

CHAPTER II

LITERATURE REVIEW

2.1 Spoilage yeast

Yeasts are unicellular fungi that have the ability to reproduce asexually essentially by budding. They are classified as heterogenic organisms because they differ from each other in terms of metabolic activity as well as structural and cultural properties. Yeasts are abundant in environments such as soil, plant, water, animals and insects. It is noteworthy to state that yeasts are also present in a wide range of fresh and processed foods and other industrial raw materials and products. Yeasts are well known for their beneficial role in the food industry such as in the production of fermented products and in the production of bread (Pitt & Hocking, 1997).

However, excessive growth of undesirable yeasts in food processing can result to complications in quality and safety with significant economic losses. Yeasts can multiply rapidly in most foods comprising of dairy products such as cheese, butter, cream and yoghurt; meat products such as sausages; sugar, syrups, honey, berries and fruit products, vegetables including pickled cabbage and pickled cucumbers; juices, soft drinks, alcoholic beverages and wines, salad dressings, mayonnaise, confectioneries, jams and jellies as well as bread (De'ak, 1991; Pitt & Hocking, 1997; Loureiro & Querol, 1999). Water activity, nutrients, and acidity are some intrinsic factors that may enhance yeast spoilage of a product (De'ak, 1991). Some of the

attributes of yeast spoilage are off-flavour (souring), production of gas (discolouration and swelling of containers) and textural changes (sediment formation and surface growth) (Loureiro & Querol, 1999).

Over 200 species of yeasts have been isolated and identified in food products (De'ak, 1991; Kurtzman & Fell, 1998; Barnett et al., 2000). However, Pitt and Hocking (1997) reported that only *Brettanomyces bruxellensis*, *Candida krusei*, *C. parapsilosis*, *Debaryomyces hansenii*, *Kloeckera apiculata*, *Pichia membranaefaciens*, *R. mucilaginosa*, *R. glutinis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Torulopsis holmii*, *Zygosaccharomyces bailii*, *Z. bisporus* and *Zygosaccharomyces rouxii* are known to act as spoilage agents when food processing is not done in accordance with good manufacturing practices. Some of these spoilage species are also known to be opportunistic pathogens which can cause several infections in vulnerable people. Usually, foods are not known to transfer yeast infections although there are some few documented evidence of gastroenteritis or allergic reactions in which the causative agent were supposed to be yeasts (Loureiro & Querol, 1999).

Spoilage of foods and beverages set in when the food products are no longer acceptable by consumer due to unpleasant odour, appearance, taste, texture, or combinations of these defects. In the handbooks of yeast taxonomy, the characteristics of 761 species of yeast were extensively described and a quarter of these yeasts can be isolated from food products (Boekhout et al., 2002). However, very few are known to play significant roles in food spoilage. Fifty years ago, there were no reports about food-borne yeast causing significant public health panic (Ingram, 1958; Walker & Ayres, 1970). However in 1980s, a report was cited referring to yeast as the main

causative agent in the development of allergies which have a negative effect in humans.

Low populations of basidiomycetous yeasts (*Cryptococcus*, *Rhodotorula*, and *Sporobolomyces* spp.) are reported to be predominant on the surfaces of fresh vegetables (Edgar & Aidoo 2001). However, most spoilage in vegetables is as a result of bacterial spoilage rather than spoilage by yeast and this may be due to the proteinaceous nature and neutral pH (Carlin, 2000). Yeast spoilage is more often related with fermented vegetables, such as the pink discolouration of sauerkraut by *Rhodotorula* species, the softening and bloating of olives and pickles by species of *Saccharomyces*, *Debaryomyces*, *Pichia*, *Candida*, and *Rhodotorula* (Fleet, 1992). Ready-to-eat vegetable salads (coleslaw, potato salad and mixed salads) mixed with vinegar-based mayonnaise or salad dressings are most prone to yeast spoilage during refrigerated storage because of their decreased pH (Fleet, 1992; Fleet, 2006; Kurtzman & James, 2006). Yeasts such as *Pichia*, *Saccharomyces*, *Rhodotorula*, *Kluyveromyces* and *Geotrichum* can survive at low pH, lower water content and even at low temperature conditions. These features make them liable to spoilage especially when the food products are preserved with weak acids, hyperosmotic condition or long-term frozen (Fleet, 2011).

2.2 *Rhodotorula* species

Rhodotorula is a widespread environmental yeast that is present in air, soil, lakes, ocean, milk, and fruit juice. It is classified under the phylum basidiomycota and has the ability to inhabit plants, humans, and other mammals. The genus *Rhodotorula*

consists of eight species, out of which *R. mucilaginosa*, *R. glutinis*, and *R. minuta* are recognized as disease causing yeast in humans (Larone, 1995). *Rhodotorula* produces pink to red colonies with blastoconidia that are unicellular lacking pseudohyphae and hyphae. *Rhodotorula* spp. was reported as evolving yeast pathogens in humans in the last two decades. They are abundant saprophytic yeasts that can be recovered from many environmental sources. This yeast has been observed to have a strong attraction for plastic, having been isolated from various medical equipments, such as dialysis equipment, fibre-optic bronchoscopes, and other environmental sources, including shower curtains, bathtubs, and toothbrushes (Kiehn et al., 1992; Hagen et al., 1995; Pfaller & Diekema, 2004).

Several authors reported the isolation of this yeast from different ecosystems, including sites with adverse conditions, such as the depths of the Baltic Sea, the high-altitude Lake Patagonia (Libkind, 2004), the soil and vegetation of Antarctica (Pavlova et al., 2001), aquatic, hypersaline (Ekendahl et al., 2003), and high-temperature environments such as the Dead Sea (Israel), Lake Enriquillo (Dominican Republic), the Great Salt Lake (USA) (Butinar et al., 2005), and beaches located in northern Brazil (Hagler et al., 1981). These authors reported that, *R. mucilaginosa* was the third most isolated yeast in seawater.

R. mucilaginosa is mostly isolated from foods and beverages. Numerous studies have described the presence of *R. mucilaginosa* in peanuts, apple cider, cherries (Venturini et al., 2002), fresh fruits, fruit juice (Las Heras-Vazquez et al., 2003; Tournas et al., 2006), cheese, sausages (Gardini et al., 2001), edible molluscs (Kajikazawa et al., 2007), and crustaceans (Eklund et al., 1965; Senses-Ergul et al., 2006). Although the

consumption of food contaminated with yeast may not have a direct role in causing opportunistic infections, there is growing concern that food may be an underestimated source of environmental pathogens (Fleet, 2003). The species of *R. glutinis* and *R. minuta* are less frequently isolated in natural environments compared to *R. mucilaginosa*. These species have been discovered in air, seawater (including deep environments), freshwater, and goat's milk (Libkind et al., 2003; Nagahama et al., 2006; Callon et al., 2007).

Environmental studies have documented the presence of *Rhodotorula* sp. in tropical fruits, sugar cane, and shrimp in the waters of Sepetiba Bay in Brazil (de Azeredo et al., 1998; Trindade et al., 2002; Pagnocca et al., 2010). In addition, environmental observation of yeasts in specific areas of hospitals showed the presence of *Rhodotorula* species in a substantial amount of air samples (Cordeiro et al., 2010). As a direct consequence of the wide exposure to *Rhodotorula* in the hospital environment, patients who have a weakened immune system can develop Rhodotorulosis, causing a variety of systemic infections. In fact, *Rhodotorula* spp. is the most common microorganism isolated from the hands of hospital employees and patients (Strausbaugh et al., 1996).

Further studies are required to explain the role of food contamination by *Rhodotorula* and the development of opportunistic fungal infections. These future studies should lay emphasis on the survival and growth of *Rhodotorula* in the gastrointestinal system and its potential ability to transfer from the gastrointestinal tract to bloodstream by understanding the ecology of *Rhodotorula* in hospitals and healthcare environments (Fleet & Balia, 2006). In this aspect, *Rhodotorula* spp. have been isolated from stool

samples, indicating that these yeasts can survive in the extreme conditions of the gastrointestinal tract, and it is still uncertain whether *Rhodotorula* is capable of passing from the gastrointestinal tract into the bloodstream (Van Uden, 1958; Silva et al., 2004). Thus, *R. mucilaginosa* from food system can lead to health problem.

Among the few references about the pathogenicity of *Rhodotorula* spp. in animals, there are several reports of an outbreak of skin infections in chickens and a report of a lung infection in sheep, both caused by *R. mucilaginosa* (Aruo, 1980; Monga et al., 1980). *Rhodotorula* was reported as the causative agent of epididymitis, skin lesions in a sea lion, and dermatitis in a cat that had crusted lesions and mastitis (Bourdeau et al., 1992; Costa et al., 1993; Kadota et al., 1995; Alvarez-Perez et al., 2010). Wirth and Goldani (2012b) conducted the first experimental study in an animal model of disseminated *Rhodotorula* infection described in the literature. Some authors have reported the *Rhodotorula* genus as a colonising agent in the oropharynx and cloaca of ostriches, in faecal samples and the cloaca of wild birds and pigeons in urban and suburban areas, in the ear canals of adult cattle with parasitic otitis, in healthy rhesus monkeys, genital tract of healthy female camels, and in healthy cats (Amaral et al., 1998; Melville et al., 2004; Brotto et al., 2005; Costa et al., 2010; Lord et al., 2010; Shokri et al., 2010). These studies indicate the pathogenicity of *Rhodotorula* is not only affected human being but animals as well.

Rhodotorula spp. is also capable of producing biofilm. Different species of *Rhodotorula* are abundant in the environment and are known to be normal inhabitants of the human body. Furthermore, they are recognized as common contaminant of skin, urine, respiratory and gastrointestinal systems in average humans (Zahra et al., 2015).

Among the species of *Rhodotorula*, the *R. mucilaginosa*, *R. glutinis*, and *R. minuta* have been reported as human pathogens. These species causes invasive Rhodotorulosis among immune-compromised patients (Fleet & Balia, 2006). Moreover, out of these three species reported as human pathogens, *R. mucilaginosa* are also able to form biofilm by adhering to surfaces (Ganttle et al., 2011).

2.3 Yeast identification

Yeast can be identified either phenotypically or genotypically. Currently, the API 20C AUX were used for phenotypic identification. The API 20C AUX (bioMérieux) contains 19 carbon assimilation tests incubated at 30°C and observe manually for turbidity. Results were obtained after 48-72h. The original API 20C introduced in 1975 had a combination of fermentation and assimilation tests in micro tubes. The microtubes were difficult to fill without trapping air bubbles. The fermentation tests required *Vaspar* seals and data analysis was tedious (Bowman & Ahearn, 1976). Fermentation and actidione tests were removed and replaced with all assimilation tests in an easier cupule format to facilitate inoculation. A computer assisted numerical approach was also introduced for rapid data analysis of octal-coded profile numbers generated from reaction patterns. Numerous evaluations of the API 20C compared to conventional methods showed correct performance ranged from 93.0% to 100.0% when used in conjunction with recommended supplemental tests such as microscopic morphology (Gündes et al., 2001).

Identification of yeast genotypically can be done by PCR-based assays which are rDNAs. Interestingly, rDNA subunits contain highly conserved domains separated by more variable domains that display species-specific sequences, making them a target of choice to design universal PCR primers and species- or genus-specific primers. The ribosomal rDNA region consists of genes encoding for small (18S), 5.8S and large (26S) subunits of ribosomal DNA separated by the internal transcribed spacers named ITS1 and ITS2 (Reiss et al., 1998; Lott et al., 1993). A multiple copy target is expected to lead to a more sensitive PCR-based assay than PCR with single-copy targets. Additionally, ribosomal DNAs are the most frequent fungal DNA sequences available in the public databases, making it easier for sequence homology searches. Several universal fungal primers representing highly conserved regions have been designed either in the 18S rDNA subunit, 5.8S rDNA subunit, or in the 26S rDNA region. These primers allow the amplification of 18S, 5.8S, 26S rDNA as well as ITS1 and ITS2 regions (White et al., 1990).

2.4 Lactic acid bacteria

Lactic acid bacteria (LABs) are Gram-positive, non-sporulating, cocci and rod, catalase-negative and facultative anaerobic bacteria with a fermentative metabolism. Most of the bacteria in the LABs group are non-pathogenic, acid resistant, bile tolerant and produce antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins (Dunne et al., 1999). However, there are some few types of LABs which have been categorised as pathogenic. Examples include *S. pyogenes*, *S. agalactiae*, *S. pneumonia* and *S. mutans* which causes dental caries. LABs have been widely used to improve the aroma and texture of fermented foods. It has also been

used in prolonging the shelf life of dairy and meat products by inhibiting the growth of spoilage microorganism.

LABs are also used commonly as starter culture for the production of fermented foods and beverages in Asia, particularly in Malaysia. The antimicrobial compounds produced by LAB enhance the immune system of human by inhibiting the growth of pathogens in the intestinal tract (Chelule et al., 2010). LABs can be isolated from different sources using selective media such as Man Rogosa and Sharpe (MRS agar) and/or LM broth. These two media are mostly used in detection and enumeration of LABs in fruit juice, dairy products and various other foodstuffs.

LABs are divided into two groups depending on their fermentation pathway. The first group are the homo-fermentative bacteria such as *Lactococcus* and *Streptococcus* which produce 95% of lactic acid, two lactates and two ATP from one glucose molecule. The second group are the hetero-fermenters such as *Leuconostoc* and *Weissella* produce one lactate, ethanol, CO₂ and one ATP out of one glucose molecule. Homo-fermentative strains of LAB usually degrade hexoses via the glycolytic pathway utilizing maltose and fructose after glucose degradation and this leads to lactate production. On the other hand, hetero-fermenters use the 6-phosphogluconate pathway to degrade carbohydrate to produce a range of metabolites such as ethanol, acetate and CO₂ in addition to lactic acid (Ganzle et al., 2007; Khalid, 2011).

Strains of LABs include *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Aerococcus* and *Pediococcus* spp. Their natural habitats are the intestine and the

reproductive tract. Out of the 16 genera of LABs, only 12 are active in food processing. Majority of LABs are mesophilic (grows at temperature ranging between 10-15°C). However, some are thermophilic which grows at temperature ranging between 40-45°C. The optimum pH of LABs growth ranges between 4.0-4.5. Some strains can withstand pH above 9.0 or as low as 3.2. Purines, pyrimidine, amino acids and vitamin B are essential for LAB growth. LABs can only produce ATP from fermentation of carbohydrates because they lack the ability to synthesize cytochromes and porphyrins (Bamforth, 2005; Khalid, 2011). LABs do not produce amino acid and hence they depend on the available amino acid present in the growth media. Amino acids need of LAB strains differ and hence, LABs have developed adaptation to rich amino acid environments through enzyme systems that can exploit the nitrogen sources present in the environment (Axelsson, 2004).

The classifications of LABs have remained controversial. However, the classification described by Orla-Jensen (1919) is still in use till this present day. Various criteria that can be used to classify LABs which include the morphology, growth at different temperature, mode of glucose fermentation, range of sugar utilization, ability to grow at high salt concentrations and acid or alkaline tolerance.

2.5 Identification of lactic acid bacteria

The identification of LAB by biochemical methods has been well studied previously (Ahmed & Kanwal, 2004; Parvathy & Puthuvallil, 2005; Isabel et al., 2000). API 50 CHL kit (BioMérieux, France) is widely used for identification of lactic acid bacteria. This method is simple, and involves the fermentation of different carbohydrates after

48 h of incubation (Conter et al., 2005). However, API test has problems such as the distinguishing between the changes of colour after the fermentation of the carbohydrate in the wells. Other issue is the number of the cell of LAB could be low and not sufficient to ferment the carbohydrate in each well (Muhialdin et al. 2011).

The molecular biology has developed very fast and this leaves a high impact on the microbiology world. Phenotypic characterization based on sugar fermentation may not always offer sufficient basis for the reliable identification of the LAB as observed by several researchers (Ashmaig et al., 2009; De Angelis et al., 2001; Muyanja et al., 2003; Nigatu, 2000). Genetic tools and techniques have been developed to identify LAB especially polymerase chain reaction (PCR) based methods such as 16S rDNA, 16S ribosomal RNA (rRNA). 16S rRNA gene sequencing has become prevalent microbiology and biotechnology as a rapid, bacterial identification accurate methods alternative to phenotypic, fingerprinting and restriction fragment length polymorphism (RFLP) in this method the restriction enzymes broke the DNA sample into pieces (digested) and the resulting restriction fragments are separated according to their lengths by gel electrophoresis (Holzapfel et al., 2001; Schleifer & Ludwig, 1995). The molecular identification is based on the similarity with other sequences within the data base in the gene bank.

2.6 Antifungal compounds produced by LAB

2.6.1 Organic acids

LABs produce organic acids as a result of carbohydrate fermentation. The main products of carbohydrate fermentation of LABs are lactic acid, acetic acid and other acids depending on the strain of LAB (El-Ziney, 1998). The acids diffuse through the membrane of the target organisms in their hydrophobic un-dissociated form and then reduce the cytoplasmic pH and thus stop the metabolic activities (Piard & Desmazeaud, 1991). Acid also helps in lowering the pH in the medium and inhibit the growth of other microorganism. Several fungi such as *Fusarium*, *Penicillium*, *Aspergillus* and *Monilia* were reported to be inhibited by a mixture of acetic, caproic, formic, propionic, butyric and n-valeric acids produced by *L. sanfrancisco* CB1 (Corsetti et al., 1998). The weak acids are more efficient at low pH conditions, and they are less active at neutral pH (Zhang et al., 2003).

Acetic acid is a well-known inhibitor due to its wide range of inhibitory activities against yeasts, molds and bacteria (Blom & Mortvedt, 1991). *Weissella cibaria* 861006 and *Weissella paramesenteroides* 860509 produced organic acids and delayed the growth of *P. oxalicum* 1092302 up to six days after *W. paramesenteroides* 860509 CFS was sprayed on the surface of grape fruit (Lan et al., 2012). Lactic acid and acetic acid can work synergistically to improve the antifungal activity at concentration of 78 mM and 31.2 mM respectively and at pH 3.4 (Leon Pelaez et al., 2012). Indeed, *L. amylovorus* DSM 19280 has been reported to produce a variety of organic acids detected by HPLC and found to be inhibitory to *Penicillium expansum*, *Penicillium*

roqueforti, *Aspergillus niger*, *Aspergillus fumigatus* and *Fusarium culmarum* (Lynch et al., 2014). Gerez et al. (2013) evaluated 91 LAB isolates for their antifungal activity and discovered that lactic and acetic acids were responsible for the antifungal activity. Lactic and acetic acid were reported to inhibit the growth of *Cladosporium* sp. YS1, *Neurospora* sp. YS3, and *P. crustosum* YS2; these acids were produced by *Lactobacillus citreum* and *Weissella* and added to rice cake as natural antifungal agents (Baek et al., 2013).

2.6.2 Carbon dioxide

Carbon dioxide is one of the end-products of hetero-fermenter LABs during the fermentation of carbohydrate (Gottschalk, 1986). Carbon dioxide operates according to a two-step approach. Firstly, it creates an anaerobic condition and replaces initial molecular oxygen in the products. Secondly, carbon dioxide has an antimicrobial activity which is important in the vegetable fermentation to prevent the growth of spoilage fungi (Lindgren & Dobrogosz, 1990). Common fruit spoilage organism such as *Botrytis*, *Rhizopus* and *Penicillium* are not inhibited by 10% carbon dioxide but concentrations between 20 and 50% which have strong antifungal activity (Clark & Takles, 1980; Bliksstad et al., 1981).

2.6.3 Hydrogen Peroxide

Most LAB species are able to produce hydrogen peroxide in the presence of oxygen (Kandler, 1983). Many researchers have widely studied hydrogen peroxide and its mode of action (Davidson et al., 1983). It is related to the strong oxidising effect on

the bacterial cell, and to the destruction of basic molecular structures of cellular proteins (Condon, 1987; Magnusson, 2003). The inhibitory activity of hydrogen peroxide was reported to be due to its strong oxidizing effect on bacterial cell. In addition, an anaerobic environment is needed for certain hydrogen peroxide-producing reaction which is an inhibitor factor for some microorganisms (Ouwehand & Versterlund, 2004). The enzyme lactoperoxidase may enhance the antimicrobial activity of hydrogen peroxide by the oxidation of thiocyanate by hydrogen peroxide and generating hypothyanite (OSCN^-) (Ouwehand & Versterlund, 2004).

2.6.4 Reuterin

Lactic acid bacteria of *L. reuteri* have been reported to produce reuterin (3-HPA) from the anaerobic fermentation of the mixture of glucose and glycerol (Axelsson et al., 1989). Reuterin is active against many kinds of microorganisms including Gram-positive and Gram-negative bacteria, yeast and fungi. Species of *Aspergillus* and *Fusarium* were inhibited by reuterin (Chung et al., 1989). Claisse & Lonvaud-Funel (2000) reported that reuterin is also produced from different LAB strains such as *L. brevis*, *L. buchneri* and *L. collinoides*. Reutericyclin is another low molecular weight antifungal compound produced by *L. reuteri*, it has a molecular weight of 349 Da, negatively charged, and highly hydrophobic (Holzapfel et al., 2001). The minimum inhibition concentration varied between 0.1 to 1 mg/L (Ganzle et al., 2000). The addition of glycerol enhanced the production of reuterin as well as their antifungal activity (Magnusson, 2003).

2.6.5 Phenyllactic acid

The early report about the production of phenyllactic acid by certain LAB and shows antifungal activity was reported by Lavermicocca et al. (2008). Phenyllactic acid isolated from LAB reported to have spectrum range to inhibit pathogen bacteria, spoilage bacteria and spoilage fungi (Strom et al., 2002; Magnusson 2003; Magnusson et al., 2003). Prema et al. (2008) reported that *L. plantarum* strain which have isolated from grass silage observed to have a broad spectrum of antifungal compound, that was due to the production of 3-phenyllactic acid which was strong inhibitor to the growth of food and feed-borne filamentous fungi in agar plate assay. Sourdough bread started with *L. plantanum* 21B that produce phenyllactic acid have inhibited the growth of the fungi *A. niger* for 7 days compared to bread started with a *L. brevis* that did not produce phenyllactic acid (Lavermicocca et al., 2000). Recently, phenyllactic acid reported to be produced by several LAB isolates and has high antifungal activity toward *A. niger* CH 101, *Penicillium* sp. CH 102, *F. graminearum* CH 103, *P. digitatum* INTA 2 and *Geotrichum citri-aurantii* INTA 1 (Gerez et al., 2009; Gerez et al., 2013).

2.6.6 Antifungal proteinaceous compounds

Several authors have reported that LAB have significant bioactive peptide producer and these peptides exhibited antifungal activity toward various spoilage and human pathogenic fungi (Strom et al., 2002; Magnusson et al., 2003; Coda et al., 2011; Gerez et al., 2013). The production of the antifungal peptides is due to two possible routes, the first mechanism of production is through their digestive enzymes. These enzymes

can break down the big proteins such as casein, α -lactalbumin, and lysozyme into small active and health promoting peptides (Christensen & Reineccius, 1995; Natteewan et al., 2010) these peptides composed of 3 to 20 amino acids. On the other hand, the cationic peptides are gene-encoded peptides composed of 12 to 50 amino acids (Touch et al., 2009). These peptides production is a result of secondary metabolites of LAB strains as part of their life cycle, these produced peptides are important for the growth of the bacteria and is part of their defence mechanisms against other microbes presence in the same environment.

Lactococcus lactis subsp. *Lactis* CHD-28.3 was studied for antifungal activity against several spoilage fungi and found to prevent the growth of such fungi in agar plate assay. The strain was isolated from cheddar cheese and produced proteinaceous compounds (Roy et al., 1996). Stiles et al. (1999) screened three strains of LAB for their antifungal activity toward *Fusarium* sp, the cell-free supernatants was neutralised and heated to eliminate the organic acids and hydrogen peroxidase but the activity was maintained and the author suggest that the activity was due to compounds other than organic acids.

A novel proteinaceous compound was isolated from *L. plantarum* VTTE 78076 and inhibited the growth of *F. avenaceum*, one of the identified compounds was cyclo (glycyl-L-leucyl) (Niku-Paavola et al., 1999). At the same year, Okkers et al. (1999) purified and characterised a protein-like peptide (pentocin TV 35b) from *L. pentosus* TV35b, which has inhibitory activity against *C. albicans*. Proteinaceous compounds produced by *L. coryniformis* subsp. *Coryniformis* strain Si3 were highly heat stable with an estimated molecular weight 3 KDa and broad spectrum (Magnusson &

Schnürer, 2001). Other kinds of antifungal dipeptides which are cyclic, cyclo (Phe-Pro) and cyclo (Phe-OH-Pro) were produced by *L. plantarum* Mi LAB 393 and *L. coryniformis* subsp. *Coryniformis* Si3 strains with broad spectrum antifungal inhibitory activity (Ström et al., 2002; Magnusson et al., 2003).

The cell free supernatant of *L. paracasei* subsp. *Paracasei* BN ATS 5w and BN ATS 7w antifungal strains was considerably inactivated by enzymes treatment and this may be due to protein nature of antifungal compounds (Gulahnadov et al., 2006). Five antifungal peptides were isolated from water-soluble extracts of sourdough fermented with *L. brevis* AM7 and *Phaseolus vulgaris* cv Pinto, they inhibited the growth of the spoilage fungi *P. roqueforti* DPPMAF1 and extended the shelf life of bread up to 21 days (Coda et al., 2008).

2.7 Anti-adhesion activity of lactic acid bacteria

LABs isolated from different sources are known to have anti-adhesion activity. Recently, Bulgasem et al. (2015) observed that LAB cell free supernatant isolated from various sources of honey sample have anti-adhesion activity against biofilm formed 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).

LAB-CFS are reported to have compounds which can decrease the 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 documented to inhibit the growth of yeast species by various ways which includes competition for adhesion sites or production of different antagonistic metabolites which inhibit the growth (Ronnqvist, 2007).

The ability of bio-surfactant to reduce pathogenic microorganisms attachment is well documented 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 is dependent on the properties of the supernatant, microorganism tested and surface properties. When the surface is acclimatized with supernatant containing bio-surfactant it becomes more hydrophilic and consequently, decrease microbial attachment (Zeraik & Nitshke, 2010). LAB strains that produce bio-surfactants can decrease microbial adhesion and reduces 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).

2.8 Bacteriocin producing lactic acid bacteria

In recent years, resistance of bacteria to antibiotic is considered as a menace due to the wide-range of use of conventional antibiotics in the treatment of human and animal diseases (Roy, 1997; Lipsitch et al., 2000; Yoneyama & Katsumata, 2006). As a result, several resistant strains have mutated and spread leading to complications and the restriction in the use of antibiotics as growth promoters. The discovery of novel antimicrobial agents has led to rise for use in medicine (Fisher et al., 2005; Kumar & Schweiser, 2005). It has also become imperative to regulate their unchecked use in food and feed products; one acceptable alternative is the use for bacterial peptides as

antimicrobial substances in place of antibiotics of human use. Among them are bacteriocins which are produced by lactic acid bacteria and have attracted growing attention because they are active in a nanomolar range and have no toxicity.

Bacteriocins are proteins or complexed proteins biologically active which possess antimicrobial action against other bacteria, particularly related species. They are produced by bacteria and are usually not called antibiotics in order to avoid misunderstanding and apprehension with therapeutic antibiotics, which can possibly cause allergic reactions in humans (Deraz et al., 2005). Bacteriocins are known to be different from most therapeutic antibiotics because of its proteinaceous nature that is can be quickly digested by proteases in the human alimentary canal. They are ribosomally synthesized peptides, and this produces the likelihood of improving their characteristics to increase their activity and range of action (Saavedra et al., 2004).

Small amount of bacteriocin in food or feed can destroy or prevent pathogenic bacteria that vie for the same ecological niche or nutrient pool. Hence it can be considered as beneficial to food and feed producers. This role is supported due to the fact that many bacteriocins have a thin host range, and it is possible to be most effective against associated bacteria with nutritive demands for the same rare resources (Deegan et al., 2006).

2.8.1 Range of activity

Considering the antimicrobial variation, producing organism, molecular weight, stability, physicochemical properties and mode of action of bacteriocins, they form a

diverse group. There are some types that are active only against homologous species, and others are active against a wide range of Gram-positive microorganisms. Nisin is a typical example of those bacteriocin that are active against a wide range of Gram-positive microorganisms and it is produced by certain strains of *L. lactis* subsp. *lactis* (De Vuyst, 1994; Rodriguez, 1996; Moreno et al., 2000). However, nisin produced by *L. lactis* subsp. *lactis*, is only effective against Gram-negative bacteria if high concentration are used or when target cells have been pre-treated with EDTA (Stevens et al, 1991).

Bacteriocins are not well known to be effective against Gram-negative bacteria. This is because the outer membrane of this bacteria type functions as a permeability barrier for the cell. This membrane prevents molecules such as antibiotics, detergents and dyes from getting to the cytoplasmic membrane (Stevens et al., 1991). However, previous studies have indicated some bacteriocin activity against this bacteria group. Examples are plantaricin 35d, produced by *L. plantarum* and active against *A. hydrophila* (Messi et al., 2001); bacteriocin ST151BR, produced by *L. pentosus* ST151BR (Torodov & Dicks, 2004) and a bacteriocin produced by *L. paracasei* subsp. *paracasei* active against *E. coli* (Caridi, 2002); thermophylin, produced by *S. thermophilus* active against *E. coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* among the Gram-negative species and against several *Bacillus* species, *L. monocytogenes* and *S. typhimurium* among the Gram-positives (Ivanova et al, 1998). Bacteriocins ST28MS and ST26MS, produced by *L. plantarum* that was isolated from molasses (Torodov & Dicks, 2005) subdued the growth of *E. coli* and *Acinetobacter baumannii* along with some Gram-positive bacteria. Lade et al. (2006) also isolated two *Lactobacillus* species (*L. plantarum* and *L. lactis*) from vegetable

waste and observed that they produced bacteriocins which inhibited the growth of *E. coli*.

2.8.2 Stability

Some studies on the classification of bacteriocins have shown that these molecules are temperature and pH dependent. Susceptibility to proteolytic enzymes indicates the proteinaceous properties of bacteriocins (De Martins et al, 2003). It was observed that the antimicrobial activity of the bacteriocins ST28MS and ST26MS produced by *L. plantarum* isolated from molasses reduced drastically after treatment with proteinase K, pronase, pepsin and trypsin. However, these bacteriocins remained stable after incubation for 2 h at pH values between 2.0 and 12.0. Also, no decline in antibacterial activity was observed after 90 min at 100°C or 20 min at 121°C (Torodov & Dicks, 2005). The thermos-tolerance feature noticed in these bacteriocins might be because of the molecular structure of the bacteriocin, usually made of small peptides without tertiary structure (Toro, 2005).

2.8.3 Bacteriocin-like substances

Lactic acid bacteria are able to produce other substances, known as bacteriocin-like substances (BLS). An example of this type of molecule is reuterin, produced by some strains of *Lactobacillus reuteri* during anaerobic fermentation of glycerol (Rodríguez et al., 2003; Pancheniak et al., 2006). It is water-soluble, pH dependent and resistant to proteolytic and lipolytic enzymes. *Carnobacterium piscicicola* L103 isolated from vacuum packed meat was reported to have the ability to produce a bacteriocin-like

substance, which caused inhibition of six strains of *Listeria monocytogenes* isolated from salmon and from human origin (Schöbitz et al., 2003). However, no significant growth of the pathogen was observed at $4 \pm 2^\circ \text{C}$ during 15 days, when compared to the salmon inoculated only with *L. monocytogenes* (Schöbitz et al., 2006).

2.8.4 Applications in food preservation

Bio-preservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocins have received a growing attention, and different methods to control pathogenic and spoilage microorganisms have been developed (Ross et al., 1999). Some LABs confirmed antagonism effect towards pathogenic and spoilage organisms. Bacteriocins are normally produced by many Gram-positive and Gram-negative species. However, those produced by LABs are essential to the food industry, since these bacteria have generally been regarded as safe. Bacterial fermentation of perishable raw materials has been used for centuries to preserve the nutritive value of food and beverages over an extended shelf life (Deegan et al., 2006). Despite the several methods other than bacteriocins are employed for the preservation of food/beverages, an increasingly, health conscious public may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives.

The production of bacteriocins by LAB is advantageous for survival of the producing bacteria in a competitive ecological niche; therefore, they could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner, which is likely to be more acceptable to consumers (Deegan et al., 2006). Minimally processed refrigerated foods have been gaining consumer acceptance in the

last years due to their natural appeal. However, the microbiological safety of these foods are of concern due to the possible presence of non-proteolytic toxic strains of *Clostridium botulinum*, able to grow at 4°C, and the post-processing contamination with psychrophilic pathogens, such as *L. monocytogenes* (De Martinis et al., 2001; Schobitz et al., 2006).

