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## *In Vitro* Study to Assess the Antifungal Effect of Lactoferrin + Econazole/ Econazole in Reduction of Biofilm formed by Fungal Culture of *Candida albicans*, *Candida tropicalis* and *Candida krusei*



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### ABSTRACT

Fungal infections caused by *Candida* species, such as *Candida albicans*, *Candida tropicalis*, and *Candida krusei*, are a major concern in healthcare settings worldwide due to their ability to form biofilms. Biofilms provide protection and resistance to antifungal treatments, leading to persistent infections. In this study, we aimed to assess the antifungal effect of Lactoferrin in combination with Econazole or Econazole alone in reducing biofilms formed by these *Candida* species. Lactoferrin is a glycoprotein with broad-spectrum antimicrobial properties, while Econazole is an antifungal agent commonly used to treat superficial fungal infections. We conducted in vitro experiments using well-established biofilm models to evaluate the viability and metabolic activity of biofilm cells and the integrity of the biofilm matrix. Our results showed that the combination of Lactoferrin and Econazole significantly reduced biofilm formation compared to Econazole alone, particularly in *Candida krusei* biofilms. Confocal scanning laser microscopy confirmed the disruption of pre-formed biofilms by the combination treatment. These findings suggest that the synergistic effect of Lactoferrin and Econazole enhances their antifungal activity and holds promise for combating *Candida* biofilm-associated infections. Further preclinical and clinical investigations are needed to explore the full therapeutic potential of this combination in managing recurrent or persistent *Candida* infections. This research contributes to the development of innovative strategies to improve patient outcomes and reduce the burden of fungal-related morbidity and mortality.

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### Introduction

Fungal infections caused by *Candida* species are a significant concern in healthcare worldwide. *Candida albicans*, *Candida tropicalis*, and *Candida krusei* are commonly found in clinical settings. These pathogens can form biofilms, which are complex structures made up of microbial cells surrounded by a protective extracellular matrix. Biofilms enhance the virulence and resistance of *Candida*, making them difficult to treat effectively [1]. Existing antifungal therapies like azoles, polyenes, and echinocandins have limited effectiveness against *Candida* biofilms, leading to recurrent and persistent infections [2]. Therefore, there is a need for new treatment strategies that specifically target and eliminate these biofilms. Lactoferrin is a versatile glycoprotein that binds to iron and exhibits broad-spectrum antimicrobial properties [3]. It is naturally present in bodily fluids such as milk, saliva, tears, and mucosal secretions, playing a role in the innate immune system. Lactoferrin disrupts microbial cell membranes, interferes with essential cellular processes, modulates immune responses, and inhibits biofilm formation [4].

Econazole, an antifungal agent of the imidazole class, is commonly used to treat superficial fungal infections. It works by inhibiting the synthesis of ergosterol, a vital component of fungal cell membranes [5]. Econazole has demonstrated potent antifungal activity against *Candida* species, even those resistant to other antifungal agents. Combining lactoferrin with econazole may produce a synergistic effect, resulting in enhanced antifungal activity and biofilm eradication [6]. This combination capitalizes on lactoferrin's ability to disrupt biofilm formation and the antifungal properties of econazole. By combining these two agents, it is hypothesized that their individual mechanisms of action can complement each other, leading to improved effectiveness against *Candida* biofilms [7].

This in vitro study aims to evaluate the antifungal effect of lactoferrin + econazole or econazole alone in reducing biofilms formed by *Candida albicans*, *Candida tropicalis*, and *Candida krusei*. Well-established biofilm models that mimic the structure and characteristics of *Candida* biofilms will be employed. Various assays will assess the viability and metabolic activity of biofilm cells, as well as the integrity of the biofilm matrix [8,9]. The findings of this

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study may contribute to the development of innovative therapeutic approaches for managing *Candida* biofilm-associated infections. If the combination of lactoferrin and econazole demonstrates superior antifungal activity against *Candida* biofilms, further preclinical and clinical investigations may be warranted. Ultimately, this combination therapy could offer a promising treatment option for patients with recurrent or persistent *Candida* infections [10].

Overall, understanding the antifungal potential of lactoferrin + econazole or econazole alone against *Candida* biofilms may lead to novel strategies for combating these challenging infections. This could ultimately improve patient outcomes and reduce the burden of fungal-related morbidity and mortality [11].

### Study Material and Animal Husbandry:

The test article utilized in this study is Lactoferrin Cream (2.0%w/w) provided by Frimline Private Limited, located at 5th Floor-51 1 Iscon Elegance Nr. Circle P, Nr. Jain Temple, Prahlad Nagar Cross Rd, Ahmedabad -380051, Gujarat, India. The cream formulation consists of lactoferrin at a concentration of 2.0% (w/w) and exhibits a light pink color. Additionally, the test item Econazole is employed in the form of white powders. To ensure the safety of the researchers and maintain laboratory standards, standard laboratory safety procedures were strictly followed during the handling of the dose formulations. This involved the use of laboratory aprons, gloves, and face masks while administering the doses. The test system and animal husbandry for this study were sourced from the Microbiology Division at CSIR-NCL, situated at Dr Homi Bhabha Rd, Ward No. 8, NCL Colony, Pashan, Pune, Maharashtra 411008, India. The selection of the test system is justified based on the rationale provided by relevant reference literature. The utilization of the in vitro disruption of fungal biofilm formation technique is recommended in the literature, establishing its suitability and applicability to this study.

### Experimental Design

Test drug were provided by the sponsor. In study *Candida albicans*, *Candida tropicalis* and *Candida krusei* were the fungal isolates used. The fungi were grown in yeast nitrogen base (0.7% wX v) with 1% glucose. The cells were harvested, washed twice with phosphate-buffered saline (PBS). Thereafter, a standard inoculum containing 1.0 X 10<sup>6</sup> cells ml<sup>-1</sup> was used for subsequent experiments.

**Biofilm formation in microtiter plates:** Biofilms were formed in flat-bottomed, 96-well microtiter plates. Standardized suspensions (100 pL) were added into the wells and the cells were allowed to adhere for 1 hr. The wells were drained, washed with PBS and 200 pL of growth medium was added. The plates were incubated for 24 - 48 hrs. After incubation, the planktonic cells were discarded and weakly adherent cells were removed by washing with PBS. Quantification of the biofilms was performed by the crystal violet method (Djordjevic *et al.* 2002). Each biofilm formation experiments were carried out in two independent sets with six replicates and the results are proportional to efficacy of crystal violet absorbance expressed as mean values of crystal violet absorbance + standard deviation (SD) from the mean [12,13]

**Disruption of preformed biofilms in microtiter plates by Econazole and Lactoferrin + Econazole:** The biofilms were individually formed in microtiter plates for 24 hrs. The residual medium was aspirated and the wells were washed with PBS. Lactoferrin (500 pg/mL) + Econazole (150 pg/mL) was added to each well from a stock solution containing 1.0 mg ml<sup>-1</sup>, in accordance

with an earlier report on the use of biofilm disruptors (Mireles *et al.* 2001). Such wells along with the culture medium were incubated further for 24 hrs and 48 hrs. And the residual biofilm was assessed. In control wells, Lactoferrin (500 qg/mL) was omitted and biofilm growth was monitored in an uninterrupted manner [14].

**Biofilm disruption on glass surface and confocal scanning laser microscopy (CSLM):** The effect of Econazole and Lactoferrin in combination of Econazole on biofilm formation was studied on glass slide surfaces. The test cultures were grown for 24 h and inoculated into petri plates containing 20 ml of YNB or LB medium, respectively. Either study arm drugs at a final concentration of Lactoferrin (500 pg/mL) and Econazole (150 kg/mL) of the medium was added to individual plates. The plates without Lactoferrin served as controls. Presterilized microscopic glass slides were immersed into the medium and the plates were incubated for 4 and 24 h. The biofilms were monitored under a CSLM after washing with PBS and staining with 0.01% acridine orange. A CSLM (Model Manual TCS SP2) equipped with DM IRE 2-inverted microscope was used to image biofilms using a 63 x 1.2 NA water immersion objective. The 488 nm Ar laser and a 500-640 nm band pass emission filter were used to excite and detect the stained cells. Images (04) were collected from the 4-hr and 24-hrs-old biofilms measuring an area of 238.1 -238.1 pm. The area of colonization with and without Lactoferrin was determined using the digital image analysis [15-18].

### RESULT

Precoating of wells with Econazole and Lactoferrin was highly effective in controlling *Candida albicans*, *Candida tropicalis* and *Candida krusei* biofilms as compared to Econazole after 24 hrs incubation period by the precoating technique. Precoating of wells with Econazole and Lactoferrin was more effective in controlling *Candida krusei* biofilms as compared to Econazole after 48 hrs incubation period by the precoating technique. There was no difference between effect of Econazole and Lactoferrin and Control (Econazole) in controlling *Candida tropicalis* biofilms. Effect of Econazole was higher in controlling *Candida albicans* biofilms as compared to Econazole and Lactoferrin (Table 1).

The effect of Econazole and Lactoferrin in disruption of preformed biofilms of *Candida albicans*, *Candida tropicalis* and *Candida krusei* was higher as compared to Econazole after 24 hrs. and 48 hrs. of incubation period. Econazole and Lactoferrin dislodged *Candida albicans* by 83.2%, *Candida tropicalis* by 74.3% and *Candida krusei* by 68.7% when compared with the control (Econazole) after 24 hrs. of incubation period. Similar results were observed in effect of Econazole and Lactoferrin in disruption of preformed biofilms of *Candida albicans*, *Candida tropicalis* and *Candida krusei* when compared to Econazole after 24 hrs. and 48 hrs. of incubation period.

The effect of co-incubation of the test cultures with Lactoferrin (500 µg/mL) + Econazole (150 µg/mL)/Econazole (150 µg/mL) in all three culture media (*Candida albicans*, *Candida tropicalis*, and *Candida krusei*) was studied by using CSLM. The effect of Lactoferrin (500 µg/mL) + Econazole (150 µg/mL)/Econazole (150 µg/mL) on biofilms of *Candida albicans*, *Candida tropicalis*, and *Candida krusei* is shown in Figure 2 to 7, respectively. In the presence of Lactoferrin (500 µg/mL) + Econazole (150 µg/mL), there was a graded decrease in the biofilm formation and a greater reduction in the area that was colonized (Figure 4, 6 & 8) as compared to Econazole (150 µg/mL) (Figures 3, 5 & 7).

Fungal Strains	Crystal violet absorbance (A595)						
	Control	24 h		48 h			
		Test	Control	Test	Control	Test	Control
<b><i>Candida albicans</i></b>	0.30 ± 0.01	0.34 ±	0.01	0.58 ±	0.01	0.53 ±	0.02
<b><i>Candida tropicalis</i></b>	0.26 ± 0.01	0.31 ±	0.01	0.36 ±	0.02	0.38 ±	0.01
<b><i>Candida krusei</i></b>	0.19 ± 0.03	0.36 ±	0.02	0.42 ±	0.01	0.57 ±	0.01

**Table 1:** Effect of Econazole and Lactoferrin + Econazole on biofilm formation.

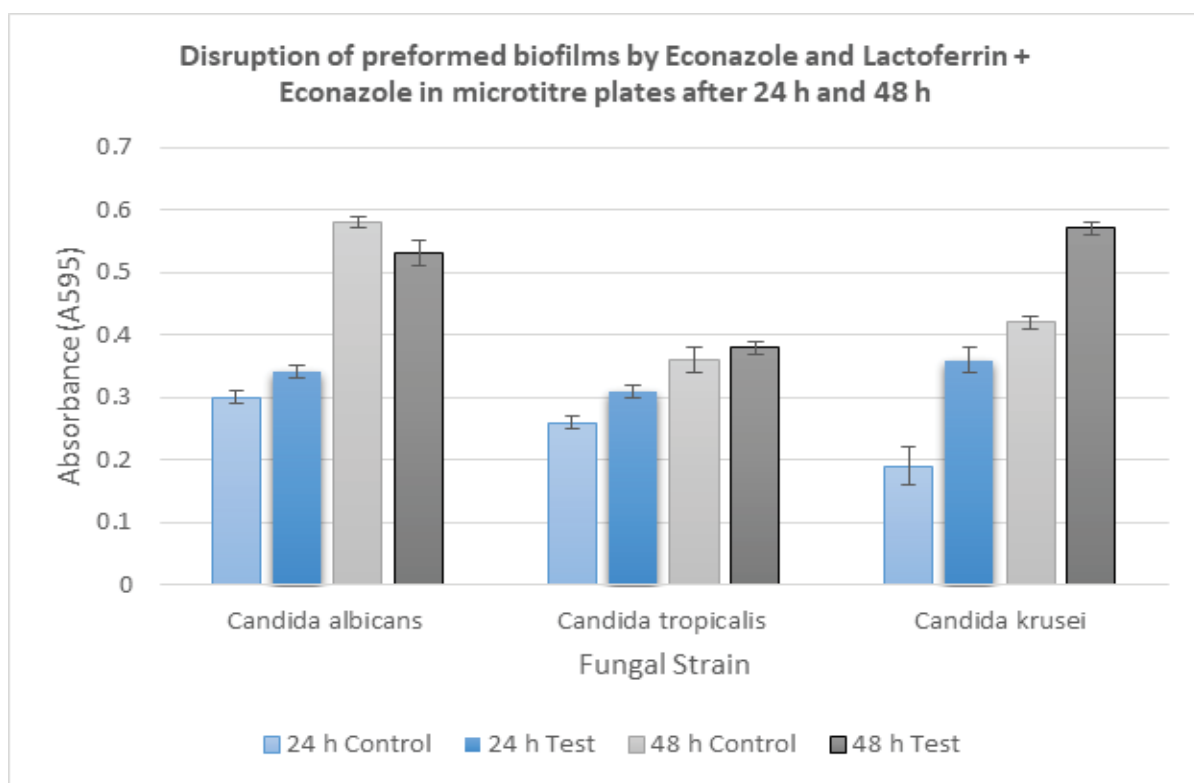


Figure 1: Disruption of Preformed biofilms by Econazole and Lactoferrin/Econazole in Microtiter Plates after 24 hrs. and 48 hrs.

Percent (%) reduction in area colonized onto glass surface after 24 h			
Treatment Groups	<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Candida krusei</i>
Econazole (150 µg/mL)	58.3	64.8	31.5
Lactoferrin (500 µg/mL) + Econazole (150 µg/mL)	83.2	74.3	68.7

Table 2: Quantitative data on biofilm disruption obtained from confocal laser scanning microscopic image analysis.

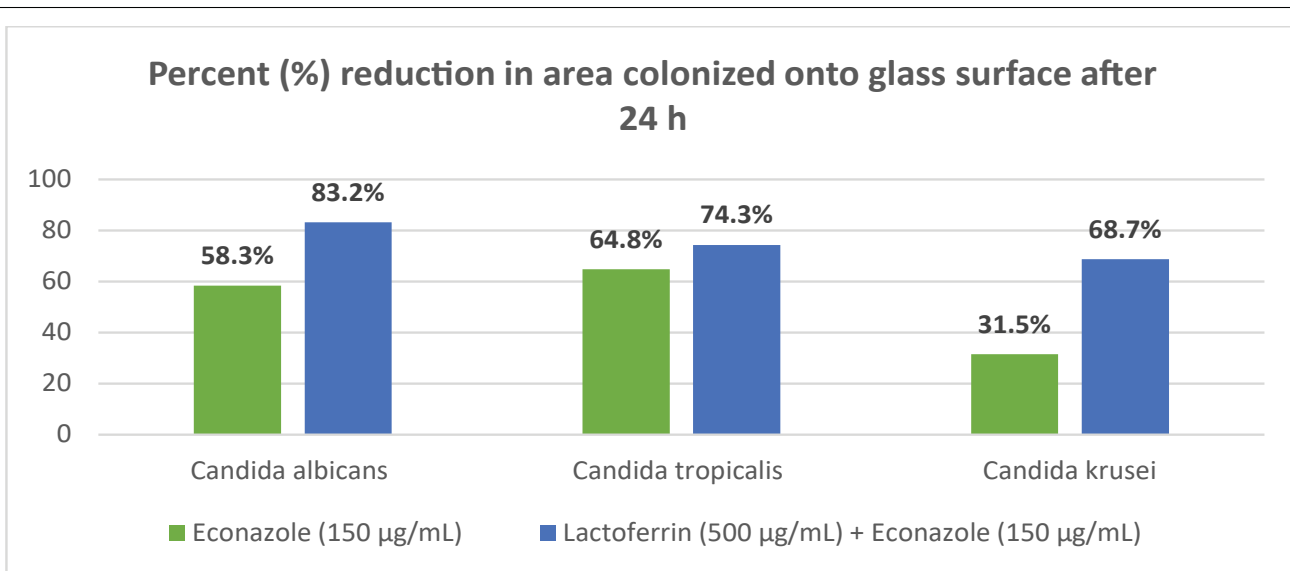


Figure 2: Percent (%) reduction in area colonized onto glass surface after 24 h.

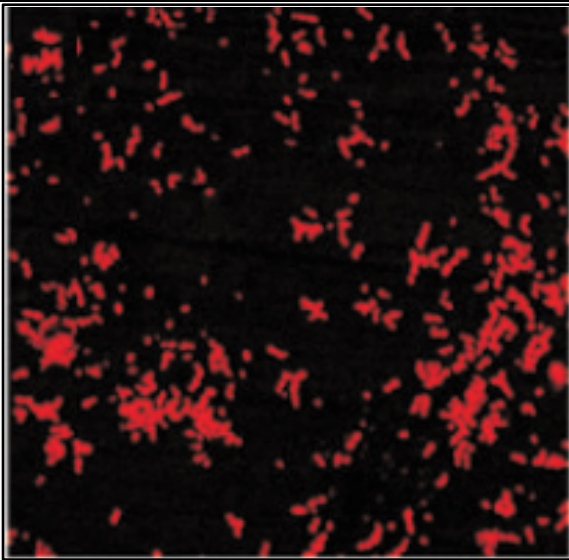


Figure 3: Econazole (150 µg/mL): *C. albicans*

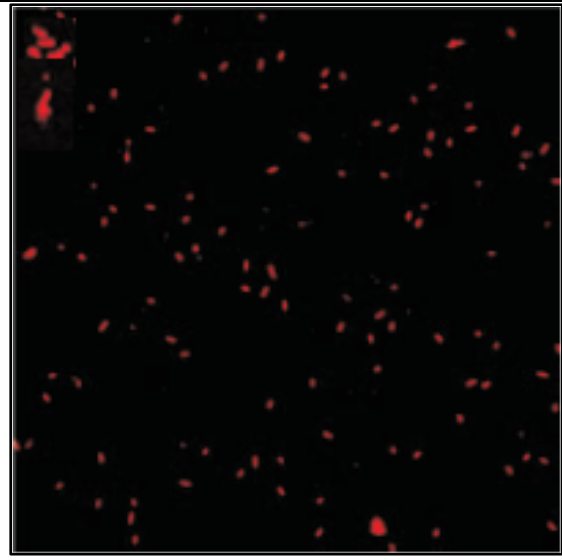


Figure 4: Lactoferrin (500 µg/mL) + Econazole (150µg/mL): *C. albicans*

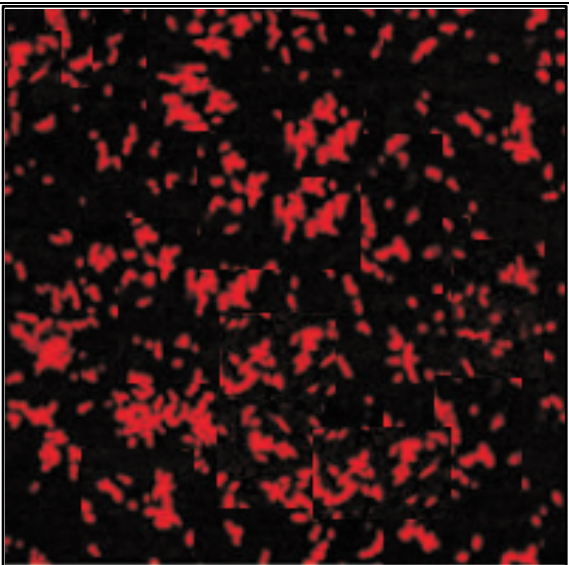


Figure 5: Econazole (150 µg/mL): *C. tropicalis*

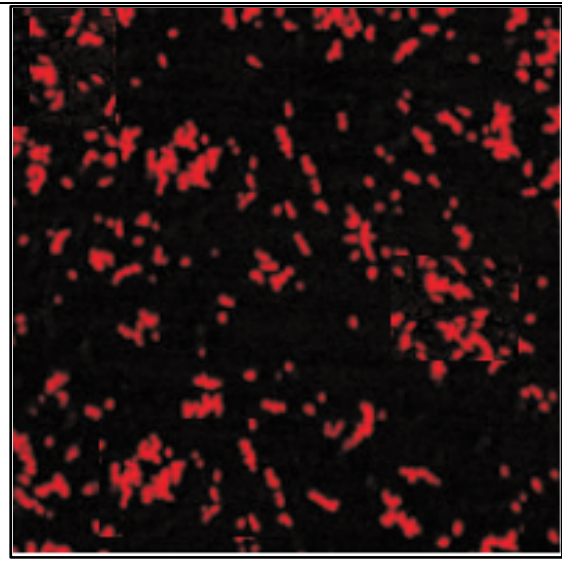


Figure 6: Lactoferrin (500 µg/mL) + Econazole (150 µg/mL): *C. tropicalis*

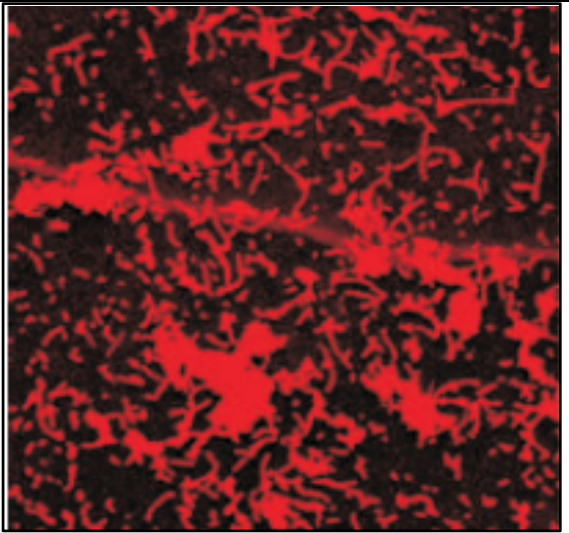


Figure 7: Econazole (150 µg/mL): *C. krusei*

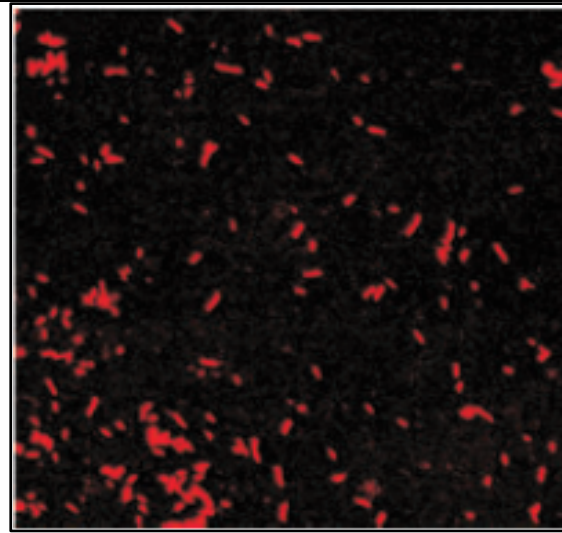


Figure 8: Lactoferrin (500 µg/mL) + Econazole (150 µg/mL): *C. krusei*

## CONCLUSION

In conclusion, study demonstrates that Econazole and Lactoferrin exhibit significant inhibitory effects on biofilm formation in representative fungal cultures under different tested conditions. Precoating with Econazole and Lactoferrin proved to be highly effective in reducing the formation of biofilms caused by *Candida albicans*, *Candida tropicalis*, and *Candida krusei* after a 24-hour incubation period [19-21]. Furthermore, it demonstrated superior control over *Candida* krusei biofilms compared to Econazole alone, particularly after a 48-hour incubation period [22]. The reduction in biofilm formation was observed to be graded across all three *Candida* species, with the combined application of Econazole and Lactoferrin resulting in a greater decrease in the colonized area. These findings indicate that the antifungal properties of Lactoferrin in conjunction with Econazole play a crucial role in inhibiting overall growth and disrupting the biofilms of the tested cultures [23,24]. Consequently, our results suggest a novel and promising application of Lactoferrin in combination with Econazole as an effective agent for biofilm disruption [25-27].

In summary, this research highlights the potential of Lactoferrin and Econazole as biofilm-disrupting agents in the context of fungal infections caused by *Candida* species. Further investigations and clinical trials are warranted to explore the full therapeutic potential of this combination, potentially paving the way for new strategies to combat biofilm-related infections.

**Acknowledgments:** Not Applicable

**Conflict of interest:** Not Applicable

**Ethical Considerations:** The research team followed CPCSE, national, and international regulations, guidelines, and ethical standards for the use of animals in research. This included compliance with relevant laws, such as the Animal Welfare Act, and adherence to the principles outlined in the 3Rs (Replacement, Reduction, and Refinement) for animal research.

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