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Original Research Article

Comparative antagonistic activity of probiotic *Lactobacillus* and *Bacillus* strains against oral *Candida* isolates: insights into cell-free supernatant and viable cell-mediated interactions

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ABSTRACT

Background: *Candida* species represent a diverse group of opportunistic yeasts, including clinically important taxa with exhibiting emerging antifungal resistance, complicating the management of oral candidiasis. Probiotic *Lactobacillus* and *Bacillus* have emerged as promising adjunctive or alternative agents for controlling oral *Candida* through antimicrobial metabolite production and competitive interactions.
Methods: The antagonistic activity of four commercial *Bacillus* strains (*B. subtilis, B. coagulans, B. clausii, B. mesentericus*) and three *Lactobacillus* strains (*L. acidophilus, L. rhamnosus, L. reuteri*) was assessed against 160 oral *Candida* isolates (*C. albicans, C. tropicalis, Pichia kudriavzevii, C. parapsilosis*). Agar overlay, agar well diffusion and spot assays were conducted to compare the inhibitory potency of viable probiotic cells and their cell-free supernatants.
Results: *B. coagulans* and *B. subtilis* demonstrated the greatest inhibitory effects across multiple *Candida* species, followed by notable inhibition from *L. acidophilus, L. rhamnosus* and *L. reuteri*. Viable probiotic cells consistently produced zones of inhibition surpassing those observed with cell-free supernatants, highlighting the importance of direct microbial presence and contact in antagonism.
Conclusions: The study establishes that probiotic antagonism against oral *Candida* is largely strain-specific and dependent on assay conditions, with active cellular interactions playing a crucial role. The findings support the therapeutic potential of viable *Bacillus* and *Lactobacillus* preparations as biotherapeutic agents in the prevention and management of oral candidiasis.

Keywords: Probiotics, *Bacillus, Lactobacillus,* Oral *Candida,* Cell- free supernatant, Antimicrobial resistance

INTRODUCTION

The escalating global burden of fungal infections, particularly those caused by *Candida* species, represents a critical challenge in modern medicine. Approximately half of all candidiasis are attributed to *Candida albicans*, while the remaining infections are caused by non- albicans *Candida* species. Among these, infections caused by *Candida tropicalis, Nakaseomyces glabratus, Candida parapsilosis, Pichia norvegensis, Candida dubliniensis,* and *Candidozyma auris* have drawn significant attention. Several of these non- *albicans* Candida species are increasingly recognized as emerging opportunistic pathogens.[1] Rising antifungal resistance exemplified by the emergence of multidrug resistant *Candidozyma auris* and the limited arsenal of conventional therapies necessitate innovative prophylactic and therapeutic strategies. Probiotic interventions, leveraging the innate antagonistic capabilities of beneficial bacteria, have emerged as a promising frontier in mycosis management.[2]

In this regard, interest in probiotics and their role in human health has increased in recent years, because of their excellent performance in preventing and treating several

diseases and the increasing demand for natural medicines by consumers.[3] Probiotics are microorganisms that have been claimed to provide health benefits when consumed. Many studies have focused on their roles and effects on the maintenance of health.[4] Current researches have shown that the balance between beneficial and pathogenic bacteria is essential in order to maintain health.[5] Probiotics confer health benefits on the host via diverse mechanisms, including preventing pathogen adherence, producing bacteriocins, changing the pH, producing vitamins and immunological modulation.[6]

So far, the best-documented probiotics are lactic acid bacteria. In contrast, the mechanisms responsible for the beneficial effects of other probiotics, especially the Bacillus species, have remained relatively unexplored until fairly recently. Recent taxonomic revisions have reclassified several *Bacillus* and *Lactobacillus* species. *Bacillus coagulans* and *B. clausii* are now recognized as *Weizmannia coagulans* and *Schouchella clausii* whereas *B.subtilis* and *B. mesentericus* remains within the genus *Bacillus*.[7,8] Likewise, *Lactobacillus rhamnosus*, and *L. reuteri* have been reassigned to *Lacticaseibacillus rhamnosus* and *Limosilactobacillus* reuteri.[9]

The study explores the *invitro* anti-*Candida* efficacy of seven bacterial taxa: *Bacillus subtilis, B. coagulans, B. clausii, B. mesentericus, L. reuteri, L. acidophilus* and *L. rhamnosus*.

METHODS

Collection of microbial strains

The present cross-sectional study was carried out at the Department of Medical Microbiology, School of Medical Education, Kottayam, Kerala from July 2024 to July 2025. A total 160 oral *Candida* isolates were collected from various diagnostic laboratories in Kerala. The isolates were further validated by subculturing in HiCrome™ *Candida* differential media, which is followed by identification using gram staining which revealed Gram-positive budding yeast cells. All of the culture media, syringe-driven filter and cellulose nitrate membrane filter paper (0.22 µm) used for the study were purchased from Hi Media, India.

Test probiotic organisms used for the analysis

The organisms used for the study were *Bacillus subtilis* HU58*, *Bacillus coagulans* (recently reclassified as *Weizmannia coagulans*), *Bacillus clausii* (O/C, N/R, SIN &T; renamed as *Schouchella clausii*), *Bacillus mesentericus* TO-A JPC, *Lactobacillus acidophilus* MTCC 10307 (procured from department store), *Lactobacillus rhamnosus* GG (ATCC 53103; renamed as *Lacticaseibacillus rhamnosus*), *L. reuteri* (taxonomically updated to *Lacticaseibacillus rhamnosus)*.

Table 1: Probiotic strains and their manufacturers used for antagonistic analysis.

Test probiotic strains	Manufacturer
Bacillus subtilis	Darolac- Mini, Aristo pharmaceuticals Pvt Ltd., India
Bacillus coagulans	Velbiom Q-Gazz, Velbiom Probiotics Private Ltd., India
Bacillus clausii	Enterogermina®, Sanofi India Ltd, India
Bacillus mesentericus	Colonise forte, Torrent pharmaceuticals Ltd, India
Lactobacillus rhamnosus	Entero plus FDC Ltd, India
Lactobacillus reuteri	VIZYLAC HP, India

Preparation cell-free supernatant

The *Bacillus* species obtained from spore germination, and *Lactobacillus* species were cultured in Mueller Hinton broth and incubated at 37°C for 24 hours. Then the broth culture was centrifuged at 9000 RPM at 4°C for 10 minutes. Supernatants were collected and filter- sterilized with 0.22 µm syringe filters.

Detection of antimicrobial activity of probiotics against Candida

The probiotic activity of both viable *Bacillus* cells as well as cell free supernatant were evaluated using two techniques; Agar overlay method and Agar well diffusion method.

Agar overlay method

The probiotic activity of *Bacillus subtilis, B. coagulans, B. clausii, and B. mesentericus*, *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. reuteri* were evaluated utilizing a modified Agar overlay method as described by Fleming *et al.*[10] Probiotic isolates were initially cultured on Mueller Hinton agar (MHA) for 24 hours at 37°C under aerobic conditions. A single colony was subsequently transferred to Mueller Hinton broth (MHB) and incubated overnight at 37°C. *Candida* spp. isolates were prepared in parallel by incubation in MHB for 24 hours at 37°C. A foundational layer of de Man, Rogosa, and Sharpe (MRS) agar was prepared for *Lactobacillus* and Mueller Hinton agar for *Bacillus spp*., and permitted to solidify. Thereafter, 5 µl aliquots of overnight the cultures and their respective cell-free supernatants were spot-inoculated onto MHA plates, spaced approximately 3 cm apart, followed by incubation for 24 hours at 37°C. Growth was observed at inoculation sites of viable probiotic cells while cell-free supernatant spots showed no visible growth. The probiotic-inoculated MHA plates were subsequently overlaid with 7 ml of molten MHA soft agar (0.75%), cooled to 40-45°C and seeded with 100 µl of *Candida* culture. Following a further

24-hour incubation at 37°C, zones of inhibition surrounding *Bacillus* and *Lactobacillus* colonies were measured in millimetres and documented for comparative analysis.

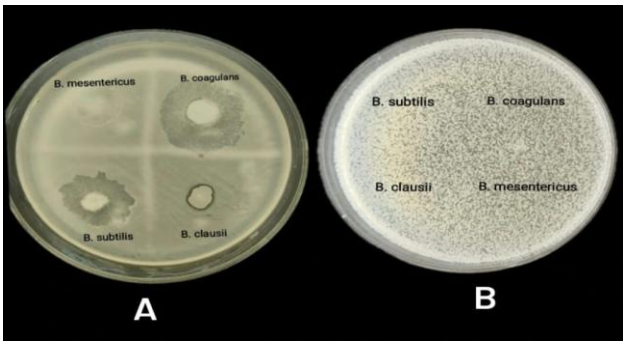

Figure 1: Agar overlay plates of (A) viable cell growth, and (B) cell free supernatants of *Bacillus* strains against *Candida* isolates.

Agar well diffusion method

The antagonistic activity of probiotic spp. against *Candida* was evaluated employing a modified agar well diffusion assay, as outlined by Nalawade et al.[11] Briefly, 50 µl of standardized *Candida* suspension was evenly inoculated onto the agar surface to establish a confluent lawn. Wells of 7 mm diameter and 4 mm depth were aseptically created at equidistant points within the agar matrix. Subsequently, 70 µl aliquots of either probiotic cell-free supernatant or viable cell suspension were introduced into each well using a micropipette. Plates were incubated aerobically at 37°C for 24 hours. Following incubation, the diameter of inhibition zones surrounding each well was measured in millimetres using a standardized scale and values were recorded.

Agar spot assay

The agar spot assay was adapted from the method of Sudan et al with minor modifications.[12] Clinical *Candida* isolates were swabbed uniformly onto Mueller Hinton agar plates. Subsequently, 5 µl aliquots of overnight cultures and corresponding cell-free supernatants of *Bacillus* and *Lactobacillus* were spot inoculated onto the agar surface. Plates were incubated at 37°C under 5–10% CO_2 for 24 hours. After incubation, zones of inhibition surrounding bacterial spots were measured in millimetres using a calibrated scale.

Statistical analysis

The study was analysed using Independent samples t test. The statistical test was used to analyze the difference in the mean growth of *Bacillus* and *Lactobacillus* strains between the *Candida albicans* and non-*albicans.* All data were processed using statistical package for the social sciences (SPSS). Differences with $p<0.05$ were considered statistically significant.

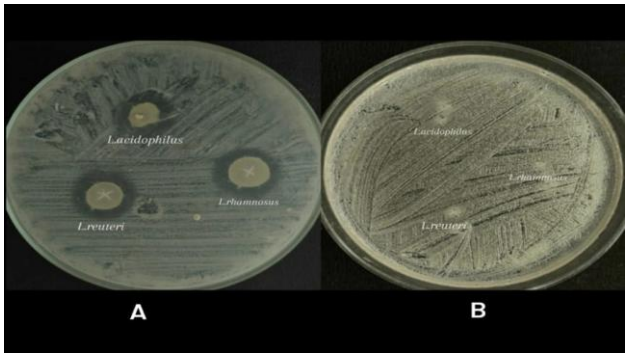

Figure 2: Agar spot assay plates of (A) viable cell growth, and (B) cell free supernatants of *Lactobacillus* against *Candida* isolates

RESULTS

Identification and characterisation of Candida and Bacillus

A total of 160 clinical *Candida* isolates obtained from diagnostic microbiology laboratories were subcultured on Sabouraud Dextrose Agar and HiCrome™ Candida differential agar for phenotypic characterization. Species identification revealed the distribution of *C. albicans* (n=90), *C. tropicalis* (n=60), *Pichia kudriavzevii* (n=4), and *C. parapsilosis* (n=6). On HiCrome™ agar, *C. albicans* colonies appeared green, *C. tropicalis* produced blue colonies, and *P. kudriavzevii* developed characteristic purple pigmentation. The identification of *C. parapsilosis* isolates was further confirmed using the VITEK MS.

Probiotic *Bacillus* and *Lactobacillus* strains utilized in this study were obtained from commercial sources and prepared under laboratory conditions. A total of four commercially obtained *Bacillus* strains; *B. subtilis, B. coagulans, B. clausii*, and *B. mesentericus* were initially cultured in Mueller Hinton Broth and subsequently subcultured onto Mueller Hinton agar for the isolation and phenotypic characterization. Parallelly, *Lactobacillus* isolates comprising *L. acidophilus*, *L. rhamnosus*, and *L. reuteri* were cultivated on de Man, Rogosa and Sharpe (MRS) agar, with visible growth obtained after 24 hours of aerobic incubation.

Antagonistic activity of viable cells

Viable cell suspensions of the tested *Bacillus* species demonstrated variable degrees of antagonistic activity against oral *Candida* isolates when screened using agar overlay, well diffusion, and spot assays. Among the strains, *B. subtilis* exhibited the highest inhibitory potential, showing strong activity against 73.75% (n=118) of *Candida* isolates (n=160), moderate activity against 20% (n=32), weak activity towards 1.25% (n=2) and no inhibition in 5% (n=8). The mean inhibition zones revealed significantly greater activity against *C. albicans* (15.74±2.3 mm) than non-*albicans Candida* (13.38±1.9 mm) ($p<0.05$). *B. coagulans* displayed strong

inhibition against 90% (n=144) of isolates, with no significant difference between *C. albicans* and non-*albicans* groups (p=0.78). In contrast, *B. clausii* exhibited minimal activity, with only 3.75% (n=6) showing strong and 10% (n=16) moderate to weak inhibition, lacking any statistically significant difference between groups. *B. mesentericus* showed negligible antagonism, with inhibition observed in only 5% (n=8) of isolates and mean zone diameters near zero (p>0.05). Overall, *B. coagulans* and *B. subtilis* emerged as the most potent *Bacillus* probiotics exhibiting effective antagonistic action against *Candida* species under *in vitro* conditions.

The antagonistic activity of *Lactobacillus* species against clinical *Candida* isolates revealed distinct species-specific inhibitory profiles. *Lactobacillus acidophilus* exhibited comparable inhibition zones against *C. albicans* (mean±SD: 14.71±3.80 mm, n=90) and non-*albicans Candida* species (15.23±3.35 mm, n=70), with no statistically significant difference between groups (Welch's t(155.45)=–0.899, p=0.370, 95% CI [–1.65, 0.62]). In contrast, *L. rhamnosus* demonstrated significantly greater antagonistic activity against *C. albicans* (13.56 ± 3.86 mm) compared to non-*albicans Candida* isolates (10.17±3.74 mm), t(158)=2.012, p=0.046, 95% CI [0.02, 2.54], indicative of a selective inhibitory effect. *L. reuteri* displayed similar inhibition against both *C. albicans* (11.76±4.49 mm) and non-*albicans Candida* (11.14±4.05 mm), with no statistically significant difference (t(158)=0.913, p=0.363, 95% CI [–0.74, 2.02]). Among the evaluated species, *L. acidophilus* showed the most pronounced antagonistic activity against *Candida*, whereas *L. rhamnosus* and *L. reuteri* exhibited comparable inhibitory effects across *Candida* groups.

Antagonistic activity of cell free supernatants

In all experimental replicates, none of the cell-free supernatant (CFS) samples produced measurable zones of inhibition against *Candida albicans* or non-*albicans Candida* species. This indicates that the extracellular metabolites present in the supernatants of the tested *Bacillus* and *Lactobacillus* strains, under the given culture and assay conditions, did not exhibit detectable antifungal activity.

Comparison of antagonistic activity of viable cell suspension and cell free supernatant

The antagonistic activity of both viable cell suspension and their corresponding CFS derived from *Bacillus* and *Lactobacillus* strains were assessed against oral *Candida* isolates. Cell free supernatants of all seven probiotic strains under study showed no antagonistic activity (mean=0). Regarding viable cell suspensions of *Bacillus* strains, *B. coagulans* (mean=20.1), *B. subtilis* (mean=14.71), *B. clausii* (mean=1.31) and *B. mesentericus* (mean=0.225) demonstrated measurable zones of inhibition with varying degrees of antagonistic effects across the *Candida* isolates.

Evidently, *B. coagulans* demonstrated the most pronounced antagonistic activity against the *Candida* isolates. This was followed by *B. subtilis*. The inhibitory effect observed with *B. clausii*, and *B. mesentericus* was relatively attenuated in comparison. Similarly, live cell suspensions of *Lactobacillus acidophilus* (mean=14.94), *L. rhamnosus* (mean=10.83), and *L. reuteri* (mean=11.48) presented considerable antagonistic effects against both *C. albicans* and non-*albicans Candida* species. *L. rhamnosus* exhibited a particularly potent inhibitory effect against *C. albicans* (p<0.05).

However, the CFS from these *Lactobacillus* strains did not produce any measurable inhibition zones across the *Candida* isolates that were tested. This would suggest that the antifungal activity seen in *Bacillus* and *Lactobacillus* probiotics is largely associated with live cells, rather than extracellular metabolites found in their supernatants.

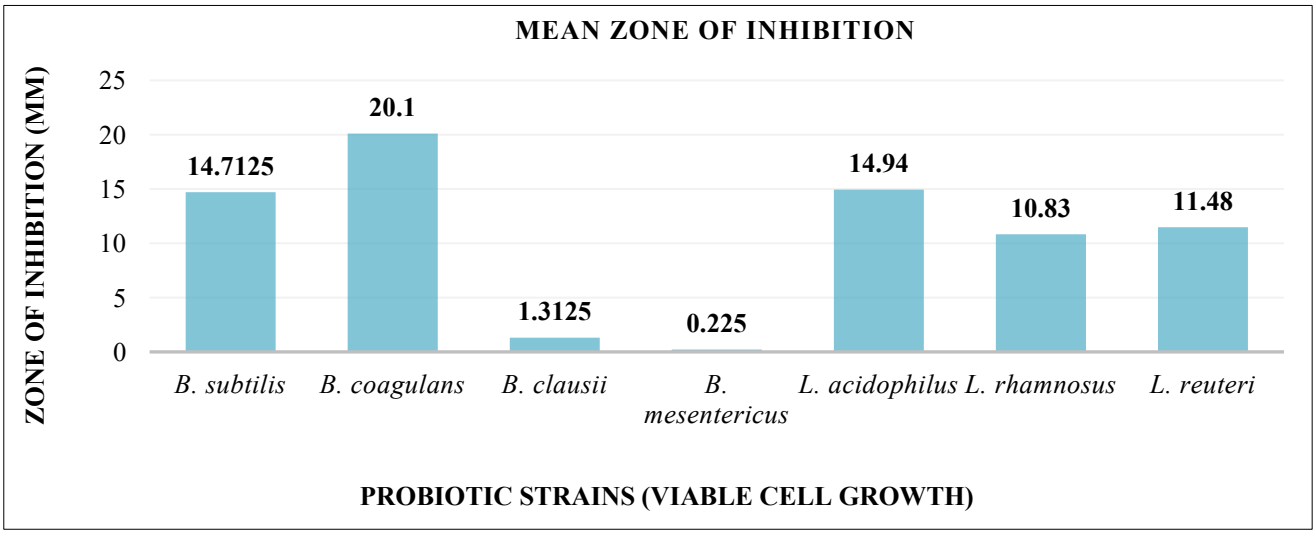

Figure 3: Graphical representation of antagonistic activity of viable cell suspension of *Bacillus* and *Lactobacillus spp.* on *Candida*.

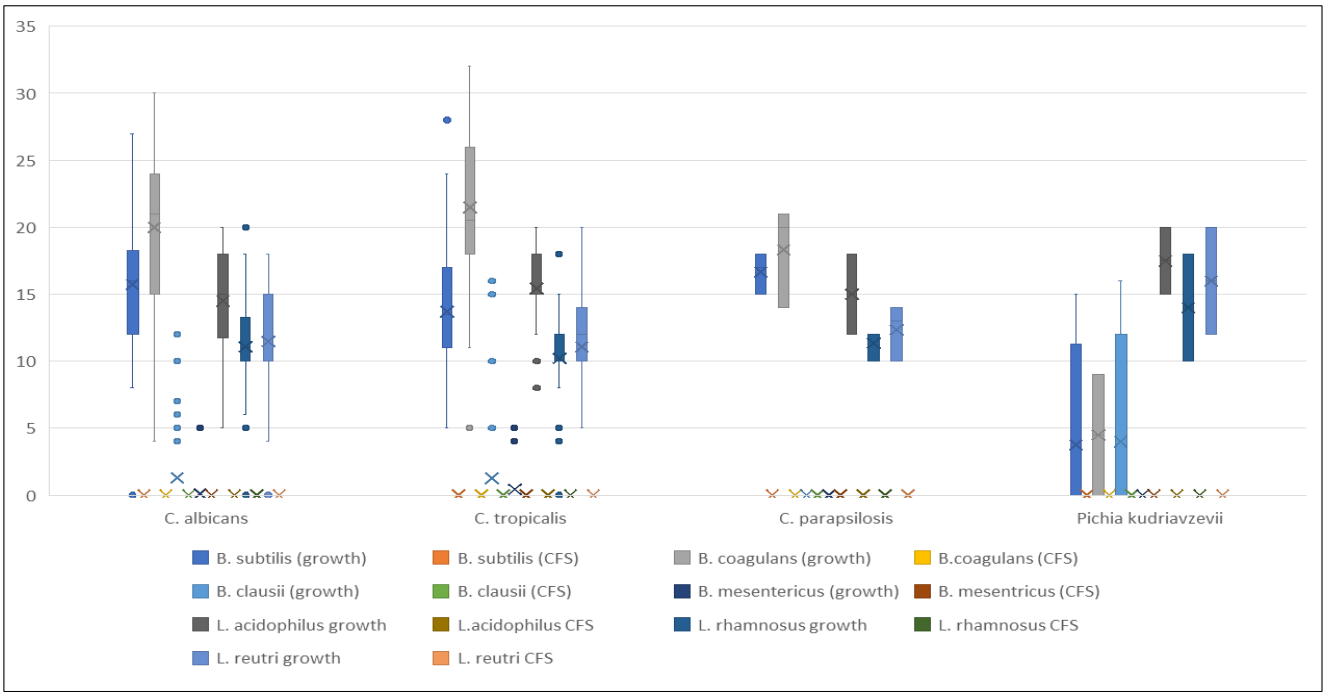

Figure 4: Graphical representation of inhibition zones across *Candida* species for all *Bacillus* and *Lactobacillus* strains (both viable cell suspensions and cell free supernatants).

DISCUSSION

Candida is a highly heterogenous genus of yeasts that include potentially pathogenic species, such as *Candida albicans, Candida glabrata, Candida tropicalis*, and *Candida parapsilosis*, which may present high prevalence of antifungal resistance.[13] *Candida* species are frequently associated with superficial and systemic invasive fungal infections, particularly in immunocompromised patients. In healthy individuals, however, they generally exist as commensal microbiota on the skin, genitals, or mucous membranes.[14] Within the *Candida* non-*albicans* species, an increasing trend of azole antifungal drug resistance including *C. tropicalis* rise in fluconazole resistance development has been reported.[15,16] The extensive use of antifungal agents, particularly fluconazole, has led to the selection of *Candida* species that exhibit intrinsic resistance to this triazole.[17] As an alternative treatment strategy, administration of natural compounds is also being investigated. We found that *Bacillus* strains may be considered as potential candidates for the treatment of candidiasis.

This investigation systematically assessed the *in vitro* antagonistic activity of four commercially available *Bacillus* strains, *B. subtilis, B. coagulans, B. clausii*, *B. mesentericus* and three *Lactobacillus* strains consisting *L. acidophilus, L. rhamnosus*, and *L. reuteri* against pathogenic *Candida* species. Consistent antifungal patterns were observed across agar overlay, well-diffusion and spot assays, with viable cell interactions producing substantially greater inhibition than cell free supernatants (CFS). Among the tested strains, *Bacillus* species notably demonstrated significant antagonism towards *Candida*. Live *Bacillus* suspensions, particularly *B. coagulans* (mean=20.1) and *B. subtilis* (mean=14.71) produced clear, reproducible inhibition zones, indicating strong antifungal activity. *B. coagulans* produced consistent and most pronounced activity against both *Candida albicans* (mean=19.98) and non- *albicans Candida* species (mean=20.25) whereas *B. subtilis* showed comparatively greater antimicrobial and antagonistic activity against *C. albicans* (mean=15.74). By contrast, viable cell suspensions of *B. clausii* (mean=1.31) and *B. mesentericus* (mean=0.225) showed only weak or inconsistent effects. *Lactobacillus* strains exhibited moderate inhibition across *Candida* taxa. *Lactobacillus acidophilus* exhibited the highest mean inhibition zone of 14.9375 mm, followed by *Lactobacillus reuteri* with a mean inhibition zone of 11.475 mm, while *Lactobacillus rhamnosus* demonstrated the lowest mean inhibition zone of 10.8375 mm. This indicates that *L. acidophilus* possesses the strongest antifungal activity among the tested *Lactobacillus* strains, suggesting its greater potential as a probiotic agent in inhibiting the growth of *Candida* species. Critically, the cell free supernatants of all tested *Bacillus* and *Lactobacillus* strains processed under controlled assay conditions were devoid of antifungal activity.

Our findings closely align with published literature regarding *Bacillus-Candida* interactions. Zhao et al demonstrated that the commercial probiotic *B. subtilis* R0179 produces robust inhibition of *C. albicans* and *C. parapsilosis* but not *P. kudriavzevii* via disc diffusion. This observation mirrors with the present work. Their study further linked antagonism to secretion of lipopeptide iturin A, validated by mass spectrometry, supporting the premise

that viable cells can actively secrete antifungal metabolites.[18] In our study cell free supernatant obtained from *B. subtilis* and other *Bacillus* spp. exhibited no detectable inhibition across all *Candida* taxa. This discrepancy may be due to difference in strain (*B. subtilis* HU58*), insufficient secretion of active compounds, or the collection of supernatants at a suboptimal growth phase, when metabolite production had not yet peaked. Similarly, Gharieb et al found that *B. subtilis* inhibited *C. albicans* through secreted lipopeptides detectable in cell-free supernatants.[19] This contradicts with our study as CFS failed to inhibit *Candida*. The contrasting results with Gharieb et al may be attributed to methodological differences. Their use of potato dextrose agar at pH 8 with 48 h incubation at 25 C may have favoured metabolic accumulation, where as our study employed MHA with 24h incubation at 37° C. In a parallel study by Spaggiari et al, both live *B. coagulans* LMG S-24828 cells and their supernatants were effective against *C. albicans* and *C. parapsilosis* using agar overlay and diffusion assays.[20] However, our CFS results showed negligible inhibitory action, likely reflecting strain differences in metabolite secretion. Regardless, both studies agree that live *B. coagulans* Cells have strong antagonistic effects.

Comparable patterns were evident among Lactic acid bacteria. The findings of present study partly consistent with those of Salari et al, who reported that *L. acidophilus* and *L. plantarum L. acidophilus* and *L. plantarum exerted antifungal activity against oral Candida* isolates.[21] Unlike their demonstration of CFS efficacy by microdilution assays, the present study imparts measurable antagonism only from viable *Lactobacillus* cells. The difference in the results may be due to assay or strain dependent variability. Jiang et al investigated six probiotic *Lactobacillus* species (including *L. rhamnosus GG* and *L. reuteri SD2112*) against oral *Candida* and the study demonstrated strong inhibitory activity of *L. rhamnosus and L. reuteri against vulvovaginal Candida.*[22] These results agree with our observation that *L. rhamnosus* exhibited stronger inhibition of *C. albicans* than *L. acidophilus* or *L. reuteri*. Both studies emphasize that acid production and local environmental pH play a critical role in antifungal efficacy.

According to Scillato et al, cell-free supernatants from *L. gasseri*, *L. fermentum*, and *L. crispatus* against multidrug-resistant urogenital bacteria produced significant antimicrobial effects although the same supernatants were less effective against *Candida* species.[23] Their results reinforce the current observation that *Candida* is comparatively resistant to soluble factors released by *Lactobacillus*, suggesting that direct cell contact or co-aggregation may be more important mechanisms for fungal inhibition than diffusible metabolites.

The absence of detectable antifungal activity in crude CFS implies that antifungal compounds present at subthreshold levels, instability of bioactive metabolites in non-living preparations, or the lack of synergistic cell–cell signalling potentially limit their efficacy against *Candida* spp. This phenomenon suggests that the antimicrobial microenvironment generated by metabolically active *Bacillus* and *Lactobacilllus* cells involves not only stable secretion but also dynamic replenishment of molecules, rapid response to environmental cues, and multifactorial interactions such as direct cell contact, competitive exclusion, and changes in local pH or redox potential that cannot be mimicked by static supernatants. Furthermore, recent advances in post-biotic research highlight that only highly concentrated or structurally preserved bioactive fractions may reproduce the effects seen with live probiotics, and that comprehensive molecular characterization is vital to harness their therapeutic potential.

Our CFS finding suggests that mere presence of extracellular metabolites in crude supernatant is insufficient for inhibition, reinforcing the superiority of live cell interactions, consistent with the majority of contemporary reports.

This study is with limitations, as it predominantly relied on phenotypic antimicrobial assays without integration of comprehensive metabolomic profiling of probiotic CFS, or genetic or transcriptomic analysis of strains during antagonism. Static monoculture assays cannot fully replicate the complexity of *in vivo* environments, including host immune responses, microbiota interactions, and dynamic factors like fluid flow, nutrient gradients, and mucosal architecture, all of which critically affect probiotic pathogen interplay and antifungal efficacy. The absence of *in-vivo* validation constrains translational interpretation. Future studies should couple multi-omics and advanced co-culture models with animal experiments to define mechanistic pathways and therapeutic potential.

CONCLUSION

The *in vitro* evaluation of antagonistic activity of *Bacillus* against oral *Candida* spp. demonstrates that *Bacillus coagulans* and *Bacillus subtilis* exert potent antagonistic effects against diverse *Candida* isolates, with viable cells outperforming cell-free supernatants. Similarly, *Lactobacillus acidophilus*, *L. rhamnosus*, and *L. reuteri* also displayed marked inhibitory activity, reinforcing the probiotic potential of lactic acid bacteria in controlling oral *Candida* overgrowth. These findings indicate that direct microbial interactions and metabolite dynamics, rather than diffusible factors alone, are critical to antifungal activity.

The species-specific variation in *Candida* susceptibility further suggests opportunities for targeted probiotic applications. Collectively, *B. coagulans*, *B. subtilis*, and selected *Lactobacillus* spp. emerge as promising biotherapeutic candidates for managing *Candida* infections, warranting further molecular characterization and *in vivo* validation to define their clinical potential.

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