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Effect of pH, heat treatment and enzymes on the antifungal activity of lactic acid bacteria against *Candida* species

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ABSTRACT

Aims: The objectives of this study were to evaluate the inhibitory activity of the cell-free supernatants (CFS) of lactic acid bacteria (LAB) isolates and determine the effect of pH, enzymes and heat treatment on the antifungal activity against *Candida* species.
Methodology and results: A total of 25 strains of LAB were isolated from honey samples from Malaysia, Libya, Saudi Arabia, and Yemen. Four from twenty-five LAB isolates showed antifungal activity against *Candida* spp. and were identified as *Lactobacillus plantarum* (HS), *L. curvatus* (HH), *Pediococcus acid lactic* (HC), and *P. pentosaceus* (HM) using 16S rDNA sequence. The CFS of these isolates were evaluated for their antifungal activity using microtiter plate assay. The antifungal activity showed significant inhibitory activity against all *Candida* spp. especially growth of *C. glabrata* ATCC 2001 was significant ($p < 0.001$) completely inhibited by CFS of HH and HM at pH 3. Similarly, growth of *C. glabrata* ATCC2001 was significantly inhibited ($p < 0.001$) when treated with previously heated CFS of *L. curvatus* HH and *P. pentosaceus* HM at 90 °C and 121 °C. While, the growth of *C. krusei* ATCC 6258 was completely inhibited by CFS of *L. curvatus* HH at 121 °C. Treatment the CFS of LAB isolates with proteinase K and RNase II increased the antifungal activity against *C. krusei* and *C. glabrata*, whereas the activity of CFS produced by *P. acidilactici* was lost when treated with RNase II, especially against *C. krusei*.
Conclusion, significance and impact of study: This study demonstrated that treated supernatant of LAB isolates with heating, adjusted pH and enzymes can be used to inhibit the growth of pathogenic *Candida* spp.

Keywords: Lactic acid bacteria, antifungal activity, pathogenic *Candida* species

INTRODUCTION

Nonpathogenic Gram-positive organisms known as lactic acid bacteria or LAB are important in food product to ferment, preserve food and vitamins during food production (Rebecca *et al.*, 2008). LAB can be found in the oral cavities, gastrointestinal tracts and vaginas of humans, they are commonly used as probiotics (De Vuyst and Leroy, 2007; Eva *et al.*, 2012). Studies on LAB antifungal activity against *Candida* spp. began with (Guillot, 1958). *Lactobacillus acidophilus* created compounds that have the ability to affect *C. albicans* (Fitzsimmons and Berry, 1993). Certain strains of LAB had the capacity to inhibt growth of *Candida* spp. (Strus *et al.*, 2005). Kariptas *et al.* (2010) discovered that *Lactobacillus paracasei* subsp M3 had an antifungal influence on *C. albicans*, *C. pseudointermedia* and *C. blankii*. Lavermicocca *et al.* (2003) stated that *L. plantarum* have antifungal compounds that could prevent fungi and yeast growth. *Lactobacillus plantarum* have antifungal activity was mentioned by other authors (Strom *et al*., 2005; Sathe *et al*., 2007; Delavenne *et al*., 2012). Of late, Kariptas *et al.* (2010) reported that *Lactobacillus* human strains have greater antifungal activity against *C. albicans* (M29, M36), *C. parapsilosis* (M25, M26, and M44), *C. famata* (M28) and *C. guilliermondii* (M38). LAB is able in producing an assortment of antimicrobial compounds that has an significant part in preventing fungal growth due to the content of organic acids, hydrogen peroxide, carbon peroxide, diacetyl and

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bacteriocins (Magnusson *et al.*, 2003; Valerio *et al.*, 2008; Gerez *et al.*, 2009; Dalié *et al.*, 2010; Muhialdin *et al.*, 2011). LAB antimicrobial compound are active in range of pH 3 to 4.5 and heat stable at 100 °C (Magnusson and Schnürer, 2001; Messens and De Vuyst, 2002; Lavermicocca *et al.*, 2003; Muhialdin *et al.*, 2011). It is necessary to search other LAB types that can inhibit the growth of pathogenic *Candida* spp. The objectives of this study were to assess the effect of pH, heat treatment and proteolytic enzymes on cell free supernatant (CFS) of LAB isolated from honey against *C. glabrata* ATCC 2001 and *C. krusei* ATCC 6258.

MATERIALS AND METHODS

Honey samples

Fifteen samples of honey were collected from Malaysia (Madu Tualang, Madu Tani, and pure Trigona honey), Libya (Al-Seder honey, Al-Hanon honey and Al-Zater honey), Yemen (Al-Seder honey and Al-Maray honey), Saudi Arabic (Al-Shifaa honey) and New Zealand (Manuka honey). All samples were stored at room temperature 28 ± 2 °C prior to the analysis. pH of honey was determined using the pH meter (METTLER TOLEDO).

Isolation of lactic acid bacteria from honey samples

Isolation of LAB from honey samples succeeding the technique defined by Bulgasem *et al.* (2015). Approximately 10 g of honey samples were suspended in 90 mL peptone water (0.1% w/v) in stomacher bags and the bags were manually agitated. Then, one mL was added to nine mL of MRS broth (Oxoid CM359) and incubated at 30 °C for 48 h until the culture broth became turbid followed by serial dilution with peptone water (0.1% w/v). A 0.1 mL of appropriate dilution was spread plated on several modified media namely, MRS agar (Oxoid) (De Man *et al.*, 1960), MRS agar with 0.8% $CaCO_3$ (Aween *et al*., 2012). MRS agar with 1% glucose, tomato juice agar with 0.8% $CaCO_3$ and tomato juice agar with 1% glucose. All plates were incubated anaerobically at 37 °C for 48 h. Isolated single colonies were tested for catalase activity with 4% H_2O_2 and were Gram stained. Catalase negative colonies were streaked on MRS agar containing 0.8% $CaCO_3$ incubated at 37 °C for 48 h to obtain pure colonies. All negative catalase and Gram positive LAB isolates were preserved in MRS broth with 15% of glycerol and stored at -20 °C for further analysis.

Culturing of *Candida* species

The *Candida* strains used were obtained from the original stock of the microbial collections at the Department of Medical Microbiology, University Putra Malaysia. The *Candida* spp. used in this study were *C. krusei* ATCC 6258 and *C. glabrata* ATCC 2001 and were cultured on Sabouraud Dextrose Agar (SDA, Oxoid) at 35 °C for 24 h, to 48 h to ensure viability and purity, and the fungal strains were maintained on SDA at 4 °C until further analysis.

Cell free supernatant preparation

The isolates of LAB were inoculated into MRS broth and incubated at 30 °C for 24 h. The CFS was prepared by centrifugation the broth at 11500 rpm at 4 °C for 10 min. (Mini Spin, Eppendorf, AG 22331, Hamburg).Then, the supernatant of the LAB isolates were filtrated using sterile filtered (0.45 µm-pore-size filter, Millipore) (Ogunbanwo, 2005), and this filtrate was utilized for analysis.

Effect of heating of LAB-CFS on antifungal activity

LAB-CFS heat treatment was done at 90 °C and 121°C for 30 min and was continuously cooled in ice water. The supernatant were tested against *Candida* spp. which are *C. glabrata* ATCC 2001, and *C. krusei* ATCC 6258 with a microtiter plate assay as done by Muhialdin *et al.* (2011). Initially, *Candida* 10^4 cell/mL was added to the prepared SDB. Next, addition of 100 µL of Sabouraud dextrose broth (SDB, Oxoid CM147) containing 10^4 CFU/mL *Candida* and 100 µL of CFS into microtiter plates wells. At that moment, 200 µL of *Candida* in SDB without adding CFS was utilized as positive control. The incubation of the plates were done at 30 °C for 24, 48 and 72 h. and the measurement of *Candida* spp.'s growth were measured using optical density (OD) at 560 nm with a micro Elisa auto reader (Model 680, BioRad). The analysis was done in duplication and mean was taken. The measurement of *Candida* spp. percentage growth was taken using the following equation:

Percentage growth of *Candida* (%) = ($OD_{560\ nm}$ after 24, 48 and 72 h – $OD_{560\ nm}$ at 0 h)/$OD_{560\ nm}$ at 0 h) × 100

Effect of different pH on LAB-CFS antifungal activity

The CFS-LAB pH isolates was adjusted at pH 3, 5, 6, 7, and 9 using 0.1 N HCl and 0.1 N NaOH. The initial LAB isolates pH was 4.1, 3.8, 3.7, and 3.9 for isolates HS, HC, HH and HM, respectively. The pH accustomed CFS were tested against *Candida* spp. *C. glabrata* ATCC 2001 and *C. krusei* ATCC 6258 in microtiter plates as defined above. A 100 µL of SDB containing *Candida*10^4 cell/ mL and 100 µL of altered supernatant were added into the wells. Later, 200 µL of *Candida* in Sabouraud broth was utilized as positive control. The incubation of the plates were at 30 °C for 24, 48 and 72 h. *Candida* growth was monitored at 24, 48, 72 h, respectively. *Candida* spp. growth percentage was measured utilizing the similar formula stated earlier.

Effect of enzymes on antifungal activity of LAB CFS

CFS proteinaceous evaluation was tested to measure their sensitivity to proteolytic enzymes proteinase K and RNase II minus the pH being adjusted. The CFS were established with enzymes proteinase K and RNase II

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independently (Aween *et al.*, 2012). First, one µL of respective enzyme were mixed into three mL of supernatant and were left 1 h at room temperature 28 ± 2 °C. Subsequently, the supernatant was tested against *Candida* spp. in microtiter plates as described above. Individual wells were injected with 100 µL of SDB that contains 10^4 cell/mL and 100 µL of supernatant with enzyme. Next, 200 µL of *Candida* in Sabouraud Broth was utilized as a positive control. The incubation of the plates was at 30 °C for 72 h. *Candida* growth was observed at 24, 48, and 72 h, respectively using optical density at 560 nm. The experiments were carried out in duplicate. *Candida* growth percentage was measured using the similar formula stated earlier.

Identification of LAB isolates by API 50 CH and 16S rDNA

The LAB isolates that show antifungal activity were identified using API 50 CHL kit assay as described by the manufacturer (Tamminen *et al*., 2004). The LAB isolates were identified using 16S rDNA gene sequences. The genomic DNA LAB of were extracted using Wizard® Genomic Gram-positive DNA purification kit (USA). The genomic DNA was amplified using polymerase chain reaction (PCR) by utilising the Fail SafeTM Pre Mix kit Epicentre® (an Illumina® company). A 1400 bp fragment of 16S rDNA gene was amplified using PCR with primers based on 16S forward (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse (5-CGGGAACGTATTCAC-CG-3) regions (Magnusson *et al*., 2003).The PCR protocols were done as follows: denaturation at 95 °C for 2 min, followed by 35 denaturation cycles at 92 °C for 45 sec, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min, and final extension at 72 °C for 5 min. Next, 5 µL of each amplified mixture were exposed to electrophoresis in 1.5% (1.5 g agarose powder with 100 mL in 1 × TEA buffer) for 45 min at 90 volts. Amplified products were sent for sequencing to the First BASE Laboratories Sdn Bhd., Malaysia. The aligned nucleotide sequence was analyzed to the Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTTN) software version 2.2.14 and was compared to other accessible isolates in the Gen Bank Database of the National Centre for Biotechnology Information (NCBI) to be confirmed.

Statistical analyses

All data were presented as mean ± standard deviation and were analyzed using one-way analysis of variance (ANOVA) for heat treatments, and two-way ANOVA for pH treatments using general linear model (GLM) procedure of SAS. Tukey's test was applied for significant means at $p < 0.05$ to evaluate the significant differences between groups. Data with zero values were transformed using root square plus one and analyzed, whereas the real values were presented.

RESULTS

Ten honey samples from fifteen were positive for LAB.The identification of four LAB isolated from honey samples that showed antifungal activity against pathogenic *Candida* spp. is presented in (Table 1). The results from API 50 CHL kit identified the LAB isolate HS from Al-Sedar honey as *Lactobacillus plantarum*2, and other three isolates of LAB HH from Al-Hanon honey, HC from Tualang honey and HM from Al-Maray were identified as *L. curvatus*. However, the results from 16S rDNA sequence were slightly different: HS was identified as *L. plantarum*, HH as *L. curvatus*, HC as *Pediococcus acidilactici* and HM as *P. pentosaceus*.

Effect of heating LAB CFS on antifungal activity

Heating the CFS of LAB (HS, HC, HH and HM) at 90 °C and 121 °C for 30 min resulted in the growth reduction of all the *Candida* spp. compared to the control after 24 h incubation (Tables 2, 3, 4 and 5). Especially the growth of *C. glabrata* ATCC 2001 was significant ($p < 0.001$) completely inhibited by the heated CFS of *L. curvatus* HH and *P. pentosaceus* HM with incubation 72 h at 90 °C and 121 °C (Table 2 and 3). Likewise, the growth of *C. krusei* ATCC 6258 was significant ($p < 0.001$) completely inhibited by the heated CFS of HH at 121 °C with 72 h incubation (Table 5).

Effect of different pH on LAB-CFS antifungal activity

The antifungal activity of CFS was observed at pH 3 to 5 but it was decreased at pH 6. The CFS of LAB isolates showed inhibited the growth of all *Candida* spp. significantly ($p< 0.05$), especially the growth of *C. glabrata* ATCC 2001 was significant ($p < 0.001$) completely inhibited by CFS of *P. pentosaceus* HM at pH 3 and pH 5 compared to the control. However, CFS of *L. curvatus* HH lead to inhibit the growth of *C. glabrata* completely ($p< 0.001$) at pH 3 with incubation 24 h. The growth of *C. krusei* was reduced at pH 3 by CFS of *L. plantarum* HS and *P. acidilactici* HC. While, was significant ($p < 0.001$)

Table 1: Similarity index of LAB isolated from honey samples as determined by API 50CHL and 16S rDNA.

Sources	Code	API CHL 50	Similarity	16S rDNA	Similarity
Al-Sedar honey, Libya	HS	*L. plantarum2*	99.4%	*L. plantarum*	99.0 %
Al-Hanon honey, Libya	HH	*L. curvatus*	99.4%	*L. curvatus*	96.0%
Tualang honey, Malaysia	HC	*L. curvatus*	99.4%	*Pediococcus acidilactici*	99.0%
Al-Maray honey, Yemen	HM	*L. curvatus*	97.4%	*Pediococcus pentosaceus*	99.0%

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completely inhibited by *L. plantarum* HS at pH 5. The CFS of LAB HS and HM lost their antifungal activity especially against *C. krusei* ATCC6258 at pH 7.

Table 2: Percentage growth of *Candida glabrata* with LAB supernatant after heat treatment at 90 °C in microtiter plates incubation at 30 °C for 72 h*.

Isolates	Time (h)		
	24	48	72
HS	12.3 ± 0.45[b]	38.7 ± .050[b]	42.8 ± 0.96[b]
HC	9.4 ± 0.51[c]	20.0 ± 0.66[c]	21.8 ± 0.55[c]
HH	3.9 ± 0.26[d]	0.00 ± 0.00[d]	0.00 ± 0.00[d]
HM	5.4 ± 0.35[d]	0.00 ± 0.00[d]	0.00 ± 0.00[d]
Control	98.70 ± 1.53[a]	100.4 ± 0.68[a]	122.6 ± 1.35[a]

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-e] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$).
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

Table 3: Percentage growth of *Candida glabrata* with LAB supernatant after heat treatment at 121 °C in microtiter plates incubation at 30 °C for 72 h*.

Isolates	Time (h)		
	24	48	72
HS	23.0 ± 0.23[b]	29.4 ± 0.85[b]	38.4 ± 0.45[b]
HC	18.6 ± 0.62[c]	20.0 ± 0.83[d]	30.8 ± 0.65[c]
HH	23.0 ± 0.26[c]	12.2 ± 0.15[d]	0.00 ± 0.00[d]
HM	10.4 ± 0.25[e]	8.6 ± 0.51[e]	0.00 ± 0.00[d]
Control	114.5 ± 0.55[a]	128.2 ± 0.70[a]	144.2 ± 0.83[a]

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-e] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$).
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

Table 4: Percentage growth of *Candida krusei* with LAB supernatant after heat treatment at 90 °C in microtiter plates incubation at 30 °C for 72 h*.

Isolates	Time (h)		
	24	48	72
HS	20.6 ± 0.85[b]	24.2 ± 0.20[b]	32.3 ± 0.73[b]
HC	16.0 ± 0.15[c]	22.6 ± 0.36[c]	33.7 ± 0.45[b]
HH	14.7 ± 0.41[d]	19.3 ± 0.35[d]	24.6 ± 0.25[c]
HM	10.4 ± 0.26[e]	13.2 ± 0.15[e]	22.7 ± 0.52[c]
Control	89.4 ± 0.50[a]	124.0 ± 0.50[a]	156.0 ± 2.51[a]

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-e] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$).
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

Table 5: Percentage growth of *Candida krusei* with LAB supernatant after heat treatment at 121 °C in microtiter plates incubation at 30 °C for 72 h*.

Isolates	Time (h)		
	24	48	72
HS	20.0 ± 0.55[c]	23.2 ± 0.32[d]	30.1 ± 0.15[c]
HC	9.0 ± 0.15[e]	26.6 ± 0.45[c]	30.7 ± 0.55[c]
HH	18.2 ± 0.20[d]	9.7 ± 0.64[e]	00.0 ± 0.61[d]
HM	27.4 ± 0.35[b]	33.8 ± 0.60[b]	45.2 ± 0.37[b]
Control	139 ± 0.64[a]	165 ± 1.52[a]	188 ± 0.57[a]

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-e] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$).
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

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Effect of enzymes on antifungal activity of LAB CFS

The LAB supernatants tested with proteinase K and RNase II showed strong inhibitory activity against most *Candida* spp. compared to the control (Tables 6 and 7). The CFS of *L. plantarum* HS showed complete inhibition against *C. krusei* when treated with proteinase K after 48 h and 72 h incubation. As well as CFS of *L. curvatus* HH and *P. pentosaceus* HM showed complete inhibition against *C. glabrata* compared to the other isolates this indicate that these isolates did not have protein (Table 8). Otherwise, when treated the CFS with RNase II the CFS of *P. pentosaceus* (HM) showed complete inhibition against *C. glabrata* compared to the other isolates and the control. However, the treated CFS with RNase II destroyed the antifungal activity of *P. acidilactici* HC and *L. curvatus* HH against all *Candida* spp. especially against *C. krusei* indicating that the CFS contained protein-like antifungal compounds therefore, show the increase in growth of *C. krusei* after 48 h and 72 h of incubation (Table 9). This observation suggests that addition proteinase K and RNase II to the CFS of LAB isolates enhanced the antifungal activity to some isolates against *Candida* spp.

Table 6: Percentage growth of *Candida* species with LAB supernatant after treatment with proteinase K in microtiter plate incubated at 30 °C for 72 h*.

		LAB				
Candida spp.	Time (h)	HS	HC	HH	HM	Control
C. glabrata	24	2.4	13.8	NG	NG	235
ATCC 2001	48	6.6	5.7	NG	NG	242
	72	17.3	4.3	NG	NG	267
C. krusei	24	1.5	7.4	6.6	10.5	121.2
ATCC 6258	48	NG	2.6	4.1	6.3	136.0
	72	NG	1.3	1.4	3.5	158.4

*The growth was measured as OD at 560 nm, NG: no growth.

Table 7: Percentage growth of *Candida* species with LAB supernatant after treatment with RNase II in microtiter plate incubated at 30 °C for 72h*.

		LAB				
Candida spp.	Time (h)	HS	HC	HH	HM	Control
C. glabrata	24	9.5	3.6	NG	4.1	158.8
ATCC 2001	48	9.0	8.7	9.3	NG	164.3
	72	5.7	14.7	16.6	NG	174.3
C. krusei	24	19.7	45.6	20.8	22.6	126.6
ATCC 6258	48	16.2	60.2	21.8	17.7	146.3
	72	13.6	103.5	28.4	11.4	151.2

*The growth was measured as OD at 560 nm, NG: no growth.

DISCUSSION

The isolation of LAB is possible from various sources and many reports were on the LAB antibacterial activity while there were fewer reports on LAB antifungal activity. LAB antimicrobial activity has been recognized to the creation of antimicrobial compounds with antifungal activities. This study observed that the four LAB isolated from honey samples supernatants (CFS), *L. plantarum* HS, *L. curvatus* HH, *P. acidilactici* HC and *P. pentosaceus* HM had good antifungal activity against pathogenic *Candida* spp. as assessed by the microtiter plates evaluation. This specifies that CFS from these LAB isolates comprise of compounds with protein-like nature. When the CFS was heated to 90 °C and 121 °C for 30 min resulted in significantly ($p < 0.05$) reduced the growth of the *Candida* spp. especially the growth of *C. glabrata* ATCC 2001 was significantly ($p < 0.001$) complete inhibited by the heated CFS of *L. curvatus* HH and *P. pentosaceus* HM as they were incubated 72 h at 90 °C and 121 °C (Tables 2 and 3). Additionally, the growth of *C. krusei* ATCC 6258 was significantly ($p < 0.001$) complete inhibited by the heated CFS of HH at 121 °C with 72 h incubation (Table 5). Similarly, Monthon (2005) observed that antifungal activity of *Lactococcus lactis* isolated from fermented food was stable during heat treatment and retaining the activity was even after autoclaving at 121 °C for 15 min against *C. albicans* DMST 5239. Moreover, Oliveira *et al.* (2008) reported that CFS of LAB isolated from vacuum packaged beef had antimicrobial activity after heating 100 °C for 10 min. The antifungal activity was enhanced after heating might be due to bacteriocins which is characterized by high heat stability indicated that the antifungal compound produced by LAB strains is a bacteriocins. In this study, when the pH of the CFS was adjusted to different value 3, 5, 6 and 7 showed significantly ($p < 0.05$) reduced the growth of *Candida* spp. whereas, the growth of *C. glabrata* was higher significantly ($p < 0.001$) inhibited by CFS of *P. pentosaceus* HM at pH 3 and pH 5 as well, the growth of *C. krusei* was significantly ($p < 0.001$) completed inhibited by CFS of *L. plantarum* HS at pH 5. This may suggest that compounds were responsible for

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Table 8: Percentage growth of *Candida glabrata* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 °C for 72*.

pH	Isolates	Time (h)		
		24	48	72
pH 3	HS	1.7 ± 0.15^{m}	36.2 ± 0.21^{g}	31.0 ± 0.17^{ij}
	HC	3.7 ± 0.20^{k}	41.5 ± 3.22^{e}	41.5 ± 0.32^{fgh}
	HH	12.1 ± 1.05^{n}	9.3 ± 0.15^{m}	0.00 ± 0.00^{n}
	HM	13.1 ± 1.59^{i}	7.5 ± 0.72^{n}	0.00 ± 0.00^{n}
	Control	178 ± 2.51^{a}	198 ± 3.00^{a}	216 ± 2.08^{a}
pH 5	HS	17.5 ± 0.31^{g}	29.8 ± 0.40^{i}	43.5 ± 0.45^{f}
	HC	13.1 ± 0.26^{i}	43.5 ± 0.36^{jk}	55.9 ± 0.75^{e}
	HH	10.7 ± 0.43^{j}	33.9 ± 0.65^{l}	30.3 ± 0.70^{j}
	HM	15.9 ± 0.62^{n}	3.5 ± 0.92^{k}	0.00 ± 0.00^{n}
	Control	69.0 ± 0.91^{b}	96.0 ± 0.75^{c}	123 ± 0.68^{b}
pH 6	HS	13.0 ± 0.53^{i}	28.0 ± 1.05^{j}	35.0 ± 0.60^{hij}
	HC	26.8 ± 1.06^{e}	$35.9 \pm 0.25g^{h}$	45.3 ± 0.85^{f}
	HH	15.2 ± 0.75^{h}	34.5 ± 0.25^{h}	36.2 ± 1.24^{ghi}
	HM	19.3 ± 0.86^{f}	38.4 ± 0.73^{f}	46.1 ± 1.13^{f}
	Control	62.1 ± 1.70^{c}	78.5 ± 0.86^{b}	87.6 ± 0.83^{c}
pH 7	HS	10.7 ± 0.83^{j}	13.2 ± 0.72^{j}	26.2 ± 0.72^{mn}
	HC	11.4 ± 1.27^{j}	$35.4 \pm 0.73g^{h}$	46.2 ± 0.66^{fg}
	HH	3.3 ± 0.70^{l}	12.8 ± 0.55^{k}	16.3 ± 1.27^{lm}
	HM	17.6 ± 0.41^{g}	28.0 ± 0.64^{l}	30.3 ± 0.58^{j}
	Control	54.2 ± 0.66^{d}	66.0 ± 0.36^{d}	76.4 ± 1.12^{d}

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-n] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$). Growth was determined by measuring OD 560 nm.
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

Table 9: Percentage growth of *Candida krusei* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 °C for 72*.

pH	Isolates	Time (h)		
		24	48	72
pH 3	HS	6.8 ± 0.81^{m}	25.6 ± 0.83^{k}	37.9 ± 0.15^{n}
	HC	3.4 ± 0.44^{n}	33.4 ± 0.74^{ij}	42.7 ± 0.35^{l}
	HH	28.7 ± 0.58^{g}	55.0 ± 0.51^{f}	56.4 ± 1.24^{i}
	HM	23.3 ± 0.96^{h}	44.7 ± 0.66^{g}	53.2 ± 1.10^{j}
	Control	124.6 ± 1.04^{a}	154 ± 0.50^{a}	178 ± 0.23^{a}
pH 5	HS	0.00 ± 0.00^{p}	27.1 ± 0.89^{k}	37.3 ± 0.90^{o}
	HC	1.7 ± 0.26^{o}	31.2 ± 1.01^{j}	44.3 ± 0.47^{k}
	HH	1.6 ± 0.46^{o}	31.7 ± 1.41^{j}	40.0 ± 1.04^{m}
	HM	49.4 ± 1.18^{d}	80.4 ± 1.24^{d}	97.3 ± 0.25^{g}
	Control	102 ± 1.15^{b}	139.5 ± 1.35^{b}	169.8 ± 1.07^{b}
pH 6	HS	11.8 ± 0.89^{l}	32.8 ± 1.96^{ij}	36.8 ± 0.92^{o}
	HC	18.7 ± 0.47^{i}	34.7 ± 0.58^{i}	40.0 ± 1.01^{m}
	HH	28.4 ± 1.33^{g}	38.9 ± 1.50^{h}	38.7 ± 1.45^{n}
	HM	48.3 ± 1.27^{d}	53.4 ± 0.21^{f}	75.0 ± 1.17^{h}
	Control	89.2 ± 1.21^{c}	125 ± 1.10^{c}	137.4 ± 0.20^{c}
pH 7	HS	35.4 ± 1.53^{f}	60.9 ± 2.56^{e}	105 ± 0.30^{f}
	HC	17.0 ± 1.04^{j}	26.6 ± 1.28^{k}	45.0 ± 0.80^{k}
	HH	16.0 ± 0.83^{k}	21.3 ± 1.27^{l}	42.6 ± 1.11^{l}
	HM	35.6 ± 0.83^{f}	65.6 ± 0.32^{e}	112 ± 1.96^{e}
	Control	46.6 ± 1.58^{e}	77.5 ± 1.32^{d}	132 ± 1.62^{d}

*The results are expressed as mean ± standard deviations of values obtained from triplicate experiments.
[a-n] Mean ± SD. Means with different superscripts in the same column differs significantly ($p < 0.05$). Growth was determined by measuring OD 560 nm.
Key: HS, *L. plantarum*; HC, *P. acidilactici*; HH, *L. curvatus*; HM, *P. pentosaceus*.

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antifungal activity in CFS of these isolates are organic acids such as lactic acid and acetic acid. The CFS of LAB isolates lost their antifungal activity against *C. krusei* at pH 7 (Table 8 and 9). Sookkhee *et al*. (2001) reported that antifungal activity of CFS of *L. paracasei* subsp. (D6 and D14) and *L. rhamnosus* isolated from healthy oral cavity of Thai volunteers was more active at acidic pH than at alkali pH against *C. albicans* DTMU2. The CFS treated with enzymes proteinase K and RNase II results were different antifungal activity against *Candida* spp. The growth of *C. krusei* was completely inhibited by CFS of *L. plantarum* HS after treated with proteinase K. Likewise, the growth of *C. glabrata* was complete inhibition by CFS of *L. curvatus* HH and *P. pentosaceus* HM. On the contrary, treating CFS with RNase II destroyed the antifungal activity of *P. acidilactici* HC and *L. curvatus* HH against *Candida* spp. especially against *C. krusei* indicating that the CFS contained protein-like antifungal compounds. Similarly, Ndagano *et al.* (2011) reported that treatment of supernatant of LAB isolated from Mill flour and fermented cassava by enzymes such as pepsine, proteinase K and α chymotrypsin showed antifungal activity. We observed that the antifungal activity in the cell free supernatants of LAB was enhanced after treatment with proteolytic enzymes (proteinase K and RNase II), suggesting that the antifungal compound could be not protein. The results obtained from present study are in agreement with (Ronnqvist *et al.*, 2007) who reported that *L. fermentum* Ess-1 isolated from human showed activity against *C. albicans* and *C. glabrata*. Adeniyi and Damsa, (2013) reported that the CFS produced by *L. plantarum* isolated from salad vegetables showed higher antifungal activity against *C. albicans* ATCC 90029 with inhibition zone 25 mm. Recently, Parolin *et al*. (2015) reported that supernatants of *L. crispatus* BC1, BC4, BC5 and *L. vaginalis* BC15 isolated from vaginal healthy woman had antifungal activity against strains of *C. albicans* and *C. lusitaniae.*

CONCLUSION

This study demonstrated that LAB isolated from honey have antifungal activity against pathogenic *Candida* spp. Supernatants produced by LAB isolates were active in pH 3 to 5 and their antifungal activity enhanced after heating the supernatants to 90 and 121 °C, and after treatment with proteinase K and RNase II. This indicated that the supernatant contains compounds which can be used to inhibit growth of the pathogenic *Candida* spp. infections.

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