CHAPTER 2
LITERATURE REVIEW

2.1 Milk and milk composition

2.1.1 Milk composition

Milk is a complex fluid containing many compounds that is secreted by female of all mammalian species. In many parts of the world, cows are the most important mammal in milk production, including in the UK, Europe and North America (Kon, 1975; Porter, 1983; Varnam and Sutherland, 1994; Aguhob and Axtell, 1996; Harding, 1999). The term of milk means a clean and clear liquid, which is distilled from udders of good healthy mammal, does not contain colostrums and is produced to bring up the newborn species (Tungjaranchai and Kavila, 1988; Potter and Hotchkiss, 1995).

The constituent of milk is largely composed of water in which is a true solution of the milk sugar (lactose), organic and inorganic salts, vitamins and other small molecules. The milk fat is emulsified and in a form of colloidal dispersion with proteins (Kon, 1975; Porter, 1983; Walstra and Jenness, 1984; Fox and McSweeney, 1998; Harding, 1999; Walstra *et al.*, 1999). The components of milk are included 85.5-89.5% of water, and 10.5-14.5% of total solids. The contents of total solids are 2.5-6.0% fat, 2.9-5.0% protein, 3.6-5.5% lactose and 0.6-0.9% minerals (Porter, 1983; Walstra and Jenness, 1984; Tungjaranchai and Kavila, 1988; Bylund, 1995; Harding, 1999).

Milk contains a high water content, at the same time water or moisture is one of the important factors for microorganisms to grow. This situation makes milk as a good growth medium for many microorganisms. For example, in nutrient broth that has 98.7% of water, the media is used to grow many types of bacteria (Suksringam, 1991; Frank, 1997). Beside that, most of bacteria can grow well at water activities around 0.90. Milk has a water activity of 0.993, which shows clearly that most of microorganisms, especially bacteria will not have any problem to grow in milk (Fox and McSweeney, 1998; Prescott *et al.*, 2002).

2.1.2 Milk fat

Fat or lipids in milk, called as milk fat, have an important function as a source of energy and an essential free fatty acid. The properties of fat will affect flavor, mouth feel and viscosity of milk and milk products (Fox and McSweeney, 1998). The milk fat consists of triglycerides, di- and mono-glycerides, fatty acids, sterols, carotenoids, vitamins and trace elements. The milk fat is present as small globules or droplets, which surrounded by a surface layer or membrane. The function of the membrane is to prevent the globules from coalescence and from the activity of lipase (Varnam and Sutherland, 1994; Bylund, 1995; Walstra *et al.*, 1999).

During storage of milk and milk products, chemical changes of milk fat can occur. Lipolysis is one of the chemical reactions that is widely recognized to be happened in milk. It is a process of enzymatic hydrolysis of glyceride ester and a production of rancid off-flavors if a prolong reaction is occurred. Some bacteria in milk, particularly psychrotrophs including *Pseudomonas* and *Alcaligenes*, can produce lipase and thus attack fat, causing rancid off-flavors. In milk products, lipolysis did not occur in the low pasteurized milk until the number of microorganisms was more than 10^7cfu/ml. In butter, the spoilage by lipolysis occurred at refrigerator temperatures. In raw milk, the lipolysis spoilage could be occurred as little as 2 days at 5°C by heat-stable lipase (Rosenthal, 1991; Varnam and Sutherland, 1994; Walstra *et al.*, 1999).

2.1.3 Milk protein

About 95% of nitrogen in milk are in the form of protein. Milk proteins are mainly divided into two parts. Casein is the first one, consists nearly 80% of the total protein in milk and precipitates upon acidification of milk at pH 4.6. The casein can be further broken down into five classes, which are α_{s1} -, α_{s2} -, β-, κ- and γ-caseins.

The other protein that remains in solution at pH 4.6, is called whey proteins or serum proteins. It consists of α-lactalbumin, β-lactoglubulin, blood serum albumin, immunoglobulins and small molecular weight peptides. The casein in milk is present in micelles and stable at temperatures up to 140°C. In contrast, whey proteins are

denatured at 80°C (Walstra and Jenness, 1984; Rosenthal, 1991; Varnam and Sutherland, 1994; Walstra *et al.*, 1999).

2.1.4 Milk carbohydrate

Lactose is the main and unique carbohydrate in milk. Lactose is a disaccharide composed of D-glucose and D-galactose, linked by a β-1,4 glycosidic bond (Fox and McSweeney, 1998; Walstra *et al.*, 1999). The sugar is a reducing sugar, and therefore several reactions related to Maillard and caramelization reactions can occur when the milk is heated. Lactose can also isomerize into lactulose (D-fructose and D-galactose) during heating at high temperatures (Walstra *et al.*, 1999).

Lactose is the main source of energy for microbial metabolism. Some bacteria contain lactase, which is an enzyme that attacks lactose, splitting the molecule into glucose and galactose. The microorganisms are then used the monosaccharides for their metabolisms and converted glucose into lactic acid. The type of bacteria that can produce lactic acid is a group of bacteria called as lactic acid bacteria. These bacteria are the main responsible bacteria when the milk goes sour (Rosenthal, 1991; Bylund, 1995; Walstra *et al.*, 1999).

2.1.5 Enzymes

Milk contains several native enzymes, which excreted by the mammary gland. Some of these enzymes are important in determining the stability of milk during storage, such as catalase. Beside these enzymes, milk may also contain enzymes secreted from microbial activities, particularly proteinase and lipase. The presence of these two enzymes can cause undesirable changes in milk and milk products, for example hydrolytic rancidity in milk and bitterness in cream and UHT milk. In contrast, they produce desirable flavors in some of milk products, especially cheese products that need to be ripened at low temperatures.

Lactoperoxidase is an important enzyme in milk. It is slightly more heat stable than most of milk native enzymes and is used as an index of high temperature pasteurization because it can be inactivated by a heat treatment at 73°C for 10 min. It is able to catalyze oxidation of unsaturated fatty acids and produce oxidation

products. In addition, it can also catalyze an oxidation of thiocyanate (CNS^-) with H_2O_2 to produce products that have antibacterial activities, such as hypothiocyanate ($OCNS^-$) (Varnam and Sutherland, 1994; Fox and McSweeney, 1998; Walstra *et al*., 1999).

Alkaline phosphatase is another enzyme that has a significant role for milk products. It is used as an indicator that a batch of milk has been properly pasteurized and not recontaminated with raw milk, because the enzyme will be destroyed by the time and temperature combinations used in the pasteurization process both batch and continuous processes (Nanasombut, 1996).

2.2 Microorganism in milk

Milk is a good source of nutrients for mammals. Therefore, it is not astonished if microorganisms can grow easily in milk. Most of bacteria and some molds and yeast can grow in milk. However, the presence of these microorganisms is generally undesirable because they can be pathogenic or produce enzymes that cause undesirable transformations in milk. There are several factors that affect the growth of bacteria in milk and milk products, including storage temperatures, initial load of microorganisms, acidity and alkalinity (pH), water availability and oxygen (Brock and Madigan, 1988). The storage temperature has a large effect on the growth rate of bacteria. Keeping milk at low temperatures will generally slow down the bacteria growth because the bacteria will need longer time for their lag phase compared to storage at high temperatures. However, the extension of milk quality at low temperatures will be depended on the type of organism that is present in milk itself. The psychrotrophic bacteria, for example, will still cause a problem for the milk quality even if the milk is stored at a refrigeration temperature.

Another factor is the initial load of microorganisms in milk. Either keeping a batch of milk at low or high temperatures, a lower initial load of microorganisms will delay spoilage in that batch of milk compared with a higher initial load. Therefore, a good quality of raw milk can be produced if the milk is directly stored at temperature ≤ 4°C after milking and the milk is processed as soon as possible because

the low storage temperature can prevent the multiplication of bacteria only for 24 h (Bramley and McKinnon, 1990; Walstra *et al.*, 1999).

Since temperature is one of the most important environmental factors for the growth and survival of microorganisms, microorganisms can be classified according to their optimum growth temperature range. These classifications are divided into several groups, including psychrophiles, mesophiles, thermophiles and hyperthermophiles (Prescott *et al.*, 2002).

2.2.1 Psychrotrophiles

The psychrotroph microorganisms are important for milk and milk products because they can affect the quality of raw milk and pasteurized milk stored at low temperatures. The microorganisms can grow at refrigerator temperatures between 0–7°C, although their optimum growth temperatures are between 20–30°C. The significance of these bacteria for milk and milk products is due to their capability to spoil the products by breaking down proteins and fat from the production of protease, lipase, phospholipase and other hydrolytic enzymes.

The predominance spoilage organisms in this class are Gram negative rods psychrotrophic species, including *Pseudomanas* spp., in which *Ps. fluorescens* is the dominance species and other species will be *Ps. putida*, *Ps. fragi*, and *Ps. aeruginosa*; *Flavobacterium*; *Alcaligenes* and coliforms. The Gram positive microorganisms will include genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus* and *Microbacterium* (Bramley and McKinnon, 1990; Varnam and Sutherland, 1994; Sorhaug and Stepaniak, 1997; Harding, 1999; Prescott *et al*., 2002).

In the review of Sorhaug and Stepaniak (1997) and Cempirkova (2002), they reported that *Pseudomonas* spp. is usually represent not more than 10% of the total bacteria in fresh milk, but this microorganism is the most psychrotrophic bacterium that was found in spoiled raw or pasteurized milk. This genus is represented by species with the shortest generation time at 0–7°C and the lowest theoretical minimum growth temperature of –10°C. Many strains of *Pseudomonas* spp. can produce proteinase to degrade casein into soluble peptides with a development of

bitter taste and lipase that is responsible for degradation of milk fat with a development of rancid and soapy flavor in milk. One of the interesting things that should be noted is the growth and metabolic activity of *Pseudomonas* spp. from a Post Processing Contamination (PPC) is the most harmful factor in the keeping quality of milk pasteurized at 72°C for 12–15 s and stored at 4–7°C. The presence of high numbers of psychrotropic organism in raw milk will also greatly affect the quality of past eurized milk. Although the pasteurization process will kill virtually all of the psychrotroph bacteria, but the heat-resistant enzymes that they produce still can be active during the storage time of pasteurized milk (Varnam and Sutherland, 1994). In raw milk, a number of more than $5x10^5$ psychrotrophs cfu/ml can be harmful, whereas a deflectable flavor can be detected when the number is over than 10^7 cfu/ml (Sorhaug and Stepaniak, 1997; Walstra *et al*., 1999).

2.2.1.1 Thermoduric psychrophiles

Chen *et al.* (2003) reported in their review that if a bacterial species survives a pasteurization process at 63°C for 30 min, it is usually referred to as being thermoduric. The group of heat resistant psychrotrophic includes *Bacillus* spp., *Arthrobacter, Microbacterium, Streptococcus, Corynebacterium* and *Clostridium*. These bacteria can produce extracellular proteases, lipase and phospholipases (lectinases). Some *Micrococcus* spp. are slightly less heat resistant, and only 1-10% of *Alcaligenes tolerans* may survive. However, only a small number, not more than 1%, of streptococci, lactobacilli and *Corynebacterium* species can survive after a process at 63°C for 30 min. The *Bacillus* spore content of raw milk rarely exceeds $5.0x10^3$ /ml and it is generally higher in winter seasons than in summer seasons. In addition to, around 12% of farm milk supplies from winter housed cows had spore counts of more than $1.0x10^2$ /ml and *Bacillus* spp. was the most common species, while *Bacillus cereus* was found only sporadically in bulk milk tank. Generally, after a heat treatment that kills the vegetative form, clostridia does not multiply in milk products (Bramley and McKinnon, 1990).

Psychrotrophic *Bacillus* spp. is aerobic bacteria that can become pathogenic microorganisms to men and animals because they form endospores that are heat resistant. *B. cereus* and *B. licheniformis* were the most commonly isolated species of

Bacillus in milk. *B. licheniformis* was present in the farm environment and was higher in raw milk than *B. cereus* during the winter months. However, *B. cereus* could come to dominate the *Bacillus* population, and reach a level of enterotoxin production, because *B. cereus* grew faster than *B. licheniformis* at ambient temperatures (Crielly *et al.*, 1994). Research showed that a low level of environmental contamination of *B. cereus* spores in raw milk would lead to a major source of psychrotrophic *B. cereus* in the pasteurized milk (Larsen and Jorgensen, 1999).

2.2.2 Mesophiles

Mesophiles are unique, present in soil and water in the tropical areas and living in men and other warm-blooded animals. They are able to grow at chill temperatures although their optimum temperatures are at 30-40°C. Most of the spoilage food and food poisoning cases are correlated with mesophillic bacteria, including coliform, *Escherichia coli*, *Bacillus subtilis*, *B. cereus*, *Clostridium perfringens*, *Clostridium botulinum* and *Staphylococcus aureus* (Garbutt, 1997; Forsythe, 2000).

Coliforms are present everywhere, including in the digestive tract. Some of these bacteria are *E. coli* and *Aerobacter aerogenes*. They can grow rapidly in milk at temperatures above 20°C, and attack proteins and lactose to produce gas and unclean flavor. Another mesophile is a group of lactic acid bacteria, which includes genera *Lactococcus lactis* subsp. *lactis* and *cremoris* and *Lactobacillus*. These microorganisms are killed by low pasteurization at 72°C for 15 s and by thermization at 65°C for 15 s (Walstra *et al.*, 1999). However, two other mesophile organisms, *Cl. perfringens* and *B. cereus*, can survive pasteurization processes. These organisms are pathogenic for human because they are able to produce toxins. *Cl. perfringens* has an ability to germinate and multiple in milk under a refrigeration storage condition. For *B. cereus*, it is well accepted as a cause of food poisoning, although general outbreaks that are caused by this organism in U.K. are associated with the consumption of cooked rice. There is a necessity to restrict the growth of *B. cereus* because the germination of the spores and its growth in pasteurized milk will lead to a

development of 'off-flavors', and an appearance that will discourage consumption. The predominance of non-pathogenic serotypes of *B. cereus* in milk is also persistence (Bramley and McKinnon, 1990).

2.2.3 Thermophiles

Thermophiles are organisms that can grow at temperatures of 55°C or higher. Their minimum growth temperature is usually around 45°C and has optimal growth temperatures between 55 and 65°C. *Bacillus stearothermophilus, Thermus aquaticus, Cyanidium caldarium* and *Chaetomium thermophile* are some microorganisms that can be categorized as thermophiles (Prescott *et al*., 2002). In milk and milk products, the thermophilic *B. stearothermophilus* is the most significance microorganism and has a high possibility to survive heat treatments, such as pasteurization. However, the organism is not able to grow at temperature below 30°C and is only caused a significant problem in hot climate countries. It has been well documented that *B. stearothermophilus* could spoil sterilized milk if they were not sufficiently heated (Varnam and Sutherland, 1994; Walstra *et al*., 1999).

2.2.4 *Bacillus* spp.

Members of the *Bacillus* spp. have characteristics of Gram positive rods, grow aerobically and produce spores. They are widely spreaded in soil and water. Most of the species are motile, some species are strictly aerobic, while others are facultative anaerobic. A few species of *Bacillus* spp. can cause disease. For example, a previous report presented an outbreak of *Bacillus* spp. food poisoning in a sanatorium with patients developed symptoms of profuse diarrhea, stomach cramps and vomiting. It was found that a spore forming *Bacillus* was isolated from meatballs that were given as part of the meal to the patients. Although the organism was firstly named as *Bacillus peptonificans*, the organism properties had a resemblance with that of *B. cereus* (Singleton and Sainsbury, 1981; Adams and Moss, 1995).

B. cereus is a facultative anaerobic organism with large vegetative cells, often motile bacilli and has typically 1.0-1.2 μm length in chains. *B. cereus* is Gram positive and catalase positive bacterium. The organism was reported to cause a food

poisoning in 1950. The outbreak was happened in Norway from a vanilla sauce, which had been prepared a day in advance and stored at room temperature before serving. Samples that was tested later on found 2.5×10^7 to 1.1×10^8 of contaminated *B. cereus* cfu/ml. The report of this outbreak described an illness in which diarrhea was the predominant symptom. After that in 1971, they were also reports regarding illness caused by *B. cereus*. The organism was identified to be responsible in two distinct types of food borne illness, which were a relatively late–onset, "diarrhea syndrome" and a rapid–onset, "emetic syndrome". Most of the time, the food poisoning outbreaks that correlated with *B. cereus* occur when the food has been received an abuse condition of time and temperature. The organism is commonly found at low levels in food around 10^2 cfu/g. However, if the food is stored at a good environment condition for *B. cereus* to multiply, then the organism will multiply to a level of more than 10^5 cfu/g, which can intoxicate the people that consumed the food (Singleton and Sainsbury, 1981; Adams and Moss, 1995; Forsythe, 2000).

Since *B. cereus* is ubiquitous in nature, it can predominantly present in milk and dairy products. In fact, spore forming bacteria that spoil dairy products usually originate from raw milk. The defect of raw milk does not correlate with the initial numbers of spore formers. The reason for this is because storage conditions of milk products can support the organisms growth. If a milk product is stored for a long period of time, the small numbers of initial microorganisms can grow and eventually cause a defect (Frank, 1997). In raw milk, *B. cereus* was more commonly found during summer months and was not detected in some winter months (Crielly *et al*., 1994; Larsen and Jorgensen, 1997).

In pasteurized milk, *B. cereus* was isolated from 56% of pasteurized milk samples, and no differences was found between full fat milk, low fat milk and double cream. The mean count of *B. cereus* was high during summer periods in the range between 10^3 and 3×10^5 cfu/ml. Researchers have reported low levels of *B. cereus* contamination in raw milk from the environment. Since the organism can survive pasteurization, this spore forming bacterium has been reported to be the major source of spoilage in pasteurized milk (Larsen and Jorgensen, 1997; Larsen and Jorgensen, 1999). Another research also confirmed that although the incidence of the vegetative cells of *B. cereus* in raw milk from the plant was low (below than 10%), the incidence

and the average count of *B. cereus* spore in raw milk were very high and similar to those of *B. cereus* vegetative cells in pasteurized milk or final products after enrichment at 8°C for 14 days (Lin *et al*., 1998).

The other *Bacillus* spp. that is important in milk and milk products is *B. licheniformis*. Both of *B. cereus* and *B. licheniformis* are capable to form endospores that are highly resistant to heat and drying. Janstova and Lukasova (2001) also found that *B. licheniformis* was the greatest heat resistance as compared to the other *Bacillus* species. The spore of this *Bacillus* can germinate after a heat treatment at 135°C. Therefore, the presence of *B. cereus* and *B. licheniformis* in raw milk becomes an important factor for the milk industries, especially when both of them can survive pasteurization. *B. licheniformis* can be found every where in the farm environment. It was reported that the count of the organism was higher during the winter months. Beside that, *B. licheniformis* can also be found in the laboratory raw milk that has been heat-treated at 80°C for 10 min. For the growth kinetics of *B. cereus* and *B. licheniformis*, it was shown that *B. cereus* grew faster than *B. licheniformis* at ambient temperatures (Crielly *et al*., 1994).

Since *B. cereus* and *B. licheniformis* can grow in dairy products, non-aseptic packaged refrigerated fluid milk can be spoiled because of the growth of these organisms. *Bacillus* spp. is present in more than 80% of raw milk samples. The spores of microorganism can germinate after pasteurization as a result of heat-shocked mechanism. In addition, the organisms can also present in milk products as a result of PPC from the processing plant. The defect characteristics in milk products as a result of *Bacillus* spp. growth are recognized as sweet curdling, coagulation by a chymosin-like protease and bitter flavor in the milk products (Frank, 1997).

2.3 Pasteurization

2.3.1 General

Louis Pasteur, who was a scientist in the 1860's, discovered the importance of pasteurization. The main purpose to pasteurize raw milk is to kill pathogens that can cause illness in humans by the heat treatment (Rosenthal, 1991). To accomplish this purpose, pasteurization is generally categorized into 2 different time and

temperature processes, which are known as High Temperature Short Time (HTST) and Low Temperature Long Time (LTLT).

2.3.2 High Temperature Short Time (HTST)

The first heat treatment is done by heating milk at 72°C for 15 s. After a heat treatment, the pasteurized milk must be cooled to 4°C and kept at temperatures less than 10°C until the product is delivered for consumption. These low storage temperatures are done to accomplish the requirements of food regulation regarding milk products, which have an aim to suppress the outgrowth of any microorganisms surviving the pasteurization process (Kon, 1975; Rosenthal, 1991; Fox and McSweeney, 1998).

2.3.3 Low Temperature Long Time (LTLT)

The other one is done by heating milk at 63°C for 30 min. It is a simple method and has been used widely in small-scale dairy industries.

In Thailand, pasteurization means heating a food product at temperatures not more than 100°C. The process can be conducted in two different time and temperature combinations. The first one is done by heating food products to a temperature not lower than 63°C for a time that is not less than 30 min. While for the second process, it can be carried out by heating food products to a temperature equal or more than 72°C for a minimum time of 15 s. The pasteurized milk either processed by the first or second combination must be cooled immediately to a temperature at or below than 5ºC (Ministry of Public Health, 2002a; Ministry of Public Health, 2002b).

Since pasteurization processes use a time and temperature combination that lower than sterilization processes, the pasteurization only destroys majority of pathogenic and spoilage microorganisms, particularly organisms that are not heat resistant. Therefore, the pasteurized milk should always be kept at a refrigeration temperature, which should not be higher than 8ºC, during filling, distribution and subsequent storage in the consumer's household. Beside this restriction, the shelf life of pasteurized milk is also limited, which is less than 10 days (Kon, 1975; Ministry of Public Health, 2002a; Ministry of Public Health, 2002b).

2.4 Nisin

2.4.1 The history of nisin

Fermentation is a traditional method to preserve food. The occurrence and process of fermentation can be naturally take place due to activities of microorganisms. The whole fermentation process mainly depends on the activity of fermented microorganisms to produce metabolites, which can prevent the growth, and survival of undesirable microflora in food. A lactic acid bacteria (LAB) group is one type of microorganisms, which can produce several microbial metabolites that contribute to the extension of the shelf life and quality of some food products, including wine, beer, cheese, yogurt and soy sauce. It was found later on that one species of LAB, *Lc. lactis* subsp. *lactis* can produce an antimicrobial compound, which is called as 'nisin'. As a result of biological metabolism, nisin has an ability to inhibit or reduce the growth of some undesirable microflora in food products (Thomas *et al.*, 2000).

Nisin was firstly discovered in England in 1928 by Rogers and Whittier during a process of cheese making. They found that a lactic streptococci species was inhibiting the growth of a milk starter culture. Shortly after that in 1933, similar problems were experienced by cheese manufacturers. The workers found that starter cultures that were added to milk failed to clot the milk within 18 h incubation time during a cheese making. Afterwards, in 1947 researchers characterized the compound and called it "nisin" because it was produced from lactic streptococci strain of the serological group N Inhibitory Substance. The suffix "-in" was commonly used for antibiotics at that time (Thomas *et al.*, 2000; Paul Ross *et al.*, 2002).

In 1953, the first commercial preparation of nisin was made in England by Aplin and Barrett, Ltd., and since then, the compound has been used in over than 48 countries all over the world to prevent clostridia spoilage in processed cheese products. In the late 1969, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additive recognized nisin as a safe and legal biological food preservative. The FAO/WHO Codex Committee on milk and milk products accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg pure nisin kg^{-1} product. This bacteriocin was also added in

the European food additive list where it was assigned with a number of E234 (Thomas *et al.*, 2000; Paul Ross *et al.*, 2002).

2.4.2 Structure of nisin

Nisin is a 34-residue long peptide belong to a family, called as lantibiotic. The lantibiotics were originally subdivided into two groups, the elongated type A group and the globular type B group. Nisin is part of the type A group because it has a linear structure rather than a circular structure (group B). It has a pentacyclic structure with one lanthionine residue ring and four β–methyllanthionine residue rings. The nature variances of nisin that have similar activities, nisin A and nisin Z have been found. Nisin Z is differed from nisin A at position 27 of amino acid residue, nisin Z is being an asparagine, but a histidine in nisin A (Breukink and de Kruijff, 1999; Paul Ross *et al.*, 2002). The structure of nisin is illustrated in Figure 2.1.

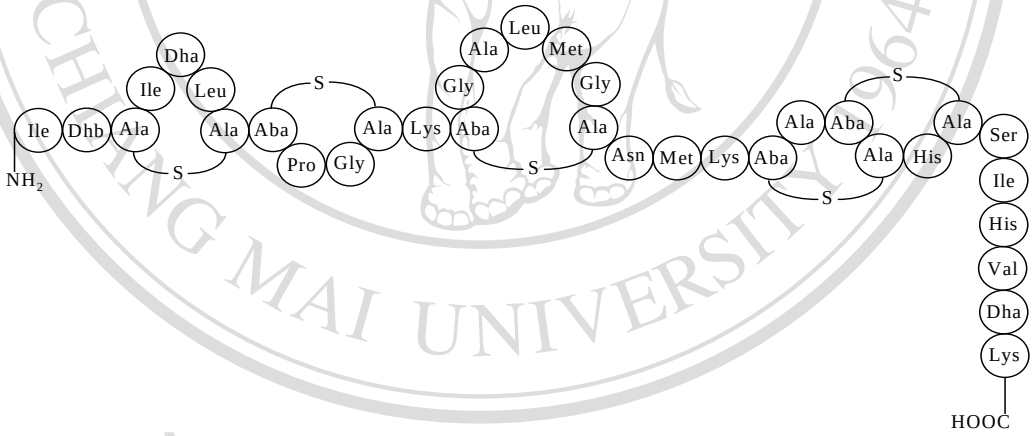

Figure 2.1 Primary structure of nisin A. Dha: dehydroalanine, Dhb: dehydrobutyrine, Ala-S-Ala: lanthionine, Aba-S-Ala: β-methyllanthionine (de Vuyst and Vandamme, 1994).

Nisin is positively charged (+4) in overall and has two rigid ring systems, one is located in N-terminal and the other in C-terminal. The nisin molecule has amphiphathic characteristics, which the half N-terminal of nisin is more hydrophobic than the other half C-terminal (Breukink and de Kruijff, 1999).

2.4.3 Activity of nisin

Nisin has a predominant activity against Gram positive bacteria. It killed its target bacteria by pore formation in the target membrane cell. Nisin made a pore in a cell membrane and caused rapid effluxes of small cytoplasm compounds, which included amino acids and ATP. If the leakage of these compounds was severe, the target cell could die. The pore formation also caused collapse of ion gradients and dispersed the proton motive force of the bacteria (Breukink and de Kruijff, 1999; Paul Ross *et al.*, 2002). Since nisin is positively charged, the compound was found to be selective to interact with negatively charged membranes and bind to membranes containing anionic lipid. Therefore, bacteria that have a high concentration of anionic lipid in their membranes will be susceptible to nisin (Breukink and de Kruijff, 1999).

Spore forming bacteria, including *Bacillus* and *Clostridium* species are very sensitive to nisin. Nisin is effective not only against the vegetative cells of these bacteria, but also against their spores. The effectiveness of nisin on vegetative cell or spores depends on several factors, including size and age of inoculum, composition of medium and pH of solution that nisin is dissolved. For example, the effectiveness of nisin in preventing the outgrowth of *Cl. botulinum* spores type A, B and E in trypticase peptone-yeast extract-glucose broth was found to be more effective in acidic substrates at pH 5.5 and 6.0 than at pH 7.0 or 8.0 (de Vuyst and Vandamme, 1994). In its action against spores, nisin does not affect the swelling of spores, which accompanies germination of *B. cereus* and *Bacillus subtilis*, but it does inhibit rupture or lysis of spore coats during emergence. Research has also similarly shown that the post-germination swelling and subsequent stages of spore development were inhibited by nisin. The degree of sensitivity of spores appears to be related to the post-germination mechanism, especially for spore that rupture their spore coats. The spores which had a lysis mechanism for rupturing their spore coats had a higher resistance to nisin than the spores that had a rupture mechanism. Therefore, the germination of spores by rupturing their coats would be more sensitive to nisin (Thomas *et al.*, 2000; de Vuyst and Vandamme, 1994).

Nisin is best added as an aqueous solution, usually mixed in the liquid part of products during the process. For example, nisin can be combined with the brine solution of a canned food and then mixed the brine into the whole product. In dairy

desserts and milk, it can be added to a small quantity of milk and then mixed the milk into the bulk part, filled and processed. Although nisin can be added as a powder, it is essential to ensure that the protein disperses thoroughly throughout the food matrix. The best time to add nisin is at the last practice stage before heat processes.

The effectiveness of nisin depends on several factors. The first factor is the length of time and the high of temperature of a heat treatment. Temperature was the most significant variable and the effectiveness of nisin became less as the temperature increased. A certain ratio of nisin is lost during a heat treatment. Therefore, the level of nisin added to a food product must be higher than the nisin effective level to compensate those that may loss during a heat treatment (Thomas *et al.*, 2000). In Ultra High Temperature (UHT) processes, the loss of nisin can reach up to 40%. However after a heat treatment, there should be a few number of spores that can survive. Thus, less concentration of nisin may be enough to prevent these spores outgrowth. Beside that, the heat treatment may damage the endospores and makes these spores more sensitive to nisin (Delves-Broughton *et al.*, 1996). Furthermore, the effect of temperature of a heat treatment is affected by the pH of the nisin solution. It was found that nisin was most stable to be autoclaved at a solution with a pH value of 3. At this pH, the activity of nisin would be lossed for less than 5% when the solution was heated at 115°C for 20 min and 15% loss when using a heat treatment at 121°C for 15 min (Davies *et al.*, 1998).

The second factor is the food matrix. This factor is important because nisin needs to be mixed properly throughout a food mixture to ensure its effectiveness. Different food matrixes may affect the uniformity of nisin distribution particularly for the spatial heterogeneity of a food matrix. If the distribution of nisin is not uniform, there is a potential that nisin fails to protect the whole food product (Thomas *et al.*, 2000). Nisin has a hydrophobic characteristic, therefore the molecule can be conjugated with lipid components in food. If this is happened, then nisin will be unavailable to function as an antimicrobial agent. Researchers had found that milk fat reduced the nisin activity. This activity would be reduced to approximately 33% in skim milk and more than 88% in milk with 12.9% fat content (Jung *et al.*, 1992). In contrast, another report showed no significant difference in the activity of nisin to inhibit *Listeria innocua* in whole and skim milk (Zapico *et al.*, 1999).

The third factor is pH values of food. Nisin is a cationic molecule due to a combination of three lysine and one or more histidine residues. It is more soluble at acid pH and becomes less soluble when the pH is increased (Thomas *et al*., 2000). This could be illustrated from the nisin activity against *B. cereus* in buffers at low pH values of 6.3 and 5.75. It was found that nisin was more effective against *B. cereus* at these two pH values compared to its activity at pH 7.0 (Periago and Moezelaar, 2001). Liu and Hansen (1990) also found that nisin had a solubility of 56 mg/ml at pH 2.2 compared to 3 and 1 mg/ml at pH 5.0 and 11.0, respectively. However, the solubility of nisin was not a main problem in food products, because in its application, nisin is often to be used in acidic foods. As an overall, it was concluded by Thomas *et al.* (2000) that nisin was effective in a wide range of pH values at 3.5-8.0.

Fourth, it is the species of bacteria in food products. Nisin can inhibit Gram positive bacteria because it is able to make pore formation and efflux small cytoplasm compounds. Gram positive bacteria have an important role in food products, including milk and milk products. Some species of the bacteria, mainly *Bacillus* spp. and *Clostridium* spp., can form heat resistant endospores. Gram negative bacteria are more resistant to nisin because they have outer membranes that prevent the binding and inserting of nisin. However, they can be more sensitive to nisin if the addition of nisin is combined with other treatments, such as hydrostatic pressure, heat treatments, freezing and thawing. These treatments will damage the outer membrane of cell wall to allow the bacteriocin having a direct access to the cell membrane (Thomas *et al.*, 2000).

The next important factor is the concentration of nisin. The concentration of nisin can be reduced due to food processing, particularly heat treatments. Therefore, the level of nisin that is added to food products should be adjusted to consider the loss during processing. For example, nisin could be lost approximately 15-20% during the melting process in a cheese making. This loss could increase depending on the degree of heat treatments, the time period of the process and the pH values of the cheese product itself (Thomas *et al.*, 2000). It is desirable to have a good retention level of nisin in the finished products to make sure that nisin is effective in preventing the spore outgrowth and inhibiting the bacteria growth.

The last factor that affects the effectiveness of nisin is the shelf life of food products. The effectiveness of nisin will be reduced during storage of a product. Most of the time, this reduction will occur slowly at low storage temperatures. A report that studied the effectiveness of nisin in processed cheese spreads at different storage temperatures found that at 30°C storage temperature, the retention level of nisin was reduced more than in the products that were kept at lower storage temperatures of 20 and 25°C (Delves-Broughton, 1990). Therefore, it is important to maintain the level of nisin above the minimum level necessary to inhibit food spoilage throughout the storage time of the nisin-added food products.

2.4.4 Safety of nisin

The production of nisin is from a fermentation of a lactoccoci culture, which is occurred naturally in raw milk and cheese. Nisin has been called as a biopreservative, that can be used as a single preservative or in a combination with other preservatives or processing methods to protect food from spoilage and health hazard problems. Nisin has been reported to inhibit both spoilage and pathogenic microorganisms (Paul Ross *et al.*, 2002). Nisin has been consumed for centuries and it is not harmful for human and animal. It is not toxic at the level that is used in food. Besides that, enzymes can degrade the compound rapidly and nisin does not alter the microflora in the intestinal tract when it is consumed orally. An International organization, the FAO/WHO Expert Committee, had made a conclusion from the available evidence that nisin at a level of 3.3×10^6 unit/kg body weight did not have any adverse effects to human. The Committee also permitted an unconditional Acceptable Daily Intake (ADI) at a level of 3.3×10^4 unit/kg body weight in 1969. In U.S.A., the U.S. Food and Drug Administration has affirmed nisin as Generally Recognized as Safe (GRAS) compound and it is allowed to be directly used as an ingredient in human food (Thomas *et al.*, 2000). All of these standards are confirming the safety of nisin and its tolerance level to use. Nowadays, more countries have permitted the use of nisin in several different products. In USA, 250 μg/g nisin is allowed to be used in some food products, including processed pasteurized cheese spread. For milk products, pasteurized and flavored milks are permitted to be added with nisin without any maximum level in some countries, such

as Abu Dhabi, Bahrain and Dubai. About more than 48 countries has approved the use of nisin in many food products. There countries are included Malaysia, Singapore, Australia, India, China, Brazil, Russia and Slovak Republic. In Thailand, nisin is approved to be used in processed cheese with a maximum level of 100 μg/g product (Thomas *et al.*, 2000).