CHAPTER IV

EFFECT OF pH, HEAT TREATMENT AND ENZYMES ON THE ANTIFUNGAL ACTIVITY OF SELECTED LACTIC ACID BACTERIA

4.1 Introduction

Previous studies explored the inhibitory activity of raw supernatant produced by LAB had inhibitory activity against the growth of *Candida* species. Monthon (2005) reported that the supernatant produced by *L. lactis* showed inhibitory activity against *C. albicans* DMST 5239. Similarly, Verdenelli et al. (2009) reported that *L. rhamnosus* and *L. paracasei* isolated from human stool had antifungal activity against *C. albicans* ATCC 10291, and Lavermicocca et al. (2003) reported that *L. plantarum* produced antifungal compounds which can inhibit the growth of fungi and yeast. Kariptas et al., (2010) showed that CFS of strains of *Lactobacillus* isolated from the stool of children had antifungal activity against *C. albicans* (M29, M36), *C. parapsilosis* (M25, M26 and M44), *C. famata* (M28) and *C. guilliermondii* (M38). Adeniyi and Damsa, (2013) reported that the CFS of LAB *L. plantarum* showed higher antifungal activity against *C. albicans* ATCC90029.

Others reports indicated that LAB isolated from different sources have antifungal activity against food spoilage fungi *P. citrinum*, *A. niger*, *A. flavus*, *A. oryzae*, (Adeboya & Aderiye, 2010; Muhialdin et al., 2011; Magnusson et al., 2003). El-Mabrok et al. (2013) also reported that CFS of both the isolates *L. plantarum* C5 and *L. pentosus* G7 had antifungal activity against *C. capsici* and *C. glosporeoides*. Okkers et al. (1999) reported that *L. pentosus* TV35b have inhibitory activity against *Clostridium sporogenes*, *Cl. tyrobutyricum*, *L. curvatus*. *L. fermentum*, *L. sake*, *Listeria innocua*,

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ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA
جامعة العلوم الإسلامية الماليزية

Propionibacterium acidipropionici, *Propionibacterium* sp. and *C. albicans*. The active compound produced was a bacteriocins-like peptide (pentocin TV35b).

The microbiocidic action of LAB is based on both competition for nutrients and the production of various compounds, such as organic acids, hydrogen peroxide, bacteriocins, and low molecular weight antimicrobial agents (Ouwehand & Vesterlund, 2004; De Keersmaecker et al., 2006; Cleusix et al., 2007; Suskovic et al., 2010). LAB produce antimicrobial compounds that enable them to have competitive advantage over other organisms such as bacteriocins that are produced by LAB. Bacteriocins exert positive influence on host's health by stimulating the immune system and have the ability to activate macrophages and lymphocytes. Bacteriocins also improve level of immunoglobulin A (IgA) and production of gamma interferon (Marteau & Boutron-Ruault, 2002). Studies on antifungal activity of LAB started with Guillot (1958) who reported that *L. acidophilus* produced compounds which have effect against *C. albicans*. Similarly, Fitzsimmons and Berry (1993) reported that *L. acidophilus* can inhibit the growth of *C. albicans*. There are many strains of *Lactobacillus* that have the inhibitory effect against urogenital candidiasis and prevent fungal contamination of food (Barousse et al., 2004).

Lactic acid bacteria produced compounds that showed antimicrobial activity against bacteria, fungi and yeast. The antimicrobial activities of LAB supernatant against bacteria and fungi are active at different range of pH, heating and treatment by enzymes (Lindgren, 1990; Magnusson & Schnurer, 2001; Messens, 2002; Lavermicocca, et al., 2003; Muhialdin et al., 2011; Aween et al., 2012). Magnusson and Schnurer (2001) observed that the activity of supernatant with antifungal activity was stable during heating at 100 °C for 60 min with maximum effect at pH 3 to 4.5.

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Therefore, the objectives of this study were to evaluate the effect of pH, heat treatment and enzymes on the antifungal activity of CFS supernatant of LAB isolated from honey against *Candida* spp. namely, *C. albicans* ATCC14053, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019, *C. glabrata* ATCC2001 and *C. krusei* ATCC6258.

4.2 Materials and Methods

4.2.1 Preparation of fungi

Cultures of *Candida* spp. were prepared as described in CHAPTER III, Section 3.2.3.

4.2.2 Preparation of supernatant

The CFS was prepared as described in CHAPTER III, Section 3.2.6.

4.2.3 Effect of heat treatment LAB CFS on antifungal activity

The CFS of LAB were heat treated at 90 °C and 121°C for 30 min and immediately cooled in ice water. The supernatant were tested against *Candida* spp. namely, *C. albicans* ATCC14053, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019, *C. glabrata* ATCC2001 and *C. krusei* ATCC6258 using microtiter plate assay as described by Muhialdin et al. (2011). A 100 µL of SDB (Oxoid CM147) contain 10^4 CFU/mL *Candida* and 100 µL of CFS were added into the wells of microtiter plates. Then, A 200 µL of *Candida* spp. in SDB without the addition of CFS was used as positive control. The plates were incubated at 30 °C for 24, 48 and 72 h. Growth of *Candida* spp. was measured by optical density at OD 560 nm using a micro Elisa auto reader (Model 680,

BioRad) and the reading was carried out in replicate. The percentage growth of *Candida* spp. was measured using the equation:

$$\% \text{ Growth of } Candida = \frac{\text{OD560 nm after 24,48 and 72 h} - \text{OD560 nm at 0h}}{\text{OD560 nm at 0 h}} \times 100$$

4.2.4 Effect of different pH adjustments on LAB CFS antifungal activity

The pH of CFS of LAB isolates was adjusted to different pH values 3, 5, 6, 7 and 9 using 0.1 N HCL and 0.1 NaOH and was determined using pH meter (Mettler Toledo). The initial pH of the CFS were 4.1, 3.8, 3.7 and 3.9 for isolates HS, HC, HH, HM, respectively. The pH adjusted CFS were tested against *Candid* spp. in microtiter plates as described above. A 100 µL of SDB contain 10^4 CFU/mL *Candida* and 100µL of adjusted CFS were added into the wells. The plates were incubated at 30 °C for 24, 48 and 72 h. Growth of *Candida* spp. were monitored at 24, 48 and 72 h. Percent growth of *Candida* was calculated as described in Section 4.2.3.

4.2.5 Effect of enzymes on antifungal activity of LAB CFS

The proteinaceous nature of CFS was evaluated by testing their sensitivity to proteolytic enzymes proteinase K and RNase II without pH adjustment. The CFS_s were treated with proteinase K and RNase II separately (Strom et al., 2002; Aween et al., 2012b). One µL of each enzyme was added to three ml of supernatant and were left for 1h at room temperature 28±2 °C. After that, the supernatant was tested against *Candida* spp. in microtiter plates, followed by incubation at 30°C for 72 h, and fungal growth was measured at optical density at 560 nm as described above. The percentage growth of *Candida* was calculated as described in Section 4.2.3.

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4.2.6 Statistical analysis

All data were presented as mean ± standard deviation. Data were analysed by 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 analysed, whereas the real values were presented.

4.3 Results

4.3.1 Effect of heating on LAB CFS antifungal activity

Heating the CFS of LAB isolates (HS, HC, HH and HM) at 90 ºC and 121 ºC for 30 min significantly ($P < 0.05$) reduced the growth of most the *Candida* spp. compared to the control after 24 h incubation (Tables 11 and 12) especially the growth of *C. glabrata* ATCC2001 was highly significant ($P < 0.001$) inhibited by heating CFS of *L. curvatus* HH and *P. pentosaceus* HM at 90 ºC for 30 min. Likewise, the growth of *C. tropicalis* ATCC750 was highly significant ($P < 0.001$) inhibited by the heated CFS of *P. acidilactici* HC with 48 h incubation the growth of *C. parapsilosis* ATCC22019 was inhibited by the CFS of *L. plantarum* HS heated at 90 ºC for 30 min (Table 11). It was observed that the antifungal activity of LAB increased when the CFS were heated to 121 ºC for 15 min especially, the growth of *C. glabrata* ATCC2001 was highly significant ($P < 0.001$) inhibited by CFS of *L. curvatus* HH and CFS of *P. pentosaceus* HM with incubation 72 h and the growth of *C. krusei* was highly significant ($P < 0.001$) inhibited by CFS of *L. curvatus* HH after incubation 72 h. The growth of *C. parapsilosis*

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ATCC22019 was highly significant (P< 0.001) inhibited by CFS of *L. plantarum* HS with incubation72 h (Table 12).

TABLE 11: Percentage growth of *Candida* spp. with LAB supernatant after heat treatment at 90 ºC in microtiter plates incubation at 30 ºC for 72 h*

Candida spp.	Time (h)	LAB				
		HS	HC	HH	HM	Control
C. glabrata ATCC2001	24	12.3 ± 0.45^{b}	9.4 ± 0.51^{c}	3.9 ± 0.26^{d}	5.4 ± 0.35^{d}	98.70 ± 1.53^{a}
	48	38.7 ± 0.50^{b}	20 ± 0.66^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	100.4 ± 0.68^{a}
	72	42.8 ± 0.96^{b}	21.8 ± 0.55^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	122.6 ± 1.35^{a}
C. albicans ATCC14053	24	22.6 ± 0.92^{b}	17.5 ± 0.65^{c}	12.5 ± 0.83^{d}	9.4 ± 0.35^{d}	130.2 ± 1.76^{a}
	48	29.4 ± 0.85^{b}	24.3 ± 0.70^{c}	5.50 ± 0.40^{e}	18.9 ± 0.92^{d}	136.4 ± 2.87^{a}
	72	51.0 ± 1.10^{b}	31.0 ± 0.72^{c}	9.30 ± 0.20^{e}	24.5 ± 0.36^{d}	160.0 ± 1.10^{a}
C. krusei ATCC6258	24	20.6 ± 0.85^{b}	16.0 ± 0.15^{c}	14.7 ± 0.41^{d}	10.4 ± 0.26^{e}	89.40 ± 0.50^{a}
	48	24.2 ± 0.20^{b}	22.6 ± 0.36^{c}	19.3 ± 0.35^{d}	13.2 ± 0.15^{e}	124.0 ± 0.50^{a}
	72	32.3 ± 0.73^{b}	33.7 ± 0.45^{b}	24.6 ± 0.25^{c}	22.7 ± 0.52^{c}	156.0 ± 2.51^{a}
C. tropicalis ATCC750	24	12.7 ± 0.32^{b}	3.4 ± 0.30^{d}	7.80 ± 0.45^{c}	11.6 ± 0.77^{b}	98.40 ± 0.81^{a}
	48	19.3 ± 0.30^{e}	0.00 ± 0.00^{c}	11.4 ± 0.51^{d}	16.5 ± 0.45^{b}	117.0 ± 1.15^{a}
	72	30.4 ± 0.83^{e}	0.00 ± 0.00^{c}	14.9 ± 0.56^{d}	15.0 ± 0.20^{b}	128.0 ± 0.47^{a}
C. parapsilosis ATCC22019	24	1.80 ± 0.30^{d}	11.9 ± 0.55^{b}	9.6 ± 0.45^{b}	5.40 ± 0.70^{c}	97.0 ± 4.35^{a}
	48	3.10 ± 0.20^{d}	13.3 ± 0.47^{c}	22.2 ± 0.30^{b}	10.4 ± 0.50^{c}	123.0 ± 3.05^{a}
	72	0.00 ± 0.00^{d}	16.3 ± 0.45^{c}	25.4 ± 0.45^{b}	16.4 ± 0.91^{c}	136.0 ± 1.52^{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 row differs significantly (P < 0.05).

TABLE 12: Percentage growth of *Candida* spp. with LAB supernatant after heat treatment at 121ºC in microtiter plates incubation at 30 ºC for 72 h*

Candida spp.	Time (h)	LAB				
		HS	HC	HH	HM	Control
C. glabrata ATCC2001	24	23.0 ± 0.23^{b}	18.6 ± 0.62^{c}	23.0 ± 0.26^{c}	10.4 ± 0.25^{e}	114.5 ± 0.55^{a}
	48	29.4 ± 0.85^{b}	20.0 ± 0.83^{d}	12.2 ± 0.15^{d}	8.6 ± 0.51^{e}	128.2 ± 0.70^{a}
	72	38.4 ± 0.45^{b}	30.8 ± 0.65^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	144.2 ± 0.83^{a}
C. albicans ATCC14053	24	46.6 ± 0.90^{b}	36.6 ± 0.60^{c}	30.5 ± 0.90^{d}	17.6 ± 0.92^{e}	150.0 ± 1.00^{a}
	48	47.3 ± 0.85^{b}	42.8 ± 1.04^{c}	35.5 ± 0.83^{d}	19.3 ± 0.30^{e}	162.0 ± 1.52^{a}
	72	59.0 ± 1.70^{b}	44.0 ± 0.58^{c}	44.0 ± 0.11^{c}	26.5 ± 0.83^{d}	184.0 ± 2.00^{a}
C. krusei ATCC6258	24	20.0 ± 0.55^{c}	9.0 ± 0.15^{e}	18.2 ± 0.20^{d}	27.4 ± 0.35^{b}	139.0 ± 0.64^{a}
	48	23.2 ± 0.32^{d}	26.6 ± 0.45^{c}	9.7 ± 0.64^{e}	33.8 ± 0.60^{b}	165.0 ± 1.52^{a}
	72	30.1 ± 0.15^{c}	30.7 ± 0.55^{c}	00.0 ± 0.00^{d}	45.2 ± 0.37^{b}	188.0 ± 0.57^{a}
C. tropicalis ATCC750	24	10.7 ± 0.50^{d}	16.8 ± 0.75^{c}	16.4 ± 1.38^{c}	34.2 ± 0.60^{b}	153.0 ± 3.90^{a}
	48	14.3 ± 0.47^{e}	33.9 ± 2.40^{c}	21.4 ± 1.17^{d}	46.8 ± 1.60^{b}	167.0 ± 4.04^{a}
	72	24.4 ± 0.90^{e}	38.8 ± 1.79^{c}	27.1 ± 0.95^{d}	51.5 ± 1.93^{b}	180.0 ± 2.51^{a}
C. parapsilosis ATCC22019	24	1.80 ± 0.35^{d}	31.9 ± 1.95^{c}	12.6 ± 1.05^{c}	22.5 ± 1.58^{b}	115.0 ± 2.00^{a}
	48	0.00 ± 0.00^{e}	43.3 ± 1.89^{c}	26.2 ± 0.97^{d}	27.4 ± 1.63^{b}	125.0 ± 2.64^{a}
	72	0.00 ± 0.00^{e}	66.3 ± 1.15^{c}	28.4 ± 1.05^{d}	36.4 ± 2.51^{b}	153.0 ± 3.60^{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 row differs significantly (P < 0.05).

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4.3.2 Effect of pH adjusted on antifungal activity of LAB supernatant

The antifungal activity of CFS was observed at pH ranged from 3 to 5, but decreased at pH 6. The CFS of LAB isolates showed significantly (P< 0.05) reduced the growth of most *Candida* spp. (Table 13, 14, 15, 16 and 17), especially the growth of *C. glabrata* ATCC 2001 was significantly (P< 0.001) inhibited by CFS of *P. pentosaceus* HM at pH 3 and pH 5 compared to the control with incubation 72 h. However, the CFS of *L. curvatus* HH lead to inhibit the growth of *C. glabrata* significantly (P< 0.001) complete inhibited at pH 3. The growth of *C. albicans* ATCC14053 was significantly (P< 0.05) reduced by CFS of *L. plantarum* HS at pH 3 and by CFS of *P. pentosaceus* HM and *P. acidilactici* HC at pH 5. Whereas, the growth of *C. tropicalis* ATCC750 was significantly (P< 0.001) inhibited at pH 3 and pH 5 by CFS of *L. plantarum* HS. The growth of *C. krusei* reduced at pH3 by CFS of *L. plantarum* HS and *P. acidilactici* HC. The CFS of LAB HS and HM lost their antifungal activity especially against *C. krusei* ATCC6258 and *C. parapsilosis* ATCC22019 at pH 7.

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TABLE 13: Percentage growth of *Candida albicans* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 ºC for 72*

pH	Isolates	Time (h)		
		24	48	72
pH3	HS	0.00±0.00^{p}	16.3±0.45^{m}	37.3±0.68^{k}
	HC	24.2±0.30^{l}	37.1±0.38^{ij}	46.5±0.79^{hi}
	HH	45.2±0.10^{e}	46.7±0.35^{fg}	48.9±1.01^{gh}
	HM	28.2±0.30^{j}	34.1±0.32^{k}	51.6±0.65e^{fj}
	Control	169±1.15^{b}	187±1.00^{a}	210±1.52^{a}
pH5	HS	1.50±0.20^{o}	12.3±0.15^{n}	17.9±0.40^{l}
	HC	0.00±0.00^{p}	10.8±0.20^{o}	13.5±0.26^{m}
	HH	7.40±0.07^{n}	42.8±0.65^{h}	47.9±0.55^{gh}
	HM	0.00±0.00^{p}	37.3±0.17^{ij}	40.3±0.15^{jk}
	Control	166±1.73^{c}	189±1.52^{c}	212±1.00^{ab}
pH6	HS	38.2±0.36^{f}	43.2±0.11^{h}	56.6±0.60^{de}
	HC	31.5±0.36^{h}	37.8±0.41^{i}	42.6±0.32^{ij}
	HH	26.3±0.17^{k}	36.5±0.26^{j}	49.9±1.01^{fgh}
	HM	23.0±0.17^{m}	31.8±0.20^{l}	49.3±0.45^{fgh}
	Control	170±1.73^{d}	198±1.00^{d}	200±0.58^{b}
pH7	HS	33.2±0.10g	46.9±0.47^{f}	61.3±0.51^{ghi}
	HC	30.0±0.64i	56.6±0.81^{e}	66.6±0.80^{d}
	HH	33.0±1.00^{g}	34.1±0.43^{k}	54.4±0.45^{ef}
	HM	31.5±0.36^{h}	45.7±0.37^{g}	67.8±0.75^{c}
	Control	154±0.57^{a}	164±0.57^{b}	178±1.00^{ab}

*The results are expressed as mean± standard deviations of values obtained from triplicate experiment

$^{a\text{-}n}$ 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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TABLE 14: Percentage growth of *Candida glabrata* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 ºC for 72*

pH	Isolates	Time (h)		
		24	48	72
pH3	HS	1.70 ± 0.15^{m}	36.2 ± 0.21^{g}	31.0 ± 0.17^{ij}
	HC	3.70 ± 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}
pH5	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}
pH6	HS	13.0 ± 0.53^{i}	28.0 ± 1.05^{j}	35.0 ± 0.60^{hij}
	HC	26.8 ± 1.06^{e}	35.9 ± 0.25^{gh}	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}
pH7	HS	10.7 ± 0.83^{j}	$13.2 \pm 0.72j$	26.2 ± 0.72^{mn}
	HC	11.4 ± 1.27^{j}	35.4 ± 0.73^{gh}	46.2 ± 0.66^{fg}
	HH	3.30 ± 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*.

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TABLE 15: Percentage growth of *Candida krusei* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 ºC for 72*

pH	Isolates	Time (h)		
		24	48	72
pH3	HS	$6.80±0.81^{m}$	$25.6±0.83^{k}$	$37.9±0.15^{n}$
	HC	$3.40±0.44^{n}$	$33.4±0.74^{ij}$	$42.7±0.35^{l}$
	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	$124.6±1.04^{a}$	$154±0.50^{a}$	$178±0.23^{a}$
pH5	HS	$15.4±0.60^{p}$	$27.1±0.89^{k}$	$37.3±0.90^{o}$
	HC	$1.70±0.26^{o}$	$31.2±1.01^{j}$	$44.3±0.47^{k}$
	HH	$1.60±0.46^{o}$	$31.7±1.41^{j}$	$40.0±1.04^{m}$
	HM	$49.4±1.18^{d}$	$80.4±1.24^{d}$	$97.3±0.25^{g}$
	Control	$102±1.15^{b}$	$139.5±1.35^{b}$	$169.8±1.07^{b}$
pH6	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	$89.2±1.21^{c}$	$125±1.10^{c}$	$137.4±0.20^{c}$
pH7	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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TABLE 16: Percentage growth of *Candida parapsilosis* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 ºC for 72*

pH	Isolates	Time (h)		
		24	**48**	**72**
pH3	HS	18.4 ± 0.50^{h}	20.6 ± 0.65^{l}	32.4 ± 0.72^{l}
	HC	14.9 ± 0.45^{j}	19.7 ± 0.35^{l}	38.3 ± 0.45^{k}
	HH	20.9 ± 0.30^{g}	28.0 ± 0.41^{j}	44.2 ± 0.34^{i}
	HM	18.0 ± 0.57^{h}	50.0 ± 0.76^{f}	78.5 ± 0.75^{ef}
	Control	76.9 ± 0.72^{b}	113 ± 0.57^{b}	188.6 ± 0.62^{a}
pH5	HS	16.9 ± 0.73^{i}	42.4 ± 0.60^{g}	53.7 ± 0.36^{h}
	HC	8.2 ± 0.15^{n}	14.3 ± 0.70^{n}	32.7 ± 0.61^{l}
	HH	21.7 ± 0.32^{g}	24.6 ± 0.21^{k}	44.4 ± 0.32^{i}
	HM	13.2 ± 0.66^{jk}	43.4 ± 0.26^{g}	75.5 ± 0.17^{f}
	Control	60.3 ± 0.62^{c}	89.5 ± 1.00^{c}	102 ± 0.45^{c}
pH6	HS	11.3 ± 0.65^{lm}	17.7 ± 0.61^{m}	23.4 ± 0.21^{m}
	HC	16.4 ± 0.65^{i}	32.5 ± 0.86^{i}	43.3 ± 0.60^{ij}
	HH	17.6 ± 0.70^{hi}	19.8 ± 0.30^{l}	41.3 ± 0.20^{j}
	HM	10.2 ± 0.45^{m}	36.5 ± 0.47^{h}	71.2 ± 1.00^{g}
	Control	125 ± 0.68^{a}	134.3 ± 0.70^{a}	144.6 ± 0.45^{b}
pH7	HS	49.4 ± 1.07^{e}	68.9 ± 0.75^{e}	83.6 ± 0.75^{d}
	HC	5.90 ± 0.61^{o}	16.4 ± 0.45^{mn}	23.4 ± 0.55^{m}
	HH	12.5 ± 0.60^{kl}	30.6 ± 0.68^{i}	31.3 ± 0.85^{l}
	HM	35.7 ± 0.90^{f}	50.8 ± 1.41^{f}	79.2 ± 0.68^{e}
	Control	57.3 ± 0.75^{d}	77.6 ± 0.32^{d}	99.6 ± 0.40^{c}

*The results are expressed as mean± standard deviations of values obtained from triplicate experiments.
$^{a\text{-}o}$ 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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TABLE 17: Growth percentage of *Candida tropicalis* with LAB supernatant pH adjusted 3, 5, 6 and 7 in microtiter plate incubated at 30 ºC for 72*

pH	Isolates	Time (h)		
		24	48	72
pH3	HS	32.4±0.35^{jk}	24.6±0.50^{h}	0.00±0.00^{n}
	HC	11.3±0.41^{h}	33.9±0.44^{g}	43.5±0.71^{f}
	HH	1.60±0.15^{m}	44.3±0.91^{f}	39.4±0.68^{g}
	HM	6.30±0.30^{k}	34.4±0.61^{g}	40.6±0.91^{g}
	Control	137.2±0.72^{b}	196±0.85^{b}	232±1.15^{a}
pH5	HS	16.2±0.42^{n}	10.7±0.44^{n}	0.00±0.00^{n}
	HC	3.20±0.20^{l}	9.70±0.35^{n}	19.3±0.41^{m}
	HH	5.90±0.32^{k}	12.2±0.55^{m}	28.5±0.55^{l}
	HM	8.30±0.46^{j}	23.3±0.46^{hi}	38.3±0.62^{h}
	Control	156±1.00^{a}	162±1.00^{a}	176±1.00^{b}
pH6	HS	7.20±0.25^{j}	13.8±0.30^{l}	36.2±0.12^{hi}
	HC	11.7±0.36^{h}	13.3±0.41^{lm}	36.7±0.56^{hi}
	HH	14.3±0.45^{g}	21.4±0.42^{j}	32.2±0.53^{jk}
	HM	10.2±0.51^{i}	17.3±0.46^{k}	37.3±0.63^{hi}
	Control	64.3±1.14^{d}	98.6±0.50^{d}	122±0.31^{d}
pH7	HS	16.0±0.10^{f}	12.5±0.56^{lm}	35.7±0.29^{i}
	HC	17.5±0.31^{e}	22.6±0.11^{ij}	33.3±0.42^{j}
	HH	18.2±0.60^{e}	51.7±0.55^{e}	63.5±0.65^{e}
	HM	13.8±0.70^{g}	17.8±0.61^{k}	31.2±1.00^{k}
	Control	166±1.15^{c}	173±2.64^{c}	214±1.00^{c}

*The results are expressed as mean± standard deviations of values obtained from triplicate experiments.
a-o 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*.

4.3.3 Effect of enzymes on antifungal activity of LAB supernatant

Generally, the treatment of CFS$_s$ with enzymes proteinase K and RNase II resulted in different antifungal activity against *Candida* spp. The LAB isolates showed various results against the tested pathogens when treated with proteinase K, compared to the control. The CFS$_s$ of HC and HM treated with proteinase K showed complete inhibition against *C. albicans*. The CFS of HH and HM showed complete inhibition against both *Candida* spp.; *C. glabrata* and *C. tropicalis* compared to the other isolates. This indicated that these LAB isolates did not have protein. While, antifungal activity of CFS

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of *L. plantarum* HS decreased when treated with proteinase K especially against *C. albicans* and *C. parapsilosis* after 72h of incubation this indicated that CFS contained protein-like antifungal compounds (Table 18). Otherwise, when treated the CFS with RNase II the supernatant (HM) showed complete inhibition against *C. albicans*, *C. glabrata* and *C. tropicalis* compared to the other isolates and the control. However, the treating supernatant with RNase II destroyed the antifungal activity of HC and HH against all *Candida* spp. especially against *C. krusei* indicated that the CFS contained protein-like antifungal compounds against fungi by the increase in the growth of *C. krusei* after 48h and 72h of incubation (Table 19). This observation suggested that addition proteinase K and RNase to the supernatants of LAB isolates enhanced the antifungal activity to some isolates against *Candida* spp.

TABLE 18: Percentage growth of *Candida* species with LAB supernatant after treatment with proteinase K in microtiter plate incubated at 30°C for 72h*

Candida species	Time (h)	LAB				
		HS	HC	HH	HM	Control
C. albicans	24	3.2	3.3	11.6	1.5	158
ATCC14053	48	6.7	NG	4.8	0.06	176
	72	24.2	NG	1.3	NG	185
C. glabrata	24	2.4	13.8	NG	NG	235
ATCC2001	48	6.6	5.7	NG	NG	242
	72	17.3	4.3	NG	NG	267
C. parapsilosis	24	4.7	7.9	10.4	11.6	126
ATCC22019	48	9.6	5.1	8.1	7.6	137
	72	22.8	2.3	6.5	3.8	149.3
C. tropicalis	24	5.6	2.7	NG	NG	147.4
ATCC750	48	12.9	1.2	NG	NG	165
	72	15.6	NG	NG	NG	178.2
C. krusei	24	1.5	7.4	6.6	10.5	121.2
ATCC6258	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.

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TABLE 19: Percentage growth of *Candida* species with LAB supernatant after treatment with RNase II in microtiter plate incubated at 30°C for 72h*

Candida species	Time (h)	LAB				
		HS	HC	HH	HM	Control
C. albicans ATCC14053	24	9.7	4.6	2.6	NG	97.2
	48	NG	14.6	8.4	NG	134
	72	NG	26.3	10.4	NG	139
C. glabrata ATCC2001	24	9.5	3.6	NG	4.1	158.8
	48	9.0	8.7	9.3	NG	164.3
	72	5.7	14.7	16.6	NG	174.3
C. parapsilosis ATCC22019	24	17.4	4.5	7.5	13.3	178
	48	13.6	7.3	12.6	11.0	186
	72	9.7	12.7	14.3	8.4	213
C. tropicalis ATCC750	24	10.3	8.2	NG	NG	254.2
	48	4.3	10.6	4.2	NG	262
	72	2.6	22.3	12.1	NG	274
C. krusei ATCC6258	24	19.7	45.6	20.8	22.6	126.6
	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.

4.4 Discussion

The antifungal activity of lactic acid bacteria (LAB) has been attributed to the production a variety of antifungal compounds that have antifungal activities. This study observed that CFS of four LAB isolated from honey samples namely, *L. plantarum* HS, *L. curvatus* HH, *P. acidilactici* HC and *P. pentosaceus* HM had good antifungal activity against five strains of pathogenic *Candida* spp. as evaluated by the microtiter plates method. This indicated that CFS from these isolates contain compounds that are protein-like nature. When the CFS was heated to 90 °C and 121 °C for 30 min resulted was significantly ($P < 0.05$) reduced the growth of most *Candida* spp. especially the growth of *C. glabrata* ATCC 2001 and was highly significant ($p < 0.001$) inhibited by the heated CFS of *L. curvatus* HH and *P. pentosaceus* HM with incubation 72 h at 90 °C and 121

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°C. Similarly, Monthon (2005) observed that antifungal activity of *Lactococcus lactis* isolated from fermented food was stable during heat treatment and the activity was retained even after autoclaving at 121 °C for 15 min against *C. albicans* DMST 5239. Oliveira et al. (2008) reported that CFS of LAB isolated from vacuum packaged beef had antimicrobial activity after heating 100 °C for 10 min. In this study, when the pH of the CFS was adjusted to different vales 3, 5, 6 and 7 showed significantly (P< 0.05) reduced the growth of most *Candida* spp. whereas, the growth of *C. glabrata* ATCC2001 and *C. tropicalis* ATCC750 were higher significantly (P< 0.001) inhibited by CFS of *P. pentosaceus* HM and CFS of *L. plantarum* HS at pH 3, and pH 5, respectively. This may suggest that compound was responsible for antifungal activity in CFS of these isolates might be organic acids (lactic acid, acetic acid). Similarly, 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. However, the growth of *C. krusei* ATCC6258 and *C. parapsilosis* ATCC22019 was difficult to inhibit when the CFS adjusted to different values especially the CFS of LAB HS and HM which lost their antifungal activity against *C. krusei* and *C. parapsilosis* at pH 7. These findings are consistent with Jin et al. (2007) who reported that growth of *C. krusei* ATCC14243 was not inhibited by *L. fermentum*, *L. crispatus* and *L. jensenni* isolated from the human vagina. The treatment of CFS with enzymes proteinase K and RNase II resulted in different antifungal activity against *Candida* spp. 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. The results obtained from present study are in agreement with Monthon (2005) who reported that the supernatant produced by *Lactococus lactis*

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showed inhibitory activity against *C. albicans* DMST 5239. Ronnqvist et al. (2007) also reported that *L. fermentum* Ess-1 showed activity against *C. albicans* and *C. glabrata*. Adeniyi and Damsa (2013) reported that the cell free supernatant of LAB isolates produced by *L. plantarum* showed higher antifungal activity against *C. albicans* ATCC90029 with inhibition zone 25mm. 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*.

4.5 Conclusion

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

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