CHAPTER IV

EFFECT OF PH, ENZYME AND HEAT TREATMENT ON INHIBITORY ACTIVITY OF *L. casei* YG1 AND *L. casei* DF22 AND *L. plantarum* C16 AGAINST *R. mucilaginosa*

4.1 INTRODUCTION

Yeasts are known to be harmless and environmental friendly microorganisms that are important fermentative organisms in the food industry. However, some yeast can be pathogenic and destructive under certain conditions (Chester & Cooper, 2011) which may result into food spoilage. Yeasts have also been reported to be present in deteriorating foods (Cassone et al., 2007). Most yeast grows within the pH range of pH 3.0 to 7.0 especially in high sugar and salt content food under optimum temperature 30 °C (Praphailong & Fleet, 1997). Many pathogenic yeast have been confirmed resistant to antifungal agents (Diekema et al., 2005). However, it can also survive at pH values as low as pH 2.0 to 2.5 (Fleet 1992; Martorell et al., 2007). These characteristics allow them to cause food spoilage especially when the food products are preserved with weak acids, hyperosmotic condition or long-term frozen storage (Fleet, 2011).

Yeasts such as *Rhodotorula* can survive at low pH, low water content and even at low temperature. The optimum growth pH for *Rhodotorula* is between 4.5 and 7.0. Preliminary screenings for anti-yeast activity using dual agar overlay method showed that *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 strongly inhibit *R.*

mucilaginosa. LAB-CFS consists of organic acid, hydrogen peroxide, bacteriocins, proteinaceous compound, fatty acids, reuterin, and diacethyle (Lindgren & Dobrogosz, 1990; Stiles, 1996; Corsetti et al., 1998; Strom et al., 2002; Messens & De Vugst, 2002; Magnusson, 2003; Dalie et al., 2009; Muhialdin et al., 2015). Thus, adjustment of pH, heat stability and its sensitivity towards proteolytic enzyme were important to screen the nature of LAB-CFS anti-yeast properties.

Antimicrobial compounds are active against bacteria and fungi at pH 3 and 4.5 and are also heat stable at 100°C (Lindgren, 1990; Magnusson & Shnurer, 2001; Messens, 2002; Lavermicocca et al., 2003). However, Yu and Yinlu (2015) reported that the anti-yeast activities of cell free supernatant of *L. plantarum*, *E. faecium* and *W. cibaria* were heat stable at 80, 100 and 121°C respectively and their activity were not affected by proteolytic enzymes treatments (proteinase K, pepsin, papain and trypsin respectively). The anti-yeast activities of cell free supernatant of *L. plantarum*, *E. faecium* and *W. cibaria* were maintained at pH ranging from pH 3.5 to 4.0. However, the activity was gradually lost with an increase in the pH from pH 4.5 to 6.0 (Yu & Yunlu, 2015).

Many chemical preservatives and antifungal agents have been used to inhibit the growth of yeast in the food industry (Cornea et al., 2013; Crowley et al., 2013). However, presently consumers are demanding for products without chemical preservative. In order to replace chemical preservatives, LAB has been reported to have a great potential to be used as bio-preservatives (Gardiner et al., 2000; Corcoran et al., 2004) because they are generally recognized as safe (GRAS) and produces many antimicrobial compound. Therefore, this study was done to investigate the bio-

preservative potential of LAB by examining the anti-yeast activity of LAB cell free supernatant in different pH conditions (3, 5, 6 and 7), heat (90 and 121°C) and enzymatic treatments (pepsin, papain, and proteinase K) against the growth of *R. mucilaginosa*.

4.2 MATERIALS AND METHODS

4.2.1 LAB and Cultures

Three LABs studied namely, *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 that were previously isolated and identified from different sources were maintained on MRS agar (Oxoid) and stored at 4°C. Yeast strains *R. mucilaginosa* CBR previously isolated from cucumber as mentioned in section 3.2.1, were grown on PDA (Oxoid) plates incubated at 30°C for 48 h and were then stored at 4°C. Prior to analysis, LAB were sub-cultured in MRS broth (Oxoid) and incubated at 30°C for 48 h, while *R. mucilaginosa* was sub-cultured in PDB (Difco) and incubated at 30°C for 24 h. Then, yeast cell counts were determined using a haemacytometer. It was adjusted to 10^6 cells/ml with sterile peptone water (0.2% w/v) which was used as an inoculum for testing (Muhialdin et al., 2011b).

4.2.2 Cell free supernatant preparation

The LABs isolates were inoculated into MRS broth (Oxoid) and incubated at 37°C for 48 h with shaking at 150 rpm anaerobically. The LAB cell free supernatant (LAB-CFS) was prepared by centrifuging the broth at 10,000 rpm, 4°C for 20 min using

Centrifuge Combi 514R (Hanil Science Industrial). The supernatant of each isolates was then filtrated using sterile filter (0.45 µm-pore-size filter, Millipore, Merk).

4.2.3 Inhibitory activity of crude LAB-CFS

Inhibitory activity of CFS on *R. mucilaginosa* CBR was carried out following the method of Gudina et al. (2010). Yeast was cultured in PDB (Difco) at 30°C for 48 h. Approximately 100 µl PDB containing yeast (10^6 cells/ml) were placed in the 96 wells micro-titer plate and 100 µl crude LAB-CFS were added into the wells. Approximately 200 µl of yeast in PDB without LAB-CFS was used as positive control while 200 µl of LAB-CFS were used as negative control. The plates were incubated at 30°C for 72 h. Inhibition activity was measured by measuring turbidity at OD_{630nm} using ELISA microplate reader Epoch (Biotex). The analysis was carried out in duplicates and the mean was taken. The growth inhibition percentage of yeast was calculated as follows:

$$\% \text{ Growth inhibition} = [1 - (A_C / A_0)] \times 100$$

Where, A_C represents the absorbance of the treatment well minus the absorbance of the negative control well and A_0 represent the absorbance of the positive control well (Gudina et al., 2010).

4.2.4 pH adjusted of LAB-CFS

The pH of LAB-CFS was adjusted to different pH values (3, 5, 6, and 7 respectively) using 0.1 N HCl and/or 0.1 N NaOH. The initial pHs of the isolates were 3.85, 3.83, 3.77 for isolates *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16, respectively. The

adjusted pH supernatant was then tested against *R. mucilaginosa* CBR in micro-titer plate assay as described in section 4.2.3.

4.2.5 Heat stability of LAB-CFS

The supernatants were heat treated at 90°C in a water-bath for 30 min and at 121°C in the autoclave for 30 min. The heat treated supernatants were tested against *R. mucilaginosa* CBR in micro-titer plate assay as described in section 4.2.3.

4.2.6 Enzymatic treatment of LAB-CFS

The effect of proteolytic enzymes on antifungal activity of LAB-CFS was investigated following the method of Magnusson and Schurer (2001). The following enzymes were used proteinase K Sigma), papain, and pepsin (Sigma). The initial pHs of the isolates were 3.85, 3.83, 3.77 for isolates *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16, respectively. The pH of the CFS was adjusted with 1M HCl and 2M NaOH to 7.6, 7.0 and 2.0 for proteinase K, papain and pepsin (Sigma), respectively. After pH adjustment of the CFS, 1 mL of the supernatants were treated with 100 µL of each enzyme and incubated at 37°C for 1 h. The reaction was stop by heating the mixture until 65°C for 30 min. The enzymatic treatments of LAB-CFS were tested against *R. mucilaginosa* CBR in micro-titer plate assay as described in section 4.2.3.

4.2.7 Screening for bacteriocinogenic LAB

LABs (*L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16) were grown in MRS broth (Difco) and incubated at 30°C for 24 h. LAB cell counts were determined using a Nanophotometer, and adjusted to 10^6 cells/ml with sterile peptone water (0.1% w/v) to be used as an inoculum for testing. Inhibition activity of the isolates was determined by the overlay method as described by Magnusson and Schnurer (2001). LAB was inoculated on MRS agar plates and incubated anaerobically at 37°C for 24 h. The plates were then overlaid with 10 ml of MRS soft agar containing 10^6 cells/ml of other LAB as indicator. *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 were used as indicator against each other following method of Saidi et al. (2011) with some modification. After 48 h of aerobic incubation at 30°C, the zone of inhibition was measured. The diameter of clear zone was measured. Inhibition tests were done in duplicate.

4.2.8 Statistical Analysis

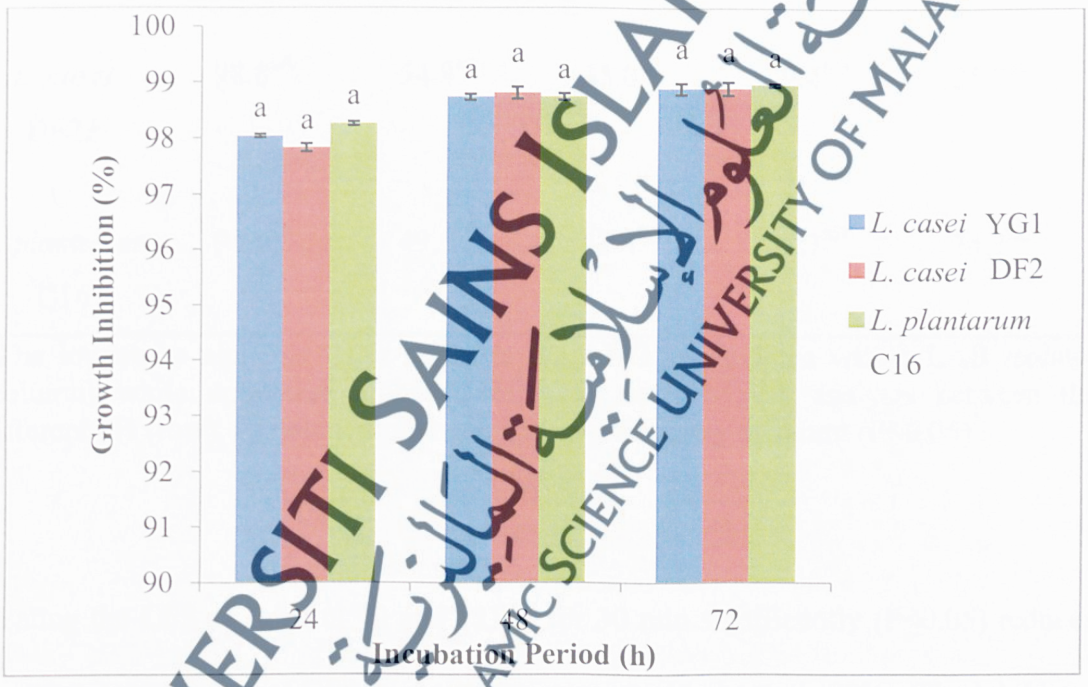
Two way analysis of variance ANOVA (Minitab16 Statistical Software., USA) was carried out to determine the statistical differences at ($p<0.05$).

4.3 RESULTS

It was observed that the three LAB isolates evaluated inhibited the growth of *R. mucilaginosa* (Figure 6). However, with longer incubation time a greater anti-yeast activity (98.8%) was observed for all LAB isolates. The anti-yeast activity of LAB

isolates was not significantly (P>0.05) affected by incubation time between 24 to 72 h at 30°C. However, slight variation in the inhibition percentages among the LAB isolates; greater inhibitory activity was observed after 48h for all the LAB isolates. The inhibition percentages at 24 h were less than at 48 and 72 h. It was observed that the inhibitory activity of *L. plantarum* C16 was nearly 99.0% after 72 h but, *L. casei* DF22 showed better inhibitory activity after 48 h against *R. mucilaginosa*.

Figure 6: Percentages inhibition of *R. mucilaginosa* by crude LAB-CFS



* different alphabet means is significantly different (P<0.05)

The initial pHs of the cell free supernatant were 3.85, 3.83, 3.77 for *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16, respectively. Adjusting the pH of CFS from pH 3.83 (control) to pH 3, 5, 6 and 7 significantly (P<0.05) affected the inhibitory activity against the yeast (Table 9). The anti-yeast activity diminished to less than 50%

inhibition after pH adjustments to pH 5, 6 and 7 for all the LAB isolates. However, no significant difference (P>0.05) was observed between the CFS of the LAB.

Table 9: Percentage of inhibition of *R. mucilaginosa* by LAB-CFS adjusted to different pH

LAB-CFS	Growth Inhibition (%)				
	Control	pH 3	pH 5	pH 6	pH 7
L. casei YG1	98.7^{aA}	51.8^{bB}	36.9^{cC}	29.4^{bD}	23.4^{cE}
L. casei DF22	98.8^{aA}	54.8^{aB}	45.0^{aC}	29.4^{bD}	25.7^{aE}
L. plantarum C16	98.9^{aA}	49.5^{cB}	42.4^{bC}	35.3^{aD}	24.3^{bE}

*The lowercase alphabet letter refers to the ANOVA analysis within LAB isolates (column) while uppercase alphabet refers to the ANOVA analysis between the different pH (row). Different alphabet means significantly different (P<0.05)

Heating the CFS of LAB at 90 and 121°C for 30 min significantly (P<0.05) reduced the inhibitory activity of CFS to about 50% compared to control (99%). The inhibitory activity of CFS of *L. casei* YG1 and CFS of *L. casei* DF22 were 59.2% and 56.6% against *R. mucilaginosa* after 48 h incubation when CFS heated at 90°C respectively (Table 10). While the growth inhibition of *R. mucilaginosa* was observed to reduce in *L. plantarum* C16 (50.2%) and *L. casei* YG1 (49.2%) after 48 h observation when the CFS was heated at 121°C compared to the CFS of LAB that was not heated. Other

CFS of LAB also showed reduction of inhibitory activity after being heated at 90°C and 121°C.

Table 10: Percentage of inhibition of *R. mucilaginosa* by heat treated (30 min) LAB-CFS

LAB-CFS	Growth Inhibition (%)		
	Control	90 °C	121 °C
L. casei YG1	98.7^{aA}	59.2^{aB}	49.2^{bC}
L. casei DF22	98.8^{aA}	56.6^{bB}	47.8^{bC}
L. plantarum C16	98.9^{aA}	52.2^{cB}	50.2^{aC}

*The lowercase alphabet letter refers to the ANOVA analysis within LAB isolates (column) while uppercase alphabet refers to the ANOVA analysis between the different pH (row). Different alphabet means significantly different ($P<0.05$)

The effect of proteolytic enzyme treatment to the CFS significantly ($P<0.05$) reduced the anti-yeast activity to about 50% compared to control (99%) (Table 11) indicating the possible presence of protein-like compounds in the MRS broth. While there was significant difference ($P<0.05$) between the effect of proteinase K and pepsin on the CFS, the anti-yeast activity was significantly ($P<0.05$) reduced by papain treatment. The activities of papain treated CFS were 26.8, 45.9 and 38.1% for *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16, respectively.

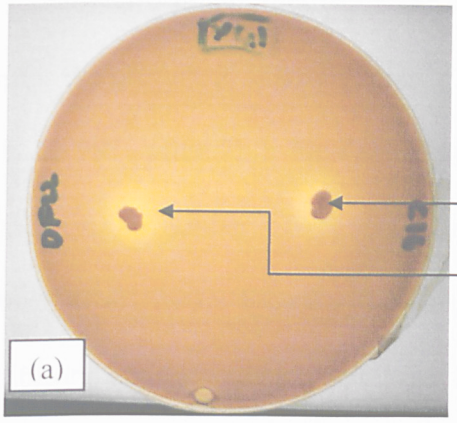
Table 11: Percentage of growth inhibition of *R. mucilaginosa* by LAB-CFS treated with proteolytic enzyme

LAB-CFS	Growth Inhibition (%)			
	Control	Proteinase K	Pepsin	Papain
L. casei YG1	98.7^{aA}	47.9^{bB}	41.3^{cC}	26.8^{cD}
L. casei DF22	98.8^{aA}	52.5^{aB}	46.5^{bC}	45.9^{aC}
L. plantarum C16	98.9^{aA}	47.9^{bB}	43.7^{aC}	38.1^{bD}

*The lowercase alphabet letter refers to the ANOVA analysis within LAB isolates (column) while uppercase alphabet refers to the ANOVA analysis between the different pH (row). Different alphabet means significantly different ($P<0.05$)

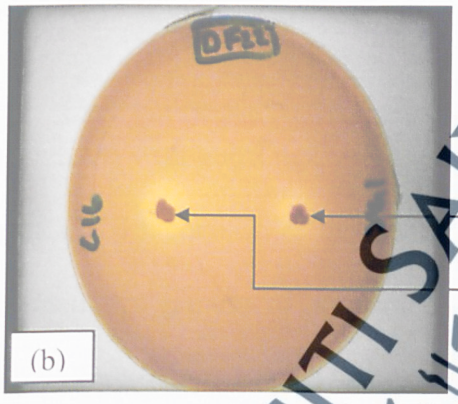
The preliminary screening of bacteriocin (Figure 7) indicated the presence of bacteriocin because the entire LAB has the ability to inhibit the growth of other LAB. *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 inhibited the growth of one another more than 10mm of inhibition zone.

Figure 7: Screening of bacteriocin production of LAB. (a) *L. casei* YG1 inhibited by *L. casei* DF22 and *L. plantarum* C16 (b) *L. casei* DF22 ihibited by *L. casei* YG1 and *L. plantarum* C16 (C) *L. plantarum* C16 inhibited by *L. casei* YG1 and *L. casei* DF22



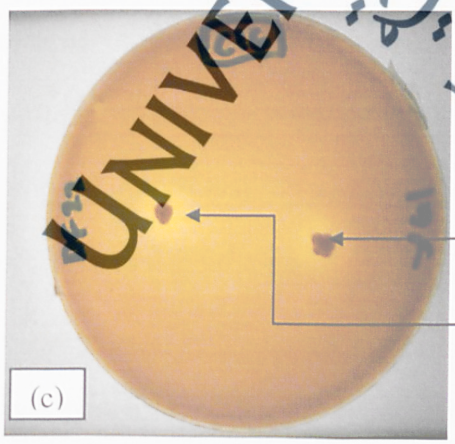
L. plantarum C16

L. casei DF22



L. casei YG1

L. plantarum C16



L. casei YG1

L. casei DF22

4.4 DISCUSSION

LAB-CFS from *L. casei* YG1, *L. casei* DF22, and *L. plantarum* C16 have strong inhibitory activity against *R. mucilaginosa* CBR as shown in Table 9. This indicates that secondary metabolite produced in the MRS broth by LAB have ability to fully inhibit the growth *R. mucilaginosa*. Growth of *R. mucilaginosa* CBR was inhibited by 97% to 99% after 72 h incubation at 30°C by crude supernatants of the three LABs evaluated. A number of reports indicated that *L. plantarum* has antifungal activity (Strom et al., 2002; Sjogren et al., 2003). *L. plantarum* isolated from yogurt showed inhibitory activity against spoilage fungi (Fowoyo & Uzoma, 2015). Similarly, *L. fermentum* Te007 was observed to give good inhibitory activity against fungi *A. niger* and *A. Oryzae*.

Other LAB with antifungal activity reported are, *L. coryniformis* subsp. *Coryniformis* strain Si3 (Magnusson & Schnurer, 2001), *L. paracaei ssp. tolerans* that completely inhibited the growth of *F. proliferatum*, and *F. Graminearum* (Yousef & Lloyd, 2008). Previously, Roy et al. (1996) reported *L. lactis subsp. lactis* inhibited the growth of *A. flavus, A. parasiticus, Fusarium* spp. Okkers et al. (1999) reported that *L. pentosus* inhibited the growth of *C. albicans*. Furthermore, Yu and Yunlu (2015) reported that cell free supernatant from *L. plantarum*, *W. cibaria,* and *E. faecium* have strong inhibitory activity against spoilage yeast such as *C. albican*, *C. Lusitaniae, C. tropicalis,* and *R. glutinis*.

It was well known that pH could affect the inhibitory activity of cell free supernatant. From this study, adjusting the pH to pH 3, 5, 6 and 7 significantly affected ($P<0.05$)

inhibitory activity of cell free supernatant of LAB against *R. mucilaginosa*. Similarly, some researcher reported that some LAB showed maximum anti-fungal activity at pH 3 to pH 4.5 (Magnusson & Schnurer, 2001). However, Muhialdin et al., (2011b) suggested that the cell free supernatant of certain LAB strain observed to be active within a wide range of pH, starting from as low as pH 3 up to pH 9 depending upon the strain.

Gerez et al. (2009) suggested that secondary metabolite produced by LAB which has the ability to inhibit microbial growth is due to the organic acid. The organic acids inhibitory activity against microorganism is related to the decrease in pH. The plasma membranes of most microorganisms restrict penetration by charged molecules. However, undissociated molecules can easily diffuse. Additionally, Yu and Yunlu (2015) reported that the inhibitory activity of LAB-CFSs against *Candida albican* was maintained at pH values range from pH 3.5 to 4.0 but was gradually lost with the increase in pH value from pH 4.5 to 6.0.

For heat treatment, the inhibitory activity of cell free supernatant from *L. casei* YG1, *L. casei* DF22, and *L. plantarum* C16 against *R. mucilaginosa* reduced significantly when treated at 90°C and 121°C for 30 min. This indicated that cell free supernatant contains compound that are protein-like in nature. Similar studies by many researchers showed that pentocin, a bacteriocin-like peptide produced by secondary metabolites of *L. pentosus* was reported to be heat stable when heated at 100°C for 30 min but lost 50% of its activity after 15 min when heated at 121°C (Okkers et al., 1998). However, Klaenhamner, (1988) reported that heating supernatants of LAB (*L. coryniformis* subsp. *coryniformis* strain Si3) at 100°C for 30 min did not destroy the inhibition

activity of LAB. Heating the supernatant of *L. citreum*, *L. mesenteroides*, *L. plantarum,* and *L. rossiae* at 100°C for 60 min did not destroy the inhibitory activity. While Muhialdin et al., (2011b) reported that *P. pentosaceus* Te010 also produced heat stable protein-like antifungal compounds at 90°C but the antifungal activity was destroyed at 121°C. New findings from Yu and Yunlu (2015) indicated that inhibitory activity of cell free supernatant of *L. plantarum*, *W. cibaria,* and *E. faecium* against *C. albicans* ATCC 90028 were resistance to heat exposure (80, 100 and 121°C for 20 min).

For enzymatic treatment, the activity of cell free supernatant of *L. casei* YG1, *L. casei* DF22, and *L. plantarum* C16 significantly reduced below 50% when treated with proteolytic enzyme. This indicates the presence of antimicrobial peptides in the LAB-CFS of all the LAB isolates. Muhialdin et al. (2011b) suggested that *L. fermentum* Te007 and *P. pentosaceus* Te010 produced antifungal compounds that were proteinaceous in nature. However, Yu and Yunlu (2015) reported that proteolytic enzymatic treatment did not affect the inhibitory activity of *L. plantarum*, *W. cibaria,* and *E. faecium* against *C. albicans,* suggesting no proteinaceous antifungal compound production.

The mechanism in which the function of papain is made possible is through the cysteine-25 portion of the triad in the active site that attacks the carbonyl carbon in the backbone of the peptide chain freeing the amino terminal portion. As this occurs throughout the peptide chains of the protein, the protein breaks apart (Amri & Mamboya, 2012). Thus, papain reduced the inhibitory of LAB-CFS significantly. Pepsin is most efficient in cleaving peptide bonds between hydrophobic and

preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Dunn, 2001). As a result, efficiency inhibitory activity of LAB-CFS affected significantly. Similarly proteinase K destructed proteins in cell lysates (Hilz et al., 1975) thus decreasing the inhibitory activity of LAB-CFS against *R. mucilaginosa*. Until recent years, the enzyme treatment and the sensitivity of the active compounds were determined at the early stages to confirm the nature of the compounds before further purification (Cizeikiene et al., 2013; Crowley et al., 2013b).

Bacteriocins from lactic acid bacteria have demonstrated as food preservatives or as therapeutics for veterinary or medical uses or as phyto-sanitary for protection of plants. Gomashe et al. (2014) defined bacteriocins as ribosomally synthesized antimicrobial peptides that are active against other bacteria either of the same species (narrow spectrum), or across genera (broad spectrum). From the initial screening of bacteriocinogenic LAB, it showed that *L. casei* YG1, *L. casei* DF22, and *L. plantarum* C16 have ability to inhibit each other species which shows the potential in producing bacteriocin. The mode of action of bacteriocins produced by LAB is the formation of holes in the membrane of the target organisms, causing leakage and destruction of the trans-membrane potential (Todorov, 2009). Jose et al. (2007) stated that bacteriocin mechanism are related to forming of pore in the membranes of the target microorganisms which cause the leakage of the cytoplasm.

Similar work by Dhewa, (2012) reported 19 isolates of LAB has the potential to produce bacteriocin and were screened from dairy and meat origin food. The isolates were confirmed as *Lactobacillus* spp., *Lactococcus* spp., and *Pediococcus* spp. Gomashe et al. (2014) also reported to have bacteriocin producing LAB isolates were

identified as *L. acidophilus*, *L. rhamnosus*, *L. leishmany*, *L. plantarum* G1, and *L. casei* G3 from food samples such as curd, milk, and probiotics which have antimicrobial effects on selected spoilage and pathogenic microorganisms in-vitro. Saidi et al. (2011) reported isolates from Algerian goat's milk were bacteriocin producing LAB and were identified as *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Streptococcus* spp., and *Pediococcus* spp.

4.5 CONCLUSION

This study showed that LAB-CFS from *L. casei* YG1, *L. casei* DF22, and *L. plantarum* C16 have strong inhibitory activity against spoilage yeast *R. mucilaginosa*. The nature of LAB-CFS is related to pH as one of inhibiting factors. LAB-CFS at pH 3 showed greater anti-yeast activity than at higher pH. LAB-CFS activities were reduced at temperature 90 and 121°C however inhibit 50% of *R. mucilaginosa*. Treatment of proteolytic enzyme suggested that protein or peptide in the LAB-CFS contribute to the inhibitory activity. The results from this study also suggest the presence of bioactive peptides in the LAB-CFS and the potential of its anti-adhesion activity should be further explore.