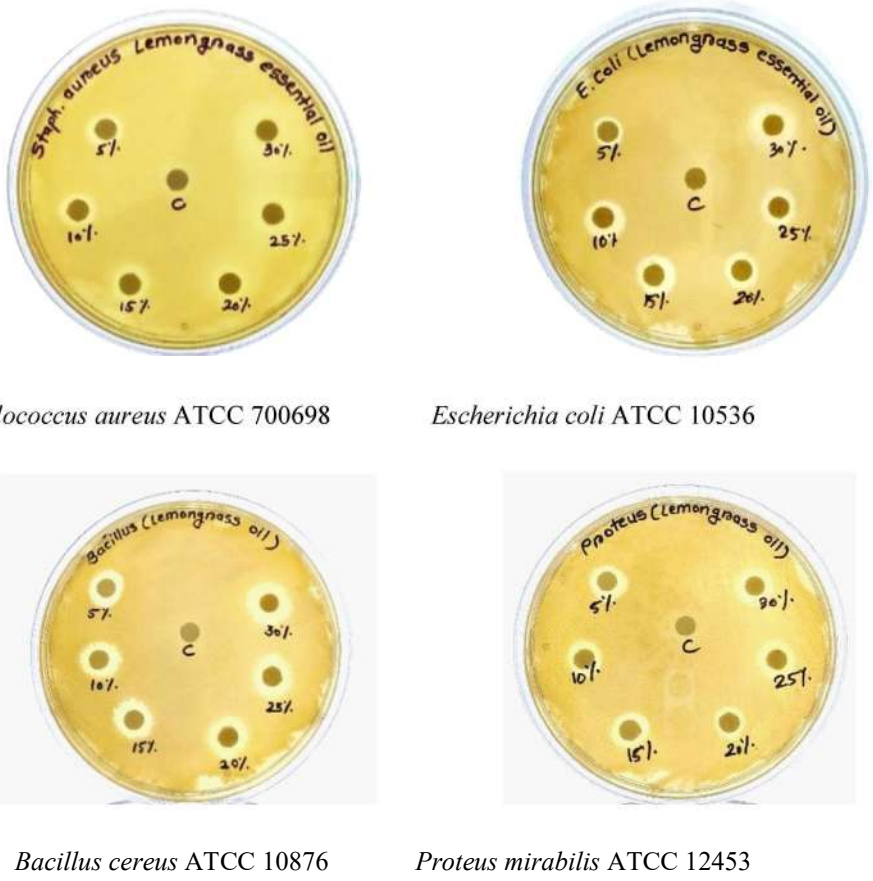


Fig.1 *E. coli* on Violet red bile Agar

Table 1: Purity testing of pathogenic strains

Sr. No.	Name of Lactic acid bacteria	Gram's staining	Catalase test
1	*Staphylococcus aureus* ATCC 700698	Gram +ve	Catalase +ve
2	*Escherichia coli* ATCC 10536	Gram -ve	Catalase +ve
3	*Proteus mirabilis* ATCC 12453	Gram -ve	Catalase +ve
4	*Bacillus cereus* ATCC 10876	Gram +ve	Catalase +ve

Fig. 2 Disc diffusion assay using LMO against pathogens



Staphylococcus aureus ATCC 700698 *Escherichia coli* ATCC 10536

Bacillus cereus ATCC 10876 *Proteus mirabilis* ATCC 12453

Table 2: Diameter of zone of inhibition of selective pathogen

Pathogen	Zone of inhibition (mm)					
	5%	10%	15%	20%	25%	30%
Escherichia coli ATCC 10536	10±0.2	13±0.4	17±0.6	18±0.2	19±0.4	20±0.3
Staphylococcus aureus ATCC 700698	14±0.5	15±0.3	18±0.4	18±0.4	18±0.5	19±0.4
Proteus mirabilis ATCC 12453	10±0.3	13±0.4	17±0.2	18±0.4	18±0.6	19±0.3
Bacillus cereus ATCC 10876	13±0.4	14±0.3	19±0.5	20±0.6	21±0.2	21±0.6

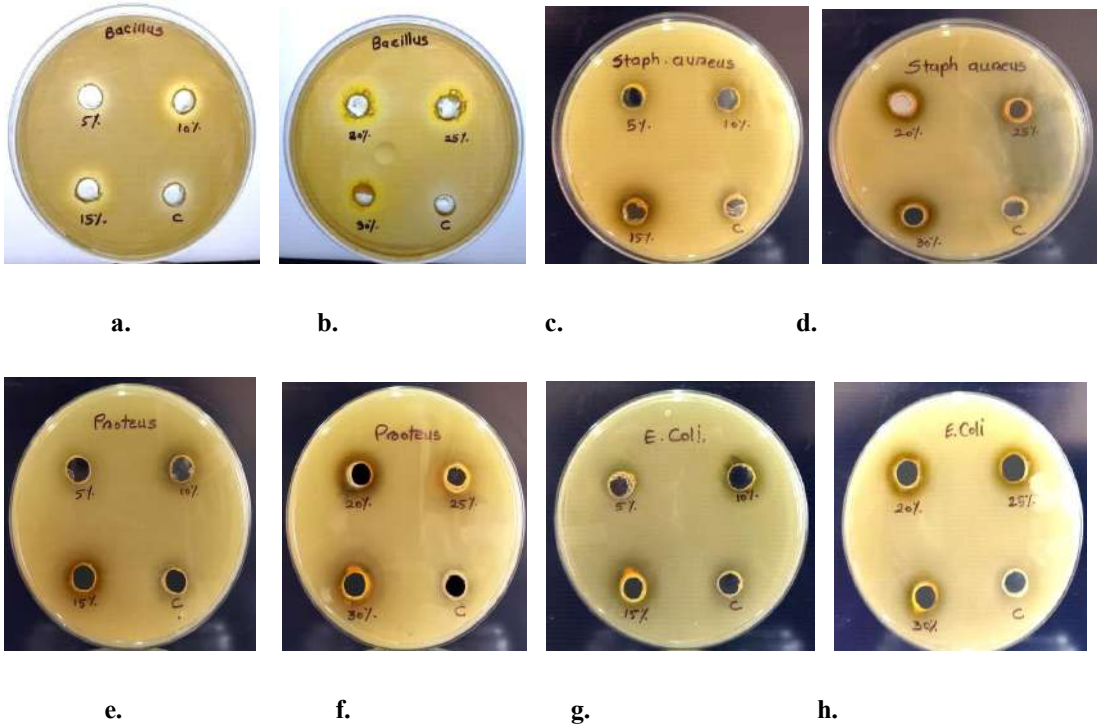
* - Average of triplicate trials

Table 3: Minimum Inhibitory Concentration (in %) of LMO against the pathogens

Pathogen	Minimum Inhibitory Concentration (MIC in % LMO)
Escherichia coli ATCC 10536	0.6
Staphylococcus aureus ATCC 700698	0.8
Proteus mirabilis ATCC 12453	0.8
Bacillus cereus ATCC 10876	0.9

Determination of Minimum Inhibitory Concentration of LMO by broth dilution method

Minimal inhibitory concentration (MIC) was performed by serial micro dilutions based method MIC values of each strain are given in Table 3. MIC of LMO tested for all 4 pathogenic bacteria ranged from 0.6 to 0.9% (V/V). Among the species majority of the strains were having MIC in the range of 0.8%. MIC of 0.9% was observed for strain *B. cereus* ATCC 10876 and 0.8% MIC was detected for *S. aureus* ATCC 700698 and *Proteus mirabilis* ATCC 12453,



a. **b.** **c.** **d.**

e. **f.** **g.** **h.**

Fig. 3 Well agar assay using different concentration (5, 10, 15, 20, 25, 30% and C-negative control) of LMO. Where, a and b - *Bacillus cereus* ATCC 10876, c and d- *Staphylococcus aureus* ATCC 700698, e and f- *Proteus mirabilis* ATCC 12453, g and h- *Escherichia coli* ATCC 10536

respectively. *E. coli* ATCC 10536 was found to be the species bearing the least MIC of 0.6%. Lemongrass oil exhibited notable inhibition of the test organisms at notably low concentrations in the broth dilution method when contrasted with the agar diffusion method. This observation coincides with the findings of Tortorano et al. (1998). Discrepancies in results between these two methods are attributed to various factors inherent to each assay, as discussed in prior research (Hili et al. 1997). These encompass variations in microbial growth, the extent of microorganism exposure to LMO, LMO solubility, and the type and quality of emulsifier employed, among other factors.

The MIC values determined in this study surpass those documented in previous research. This variation can be attributed to a multitude of factors, including disparities in plant cultivation conditions, specific plant material used, the technique employed for essential oil extraction, and the choice of solvent (Burt, 2004; Alma et al. 2007; Guan et al. 2007; Polatoglu et al. 2010). Furthermore, variations strains of *S. aureus* also contribute to this diversity. Fluit et al. (2001) reported a broad spectrum of antimicrobial susceptibility among 3,051 *S. aureus* isolates, exhibiting varying MIC levels for gentamicin (ranging from 0.12 to >8 mg/L).

Time kill analysis of planktonic cells of pathogens by using LMO (0.8%) in broth medium at their stationary phase of growth

Time kill analysis with all the four planktonic pathogens in broth medium by using 0.8% LMO (selected based on MIC) was checked in this experiment at their stationary growth phase.

As shown in Figure 4, the logarithmic value of all pathogens in LMO treated broth started to reduce at 20 minutes onwards. Increasing the contact time with LMO from 0 to 1440 min (24 h) increased cell death. For LMO treated *S. aureus* ATCC 700698 in broth medium growth inhibition was observed as 6, 26, 55, 71, and 100% after 20, 40, 60, 80 min and 24 h, respectively. *E. coli* ATCC 10536 was found to be most sensitive and got inhibited within 20 min at 0.8% concentration of lemongrass essential oil. For LMO treated *B. cereus* ATCC 10876 in broth medium growth inhibition was observed as 13, 15, 24, 27, 100% after 20, 40, 60, 80 min and 24 h, respectively. Similarly, for LMO treated *Proteus mirabilis* ATCC 12453 in broth medium growth inhibition was observed as 7, 39, 45, 53 and 100% after 20, 40, 60, 80 min and 24 h, respectively.

Time kill analysis of pathogenic biofilm formed on stainless steel chip by using LMO (0.8%)

Time kill analysis with one Gram's positive (*S. aureus* ATCC 700698) and one Gram's negative (*E. coli* ATCC 10536) pathogenic biofilms formed on stainless steel chip after overnight growth at 37°C by using 0.8% LMO (selected based on MIC) was checked in this experiment. As shown in Figure 5, after 24 h treatment of LMO, *E. coli* ATCC 10536 reduced by 8.10 log showing 100% inhibition and for control, it cells number increased by 0.17 log value. Similarly, for *S. aureus* ATCC 700698 reduced by 8.08 log showing 100% inhibition and for control, it cells number increased by 0.52 log value.

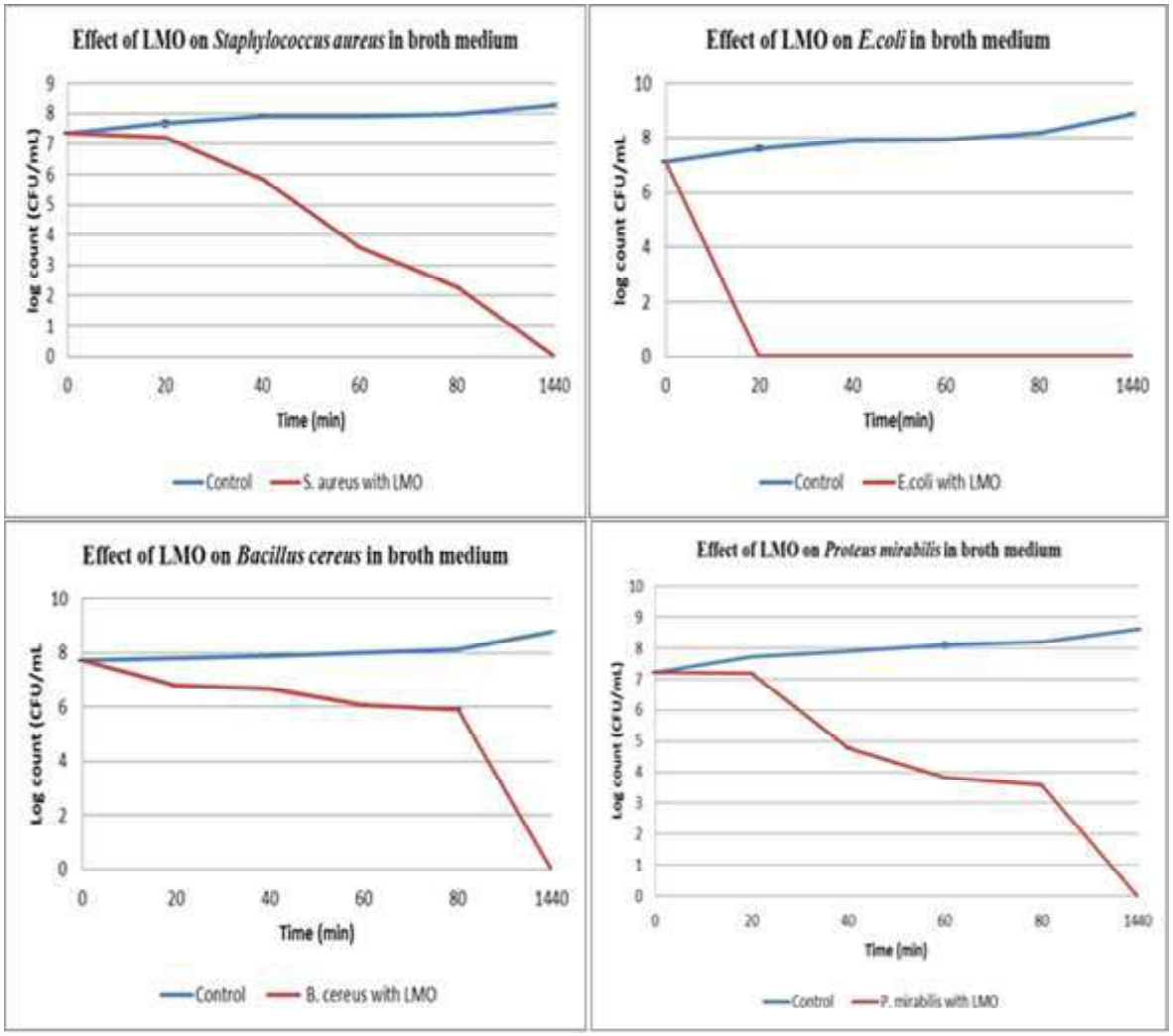
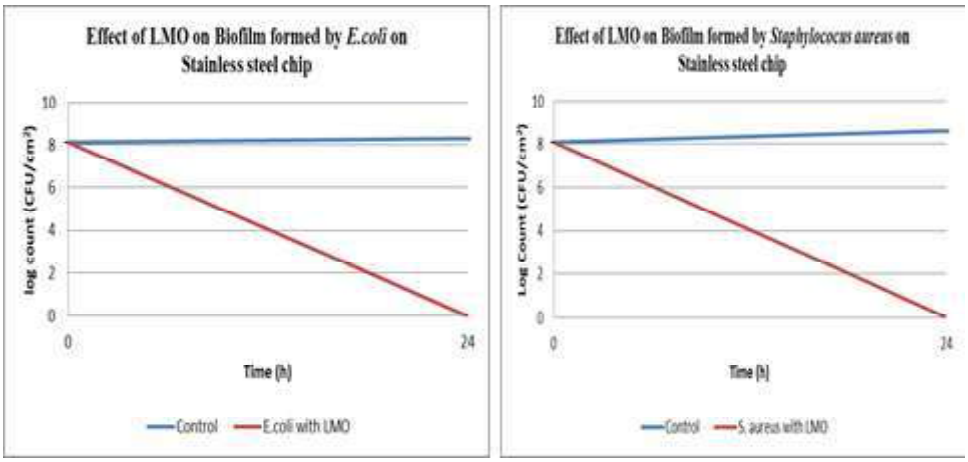


Fig. 4 Time kill analysis of planktonic cells of *S. aureus* ATCC 700698, *E. coli* ATCC 10536, *B. cereus* ATCC 10876 and *P. mirabilis* ATCC 12453 by using 0.8% LMO treatment in broth medium at their stationary phase of growth

Fig. 5 Antimicrobial effect of LMO (0.8%) on *E. coli* ATCC 10536 and *S. aureus* ATCC 700698 biofilm grown on stainless steel chip



This study stands among the limited research endeavors exploring the anti-biofilm characteristics of lemongrass essential oil concerning biofilms formed by *S. aureus* and *E. coli* on stainless steel chips. Prior research (Bearden et al. 2008) focused on examining commercial formulations containing essential oils in relation to MRSA.

Conclusions

In conclusion, as far as our understanding extends, this study represents the inaugural comprehensive examination of the anti-biofilm attributes of LMO in the context of biofilms formed by *S. aureus* and *E. coli* on stainless steel chips. The findings of this study underscore the potential of LMO as a dependable disruptor of foodborne pathogen biofilms. This treatment presents a noteworthy alternative to conventional sanitizers and holds promise for addressing biofilm concerns in food processing facilities, while also economically safeguarding food products against cross-contamination.

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Ethical Considerations: This article does not involve any studies involving human or animal subjects.

Declaration of Conflicts of Interest: The authors confirm that there are no conflicts of interest to disclose.

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