

CHAPTER V

ANTI-ADHESION ACTIVITY OF LACTIC ACID BACTERIA SUPERNATANT AGAINST BIOFILM PRODUCE BY *R. mucilaginosa*

5.1 INTRODUCTION

Yeast biofilms contribute to quality impairment of industrial processes and also play an important role in clinical infections. Biofilms are aggregates of microorganisms, which are formed due to the attachment of cells to host surface in an aqueous environment (Lynch et al., 2003). Little is known about biofilm formation and their treatment. The presence of biofilms is common in food industry. Biofilms can exist on all types of surfaces in food plants ranging from plastic, glass, metal, wood, to food products (Chmielewski & Frank, 2003). Poor hygiene practices in food processing plants may result in the contamination of food products with pathogens, which can cause a serious health risk for consumers. Moreover, the complete elimination of pathogens from food processing environments is a difficult task, in part because bacteria can attach to food contact surfaces and form biofilms, where they survive even after cleaning and disinfection (Brooks & Flint, 2008).

Biofilms are the most common bacterial lifestyle in nature. After initial attachment of cells to a surface, they start to multiply and secrete a consistent matrix of extracellular polymeric substances (EPS) in which cells are wrapped. Biofilms are a serious problem in many food industry sectors. In dairy environments, the microorganism

most commonly associated with food contact surfaces include *Enterobacter*, *Lactobacillus*, *Listeria*, *Micrococcus*, *Streptococcus*, *Bacillus*, *Staphylococcus*, and *Pseudomonas* (Bremer et al., 2006). The attachment of the microorganism to the food product or the product contact surfaces leads to serious hygienic problems and economic losses due to food spoilage. For these reasons, it is considered that the presence of biofilms in the food systems is a serious public health risk.

One of the main problems of the food industry is characterized by survival of foodborne pathogen or spoilage microorganisms due to an insufficient disinfection of surfaces or instruments that come in contact with food (Carpentier & Cerf, 1993). *Rhodotorula* spp. has the ability to produce biofilm (Nunes et al., 2013). The different species of *Rhodotorula* are living in the environment and considered as normal inhabitants in the human body. In addition, they are found as common contaminant of skin, urine, respiratory and gastrointestinal systems among normal individuals (Zahra et al., 2015). Among the species of *Rhodotorula*, the *R. mucilaginosa*, *R. glutinis*, and *R. minuta* are considered as human pathogens. These species causes invasive Rhodotorulosis among immunocompromised patients (Fleet & Balia, 2006). Furthermore, *R. mucilaginosa* are able to form biofilm by adhering to surfaces (Ganttle et al., 2011).

Lactic acid bacteria are reported to help in the maintenance of human health by producing various compounds such as organic acid, hydrogen peroxide, diacetyl, bacteriocins and biosurfactants which inhibit the growth of pathogenic bacteria (Pascual et al., 2008). LAB isolates and LAB-CFS is well known for their antibacterial activity (Ammor et al., 2006; Awaisheh & Ibrahim, 2009), fungi (Cortés-

Zavaleta et al., 2014) and yeast (Yu & Yunlu, 2015). Additionally, LABs are also well documented to have anti-adhesion activity (Gudina et al., 2010). Recently, Bulgasem et al. (2015) documented that LAB cell free supernatant isolated from various sources of honey sample have the anti-adhesion activity against biofilm produced by several types of *Candida* species.

Lactic acid bacteria are also capable of interfering with the pathogens adhesion on epithelial cells of urogenital and intestinal tract (Otero & Nader-Macias, 2007). Supernatants produced by LAB contain compounds which can reduce adhesion of pathogenic micro-organisms to glass (Velraeds et al., 1996), silicone rubber (Busscher et al., 1997), surgical implants (Tamang et al., 2005) and voice prosthesis (Rodrigues et al., 2004). Many LABs are known to inhibit the growth of yeast species by different ways such as competition for adhesion sites or production of different antagonistic metabolites which inhibit the growth (Ronnqvist, 2007). However, limited studies are reported on the anti-adhesion properties of LAB cell free supernatant against biofilm produced by *R. mucilaginosa*. Therefore, this study aimed to determine the anti-adhesion stability of cell free supernatant produced by LAB against biofilm produced by *R. mucilaginosa* CBR.

5.2 MATERIALS AND METHODS

5.2.1 Cultures preparation

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 strain *R. mucilaginosa* CBR previously isolated from cucumber as mentioned in section 3.2.1, was grown on Potato Dextrose Agar, PDA (Oxoid) plates incubated at 30°C for 48 h and were then stored at 4°C. Prior to analysis, LAB were subcultured in MRS broth (Oxoid) incubated at 30°C for 48 h, while *R. mucilaginosa* was subcultured in potato dextrose broth (Difco) incubated at 30°C for 24 h.

5.2.2 Cell free supernatant preparation

The LABs isolates were inoculated into MRS broth (Oxoid) and incubated at 37°C for 48 h anaerobically. The LAB cell free supernatant from *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 were prepared by centrifuging the broth at 10,000rpm, 4°C for 20 min using Centrifuge Combi 514R (Hanil Science Industrial). Then the supernatant of each isolates was filtrated using sterile filter (0.45 µm-pore-size filter, Millipore).

5.2.3 Determination of the anti-adhesion activity of LAB supernatants

The anti-adhesion activity of the LAB CFS against *R. mucilaginosa* CBR was done by using pre-coating and co-incubation methods. The pre-coating experiment was carried out as described by Gudiña et al. (2010). A 96-wells microtiter plates were coated with different CFS. A 200 µL of CFS was pipetted into the wells and the microtiter plates were incubated at 37°C for 24 h. The CFS was then removed and the plates washed twice with 100 µL of phosphate buffer saline (PBS) pH 7.2 to remove non-adhering supernatant. After that, 150 µL of 24 h culture *R. mucilaginosa* CBR suspension (1.5×10^7 CFU/mL) cultured in PDB (Difco) was added to each well then

the microtiter plate was again incubated at 30°C for 24 h. Non-adhering cells were removed by gently washing twice the wells with phosphate buffer saline (PBS) pH 7.2.

Quantification was done using the crystal violet assay (Peeters et al., 2008; Fracchia et al., 2010). The biofilm was fixed for 15 min by adding 100 µL of 99% methanol to each well and the plate was air dried. After that, 100 µL of crystal violet (2% w/v) was added and held for 20 min then the excess crystal violet was removed by pipette and, residue in the wells was washed with tap water. The stain bound to the adherent fungi was solubilized with 100 µL of 33% glacial acetic acid per well and the optical density readings of each well was measured at 595 nm using Elisa reader (Epoch). *R. mucilaginosa* suspension without CFS was prepared as control. The anti-adhesion activity was calculated as follows:

$$(\%) \text{ Anti-adhesion Activity} = [1 - (A_C / A_0)] \times 100$$

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

However, in co-incubation experiment, suspension of 24 h culture *R. mucilaginosa* in PDB (1.5×10^7 CFU/ mL) were added to each well together with different LAB-CFS (100 µL supernatant: 100 µL *R. mucilaginosa* culture), and incubated at 30°C for 24 h. Determination of percentage adhesion was carried following the method described above. Then, the CFS was removed and the plates washed twice with 100 µL of phosphate buffer saline (PBS) pH 7.2 to remove non- adhering supernatant. After that, 150 µL of each 24h culture *R. mucilaginosa* CBR suspension (1.5×10^7 CFU/mL) cultured in PDB were added to each well, then the microtiter plate was again

incubated at 30°C for 24 h. Non-adhering cells were removed by gently washing twice the wells with PBS pH 7.2. Quantification was done using the crystal violet assay (Peeters et al., 2008; Fracchia et al., 2010). The biofilm was fixed for 15 min by adding 100 µL of 99% methanol to each well and the plate was air dried. After that, 100 µL of crystal violet 2% was added and held for 20 min then the excess crystal violet was removed by pipette and, residue in the wells was washed with tap water. The stain bound to the adherent fungi was solubilized with 100 µL of 33% glacial acetic acid per well and the optical density readings of each well was measured at 595 nm using Elisa reader (Epoch). *R. mucilaginosa* suspension without CFS was prepared as control. The analysis was carried out in duplicates and the mean was taken. The anti-adhesion activity was-calculated as follows;

$$(\%) \text{ Anti-adhesion Activity} = [1 - (A_C / A_0)] \times 100$$

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

5.2.4 Heating effect on LAB-CFS anti-adhesion activity

The CFS of LAB isolates was heat treated at 90 and 121°C for 30 min, and then the samples were cooled in ice water. The CFS was then tested against *R. mucilaginosa* CBR biofilm by using pre-coating experiment method as described in section 5.2.3. Cell free supernatants of LABs without treatment were used as control.

5.2.5 PH adjustments on LAB-CFS anti-adhesion activity

The pH of LAB-CFS was adjusted to different pH values of 3, 5, 6, and 7 using 0.1 N HCL or 0.1 NaOH and read by pH meter (Mettler Toledo). The CFSs were then tested against *R. mucilaginosa* biofilm using pre-coating experiment as described in 5.2.3. Cell free supernatants of LABs without treatment were used as control.

5.2.6 Effect of proteolytic enzyme on LAB-CFS anti-adhesion activity

The effect of proteolytic enzymes on anti-adhesive activity of LAB-CFS was investigated following the method of Gudina et al. (2001). The following enzymes were used: proteinase K (Sigma), papain, and pepsin (Sigma). The initial pH of the CFSs were 3.85, 3.83, 3.77 for isolates *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16, respectively. The pH of the CFSs were 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 adjustments, 1 mL of the supernatants were treated with 100 μ L of each enzyme and incubated at 37 °C for 1 h. The reaction was stopped by heating the mixture at 65°C for 30 min. Then the CFSs were tested against *R. mucilaginosa* biofilm using pre-coating method as described in section 5.2.3. Cell free supernatants of LABs without treatment were used as control.

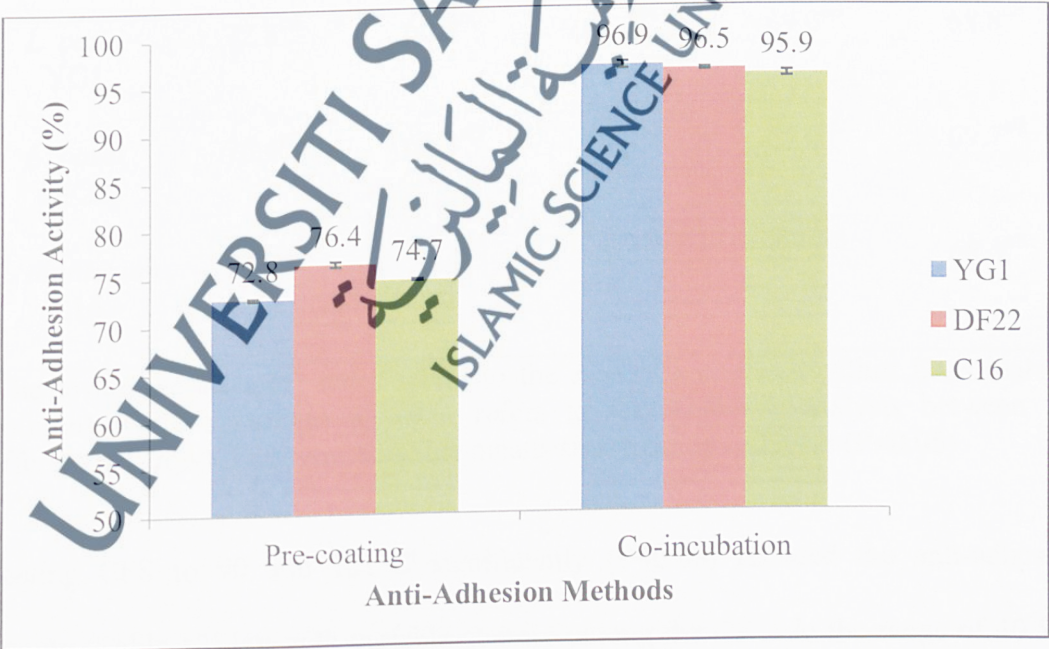
5.2.7 Statistical Analysis

Data were analysed using Minitab 16. ANOVA analysis was carried out to determine the level of significance of the anti-adhesion activity at $P < 0.05$.

5.3 RESULTS

Results of the ability of the cell free supernatant of LAB to produces anti-adhesion properties against biofilm formation *R. mucilaginosa* CBR with variable anti-adhesion activity is shown in Figure 8. Anti-adhesion activity for pre-coating method were significantly lower ($P<0.05$) than those of co-incubation method. Pre-coating of the polystyrene wells with CFS of *L. casei* DF22 showed significantly ($P<0.05$) higher (76.4%) anti-adhesion activity against *R. mucilaginosa* CBR than CFS *L. casei* YG1 (72.8%) and *L. plantarum* C16 (74.7%) respectively. Higher anti-adhesion activity on the yeast was observed by co-incubation assay for all CFS with values of more than 95%.

FIGURE 8: Anti-adhesion activity of LAB cell free supernatants against *R. mucilaginosa* CBR evaluated by pre-coating and co-incubation method



Pre-coating the wells of titer plates with CFS (pH 3.79 to 3.83) showed anti-adhesion activity of 72.8, 74.7, and 76.4 % for *L. casei* YG1, *L. plantarum* C16 and *L. casei* DF22, respectively. Adjusting the pH of CFS to pH 3, 5, 6, and 7 significantly ($P<0.05$) reduced the anti-adhesion activity (Table 12) but with variable activity among the CFSs in the range of 50.4 to 69.7 %. The anti-adhesion activity was significantly ($P<0.05$) reduced to 50.4% for *L. plantarum* C16-CFS at pH 3 compared to other pHs. Similarly, reduced activity of 58.95 % was observed for *L. casei* DF22 at pH 5. However, no significant difference ($P>0.05$) for *L. casei* YG1-CFs to reduce anti-adhesion activity was observed at pH 3 and 5 as well as at pH 6 and pH 7.

Table 12: Percentages anti-adhesion activity of LAB-CFS against *K.mucilaginos* CBR at different pH

LAB-CFS	Anti-adhesion (%)				
	Control	pH 3	pH 5	pH 6	pH 7
<i>L. casei</i> YG1	72.8 ^{cA}	60.0 ^{bC}	61.1 ^{bC}	69.0 ^{aB}	68.8 ^{aB}
<i>L. casei</i> DF22	76.4 ^{aA}	63.3 ^{aD}	59.0 ^{bE}	67.0 ^{aC}	69.7 ^{aB}
<i>L. plantarum</i> C16	74.7 ^{bA}	50.4 ^{cC}	69.6 ^{aB}	69.4 ^{aB}	68.0 ^{aB}

*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 CFS to 90 and 121°C significantly ($P<0.05$) reduced the anti-adhesion activity (Table 13) but with variable activity among the CFSs in the range of 40.5 to 66.4%. The anti-adhesion activity was significantly reduced to 40.5% for *L. casei* YG1-CFS at 90°C. Similarly, reduced activity of 54.6% was observed for *L. casei*

YG1-CFS when heated to 121°C. However, there was no significant difference (P>0.05) was observed for anti-adhesion activity for *L. casei* DF22 and *L. plantarum* YG1 when the supernatant was heated at 90 and 121°C. The anti-adhesion activity of *L. plantarum* C16-CFS was heat stable but with reduced activity when heated at 90 and 121°C.

Table 13: Percentages anti-adhesion activity of LAB-CFS against *R.mucilaginosa* CBR at 90 and 121°C

LAB-CFS	Anti-adhesion (%)		
	Control	90 °C	121 °C
<i>L. casei</i> YG1	72.8 ^{aA}	40.5 ^{bC}	54.6 ^{bB}
<i>L. casei</i> DF22	76.4 ^{bA}	61.3 ^{aC}	63.3 ^{aB}
<i>L. plantarum</i> C16	74.7 ^{cA}	66.4 ^{ab}	63.0 ^{aB}

*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)

Treating the LAB-CFS with pepsin, papain and proteinase K significantly reduced (P<0.05) the anti-adhesion activity (Table 14) but with variable activity in the range of 26.2 to 60.6%. However, treatment with papain, pepsin and proteinase K significantly reduced (P<0.05) the anti-adhesion activity of *L. casei* YG1-CFS and *L. casei* DF22-CFS. However, the reduction in anti-adhesion activity varies with the enzyme used. Treatment with papain reduced CFS of *L. casei* YG1-CFS and *L. casei* DF22-CFS from 72.8 to 26.2% and 76.4 to 39.0%, treatment with pepsin significantly (P<0.05) reduced the anti-adhesion activity of from 72.8 to 58.4% and 76.4 to 57.6%,

respectively and treatment with proteinase K significantly ($P<0.05$) reduced the anti-adhesion activity from 72.8 to 60.6% and 76.4 to 57.9%, respectively. Although there was a reduction in anti-adhesion activity, there was no significant difference ($P>0.05$) in the activity of pepsin and proteinase k treated CFS of *L. casei* YG1-CFS and *L. casei* DF22-CFS. It is possible that the two isolates are of similar identity. The anti-adhesion activity of *L. plantarum* C16-CFS was significantly reduced ($P<0.05$) from 74.7 to 43.3% when treated with proteinase K, but not significantly different ($P>0.05$) when treated with pepsin and papain; the anti-adhesion activity was reduced to 47.4% and 48.2%, respectively.

Table 14: Percentages anti-adhesion activity of LAB-CFS after treated with enzyme against *R. mucilaginosa* CBR

LAB-CFS	Anti-adhesion (%)			
	Control	Pepsin	Papain	Proteinase K
<i>L. casei</i> YG1	72.8 ^{aA}	58.4 ^{aB}	26.2 ^{cC}	60.6 ^{aB}
<i>L. casei</i> DF22	76.4 ^{bA}	57.6 ^{aB}	39.0 ^{bC}	57.9 ^{aB}
<i>L. plantarum</i> C16	74.7 ^{aA}	47.4 ^{bB}	48.2 ^{aB}	43.3 ^{bC}

*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 ($P<0.05$)

5.4 DISCUSSION

Biofilms produced by microorganism have quality issues in food industry. Formation of biofilms on critical locations in processing plants of the food and beverage industries resulted from inadequate cleaning, disinfection and sanitation. When this happens it can lead to a number of problems such as product spoilage, food safety problems, and loss of production efficiency (Srey et al., 2013). A number of ways were reported to prevent or remove biofilms such as using chemical based control like sodium hypochlorite, hydrogen peroxide and peracetic acid (Chemat, et al., 2011; Oulahal-Lagsir et al., 2000). Ultra-sonication was reported as an efficient biofilm removal method and is a well known technique used in various food industry processes namely freezing, cutting, drying, tempering, bleaching, sterilization, and extraction (Piyasena et al., 2003). Recent study demonstrated that gemini surfactant successfully inhibited fungal cell adhesion to polystyrene and silicone surface. The surfactant reduced *C. albicans* filamentation and eradication of *C. albicans* and *R. mucilaginosa* biofilms (Oblak et al., 2015). Bio-surfactant has the ability to remove biofilm formation by bacteria (*E. coli*, *S. aureus*, *S. epidermidis*, and *S. agalactiae*) and *C. albicans* (Gudina et al., 2010). Some lactic acid bacteria have the ability to produce bio-surfactant that can remove biofilm produced by bacteria and *C. albicans* (Gudina et al., 2010; Salman & Alimer, 2014).

This study observed that the supernatants of *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 have good anti-adhesion activity against biofilm produced by *R. mucilaginosa* CBR as evaluated by pre-coating and co-incubation experiments. The anti-adhesion activity was observed more than 72.0% against *R. mucilaginosa* CBR

when the micro-titer plates were pre-coated with CFS of the entire LAB evaluated. In contrast when the CFS was evaluated using co-incubation study, the anti-adhesion activity was observed more than 95%. Similarly, Fracchia et al. (2010) reported anti-adhesion activity *L. brevis* CV8LAC against two *C. albicans* in pre-coating and co-incubation experiments and indicated that bio-surfactant was produced by this LAB. In co-incubation assay, the supernatant is in direct contact with the target organisms and inhibited or reduced the ability of the live cell to attach to the surface to form biofilm. Lee and Puong (2002) proposed that the degree of adherence of two competitor bacteria depends on the affinity of adhesive molecules expressed on the surface of bacteria to adhesion receptor present on the surface of host cells that they are competing for. In addition, the adsorption of a bio-surfactant on the surface was found to change its hydrophobicity, which might cause interference in the adhesion and desorption processes (Fischer, 1996).

The anti-adhesion activity of LAB against yeast and bacteria varies with the LAB and target organisms evaluated. Gomaa (2013) reported that CFS *L. fermentum* showed the highest anti-adhesion activity (84.69%) against *C. albicans* ATCC70014. Brozowski et al. (2011) reported that *L. fermenti* 126 and *L. rhamnosus* CCM1825 produced bio-surfactant that inhibit the cell adhesion of *E. coli*, *K. pneumonia* and *P. aeruginosa* while Salman and Alimer (2014) reported that biofilm formation by *S. aureus*, *E. coli* and *K. pneumonia* using titer plates assay could be reduced by the bio-surfactant produced by *L. rhamnosus*. Similarly, Madhu and Prapulla (2014) reported that bio-surfactant from *L. plantarum* showed anti-adhesive property against *E. coli* ATCC 31705, *E. coli* MTCC 108, *S. typhi*, *Y. enterocolitica* MTCC 859, and *S.*

aureus F 722. However, *L. paracasei* ssp. *paracasei* A20 showed lower anti-adhesion activity of 29.5% against biofilm produced by yeast *C. albicans* (Gudina et al., 2010).

The anti-adhesion activity of CFS from *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 were stable after heating at 121°C for 30 min with anti-adhesion activity of 54.58, 63.28 and 63.01% respectively. Adjusting the pH CFS to stable pH 3, 5, 6 and 7 resulted in the reduction of anti-adhesion activity (Table 11). Similarly, Gudina et al. (2010) reported that anti-adhesion activity of bio-surfactant produced by a *L. paracasei* strain isolated from Portuguese dairy was stable at different pH values, being more effective at pH 7. Similar finding was reported by Bulgasem et al. (2015) on LAB isolated from different honey samples on the anti-adhesion activity against five *Candida* spp. This study found that the anti-adhesion activity of CFS of *L. curvatus* HH isolated from honey was also heat stable at 121°C and significantly reduced the biofilms formation of *C. albicans* and *C. glabrata*. However *L. curvatus* HH were not stable under acidic condition (Bulgasem et al., 2015).

Cell free supernatants were treated with proteolytic enzymes to determine their sensitivity to the enzymes and the possibility of generating compounds with better anti-adhesion activity. It was observed that the anti-adhesion activity of CFS of the isolated LABs reduced after enzyme treatment (Table 13). Shokouhfard et al. (2015) suggested that bio-surfactant from *L. acidophilus* that have high protein compound showed anti-adhesion against *S. marcescens*. Velraeds et al. (2014) demonstrated that bio-surfactants from *L. acidophilus* RC14 and *L. fermentum* B54 were richer in protein and also had less polysaccharide than bio-surfactants from *L. casei* subsp. *ramnosus* ATCC 7469.

Thus, the anti-adhesion activity of CFS of LAB against *R. mucilaginosa* CBR may be due to the presence of bio-surfactant that strongly prevented the adherence of the biofilm formation by the yeast. Several researcher reported that bio-surfactant produced by LAB have anti-adhesion properties that can inhibit the adherence of biofilm to the surfaces (Fracchia et al., 2010; Gomaa, 2013). Rodrigues et al. (2006) postulated that BS reduces hydrophobic interactions and consequently microbial adhesion. Hydrophobic surfaces have shown to be particularly colonized by microorganisms, probably because these surfaces facilitate the close approach between microorganism and solid substratum, favouring the elimination of interfacial water present in the interacting surfaces. Consequently, when a surface is conditioned with bio-surfactant, it becomes more hydrophilic, with an expected decrease of microbial attachment (Rodrigues et al., 2006).

However, anti-adhesion of CFS of LAB against biofilm *R. mucilaginosa* have not been well documented. The anti-adhesion activity of LAB may be due to the presence of bio-surfactant properties in the cell-free supernatant. The ability of bio-surfactant to decrease pathogenic microorganisms attachment was observed by many researchers (Fracchia et al., 2010; Gudina et al., 2010; Gudina et al., 2010b; Ali, 2012; Zakaria, 2013; Abedi et al., 2013). The effect of CFS as anti-adhesion depends on the properties of the supernatant, microorganism tested and surface properties. When the surface is conditioned with supernatant containing bio-surfactant it becomes more hydrophilic and consequently, decrease microbial attachment (Zeraik & Nitschke, 2010). LAB strains that produce bio-surfactants can reduce microbial adhesion and combating colonization by pathogenic microorganisms not only in the biomedical field, but also in food industry (Rodrigues et al., 2006; Nitschke & Costa, 2007; Singh

et al., 2007). The current results indicated that CFS of *L. casei* YG1, *L. casei* DF22 and *L. plantarum* C16 had a significant anti-adhesion activity against biofilm produced by *R. mucilaginosa* CBR. The result is supported with studies of Rodrigues et al. (2006) and Falagas and Makris (2009), who reported that bio-surfactant isolated from *Lactobacillus* play an important role in care equipment such as catheters and other medical devices in hospitals.

5.5 CONCLUSION

This study showed that cell free supernatant produced by LAB has anti-adhesion activity against *R. mucilaginosa*. However, the activity of LAB-CFS significantly affected when treated by pH, heat and enzyme. The compound present in CFS is may be due to the bio-surfactant which have the anti-adhesion properties and can be used in food industry to avoid biofilm produced by *R. mucilaginosa*.