Identification of antifungal molecules from novel probiotic *Lactobacillus* bacteria for control of *Candida* infection

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KEY MESSAGES

1. Probiotic lactic acid bacteria were identified to have antifungal properties against *Candida albicans*.
2. The cell-free supernatant of the lactic acid bacteria was successfully fractionated and purified using fast performance liquid chromatography.
3. Eight of the 41 fractions containing the antifungal components exhibited a growth inhibitory effect against *C albicans*.

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Introduction

Candida is a commensal fungus that inhabits various niches of the human body and can cause infection under certain circumstances, ranging from superficial mucous membrane infection to systemic disease with high mortality.[1] Rising drug resistance and undesirable side effects of currently available antifungal agents have prompted research of alternative therapeutic strategies.[2] Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts in food or as a dietary supplement, according to the Food Agricultural Organization/World Health Organization. Probiotics, such as lactic acid bacteria (LAB), are well-recognised for their health benefits and have been shown to improve various medical conditions, including gastrointestinal disorders, cancer, and infectious disease.[3] Probiotic-containing products can reduce *Candida* infection such as oral candidiasis and vulvovagional candidiasis.[4] Probiotic products have preferable properties as antifungal agents for control of *Candida* infection, because they are natural, effective, and safe.

In a preliminary study, we found that LAB had antifungal properties. Subsequent experiments demonstrated that culture supernatant of LAB inhibited the hyphal formation of *C albicans* in a dose-dependent manner, and exhibited fungicidal acidity at slightly higher concentrations. LAB supernatant was effective in preventing *C albicans* infection in vitro. The present study aimed to identify the inhibitory antifungal molecules in the LAB supernatant using standard methodology.

Methods

This study was conducted from October 2012 to September 2013.

Preparation of cell-free supernatant

Lactobacillus species were cultured in Man Rogosa Sharpe broth (Difco Laboratories, USA) anaerobically at 37°C to the stationary phase. Cultures were centrifuged at 9500 × g for 15 min and filter-sterilised through a 0.45 μm pore filter (Advantec MFS, Japan). The cell-free culture filtrate was then used for downstream experiments.

Fractionation and purification of LAB supernatant

Proteins of the culture supernatant were purified using standard methodology. In brief, proteins in the cell-free supernatant were precipitated with ammonium sulfate, dissolved in sodium phosphate buffer and desalted by overnight dialysis in sodium phosphate buffer at 4°C. The mixture of proteins was then fractionated in a DEAE Sepharose Fast Flow column (HiPrep 16/10 DEAE-FF, GE Healthcare) mounted in a fast performance liquid chromatography (FPLC) system (Akta purifier, GE Healthcare). Activity was eluted at a flow rate of 1 mL/min using a running buffer of 0.05M Tris (pH 7.5) and an elution buffer of 0.05M Tris (pH 7.5) and 2M NaCl. A gradient of NaCl was applied linearly from 0 to 40% (elution buffer). A total of 41 fractions (5 mL each) were collected. The total protein concentration of each sample was measured using a 2D Quant kit (GE Healthcare).

Antifungal activity of the fractions

All fractions were measured for their antifungal activity against *C albicans* using a broth microdilution assay in a time-dependent manner. Inocula from

TABLE. Antifungal activity of the fractions of supernatants derived from lactic acid bacteria*

Fraction	Time-interval		
	24 h	48 h	72 h
A1	×	×	×
A2	×	×	×
A3	×	×	×
A4	×	×	×
A5	×	×	×
A6	×	×	×
A7	×	×	×
A8	100%	100%	50%
A9	100%	50%	×
A10	100%	100%	50%
A11	×	×	×
A12	100%	×	×
B1	×	×	×
B2	×	×	×
B3	×	×	×
B4	×	×	×
B5	×	×	×
B6	100%	50%	50%
B7	100%	50%	50%
B8	100%	100%	100%
B9	100%	100%	100%
B10	×	×	×
B11	×	×	×
B12	×	×	×
C1	×	×	×
C2	×	×	×
C3	×	×	×
C4	×	×	×
C5	×	×	×
C6	×	×	×
C7	×	×	×
C8	×	×	×
C9	×	×	×
C10	×	×	×
C11	×	×	×
C12	×	×	×
D8	×	×	×
D9	×	×	×
D10	×	×	×
D11	×	×	×
D12	×	×	×

* Complete (100%), partial (50%), or no (x) inhibition of growth was recorded for each fraction at each time point. Eight of the fractions displayed an inhibitory effect against *Candida albicans*. Four of them exhibited a complete inhibitory effect (100%) for 48 hours or more

24-hour *C albicans* cultures were harvested and suspended in RPMI 1640 medium with turbidity equivalent to McFarland standard 0.5 (1×10^6 cells/mL) and then diluted to approximately 0.5×10^3 to 2.5×10^3 cells/mL. The test was performed in pre-sterilised, flat-bottom 96-well polystyrene plates (Iwaki, Japan), with each well filled with 0.1 mL of *C albicans* suspension and 0.1 mL of the fraction. The plates were then incubated at 37°C. The inhibitory activity of the fractions was recorded by visual observations of growth inhibition after 24, 48, and 72 hours. To verify the results, at the end of the 72-hour incubation, aliquots of broth culture were plated on the Sabouraud Dextrose Agar and the number of colonies was recorded. Fractions with significant antifungal activity as shown in the aforementioned assay were used for downstream proteomic studies to identify the antifungal protein components.

Results

A total of 41 fractions were collected from the FPLC purification of the cell-free LAB supernatant. The antifungal activity of these fractions is shown in the Table. Interestingly, eight of the 41 fractions

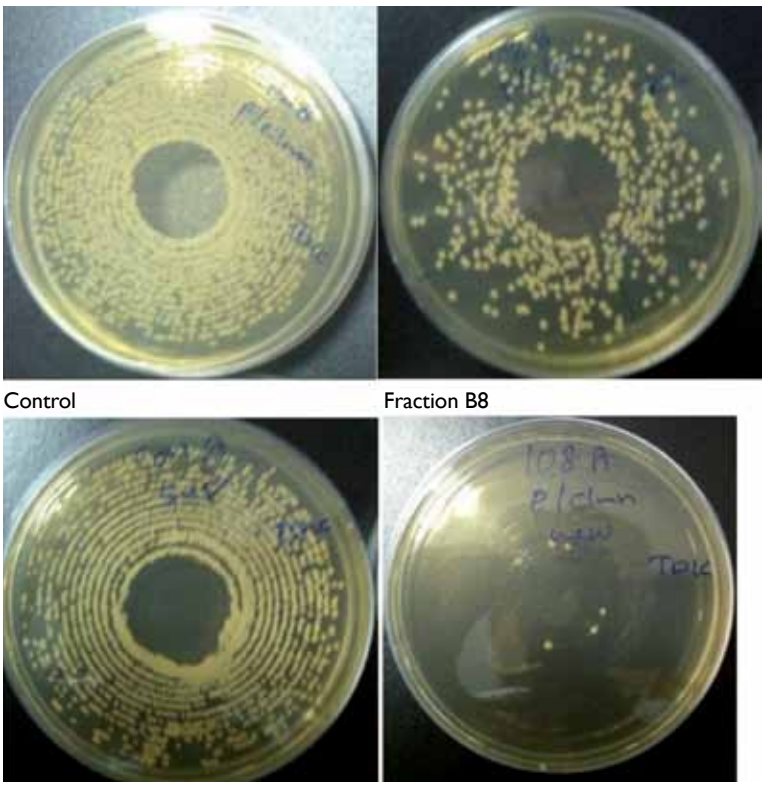

FIG. Growth inhibitory effect of each fraction was further evaluated by plating on Sabouraud Dextrose Agar and counting of the colony forming units (CFUs): the number of CFUs in Fractions B7 and B8 was significantly lower than that in controls, with 50% and 100% of the growth inhibitory effect against *Candida albicans*, respectively.

displayed a growth inhibitory effect against *C albicans*. Among these eight fractions, four (A8, A10, B8, and B9) exhibited a complete growth inhibitory effect (100%) in the broth microdilution assay when incubated with *C albicans* for 48 hours or more. This finding indicated that the antifungal components of the supernatant were successfully fractionated. The growth inhibitory effect of these fractions was further confirmed by plating on Sabouraud Dextrose Agar and counting of the colony forming units (Fig). These fractions were selected for further protein identification using liquid chromatography-mass spectrometry.

Discussion

Fungal infections, of which candidiasis is the most common form, have become a significant problem in the clinical setting worldwide, due to an increase in immunocompromised populations. In this study, we successfully identified LAB strains with antifungal activity against *C albicans*. The antifungal compounds of LAB are complex, and their isolation and purification from LAB are difficult.[5] Despite this, we successfully established a protocol for fractionation using FPLC, and isolated the working fractions from the probiotic supernatant that exhibited a growth inhibitory effect against *C albicans*. We are working on identification of the antifungal components in the selected fractions using a proteomics approach.

Conclusions

Candidiasis places a huge burden on public health, especially in immunocompromised populations. Prophylactic antifungal treatment is associated with undesirable side effects. Probiotic products, such as LAB, have been proven to be effective and safe to prevent and treat candidiasis. Therefore, it is important to identify appropriate probiotics and associated products. We successfully fractionated and purified the cell-free supernatant of LAB with antifungal activity using fast performance liquid chromatography, and eight of the 41 fractions exhibited antifungal effects in the broth microdilution assay against *C albicans*. Our findings facilitate further investigation of the molecular mechanisms of the antifungal activity of LAB.

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