CHAPTER 2

REVIEW OF LITERATURE

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be safe to eat, i.e. they may not cause illness because there are no pathogens or toxins present, but changes in texture, smell, taste, or appearance cause them to be rejected. Some ecologists have suggested these noxious smells are produced by microbes to repulse large animals, thereby keeping the food resource for themselves (Sherratt et al., 2006).

Food loss, from farm to fork, causes considerable environmental and economic effects. The USDA Economic Research Service estimated that more than ninety-six billion pounds of food in the U.S. were lost by retailers, foodservice and consumers in 1995. Fresh produce and fluid milk each accounted for nearly 20% of this loss while lower percentages were accounted for by grain products (15.2%), caloric sweeteners (12.4%), processed fruits and vegetables (Kantor et al., 1997). Some of this food would have been considered still edible but was discarded because it was perishable, past its sell-by date, or in excess of needs. There are also environmental and resource costs associated with food spoilage and loss. If 20% of a crop is lost, then 20% of the fertilizer and irrigation water used to grow that crop was also lost.

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, including species of Lactobacillus, Pediococcus, Leuconostoc some of which are useful in producing fermented foods such as yogurt and pickles. However, under low oxygen, low temperature, and acidic conditions, these bacteria become the predominant spoilage organisms on a variety of foods. Other thermophiles (Bacillus and Geobacillus spp.) cause a flat sour spoilage of high or low pH canned foods with little or no gas production, and one species causes ropiness in bread held at high ambient temperatures. Mesophilic anaerobes, growing at ambient temperatures, cause several types of spoilage of vegetables (Bacillus spp.); putrefaction of canned products, early blowing of cheeses, and butyric acid production in canned vegetables and fruits. While others produce off-odors and gas in vacuum-packed, chilled foods and milk (Bacillus spp.).
Listeria monocytogens is a small, non-spores forming rod gaining public awareness because of its presence in food products. Contamination of food products with Listeria monocytogens has resulted in the closing of business or discontinued production of profitable food products (Kellum et al., 2002). Listeria is widely found on foods and most raw foods are likely to be contaminated.

Listeria is easily killed by heat although cooked foods can easily become decontaminated through poor handling. This is one of the few pathogens that can grow in the refrigerator. Although it can grow in the fridge, it will do so only vary slowly so make sure your refrigerator is keeping your food at or less than 5°C (Bunning et al., 1986). Listeria is widespread in nature, living closely associated with soil and plant matter. This organism has been found in feces of humans and animals, soil leafy vegetables decaying corn and soybeans, raw and treated sewage, effluent from poultry and meat processing facilities, normal and mastitis milk and improperly fermented silage (Catherine, 1987). Listeriosis is rare form food borne illness, but it may be a serious diseases in a small group of individuals, those who pregnant, immune-compromised and young children and every year a few people die. Due to this problem it has also caused occasional outbreak of mild gastroenteritis in healthy people. The symptoms are usually described as ‘flu-like’, although vomiting and discovered urine can occur. Miscarriage can result if pregnant women is infected, even she does not show the symptoms. The time from infection to symptoms can be anywhere 8 to 90 days (Dalton et al., 1977). Pseudomonas and related genera are aerobic, Gram-negative soil bacteria, some of which can degrade a wide variety of unusual compounds. They generally require a high water activity for growth (0.95 or higher) and are inhibited by pH values less than 5.4. Some species grow at refrigeration temperatures (psychrophilic) while other are adapted for growth at warmer, ambient temperatures.

2.1 Bakery Products

Bakery products are subjected to spoilage problems. These include physical, chemical and microbial spoilage. Since the most common factor of bakery products is water activity, microbiological spoilage, in particular mould growth is the major economic importance of bakery products. Mould spoilage is a serious and costly problem for bakeries. Bacterial contamination is more dangerous because very often the food does not look bad, even though
severely infected, it may appear quite normal. The presence of highly dangerous toxins and bacterial spores is often not detected until after an outbreak of food poisoning (Sockett, 1991).

Cakes undergo bacterial spoilage due to their usually high concentration of sugars, which restrict the availability of water. The baking process is generally sufficient to destroy microorganisms. Microorganism may enter baked cakes from handling also from air. Growth of microorganisms on the surface of cakes is favoured by high humidity and growth of moulds on cakes results in a hardening of the product (Bamford, 1973). Food spoilage can be defined as disagreeable changes in food’s normal state; such changes can be detected by smell, taste, touch or sight. These changes are due to number of reasons such as air, moisture, light, microbial growth and temperature.

Main single cause of food spoilage is invasion by microorganisms such as bacteria, yeasts and moulds. Molds are the most important contaminants because of the low moisture levels in grains, but molds do require some moisture so efficient drying and good storage facilities are necessary to prevent their growth. Microbial populations decrease during milling and storage of grain. Molds cause spoilage by altering the appearance of grains and flours, and some species also synthesize toxic secondary metabolites called mycotoxins. Molds are also the primary spoilage organisms in baked goods, with *Aspergillus*, *Penicillium*, and *Eurotium* being the most commonly isolated genera. *Penicillium* tends to be the more important in sourdough breads and in breads stored at cooler temperatures. Freshly baked breads do not contain viable molds but soon become contaminated upon exposure to air and surfaces (Smith *et al.*, 2004). *Bacillus* spores are strongly heat resistant and can survive baking in the interior of bread loaves and then germinate and start growing as the bread cools. Some strains cause a defect called ropiness, a soft sticky texture caused by starch degradation and slimy exopolysaccharides often accompanied by a fruity odor (Pepe *et al.*, 2003). Yeasts may also be involved in spoilage of some breads and fruitcakes, causing a chalky appearance on surfaces and off odors. High sugar content and low water activity of cakes also favors molds over other spoilage microbes but some species of yeasts and bacteria (*Bacillus* and *Pseudomonas*) may also attack cakes. Bakery products containing cream, custard or fruit filling are targets of additional spoilage organisms.

### 2.2 Dairy Products
Dairy products like soft or unripened cheeses, which generally have the highest pH values, along with the lowest salt to moisture ratios, spoil most quickly. In contrast, aged, ripened cheeses retain their desirable eating qualities for long periods because of their comparatively low pH, low water activity, and low redox potential. Some of the spoilage microorganisms were able to grow at relatively low pH values (4.6-4.7) when incubated at 7°C and were able to grow at pH 3.6 when grown in media at 20°C. Spoilage problems in cheese can sometimes be traced to low quality milk but may also result from unhygienic conditions in the processing plant. Some coliforms and *Clostridium spp.* that cause late gas blowing can grow under these conditions as can several species of molds.

Soft cheeses with a higher pH of 5.0-6.5 and a moisture content of 50-80% may be spoiled by *Pseudomonas, Alcaligenes,* and *Flavobacterium.* *Clostridium sporogenes* has been found in spoiled processed cheese, where it produces gas holes and off-flavors (Lycken and Borch, 2006).

Milk is an excellent medium for growth for a variety of bacteria (Boor k and Fromm, 2006). Spoilage bacteria may originate on the farm from the environment or milking equipment or in processing plants from equipment, employees, or the air. LAB are usually the predominant microbes in raw milk and proliferates if milk is not cooled adequately. When populations reach about 10^6 cfu/ml, off-flavors develop in milk due to production of lactic acid and other compounds. Pasteurization kills the psychrophiles and Mesophilic bacteria (LAB), but heat-tolerant species (*Alcaligenes, Microbacterium,* and the sporeformers *Bacillus* and *Clostridium*) survive and may later cause spoilage in milk or other dairy products (Dogan B and Boor KJ, 2003) Immediately following pasteurization, bacterial counts are usually <1000 cfu/ml. However, post-pasteurization contamination of milk, particularly with *Pseudomonas* and some Gram-positive psychrophiles does occur. Spoilage problems in cheese can sometimes be traced to low quality milk but may also result from unhygienic conditions in the processing plant.

Hard and semi-hard cheeses have a low moisture content (<50%) and a pH ~5.0, which limits the growth of some microbes. Some coliforms and *Clostridium spp.* that cause late gas blowing can grow under these conditions as can several species of molds. Other psychrotrophs produce biogenic amines, particularly tyramine, during storage of cheese. Soft cheeses 50-80% may be spoiled by *Pseudomonas, Alcaligenes,* and *Flavobacterium.* *Clostridium sporogenes* has been
found in spoiled processed cheese, where it produces gas holes and off-flavors. Yeasts and molds are the main spoilage organisms found in cultured milks (yogurt, sour cream and buttermilk) because the higher acidity in these products inhibits many bacteria. *Pseudomonas*, yeasts and molds can spoil butter and light butters. Since the light butters have a higher moisture content than butter, they can support more microbial growth. Cream may become rancid when populations of *Pseudomonas* and *Enterobacter* proliferate.

### 2.3 Bio-preservative

Bio-preservation systems are of increasing interest for food industry and consumers. The interest in bio-preservation of food has prompted the quest for new natural antimicrobial compounds from different origins that have been widely recognized as natural food biopreservatives. Bio-preservation is an innocuous and ecological approach to the problem of food preservation and has gained increasing attention in recent years. Foods preserved with natural bio-preservatives have become popular due to greater consumer awareness and concern regarding synthetic chemical additives. Lactic Acid Bacteria (LAB) have been shown a major potential for use in bio-preservation because of safety for human consumption (GRAS status) (Vignolo et al., 2008).

Recently, the food industry and food research have intensified on natural antimicrobial compounds owing to the consumers’ ban against chemical preservatives (Devlieghere et al., 2004). They also decrease the outbreak caused by foodborne illnesses (Ray, 2004). So now days, novel technologies such as ‘biopreservation’ have attracted great attention as natural means for controlling the shelf-life and safety of food products. Bio-preservation systems are of increasing interest for food industry and consumers. The application of bio preservatives to ensure the hygienic quality is a promising tool although, it should be considered only as an additional measure to good manufacturing, processing, storage and distribution practices (Mataragas et al., 2003).
2.4 Bacteriocins

The bacteriocins were first characterized in Gram-negative bacteria that are colicins of *E. coli* (Lazdunski, 1988). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract (Cotter *et al.*, 2005). They are generally recognized as “natural” compounds able to influence the safety and quality of foods (Saavedra *et al.*, 2004). Bacteriocin production has been found in numerous species of bacteria, among which, due to their “generally recognized as safe” (GRAS) status, LAB have attracted great interest in terms of food safety.

Bacteriocins are ribosomally synthesized, extracellular released low molecular-mass peptides or proteins (usually 30-60 amino acids), which have a bactericidal or bacteriostatic effect on other bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum) (Tagg *et al.*, 1976). Bacteriocins, particularly lantibiotics, inhibit target cells by forming pores in the membrane, depleting the trans membrane potential and the pH gradient, resulting in the leakage of cellular materials. Early studies suggest that in order for nisin to form pores, target cells require (inside negative) and ΔpH (inside alkaline) (Okereke and Montville, 1992).

2.4.1 Classification of bacteriocins

Bacteriocins are commonly divided into three or four groups (ZKlaenhammer *et al.*, 1993) Nisin was discovered in 1928 (ZHurst, 1967) and subtilin, a nisin analogue differing by 12 amino acid residues, was discovered in 1948 (ZHan sen, 1993). Both belong to Class I, termed lantibiotics. Class I is being further subdivided into Class Ia and Class Ib. In general, Class I peptides typically have from 19 to more than 50 amino acids. Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyryraine and dehydroalanine.

Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid class Ib. Class Ib bacteriocins, which are globular peptides, have no net charge or a net negative charge (Altena *et al.*, 2000). Class II contains small heat-stable, non-modified peptides, and can be
further subdivided. According to conventional classification, Class IIa includes Pediocin-like Listeria active peptides with a conserved N-terminal sequence Tyr–Gly–Asn–Gly–Val and two cysteines forming a S–S bridge in the N-terminal half of the peptide. Bacteriocins composed of two different peptides comprise Class IIb. The two peptide bacteriocins need both peptides to be fully active. The primary amino acid sequences of the peptides are different. Though each is encoded by its own adjacent genes, only one immunity gene is needed. Class IIc was originally proposed to contain the bacteriocins that are secreted by the general sec-system (Nes et al., 1996). The large and heat labile bacteriocins make up the Class III bacteriocins for which there is much less information available. A fourth class consists of bacteriocins that form large complexes with other macromolecules, has been proposed (Klaenhammer, 1993).

2.4.2 Mode of action

Bacteriocins, particularly lantibiotics, inhibit target cells by forming pores in the membrane, depleting the transmembrane potential. Bacteriocins are positively charged molecules with hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane (Chen et al., 1997). The association of hydrophobic patches of bacteriocins with the hydrophobic membrane has also been modeled using computer simulation to predict the most favorable interaction (Ziney et al., 1999). It is likely that the hydrophobic portion inserts into the membrane, forming pores. According to the AwedgeB model, after a critical number of nisin molecules associate with the membrane, they insert concurrently, forming a wedge (Driessen et al., 1995). More recent studies demonstrate the complexity of bacteriocin activity, where nisin must bind to lipid II on the susceptible cell membrane in order to kill (Breukink et al., 1999). For other cationic peptides, the peptide concentration required to cause membrane depolarization does not always correspond with the minimal inhibitory concentration and does not necessarily cause cell death (Friedrich et al., 2000).

2.5 Nisin

Nisin was composed of 34 amino-acid with molecular mass of 3354 Da and was produced by certain strains of Lactococcus lactis subsp lactis. Nisin was effective against a wide range of
spoilage and pathogenic gram-positive bacteria. Many recent studies reported that nisin inhibited growth of gram positive bacteria, such as *Bacillus cereus* *Staphylococcus aureus*, *Listeriamonocytogenes*, *Lactobacillus plantarum*, *Micrococcus luteus*, *Micrococcus flavus* and *Brochothrix thermosphaeta* (Padgett et al., 1998). It prevented the growth of germinating *Bacillus* and *clostridial* spores and through the addition of calcium chelator (Stevens et al., 1991).

### 2.5.1 Mode of action

Nisin provides a paradigm for studies of lantibiotic structure, biosynthesis and mode of action of antimicrobial peptides, and is often referred to as the “prototypical” lantibiotic. Nisin has different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions (Pag and Sahl, 2002). Nisin binds with high affinity to the Lipid II molecule, a hydrophobic carrier for peptidoglycan monomers, using this compound as a specific receptor to integrate into the bacterial membrane and to form pores that increase membrane permeability; nisin-Lipid II interaction compromises the incorporation of precursor units, blocking the biosynthesis of bacterial cell wall (Breukink et al., 1999). Lipid II has also been recognized as the primary target for antibiotics (e.g. ramoplanin and vancomycin), and other bacteriocins, pediocin, subtilin, galidermin and epidermin.

The interaction between nisin and Lipid II starts specifically with the high affinity binding between nisin’s N-terminus with the pyrophosphate from Lipid II, while the C-terminal region of the bacteriocin inserts into the cell membrane (Hsu et al., 2002). The interaction between nisin-Lipid II complexes with the cell membrane results in the formation of complexes that consist of several nisin and Lipid II molecules, which assemble further into larger complexes; the conversion of the large complexes into a pore requires the cooperative insertion of several nisin molecules into the lipid bilayer. The final pore structure is believed to have a stoichiometry of eight nisin and four Lipid II molecules (Hasper et al., 2004). In 1969 nisin peptide was approved by F.A.O and W.H.O (Food and Agriculture Organization / World Health Organization) to use as a preservative in food. Nisin may be used in hurdle technology, in which synergistic preservation methods such as low pH and high salt concentrations are combined (Ariyapitipun et al., 1999). The primary target of Nisin’s antimicrobial action is the cell membrane. It has been studied that
Nisin interferes the energy supply of the cell by creating pores in the membrane (Sahl et al., 1995).

Nisin is used, often in combination with other preservation methods, for extending the shelf-life of certain bakery products due to its inhibition of certain spoilage and food poisoning bacteria. Nisin binds to the anionic phospholipids (including lipid II) of the cell membrane and are subsequently inserted into the membrane (Bonev et al., 2004). It has been studied to evaluate the combined effect of a heat pretreatment with the use of antimicrobials Nisin and carvacrol against *Listeria monocytogenes*. Studied that comparative evaluation of the preservative effect of benzoate, sulphite and Nisin on the quality of white layer cake.

2.6 *Lactobacillus reuteri*

*Lactobacillus reuteri* belongs to the obligate heterofermentative group of lactobacilli, and use the phosphoketolase-based metabolic pathway to utilise available carbohydrates (Axelsson et al., 1998). It can ferment glucose alone and produce lactate, ethanol and CO$_2$ as end-products; but an essential characteristic of *L. reuteri* is its ability to utilise glycerol (Talarico et al., 1988).

2.6.1 Historical background

*Lactobacillus reuteri* was recorded in scientific classifications of lactic acid bacteria as early as the beginning of the 20th century (Orla-Jensen et al., 1919), though at that time it was undistinguished from *L. fermentum*. In the 1960s, a German microbiologist Gerhard Reuter isolated *L. reuteri* from human faecal and intestinal samples, and subsequently separated it from *L. fermentum* and re-classified it as *L. fermentum* biotype II (Reuter et al., 1965). Eventually identified *L. reuteri* as a distinct species based on phenotypical and genetic characteristics, and proposed it being a new species of heterofermentative lactobacilli. Later, modern technologies have further confirmed the identity and clearly separated the two species. Since 1980, *L. reuteri* has been classified as a distinct species in the *Lactobacillus* genus (Kandler et al., 1980).

2.6.2 Morphology
*Lactobacillus reuteri* strains are Gram-positive, lactic acid-producing bacteria, their cells are slightly irregular, bent rods with rounded ends, generally 0.7-1.0 x 2.0-3.0 μm in size (Kandler and Weiss, 1986) and occurring singly, in pairs and in small clusters.

### 2.6.3 Biochemistry

*Lactobacillus reuteri* belongs to the obligate heterofermentative group of lactobacilli, and uses the phosphoketolase-based metabolic pathway to utilise available carbohydrates (Axelsson, 1998, Casas and Dobrogosz, 2000). It can ferment glucose alone and produce lactate, ethanol and CO2 as end-products; but an essential characteristic of *L. reuteri* is its ability to utilise glycerol (Talarico et al., 1988; El-Ziney et al., 1998; Luthi-Peng et al., 2002). When glycerol is added as a substrate, the end-products change to more acetate/less ethanol, and the NADH formed during glycolysis is reoxidized by glycerol rather than in the ethanol pathway (Talarico et al., 1990). In the process involving glycerol, *L. reuteri* takes the phosphoketolase pathway (PKP) to produce lactate; in parallel with lactate formation, glycerol successfully competes with acetylphosphate as a preferred terminal hydrogen acceptor to recycle NAD+, and this results in the release of the highly energetic acetylphosphate which is subsequently channeled into the acetate kinase reaction for more ATP production. As a consequence, this glycerol metabolism allows a higher ATP generation and greater acetate production, and ultimately a higher cell growth rate and more biomass are achieved. Hence, it is more favourable for *L. reuteri* to utilise glycerol at the later stages of glycolysis. It should be noticed that glycerol utilisation is only initiated by completion of the first few steps of breaking down fermentable carbohydrates (*e.g.* glucose). (Talarico *et al.*, 1990) postulated that *L. reuteri* cannot grow on glycerol alone, so this favourable process can occur only in the presence of fermentable carbohydrates. At the end of this featured glycerol metabolism, a series of end-products, including SCFA and other organic compounds, were produced. Among these, 3-HPA (3-hydroxypropionaldehyde) has drawn the most attention, and has been found to be a potent antimicrobial substance. 3-HPA is also termed reuterin (Talarico *et al.*, 1988).

### 2.6.4 Production of reuterin
Reuterin was produced as per the method reported by (Chung et al., 1989) with a slight modification. The harvested *Lactobacillus reuteri* (250 mg wet weight) were suspended in 5 ml of 250 mM glycerol in distilled water and incubated for 2 h under anaerobic conditions. After fermentation, the cells were collected by centrifugation at 4000×g for 10 min. The supernatant fraction was filtered using a filter membrane (0.45 μm pore size, nylon, Millipore) and stored at 4°C in a container for purification. It is a potent antimicrobial agent active against Gram positive and Gram negative bacteria, as well as yeasts, moulds and protozoa (Axelsson et al., 1989).

Reuterin is synthesized in vitro under pH, temperature and anaerobic conditions similar to those of the gastrointestinal tract (Chung et al., 1989). Reuterin is a natural substance with no health risk, active in wide pH range, has no corrosion effect or foaming production, and it does not affect water hardness or organic food residuals which makes it good for future applications. More trials at pilot-plant scale should be conducted and the synergistic effect of reuterin and nisin must be carefully investigated. In case of bio preservation the addition of lacto peroxidase to reuterin, has enhanced its bactericidal effect against Gram-negative pathogens in milk (Arques et al., 2007).

### 2.6.5 Importance of *Lactobacillus reuteri*

*L. reuteri* was first introduced into the human functional foods market in Sweden in 1991 as a mixture of cultures (*Bifidobacterium animalis, L. reuteri* and *L. acidophilus*) in milk. From then on, *L. reuteri* has been incorporated into various food and drinks as a potent bioactive ingredient throughout the western world. Now, all clinically tested *L. reuteri* strains are available commercially as functional food ingredients (Casas and Dobrogosz, 2000). BioGaia, a company based on strong scientific research of *L. reuteri*, is a worldwide producer of *L. reuteri*-containing products from strains with clinically approved efficacy (Vollenweider, 2004; Wikipedia.org, 2007; BioGaia, 2008). *L. reuteri* is also reported to be a possible alternative to bio-feed in certain types of animal farming, given its proven safe usage and significance in improving animal growth (Casas and Dobrogosz, 2000). It can be foreseen that potential replacement of antibiotics with host-specific probiotic feed in animal farming will largely reduce the threat of
growing antibiotics-resistance. Recently, more attention has been put on the applications of purified reuterin. As a potent natural antimicrobial agent with low cytotoxicity, it has been used in sanitization of biological tissues (Sung \textit{et al.}, 2002; Chen \textit{et al.}, 2002; Liang \textit{et al.}, 2003), and treatment of food-borne pathogens as a microbial preservative (Daeschel, 1992; El-Ziney and Debevere, 1998; El-Ziney \textit{et al.}, 1999; Kuleasan and Cakmakci, 2002; Arques \textit{et al.}, 2004). A very recent proposal incorporates reuterin into modified atmosphere packaging in food preservation (Lu, 2007).