

Evaluation of the antimicrobial activity of *Lactobacillus acidophilus* LA-5 against *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus aureus*: An in-vitro study

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Article

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Abstract

Aim

This study aimed to evaluate whether Lactobacilli probiotics cell-free supernatant (CFS) exhibit antibacterial activity comparable to chlorhexidine against *Enterococcus faecalis* (*E. faecalis*), *Candida albicans* (*C. albicans*) and *Staphylococcus aureus* (*S. aureus*).

Materials and Methods

This study followed the Preferred Reporting Items for Laboratory Studies in Endodontology PRILE 2021 guidelines and used an in vitro agar well-diffusion assay to assess antimicrobial activity. Standardized 0.5 McFarland inocula of *E. faecalis*, *C. albicans*, and *S. aureus* were lawn-cultured on Mueller–Hinton agar. *Lactobacillus acidophilus* (LA-5) was cultivated to obtain cell-free supernatant (CFS), serving as the experimental biological antimicrobial agent. CFS solutions (300–50 mg/mL) were tested alongside chlorhexidine and sterile water controls and incubated for 18–24 h at 37°C. Zones of inhibition were measured in triplicate by two calibrated observers for statistical analysis.

Results

Intergroup analysis showed significant differences in antimicrobial activity across all groups ($P < 0.05$). *E. faecalis* and *C. albicans* exhibited no inhibition with any CFS concentration, while chlorhexidine produced large zones. *S. aureus* showed measurable inhibition with CFS at 300 and 200 mg/mL, though chlorhexidine remained most effective. Intragroup comparisons confirmed that only *S. aureus* responded to Lactobacillus CFS, demonstrating selective antibacterial activity.

Conclusion

Lactobacillus acidophilus CFS showed concentration-dependent antimicrobial activity, with the higher doses (300 and 200 mg/mL) producing measurable inhibition exclusively against *S. aureus*. No inhibitory effect was observed on *E. faecalis* or *C. albicans*, for which chlorhexidine remained significantly more effective. These findings indicate that CFS has limited but selective antibacterial potential, primarily evident at higher concentrations.

Introduction

Microorganisms like *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus aureus* are frequently blamed for persistent endodontic infections, which are a significant contributor to the failure of root canal therapy. Eradication is difficult because these organisms can develop biofilms, infiltrate dentinal tubules, and resist traditional intracanal medications (Siqueira and Rôças, 2008).

Despite their effectiveness, traditional antimicrobial treatments like sodium hypochlorite and chlorhexidine have drawbacks such as cytotoxicity, tissue irritation, and insufficient eradication of resistant bacteria (Haapasalo et al., 2014). Furthermore, it has recently been demonstrated that high sodium hypochlorite concentrations considerably weaken root dentin's fracture strength and erode intertubular dentin, causing damage that can reach depths of up to 400 µm, potentially jeopardizing tooth integrity (Clarkson et al., 2023). Although chlorhexidine is frequently used to control plaque, it has also been shown to cause significant changes in the oral microbiome, such as loss of diversity, decreases in beneficial taxa (such as nitrate-reducing bacteria), and an increase in acidic byproducts like lactate, which may have numerous negative effects on oral and systemic health (Bescos et al., 2020).

These disadvantages emphasize how crucial it is to look into substitute environmentally friendly materials. Antimicrobial metabolites such as bacteriocins, organic acids, and hydrogen peroxide are known to be produced by probiotics, especially *Lactobacillus* species (Ouweland and Vesterlund, 2004). When separated as cell-free supernatants, these metabolites might have antibacterial properties without the dangers of living bacteria. Studies in medical microbiology have demonstrated promising effects of probiotic supernatants against various pathogens (Tejero-Sariñena et al., 2012), but evidence in endodontic infections remains limited.

Examining *Lactobacillus* supernatants may be a potentially useful and safer biological substitute for traditional irrigants. Additionally, this strategy is in line with the increasing need for environmentally safe and biocompatible endodontic materials (Brito et al., 2014). Finding novel treatment approaches may be aided by assessing their in vitro antibacterial efficacy against common endodontic infections.

This study attempts to close the gap between probiotic research and clinical endodontics by investigating these natural metabolites. If effective, it might open the door for root canal disinfection procedures to use chemicals derived from probiotics.

The current study's null hypothesis was that Lactobacilli probiotics would have the same antibacterial activity as chlorhexidine against *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus aureus*.

Materials and methods

The Fayoum University Supreme Committee for Scientific Research Ethics' institutional review board has authorised the current study with the number EC25140. The reference (Shaaban et al., 2022) was used to calculate the sample size. This study found that when the power was 80% and the type I error probability was 0.05, the minimum acceptable sample size was 4 per group for each concentration. The mean $\pm$ standard deviation in Group I was 10.52 ± 0.71 , whereas in Group II it was 8.5 ± 0.71 , with an effect size of 2.84. To account for the 30% dropout rate, the total sample size was increased to six (24 for varying doses for each microbe). G. Power 3.1.9.4 was used to do the t test.

• Study design

The Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 standards (Nagendrababu et al 2021) were followed in the design and reporting of this in vitro experimental laboratory investigation. Antimicrobial activity was evaluated by quantifying inhibition zones using method of agar well diffusion, and all experiments were carried out in triplicate for each concentration. Measurements were taken independently by two calibrated observers, and results were recorded as mean values in millimeters for subsequent statistical analysis.

• Microbial Strains:

Among the test strains are:

- ATCC 19433 *Enterococcus faecalis*
- ATCC 10231 *Candida albicans*
- ATCC 29213 *Staphylococcus aureus*

• Preparation of Inoculum:

The three strains were preserved frozen in sterile glycerol broth safe-lock Eppendorf tubes. Each of them was then refreshed to ensure purity and viability, *Enterococcus faecalis* on bile esculin agar, *Staphylococcus aureus* on nutrient agar, and *Candida albicans* on SDA. Each organism was then suspended in sterile 0.9% normal saline. Each suspension was adjusted to achieve a turbidity comparable to the 0.5 McFarland standard, which was determined by measuring optical density at 600 nm using a spectrophotometer. At this level, the estimated cell density was approximately 1×10^8 CFU/mL for bacterial isolates and about 1×10^6 CFU/mL for *Candida albicans*.

• Agar Plate Preparation and Inoculation:

Sterile 90 mm Petri dishes were filled with Mueller-Hinton Agar (MHA). (~25 mL per plate) and allowed to solidify under aseptic conditions. The three prepared microbial suspensions— *Candida albicans*, *Staphylococcus aureus*, and *Enterococcus faecalis* — were standardized to the 0.5 McFarland turbidity, and are used to inoculate individual agar plates. Inoculation was performed using the lawn culture technique, by evenly spreading the microbial suspension over the entire agar surface in three directions, rotating the plate approximately 60° between each pass to ensure uniform distribution.

• Well Diffusion Assay: (Balouiri et al., 2016)

Six wells (diameter of 6 mm) were inserted aseptically into every agar plate that had been inoculated using a sterile pipette tip. Each well will be loaded with 100 µL of the test agent in its respective concentration.

• Preparation and Testing of *Lactobacillus acidophilus* Cell-Free Supernatants (CFS) then comparing it with chlorhexidine as a positive control and Sterile water as a negative control:

• Cultivation of *Lactobacillus acidophilus* (LA-5)

Lactobacilli were grown in De Man, Rogosa, and Sharpe (MRS) broth for 48 hours at 37°C under microaerophilic conditions. After adjusting fresh cultures to an optical density of 0.6 at 600 nm (OD₆₀₀), they were propagated in 10 mL of MRS broth and incubated at 37°C for 24 to 48 hours.

• Supernatant Extraction and Lyophilization

Centrifugation of the cultures was performed at 4°C with a speed of 8000 rpm for 10 minutes. Filtering of the cell free supernatant (CFS) with sterile filter of 0.45 µm in size was achieved followed by using a freeze dryer vacuum for lyophilizing the CFS. The lyophilized powder was kept till use at a temperature of – 20°C. (Sornsenee et al., 2021)

• Preparation of CFS Working Solutions

Four test concentrations were prepared by reconstituting CFS in sterile distilled water: 300, 200, 100, and 50 mg/mL. To guarantee total dissolution, each solution was combined using a vortex mixer.

• Antimicrobial Testing of CFS

The antibacterial activity of each CFS concentration against *C. albicans*, *S. aureus*, and *E. faecalis* was assessed using the agar well diffusion assay and compared with sterile water and chlorhexidine. In each inoculated plate, six wells were prepared using sterile pipette tips and filled with 100 µL of each test concentration:

- 4 wells for the above mentioned CFS concentrations 300, 200, 100, and 50 mg/mL.
- 1 well for chlorhexidine (positive control)
- 1 well for sterile water (negative control)

(Meena et al., 2025)

The plates were left at room temperature for 15–20 minutes to allow for compound diffusion, then incubated at 37°C for 18–24 hours under aerobic conditions.

• Assessment of the antimicrobial activity of CFS by measurement of Inhibition Zones

Following 24 hours incubation, the diameter of each well's zone of inhibition (Z.O.I.) was measured. in millimetre's using a sterile ruler by two independent calibrated observers.

Statistical analysis

Microsoft Excel 2016, Graph Pad Prism®, and SPSS 27® were used for statistical examination. The Normality tests of Shapiro-Wilk and Kolmogorov were employed to assess each data set's normalcy., and the results showed that all of the data came from non-parametric distributions. As a result, test of Kruskal Wallis was used to compare groups, and test of Friedman was used to compare species. $P < 0.05$ was chosen as the significant level.

1. IBM, USA: Statistical Package for Social Science.

2. USA: Graph Pad Technologies,

3. USA: Microsoft Cooperation,

No zones of inhibition were observed around the wells in plates (b) and (c) at any tested concentration (300, 200, 100, 50 mg/mL), indicating no detectable antimicrobial effect against *E. faecalis* and *C. albicans*. In plate (a), *S. aureus* exhibited weak sensitivity at higher concentrations, with inhibition zones of 5 mm and 3 mm observed at 300 mg/mL and 200 mg/mL, respectively. No activity was detected at lower concentrations.

Results

• Intergroup comparison (comparison between groups):

Descriptive results and comparison between groups was presented in Table 1 and Fig. 4, and was performed by using Kruskal Wallis test which revealed that there was a significant difference between groups ($P < 0.05$) as:

In *E. faecalis*, all concentrations revealed absence zone of inhibition as mean $\pm$ standard deviation was (0 ± 0), except chlorohexidine 2% +ve control was (25.17 ± 0.75).

In *S. aureus*, the highest zone of inhibition was observed in chlorohexidine 2% +ve control group (23.17 ± 0.75), followed by *Lactobacillus* supernatant 300 conc. group (11.17 ± 0.75) and *Lactobacillus* supernatant 200 conc. group (9.17 ± 0.75) with insignificant difference between them, while other groups revealed no zone of inhibition (0 ± 0).

In *C. albicans*, all concentrations revealed absence zone of inhibition as mean $\pm$ standard deviation was (0 ± 0), except chlorohexidine 2% +ve control was (22.00 ± 0.63).

• Intragroup comparison (comparison between different species within each group):

Comparison between different species was performed by using Friedman's test which revealed that there was a significant difference between species regarding *Lactobacillus* supernatant 300 conc. group, *Lactobacillus* supernatant 200 conc. group, and chlorohexidine 2% +ve control group ($P < 0.05$) as:

In *Lactobacillus* supernatant 300 conc. group: *S. aureus* demonstrated (11.17 ± 0.75) zone of inhibition while *E. faecalis* and *C. albicans* showed no zone of inhibition (0 ± 0)

Table 1
Zone of inhibition regarding different concentrations of lactobacillus supernatant, +ve control, and -ve control groups

Organism	lactobacillus supernatant 300 conc.		lactobacillus supernatant 200 conc.		lactobacillus supernatant 100 conc.		lactobacillus supernatant 50 conc.		Chlorohexidine 2% +ve control		Saline -ve control		P value
	Min/Max (Med)	Mean $\pm$ SD	Min/Max (Med)	Mean $\pm$ SD	Min/Max (Med)	Mean $\pm$ SD	Min/Max (Med)	Mean $\pm$ SD	Min/Max (Med)	Mean $\pm$ SD	Min/Max (Med)	Mean $\pm$ SD	
E. faecalis	0/0(0)	0.00 aA $\pm$ 0.00	0/0(0)	0.00 aA $\pm$ 0.00	0/0(0)	0.00 a $\pm$ 0.00	0/0(0)	0.00 a $\pm$ 0.00	24/26(25)	25.17 bA $\pm$ 0.75	0/0(0)	0.00 a $\pm$ 0.00	0.0001*
S. aureus	10/12(11)	11.17 aB $\pm$ 0.75	8/10(9)	9.17 aB $\pm$ 0.75	0/0(0)	0.00 c $\pm$ 0.00	0/0(0)	0.00 c $\pm$ 0.00	22/24(23)	23.17 bAB $\pm$ 0.75	0/0(0)	0.00 c $\pm$ 0.00	0.0001*
C. albicans	0/0(0)	0.00 aA $\pm$ 0.00	0/0(0)	0.00 aA $\pm$ 0.00	0/0(0)	0.00 a $\pm$ 0.00	0/0(0)	0.00 a $\pm$ 0.00	21/23(22)	22.00 bB $\pm$ 0.63	0/0(0)	0.00A $\pm$ 0.00	0.0001*
< 0.0001*		< 0.0001*		-----		-----		0.001*		-----			

*Significant difference as $P \leq 0.05$.

Means with different superscript letters (Small per row / capital per column) were significantly different as $P \leq 0.05$.

Discussion

Probiotics are live microorganisms, primarily bacteria, that are found naturally in a variety of dietary products and have been shown to improve human health. The World Health Organization (WHO) referred Probiotics as being a living microorganisms when taken in proper amounts as dietary supplements or in food they achieve healthy benefits for the host. The most popular probiotic strains are Bifidobacteria and Lactobacilli (Shaaban et al., 2022). Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus paracasei, and Lactobacillus reuteri are the species of Lactobacilli that have been studied the most. Scientific research has also looked at members of the Bifidobacterium genus and additional probiotic microbes including Bacillus, Saccharomyces, Lactococcus, Pediococcus, and Enterococcus, albeit to a lesser degree (Fijan, 2016).

Probiotics, or live microbes, have long been used to treat gastrointestinal disorders. Numerous digestive problems, such as infantile diarrhoea, necrotising enterocolitis, diarrhoea associated with antibiotics, diarrhoea related to Rotavirus, infections with Helicobacter pylori, and traveller's diarrhoea, have long been treated with probiotics (Sullivan et al., 2005).

The creation of antibiotic chemicals, competition with pathogens for colonisation sites, and thickening of the intestinal barrier are just a few of the mechanisms linked to the biological functions of probiotics. Probiotic strains are frequently able to produce several compounds of antimicrobial nature at once, which may work in concert to inhibit a wider variety of pathogens (Papadimitriou et al., 2015). Numerous antimicrobial compounds, including formic acid, acetic acid, phenyllactic acid, lactic acid, benzoic acid, and other organic acids, as well as hydrogen peroxide, short chain fatty acids, acetaldehyde, carbon dioxide diacetyl and acetoin, are known to be produced by beneficial microbes. Additionally, they make bacteriocins and inhibitory compounds that resemble bacteriocins. Lactocin, pediocin, pisciolin, enterocin, reuterin, plantaricin, enterolysin, and nisin are some of the most often reported bacteriocins (Fijan, 2016).

In this study, we looked forward to know more about the *Lactobacillus acidophilus* antimicrobial activity against some pathogens (ATCC 29213 *Staphylococcus aureus*, ATCC 10231 *Candida albicans* and ATCC 19433 *Enterococcus faecalis*). *L. acidophilus*'s antibacterial efficacy against these microorganisms was assessed by agar well diffusion. *Staphylococcus aureus* growth was inhibited at CFS doses of 300 and 200 mg/ml, according to the agar well diffusion experiment. Lactobacillus species have strong antibacterial action against clinically significant infections, including *S. aureus*, according to several studies using the same agar well diffusion method, such as the study of (Gharaei-Fathabad and Eslamifar 2011). Furthermore, Lactobacillus acidophilus showed antibacterial activity against *S. aureus*, according to (Mobarez et al. 2008). These results imply that lactobacilli probiotics is a viable antibacterial agent that could be used as an intracanal drug and irrigant. However, Ayantola et al. (2016) found that *L. acidophilus* supernatant had no inhibitory effect on *S. aureus*.

Due to its virulence characteristics, *Enterococcus faecalis* (*E. faecalis*) is one of the most often recovered bacteria following unsuccessful root canal treatments. Gram-positive facultative anaerobes with strong survival strategies include *E. faecalis*. It creates a resilient biofilm that penetrates the dentinal tubules deeply and can tolerate prolonged periods of malnutrition while adapting to harsh environmental conditions. The main defence strategy of *E. faecalis* against numerous intra canal medications and chemicals is thought to be the existence of a proton pump (Shaaban et al., 2022).

With no inhibitory zones in agar well diffusion, this investigation demonstrated that CFS of Lactobacillus acidophilus had no antibacterial action against *E. faecalis*. Variations in colony forming units or the requirement for higher CFS concentrations may account for this. Furthermore, the CFS was utilized in solution form in this study; future research may employ gel form, which may be more efficient than the solution. This aligns with the results of Hammad et al. (2014) that CFS had no antibacterial impact on *E. faecalis*. However, Shaaban et al. (2022) discovered that CFS was more effective than Ca(OH)_2 at having an antibacterial impact on *E. faecalis*.

Additionally, the study showed that probiotic CFS had no antifungal impact on *Candida albicans*. But according to Akano et al. (2023), lactobacilli metabolites suppress *Candida albicans* at several phases.

To assess the CFS in gel form and at larger doses, more research is required. Additionally, tooth models and other more precise tests, like as the time kill assay, can be used to evaluate the antibacterial action of CFS. It is preferable to compare the agar well diffusion test with other tests because it is regarded as one of the subjective tests.

Conclusion

Lactobacillus acidophilus CFS demonstrated limited, concentration-dependent antimicrobial activity, showing measurable inhibition only against *S. aureus* at 300 and 200 mg/mL. No inhibitory effect was observed against *E. faecalis* or *C. albicans*, while chlorhexidine remained significantly more effective across all organisms. Overall, CFS shows selective but weak antibacterial potential compared to conventional disinfectants.

Declarations

Ethics approval:

Fayoum University Supreme Committee for Research Ethics and Scientific integrity (FU-SCRESI) (code number of the proposal: EC25140).

Consent to participate:

· Ahmed maged negm: writing the original draft, formal analysis, – review and editing.

· Doaa nader: Methodology, writing, formal analysis, – review and editing.

o Sara El mallah: Conceptualization, Methodology and writing

Consent for publication:

not applicable

Conflict of Interest:

The authors report no conflicts of interest.

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Author Contribution

Ahmed maged negm: writing the original draft, formal analysis, – review and editing. Doaa nader: Methodology, writing, formal analysis, – review and editing. Sara El mallah: Conceptualization, Methodology and writing

Acknowledgments:

Not applicable.

Data Availability

All data analysed during this study are included in the article.

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Figures



Figure 1

LA-5 cultures in MRS broth, prior to propagation into larger volumes.

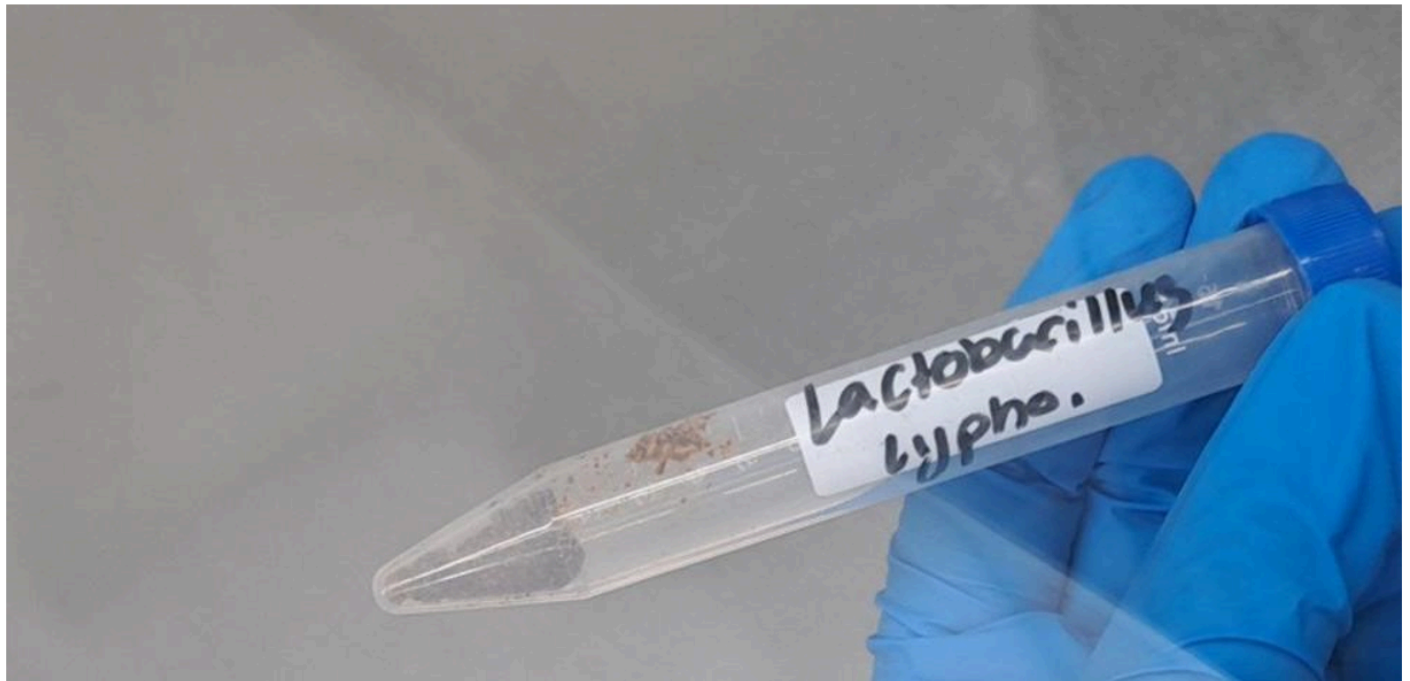


Figure 2

Freeze-dried Lactobacillus supernatant (CFS) stored in a sterile Falcon tube.

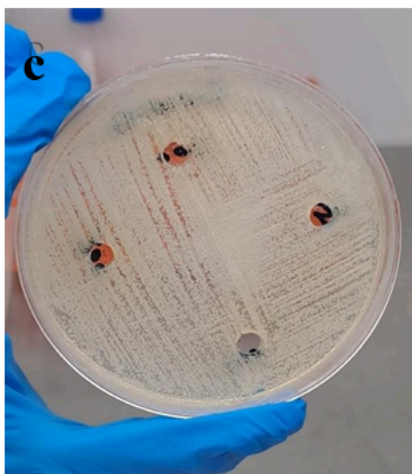
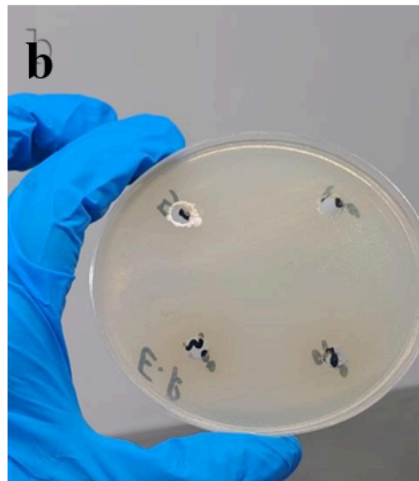
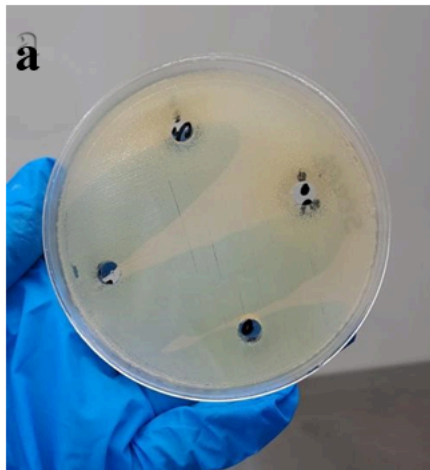


Figure 3

Antimicrobial activity of cell-free supernatants (CFS) of LA-5 against clinically relevant pathogens using the agar well diffusion method. (a) *Staphylococcus aureus*; (b) *Enterococcus faecalis*; (c) *Candida albicans*

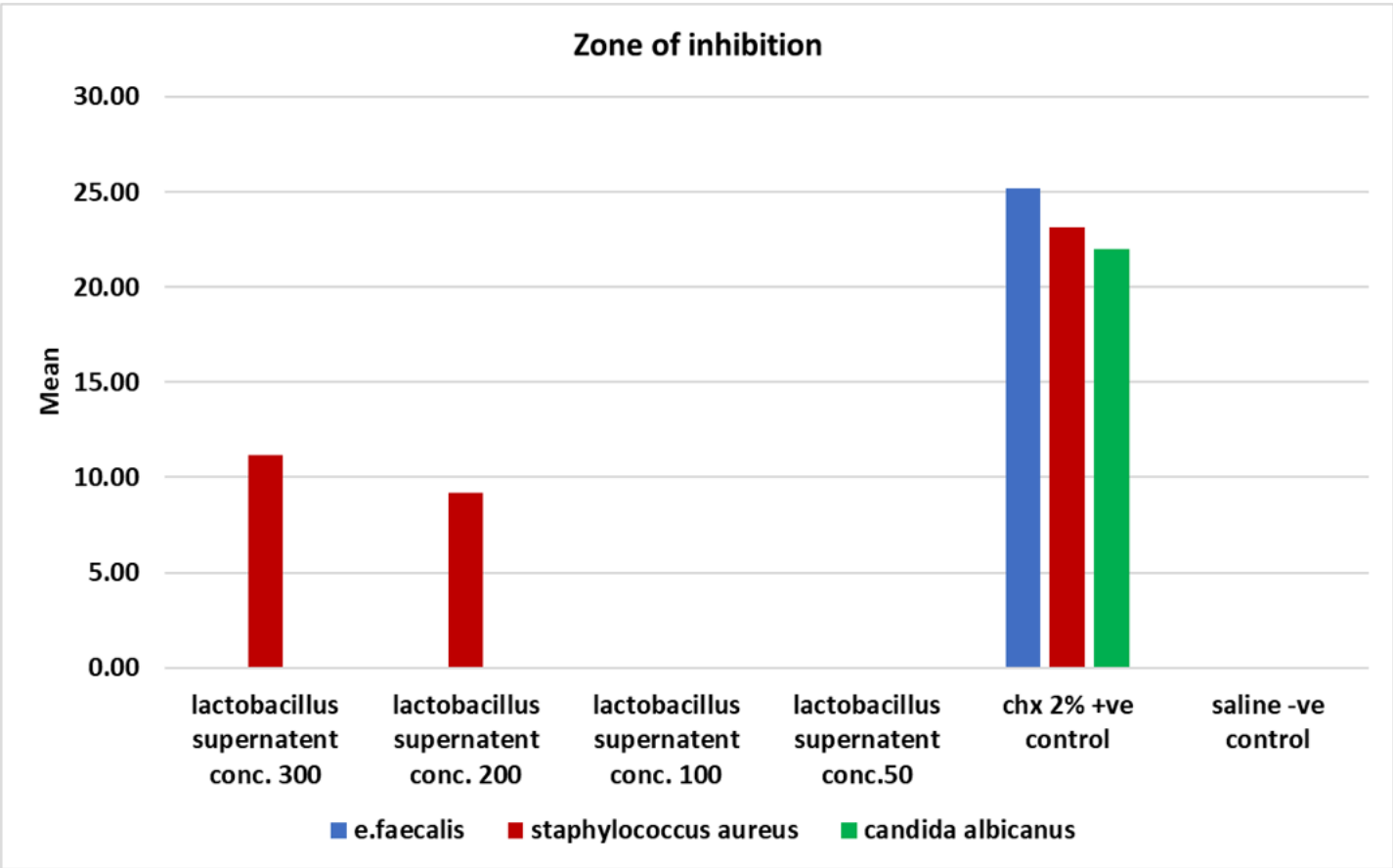


Figure 4

Bar chart represents zone of inhibition of all groups.