CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

2.1.1 Characteristics of Lactic Acid Bacteria

Lactic acid bacteria (LAB) are Gram-positive bacteria that belong to a number of diverse genera including *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, *Weisella* and *Lactobacillus* (Axelsson, 2004). The common characteristics of these bacteria are nonspore forming, nonrespiring, cocci or rods in shape with nonaerobic habit but aerotolerant that have ability to metabolise lactose sugar into lactic acid during fermentation of carbohydrates as the major end product (Halász, 2009) (Figure 2.1).

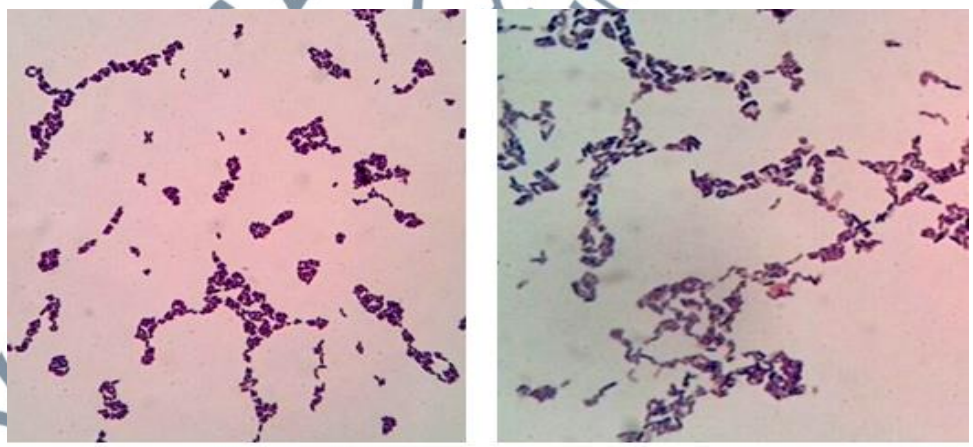


Figure 2.1: Morphology of Lactic Acid Bacteria in Cocci and Rod Shapes

The first pure culture of a LAB previously named "*Bacterium lactis*" was obtained by Joseph Lister in 1873 ten years after Louis Pasteur studied lactic acid fermentation. First monograph of LAB established by Orla-Jensen in 1919 found great impact on the systematic development of LAB had been used until now (König & Fröhlich, 2009). According to Orla-Jensen's monograph, the point should be considered to classify LAB are the morphology (cocci or rods, tetrad formation), glucose fermentation mode (homo- or heterofermentative), growth at certain "cardinal" temperatures (e.g: 10 ºC and 45 ºC), and lactic acid production form (D, L, or both) (Khalid, 2011; Okano et al., 2009).

The morphology of LAB varies from long, slender rods to cocci in structure which frequently form chains or in tetrad formation (Khalid, 2011). The mode of glucose fermentation by LAB was based on the sole end product of glucose called lactic acid or lactate. The homofermentative species of LAB produce lactic acid (< 85%) while the heterofermentative species of LAB produce lactic acid, carbon dioxide, and ethanol/acetate as their sole end product (König & Fröhlich, 2009). Not all genera of LAB are able to grow at the same temperature. Some LABs have their own optimum temperature growth such as at 8, 10, 15, or 45 ºC (Carr et al., 2002). The other characteristics to classify the genera of LAB are isomer forms of lactic acid which consist of two optical isomers of lactic acid which are L(+)-lactic acid and D(–)-lactic acid but in some cases mixture of DL-lactic acid (Okano et al., 2009). Then, researchers added other characteristics of LAB should be considered to classify LAB such as ability of LAB to grow at high salt concentrations and acid/alkaline tolerance, but these four characteristics studied by Orla-Jensen are still very important and fundamental for LAB classification. After that, the core of LAB represented for four genera; *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Khalid, 2011).

2.1.2 Source of Lactic Acid Bacteria

The LABs are ubiquitous in nature and their presence are generally from plants such fruits and vegetables, animals, and other sources (Table 2.1). For examples, 205 and 496 LAB strains were successfully isolated from fruits and vegetables, respectively (AbuBakr et al., 2012b; Trias et al., 2008). Previously, 75 LAB strains were isolated from meats (lamb, beef, and pig) and numerous LAB dominated with *L. plantarum* were isolated from various samples of fish and prawn (Jones et al., 2008; Nair & Surendran, 2005). In fact, three LABs identified as *L. lactis*, *Pediococcus pentosaceus*, and *Lactobacillus curvatus* were also successfully isolated from *Polygonum minus* leaves (Malaysian local herbs) using 16S rDNA genotype-based methods (Baradaran et al., 2012).

In general, foods consist of an abundance of LABs. A total of 41 LAB strains were successfully isolated from food samples included raw meat, fruit, vegetables, and herbs collected from Stillwater, Oklahoma State (Henning et al., 2015). Furthermore, Pundir et al. (2013) reported that 26 LAB strains was successfully isolated from vegetables, fruits, and ready-to-eat foods collected from Ambala, Haryana. Besides, 32 LAB strains were successfully isolated from honey produced in Malaysia, Libya, and Saudi Arabia (Aween et al., 2012).

Indeed, LABs were not only found in nature but may also abundantly available in the most fermented foods such as in Korean fermented vegetables known as Kimchi (Cho et al., 2013), fermented chili fruits (Husain et al., 2017), fermented milk products like yogurt, Laban and Dahi (Ali, 2011; Chammas et al., 2006; Harun-ur-Rashid et al., 2007). Moreover, the LABs can also be found in a sourdough because these bacteria were responsible for the rheology, flavour, and nutritional properties of sourdough-based baked products (Gobbetti et al., 2005; Manini et al., 2014). Other than that, the

LABs were discovered in other sources such as in soils and faeces. Previous studies reported that 14 LAB strains were found from local Malaysian soils while 14 LAB strains were successfully isolated from newborn baby faeces obtained from a hospital at Jeonju, Korea (Husain et al., 2017; Park et al., 2002).

Table 2.1: Sources of Lactic Acid Bacteria Isolates

Source	Name and Part	Total of LAB	References
Animal	Meats of lamb, beef and pig	75	Jones et al., 2008
Animal	Fish and prawn	337	Nair and Surendran., 2005
Beef cattle rumen Feces Fruits Herbs Raw meat Raw milk Vegetables		41	Henning et al., 2015
Bread making	Sourdough		Manini et al., 2014
Feces		14	Park et al., 2002
Fermented food	Dahi	266	Harun-ur-Rashid et al., 2006
Fermented food	Fermented chilli	7	Husain et al., 2017
Fermented food	Kimchi	106	Cho et al., 2013
Fermented food	Laban	96	Chammas et al., 2006
Fermented food	Yogurt	303	Ali, 2011
Fruits		205	Abubakr et al.(b), 2012
Fruits Vegetables		484	Trias et al., 2008
Fruits Ready-to-eat foods Vegetables		26	Pundir et al., 2013
Herb	Kesum leaves	3	Baradaran et al.,2012
Honey		32	Aween et al., 2012
Soils		14	Husain et al., 2017

2.1.3 Benefits of Lactic Acid Bacteria

It is well-known that LABs are generally recognised as safe (GRAS), and these bacteria were already used a long time ago during an ancient time in food products

especially as starter cultures in the making of fermented foods such as fermented vegetables, meats, dairies products, alcoholic beverages, and have also been used in bread making to ferment dough (Carr et al., 2002). The LABs were not only one of the factors of food fermentation to succeed, but also may contribute for a nice flavour, aroma, and prolong the shelf-life of some food products (Kunene et al., 2000).

During ancient times making varieties of foods, ancestors may not realise implementing LAB into their cooking such as in the making of yogurt and cheese. As food knowledge and technology developed, scientists found that the LAB may be responsible for some formation of food products mainly in fermented foods. Through the years, more studies have been done and the field work of LABs varied, evolved, and went deeper due to their uniqueness. The implementation of LABs into certain products and its application has increased tremendously, especially in food industry. These also increased public concern on scientific research regarding the LABs whether these bacteria may harm or improve health. Interestingly, LAB found upgrade certain foods to become more valuable include improve health by overcoming or reducing certain diseases because of their potential related to health benefits (Soomro et al., 2002).

Some species of LABs may contribute and improve nutritional value of certain foods, control of intestinal infections, improved digestion of lactose, control of some types of cancer, and control of serum cholesterol in blood levels. These potential benefits of LABs may result from growth and action of the certain LABs during manufacture of cultured foods. In other cases, the potential benefits of LAB may result from growth and action of certain LAB in the intestinal tract following ingestion of foods containing them (Gilliland, 1990). Therefore, in order to obtain the specific benefits of LAB, the selection of LAB strains within a given species should be selected specifically.

2.1.3.1 Proteolytic System of Lactic Acid Bacteria

In the making of fermented foods including dairy products, several metabolites' products of LAB were applied to give impact of special functions such as flavour development, and in ripening process of dairy products such as in cheese or yogurt making (Kunji et al., 1996). One of the LAB metabolites is through proteolytic system. Proteolysis or proteolytic activity is a protein degradation that hydrolyses one or more peptide bonds in a protein either through catalysis of proteolytic enzymes, or non-enzymatic activity such as due to very low, or very high in pH (Varshavsky, 2001).

According to Savijoki et al. (2006), proteolytic activity of LAB in casein resulted in, and came out with a model for a proteolytic system of LAB consist of casein proteolysis, transport, peptidolysis, and its regulation when using *L. lactis* as baseline study. Based on structural components of LAB, the proteolytic system of LAB comprises three major components which were (i) cell-wall bound proteinase that initiates the degradation of extracellular casein (milk protein) into oligopeptides, (ii) peptide transporters that take up the peptides into the cell, and (iii) various intracellular peptidases that degrade the peptides into shorter peptides and amino acids (Kunji et al., 1996; Liu et al., 2010).

For example, during milk fermentation, the milk proteins are commonly hydrolysed by proteinase and peptidase enzymes of LAB that enhance the amount of free amino groups and peptides. The ability to hydrolyse proteins may improve the quality of fermented foods such as prolonging the shelf-life of cheese during storage and may generate other beneficial effects of bioactive peptides (Donkor et al., 2007). By manipulating the pathways of protein and peptide degradation, the proteolytic system of LAB has become a great source and trending research due to their ability to hydrolyse protein into simple peptides and amino acids for further research. These

developments have paved the way to new, more economical, and better quality food products (Farahani et al., 2017; Kunji et al., 1996).

2.1.3.2 Lactic Acid Bacteria as Probiotic Bacteria

One of the biggest benefits of most LABs was their properties as probiotic bacteria. During expert consultation that was held at Cordoba, Argentina on Probiotics in Food, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) expert consultation defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). The probiotic properties of these microorganisms were able to tolerate to acid and bile, ability to adhere into mucosal and epithelial surfaces for immune modulation, have antimicrobial activity against pathogenic bacteria as well as are able ty to hydrolyse bile salt (Kechagia et al., 2013).

The most common microorganisms used as probiotics available in the market nowadays are from the *Lactobacillus* and *Bifidobacterium* genera (Figure 2.2). While other bacterial genera of LABs found to be potential as probiotic also *Enterococcus*, *Streptococcus*, and *Escherichia* (Mack, 2005).

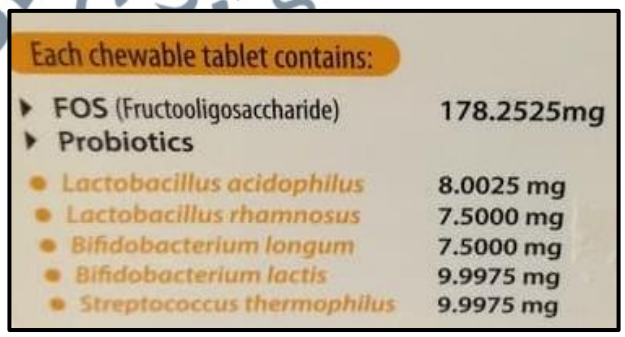


Figure 2.2: The Examples of Probiotic LAB Species in a Health Supplement

Nonbacterial organisms such as a nonpathogenic yeast *Saccharomyces boulardii* have also been used and considered as probiotics and had been applied in yogurt making (Szajewska et al., 2006). Furthermore, the probiotic LAB can be found in feed, health supplement, and fermented foods especially in fermented milk products such as cheese making, cultured drinks, and yogurt. The most probiotic LABs seen in food labeling were *L. plantarum, Lactobacillus bulgaricus,* and *Lactobacillus casei.*

Historically, in 1908, the Russian Nobel Laureate immunologist named Elie Metchnikoff suggested that consuming large amounts of fermented milk products could prolong the life of Bulgarians. His idea created great interest in the role of healthy gut microbiota to improve lifestyle (Binns, 2013). The potential of probiotics to improve certain health issues are still in interest such as implementing probiotics to prevent bowel disease, improve immune system, treat lactose intolerance, balance intestinal microbiota, exhibit antihypercholesterolemic as well as antihypertensive effects, and reduce diarrhoea (Ghosh et al., 2019; Kechagia et al., 2013; Wedajo, 2015). Probiotic bacteria may also regulate brain processes through a bidirectional communication network known as the gut microbiota brain axis (GBA). These clinical studies suggested that the microbiota and its metabolites may affected the behaviours and brain processes via GBA such as stress responsive, anxiety, depression and pain modulation for those with fibromyalgia and irritable bowel syndrome (Pinto-Sanchez, 2017; Roman et al., 2018).

Previously, LAB with probiotic properties isolated from piglet faeces identified as *Pediococcus acidilactici* FT28 may have antioxidant properties when compared to the control (without microorganisms) and *L. acidophilus*. By running an erythrocytic antioxidant profile test, the finding suggested that supplementation of swine origin with *P. acidilactici* FT28 may improve antioxidant value to combat weaning stresses in

piglets (Dowarah et al., 2018). To date, studies on health effects of probiotics on humans and animals are still undergoing and evolves, thus, it can be contributed to society in many ways.

2.1.4 Identification of Lactic Acid Bacteria

Identification of LAB strains were established a long time ago. It is important to identify the strains because each LAB may have different characteristics that should be considered and, thus, allow the industry to manipulate the LAB's properties and benefits to society. Previously, identification of LAB using phenotypic and chemicals methods which depend on the general characteristics of LAB such as different carbohydrate fermentation, hetero or homo fermentation, gas production, motility, and either spore or nonspore producing bacterial types (Astuti, 2016; Khalil and Anwar, 2016). These can be done by isolation of LAB colonies at a specific media, characterise the pure culture of LAB morphologically and biochemically, and proceed with identification technique.

In fact, identification of LAB was developed many years ago and researchers came out with more selective and sophisticated techniques to identify these bacteria. For example, rapid identification of LAB through biochemical tests miniature which only using Analysis Profile Index (API) kits such as API 38 CHL strips, API 20 STREP, and API 50 CHL (Khalil & Anwar, 2016; Lu et al., 2008; Soda et al., 2003; Suhartik et al., 2014; Yuliana & Dizon, 2011). The principle behind each test kits was based on the carbohydrate fermentation which is provided in each well and this took between 24 and 48 h incubation time, which is faster than other chemical methods. Using these kits, the identification of LAB is more reliable with less time consuming compared to conventional methods.

However, in microbiology field, the identification of microorganisms including LAB should be supported with molecular biological tools which are based on nucleic acids, and other macromolecules. The genotypic identification by nucleic acid-based tools is more commonly used for the last decades because of the accuracy and efficiency potential outcome is high provided using PCR amplification or ex situ/in situ hybridisation either with DNA, RNA, or peptide nucleic acid probes (Ben Amor et al., 2007; Tilahun et al., 2018). As a result of the widespread use of PCR and DNA sequencing, the 16S rDNA sequencing has played a vital role in the accurate identification of bacterial isolates and the discovery of novel bacteria in the microbiology field (Woo et al., 2008).

In genotypic identification, usually universal primers are used for bacterial identification include LAB. Usually, two universal primers based 16S rDNA sequence specific primers which are 27f forward (5'-AGAGTTTAGTCCTGGCTCAG-3') and 1492r reverse (5'-GGTTACCTTGTTACGACTT-3'), two specific primers of 16S forward (5'-AGAGTTTGATCCTGGCTC-3') and 16S reverse (5'-CGGGAACGTATTCAC-CG-3') are used in identification (Lawalata et al., 2011; Maryam & Wedad, 2017). The genotypic identification of LAB will then compare through profile matching based on the phenotypic characteristic traced using Bergey's Manual of Determinative Bacteriology to estimate the possibility of the bacterial genus and species based on the referenced LAB's genus and species (Astuti, 2016).

2.2 Milk

Milk is a white liquid produced by the mammary glands of mammals (Hashmi & Saleem, 2015). It is the first most complete food for mammals that supplies all the energy, high in nutritious property and considered as an important constituent of a

balanced diet needed for the proper growth and development of the neonate. For all mammals, the consumption of milk ends at the weaning period except for humans that continue consuming milk throughout their life depending on human own demand (Hashmi & Saleem, 2015; Hsieh et al.,2014). The major components of milk are water, fat, lactose, and protein (Stokes et al., 2000). Furthermore, milk is also a source of many bioactive components in proteins, lipids, carbohydrates, lactose, vitamins, minerals, enzymes, hormones, immunoglobulins, and growth factors (Hseih et al., 2015).

Each year, the world dairy market is constantly evolving, and the milk production is consistently increasing to fulfill worldwide requests. The production of cow milk represents 84.0 % of total world milk production followed by buffalo (13.0 %), goat (2.2 %), sheep (1.3 %), and camel (0.2 %) milk (International Dairy Federation, 2009). These percentages were slightly changed into 85.0 % for cow milk followed by 11.0 % for buffalo milk and buffalo milk demand was still in second place (Gerosa & Skoet, 2012). Generally, the world demands for milk production are whole milk powder, cow milk cheese, liquid milk, butter, skim milk powder, condensed milk, whey products, and casein (The World Dairy Situation, 2010). In the year 2014, the total milk production had increased up to 3.3 % compared to 2013 and was estimated at around 802 million tonnes (The World Dairy Situation, 2015).

Increasing demand for milk and dairy products in every year has lead scientists to study the composition and physicochemical of milk, the microflora existence in milk, the nutritional values of milk, the health benefit contribution of milk and many more approaches (Gakkhar et al., 2015; Stokes et al., 2000; Soliman, 2005; Mahmood & Usman, 2010; Samarźija et al., 2012; Hashmi & Saleem, 2015; Jenkins & McGuire, 2006; Bhopale, 2016). Most milk research focuses on cow milk as a representative milk

apparently because of a larger number of cow milk production compared to other types of milk and can be acquired in any country (Ng-Kwai-Hang et al., 1984; Stokes et al., 2000). Despite the huge difference between the world production of cow milk and buffalo milk, the percentage for the buffalo milk production has constantly increased year by year (The World Dairy Situation, 2015).

2.2.1 Buffalo Milk

Domesticated buffalo or scientifically known as *Bubalus bubalis* is generally categorised as Asian and Mediterranean buffaloes with two main sub species; River (Murrah group), and Swamp buffaloes that are 95 % present in Asia (Amano et al., 1980; Pasha, 2013) (Figure 2.3). In 1998, the total population of buffaloes in Malaysia was about 170, 000 and 60 % were mostly concentrated in the rice growing states of Kelantan, Terengganu, Kedah, and Pahang in West Malaysia (Borghese & Mazzi, 2005). The buffaloes are also referred to as a triple purpose animal because it provides leaner meat, nutritious milk, and mechanical power to humankind that offers massive potential for the improvement of livelihood (Pasha & Hayat, 2012). Besides, the buffaloes were used for farming has long been preferred because of their efficient application of low quality, high roughage, resistance to parasites, quick and easy calf growth, good meat quality as well as rich in milk and milk products (El-Salam & El-Shibiny, 2011).

Instead of cows, buffaloes play an important role in the milk production system in most of Asian countries including Malaysia (Pasha & Hayat, 2012). Buffalo milk is one of the valuable milks and has become one of the research subjects in milk and dairy industry because of its richness in nutrient contents with a great economic value (Mahmood & Usman, 2010; Ng-Kwai-Hang et al., 1984; Rizqiati et al., 2015). The

buffalo milk is pure white in colour with thick viscosity and rich in taste (Ng-Kwai-Hang et al., 1984; Soliman, 2005). The composition of buffalo milk was similar to other types of milk produced by cow, goat, sheep, camel, and human milk, which was fat, protein, lactose, total dry matter, vitamins, and minerals but with different percentages or ratios (Atanasova & Ivanova, 2010; Hseih et al., 2015; Jrad et al., 2014; Murtaz et al., 2014; Ng-Kwai-Hang et al., 1984; Soliman, 2005).

Figure 2.3: Murrah Buffaloes Located at Bangi Buffalo Farm

Compared to other milk, buffalo milk has higher protein content than other types of milk including human, cow, camel, and goat milks (Hashmi & Saleem, 2015; Mahmood & Usman, 2010; Soliman, 2005). Generally, milk proteins consist of approximately 80 % casein, and 20 % whey proteins. The separation of casein and whey proteins will be accomplished through acid or rennet precipitation (O'Mahony & Fox, 2014). Caseins in milk is divided into subclasses of α-, β-, and k- caseins (Mohanty et al., 2015). In buffalo milk, almost all caseins are presented in micellar form rather

than submicelles. In contrast, casein micelle in cow milk were only 90 to 95 % of the casein while the rest is presented in serum phase (Arora & Khetra, 2017; Khedkar et al., 2016). Micellar casein is important because is a slow-digesting protein which releases the amino acids at slower rate that may contain bioactive peptide (Fekete et al., 2015).

Through casein precipitated, whey remained soluble in liquid form after reaching isoelectric precipitation of milk at pH 4.6 (O'Mahony & Fox, 2014). While whey proteins in milk comprised of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, glycomacropeptides, and other minor proteins such as lactoperoxidase, lysozyme and lactoferricin (Atanasova & Ivanova, 2010; O'Mahony & Fox, 2014; Mohanty et al., 2015). Basically, the separation of whey based on differences in electrical charge by electrophoresis or in molecular size by microfiltration and ultracentrifugation with the isoelectric point of whey at pH 4.5 to 5.35 (Lecoeur et al., 2010).

In the context of fat content, buffalo milk is higher in total solid fat than in cow milk which the fat content could interrupt casein and whey separation. Whey is also preferred as a by-product in cheese making that had been made through a process of agglomeration of casein micelles in milk (Lappa et al., 2019). To obtain a complete separation of casein and whey of milk protein in buffalo milk, milk fat globules should be removed first.

2.2.2 Milk Fermentation

Fermentation originally comes from the Latin word *fervere,* which was defined by Louis Pasteur as "La vie sans l'air" which means life without air. From a biochemical view, fermentation was defined as a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidising agent (Bourdichon et

al., 2012). The meaning of fermentation can be simplified as a process of breaking down larger complex molecules such as starch or sugar into smaller molecules of by the action of microorganisms such as yeast and/or bacteria under anaerobic conditions (Vuppala et al., 2015; Tamime, 2002).

Since ancient times, ancestors applied fermentation concepts into foods to preserve and prolong the shelf-life of foods. Several fermented foods from traditional to modern approaches available nowadays are based bean, grain, vegetable, fruit, honey, dairy, meat, fish, and tea sources (Vuppala et al., 2015). One of the most popular fermented foods is milk or dairy fermentation. Milk from species of domesticated mammals such as cow, buffalo, sheep, goat, and camel has been used to make traditional fermented milk products. In fact, it has been practised by human beings throughout the world for thousands of years ago (Tamime, 2002). The evidence from archaeological studies suggested that certain civilizations such as Sumerians, Babylonians, Pharos, and Indians were well advanced in agriculture and in the production of fermented milks. They also suggested that the origin of these fermented milks may have started from the Middle East and the Balkans, and, then evolved through the ages and was dependent on the culinary skills of the inhabitants living in these regions (Kroger et al., 1992; Tamime, 2002).

The process of fermenting milk basically is practiced for extending the shelf-life of milk and one of the oldest methods for milk preservation. Compared to fermented milk, the characteristics of natural milk are highly perishable, therefore, the main purpose of fermenting milk using microorganisms especially LAB species is to prolong its shelf-life (Widyastuti et al., 2014). Historically, milk and fermented milk products have been consumed as beverages or added ingredients in making foods. But, as time passed, a variety of fermented milks reacted such as snackers and grazers especially in

the dessert and confectionery categories depending on the food technologists and food innovators in that era (Kroger et al., 1992). The combination from the previous art of ancient craft with the application of sciences (microbiology and enzymology, physics and engineering, and chemistry and biochemistry) produced numerous types of fermented milks that benefit the consumers (Tamime, 2002).

From a biological standpoint, fermented milks are characterised by the accumulation of microbial metabolic products of lactic acid, ethyl alcohol and other chemicals (Kroger et al., 1992). Microorganisms used in milk fermentation mostly the LAB, yeast, mould, and the combination of these three (Tamime, 2002). In fact, the LABs have also been widely applied in other food fermentation worldwide due to their well- known status as GRAS microorganisms (Widyastuti et al., 2014).

Naturally, the milk itself is one of the famous habitats for LAB. Several studies successfully isolated LAB from milk sources (Franciosi et al., 2009; Henning et al., 2015; Puniya et al., 2016). From cow milk samples, LAB identification belongs to six genera of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Franciosi et al., 2009). While two LAB strains isolated from raw cow milk and identified as *E. faecium*.(Henning et al., 2015). In the study of isolation of LAB from raw and fermented milk, about 50 LAB strains were found in cow, goat, and buffalo milk while another 15 LAB strains from fermented milk of Dahi and Lassi (Puniya et al., 2016).

In the food industry, the LAB are mostly chosen as starter cultures in the making of dairy products like cheese, yogurt, and kefir. One of the purposes of LAB inoculated in the milk is to accelerate and steer the fermentation process of milk to produce the desired products (Leroy & De Vuyst, 2004). Then, as the research field of LAB

developed, the fermented milk inoculated with LAB generated peptides with many potentials of biological health (Korhonen & Pihlanto, 2006).

2.3 Bioactive Peptides

2.3.1 Bioactive Peptides in Milk

Protein is a macronutrient that provides essential amino acids for growth and maintenance of physiological functions. Consumers may gain protein from different food sources including meat, fish, milk, nuts, beans, and seeds. However, milk and other dairy products provide more than basic nutrition of protein. Several studies revealed that milk protein offered bioactive peptides with wide range of biological functions (Abubakr et al., 2012a; Bhopale, 2016; Nandhini et al., 2012; Szwajkowska et al., 2011; Unal & Akalin, 2012).

Some of the milk proteins were hydrolysed into biologically active peptides responsible as antihypertensive (Bhopale, 2016), antimicrobial properties (Bhopale, 2016), anticancer (Bhopale, 2016), immunomodulatory (Mohanty et al., 2015), angiotensin-converting enzyme (ACE) inhibitory (Nandhini et al., 2012), and antioxidant activities (Abubakr et al., 2012a). These bioactive peptides contribute to better health to the physiological system through the cardiovascular, nervous, digestive, and immune systems (Korhonen & Pihlanto, 2006) (Table 2.2).

Indeed, some of the biological activity of milk protein components is latent within the sequence of the parent protein molecule but it released upon degradation of the native protein structure into specific peptides and amino acids (Atanasova & Ivanova, 2010; Bhopale, 2016; Korhonen & Pihlanto, 2003). This degradation may be a consequence of enzymatic hydrolysis by digestive enzymes (Jrad et al., 2014), fermentation of milk with proteolytic starter cultures (Virtanen et al., 2007), and

proteolytic activity by enzymes derived from microorganisms (Abubakr et al., 2012a) or plants (Mahajan et al., 2015).

Table 2.2: Physiological Functionality of Milk Derived Peptides

Physiological system	Function
Cardiovascular system	Antihypertensive Antioxidative Antithrombotic Hypocholesterolemic
Nervous system	Opioid agonist activity Opioid antagonist activity
Gastrointestinal system	Mineral binding Anti-appetizing Antimicrobial
Immune system	Immunomodulatory Cytomodulatory

Source: Korhonen & Pihlanto (2006)

Some of the peptides released during food processing because of some chemical or structural changes occurred inside food protein and may be ingested as one of components in food products (Bhopale, 2016). During milk fermentation with certain dairy starter cultures, peptides with various bioactivities are released and detected in an active form even in the final products (Korhonen & Pihlanto, 2003). A variety of naturally formed bioactive peptides have been found in the final products of fermented milk such as in yogurt, sour milk, and cheese (Atanasova & Ivanova, 2010). After release from native protein structure, these bioactive peptides may exert various functions on the metabolism (Table 2.2).

2.3.2 Production of Bioactive Peptides from Milk

2.3.2.1 Enzymatic Hydrolysis by Digestive Enzymes

The hydrolysis of whole protein molecules in milk inside the gastrointestinal tract can be released into useful bioactive peptides by action of digestive enzymes (Bhopale, 2016). The most prominent digestive enzymes for protein in gastrointestinal tract that have been proven able to release numerous biological active peptides are pepsin and pancreatic enzymes of trypsin, chymotrypsin carboxy, and aminopeptidases (Bhopale, 2016; Szwajkowska et al., 2011).

Most researchers used pepsin and chymotrypsin on the production of bioactive peptides hydrolysed from latent protein. Other than that, the digestive enzymes and combinations of proteinases (alcalase, chymotrypsin, pancreatin, pepsin, thermolysin) are also used for the production of bioactive peptides from various sources (Szwajkowska et al., 2011). For example, pepsin and pancreatin enzymes hydrolyse camel milk casein mimicking human gastrointestinal digestion to generate bioactive peptides responsible for antioxidative activity. After undergoing digestion process using pepsin and pancreatin, the scavenging activity of the casein peptides was more competent than digestive hydrolysates of camel milk, colostrum, and whey proteins (Jrad et al., 2014). While pepsin, trypsin, α-chymotrypsin, and β-chymotrypsin enzymes were applied in the bovine casein to access bioactive peptides responsible for antibacterial activity (McCann et al., 2006).

2.3.2.2 Microbial Fermentation

Starter cultures are widely used in many assorted types of fermentation such as in beverage of wine, production of sourdough in bread making, application in fermented fish, meat, and milk (Banaay et al., 2013; Bromberg et al., 2004; Manini et al., 2014;

Romano & Capece, 2017; Tamime, 2002). Some fermented products used spontaneous fermentation like vinegar production or sauerkraut fermentation but mostly the industries preferred using microbial starter cultures to accelerate the fermentation process of various food and beverage products (Hammes, 1990).

The microbial starter cultures were microorganisms used in food fermentations and were also called microbial food cultures (MFC). The European Food and Feed Cultures Association (EFFCA) defined MFC as "live bacteria, yeasts, or molds used in food production". From this definition, the starter cultures used in food fermentation were divided into bacteria and fungi. The bacteria starter cultures consisted of actinobacteriaceae, firmicutes, and proteobacteriaceae while fungi consisted of yeasts, and filamenteous fungi. The application of starter cultures in food fermentation may use one or more microbial species and/or strains depending on their purpose and suitability of the end products (Bourdichon et al., 2012).

In the food industry, microbial fermentation by LAB was widely selected because this process is considered as eco-friendly, cheap, and easy in order to produce metabolites with a food-grade quality (Venegas-Ortega et al., 2019). For example, in dairy industry, microbial starter cultures responsible for milk fermentation are mainly from LAB species generally not only LAB doing this action, but, yeast, and mould had also been found in fermented milk action (Wouters et al., 2002).

The application of LAB as starter cultures in milk is usually high in proteolytic activity. This is important because the peptides and amino acids released from degradation of milk proteins during fermentation contributed to the typical flavour, aroma, and texture of each dairy product (Vercruysse et al., 2009). Proteolytic LAB able to degrade protein into bioactive peptides should also remain their functionality until last stage of making products and, thus, adding criteria of probiotic LAB as starter

cultures in milk fermentation is crucial to conserve these bioactive peptides until reach consumption to attain those benefits (Donkor et al., 2007). These two criteria of proteolytic and probiotic LAB are vital because instead of providing quality of dairy products, these fermented milk products contributed to the activation of bioactive peptides with biological functions such as antihypertensive, antimicrobial, anticancer and antioxidative peptides (Bhopale 2016; Daliri et al., 2017; Virtanen et al., 2007).

2.3.2.3 Enzymes Derived from Proteolytic Microorganisms or Plants

Generation of bioactive peptides from milk fermentation could be produced through enzymatic hydrolysis of the whole protein molecules. Enzymes originated from microorganisms, either bacteria or fungi sources have also been utilised to generate bioactive peptides from various proteins (Mohanty et al., 2015). For example, crude alkaline protease from marine bacterium SD11 isolated from sea muds in China has potential to generate peptides from various sources including milk (Cui et al., 2015). Moreover, the alkaline protease from *Bacillus licheniformis* (*B. licheniformis*) ATCC 21424 was discovered to be thermally stable at alkaline pH (Bezawada et al., 2011). According to Mahajan et al. (2015), protease enzymes derived from Euphorbian plant latex were found and observed as a proteolytic enzyme.

2.3.2.4 Food Processing

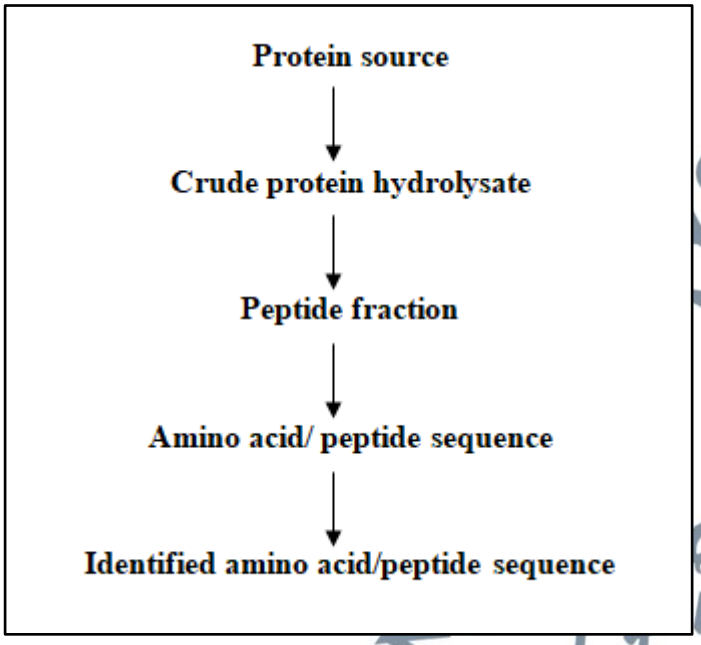
During food processing for ingredients containing protein, some changes occurred inside the food where the structural and chemical food proteins were disturbed and hydrolyse to release bioactive peptides (Bhopale, 2016). The release of bioactive peptides during processing of foods may be caused by chemical, physical, and

enzymatic treatments either the existence of enzymes inside foods naturally exist or derived from exogenous or microbial sources (Smacchi & Gobbetti, 2000).

In making dairy products of cheese, sour milk, or yogurt, peptides can be generated during manufacturing and without notice may thus be ingested as one of the food components (Atanasova & Ivanova, 2010). For example, an evaluation of angiotensin I convert enzyme (ACE) inhibitory activity during cheese ripening found active ACE inhibitor peptides naturally formed in cheese (Gupta et al., 2013). This may be due to the existence of phosphopeptides as natural constituents and secondary proteolysis during the cheese ripening process (Bhopale, 2016). In the study of processing eggs, peptides were found in eggs that undergo cook, fry, and boil processes. Among all treatments, boiled egg white hydrolysate found the highest antioxidative activity and further identified these peptide sequences resulted in finding 63 peptides (Remanan & Wu, 2014).

2.3.3 Isolation, Characterisation, and Identification of Bioactive Peptides

There are several procedures that take place for peptides determination after successfully hydrolysed (Figure 2.4). Protein from various sources can be hydrolysed in many ways as mentioned previously to generate peptides with different capability/activity (Korhonen & Pihlanto, 2006). The process of isolation, characterization, and identification of bioactive peptides are important to produce optimal peptides with their specific bioactivities (Herraiz, 1997; Lu et al., 2009; Mahdi et al., 2018). In milk and dairy cases, the activated bioactive peptides from milk products either by microbial fermentation, enzymatic hydrolysis, or through proteolysis produced crude protein hydrolysates, wheys, or caseins with various bioactivities (Hafeez et al., 2014; Nielson et al., 2017).



Source: Ryan et al. (2011)

Figure 2.4: General Flow of Isolation, Separation, and Identification of Peptides

Preparation of protein hydrolysates into peptide fractions commonly involved with the precipitation process. Selection of precipitation process is a crucial step which involved in choosing either organic solvents (methanol, ethanol, or acetone) or acids (trichloroacetic acid), also by high concentration of salts (ammonium sulphate), or by adjusting the pH to the isoelectric point. Selective fractionation of peptides is dependent with their solubility in precipitation agents in the solutions (Herraiz, 1997). In precipitation solution, the insoluble material is centrifuged and filtered to separate precipitate peptides, or proteins (Abubakr et al., 2012a; Li et al., 2013). Once the peptides are selectively fractionated into insoluble and soluble peptides, both fractions can be further analysed.

Fractionation of peptides can be obtained through ultrafiltration, ion exchange chromatography, reverse phase liquid chromatography, and via gel electrophoresis (Dib et al., 2014; Finoulst et al., 2011; Herraiz, 1997). For example, precipitation of goat

milk yogurt using ultrafiltration through membrane 3 kDa molecular weight cut of indicated that protein or peptide fraction is considered as ≤3 kDa in M_w was hydrolysed by LAB when compared to fresh goat milk. The peptide fraction was further analysed by liquid chromatography–tandem mass spectrometry (LC MS/MS) resulted in three bioactive peptides with amino acid sequences of LYQEPVLGPVRGPFPI, YQEPVLGPVRGFPIL, and VQSWMHQPPQPLSPT, respectively. These three sequences were continued with Blast for identification based on peptide sequence in the database and found that these three sequences had a low molecular mass between 1731.84 and 1780.90 Da (Mahdi et al., 2017).

Indeed, using gel electrophoresis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) can purify and visualise peptide fractions into band formation (Dib et al., 2014). The SDS PAGE gel electrophoresis approach is peptides are fractioned by gel electrophoresis based on the sizes, shapes, and net charges of the macromolecules (Garfin, 1990). Electrophoresis is a method to separate a complex mixture of proteins while SDS PAGE is a technique used to move charged molecules through a gel matrix by means of an electric current (Garfin, 1990; Manns, 2011). Through SDS PAGE assay, the peptides can be identified, and monitored during purification, as well as can assess the homogeneity of purified fractions because the separation of peptide mixtures is based on charged molecules by their molecular masses in an electric field of gel (Manns, 2011). Thus, the SDS PAGE can be able to estimate protein subunit by molecular weight (M_w) size which, then, able to determine the subunit compositions of purified proteins (Garfin, 1990).

2.4 Oxidative Stress

Oxidation generally is referred to a complete removal of one or more electrons from a molecular entity where oxidative stress is a situation between the imbalance amounts of oxidant produced with antioxidant defense mechanism (Esfandi et al., 2019; Pizzino et al., 2017; Silverstein, 2011). Oxidant can be divided into endogenous, and exogenous oxidants which derived from both endogenous (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells etc.), and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like halothane, paracetamol, and radiation) (Birben et al., 2012; Phaniendra et al., 2015).

The main oxidants are in the form of reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Esfandi et al., 2019). Both ROS and RNS are activated forms of oxygen and nitrogen including free radicals, and non-radicals (Osuntoki & Korie, 2010). Example for free radicals are superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) while for non-radicals are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), di-nitrogen trioxide (N_2O_3), and hydroperoxide (ROOH) (Esfandi et al., 2019; Li et al., 2016). Both ROS and RNS could be harmful (toxicity), or benefit to others depending on their balance with current antioxidants (Pham-Huy et al., 2008). At low or moderate levels, ROS or RNS exert beneficial effects on cellular responses and immune function. However, high concentration of ROS or RNS, resulted in imbalance of oxidants and antioxidant that generate oxidative stress or nitrosative stress in the system which caused damage to the biomolecules (Pham-Huy et al., 2008; Phaniendra et al., 2015).

In biology and medicine studies, terminology of ROS, RNS, and other oxidants is referred to as free radicals (Li et al 2016). These free radicals may attack cellular components which lead to the oxidation of lipids, proteins, and DNA that cause structural and functional changes to these molecules (Bandyopadhyay et al., 1999). In food industry, food quality also affected by an unexpected oxidation process occurred during manufacturing which tends to reduce the nutritional value and food safety by producing undesirable flavours, and toxic substances (Osuntoki & Korie, 2010).

In human body, the free radicals induced oxidative stress were believed to be associated to the development of chronic and degenerative illness such as acute respiratory distress syndrome, asthma, atherosclerosis, cancer, cardiovascular, cataract, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, multiple sclerosis, neurodegenerative disease, psoriasis, rheumatoid arthritis, systemic lupus erythematosus (SLE), and scleroderma (Birben et al., 2012; Pham-Huy et al., 2008; Santos-sánchez et al., 2019). The sources of free radicals that affected humankind may happen due to a combination of unhealthy lifestyles such as inappropriate nutrition in food intake, lack of exercise, exposure to air pollution, and/or smoking (Ramesh et al., 2011). Thus, consumption of synthetic or natural antioxidants are believed to prevent oxidative stress, and its deleterious effects (Sarmadi & Ismail, 2010). Foods containing antioxidative materials are important to be included in daily human intake to reduce, or eliminate the chances of getting these diseases (Shebis et al., 2013).

2.5 Antioxidant Activity

Antioxidant was defined as "any substance that delays, prevents, or removes oxidative damage to a target molecule" (Halliwell, 2007). In addition, antioxidant is a substance that at low concentrations delays, or prevents oxidation of a substrate (Santos-

sánchez et al., 2019). It is a stable molecule that able to donate an electron to fight loads of free radicals, and by neutralising the process, thus, reducing its capacity to damage or inhibit cellular damage (Lobo et al., 2010; Sarkar & Ghosh, 2016).

Antioxidants available in industries normally used synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxyltoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) (Race, 2009). These synthetic antioxidants are usually implemented in food, cosmetic, and pharmaceutical industries as a supplement, or as a preservative to prevent oxidation occurring in food products, and to stabilise therapeutic agents of pharmaceutical products that are susceptible to chemical degradation caused by oxidation (Shebis et al., 2013).

The usage of both natural and synthetics antioxidants in industries has their own advantages and disadvantages (Table 2.3). The safeness of application of synthetic and natural antioxidants in certain products has been done throughout the decades (Augustyniak et al., 2010; Caleja et al., 2017; Mbah et al., 2019; Race, 2009). Several studies reported that the carcinogenesis of using synthetic antioxidants such as BHA, BHT, and TBHQ (Ito et al., 1985; Ito & Hirose, 1989; Thamavit et al., 1985). However, several researchers reported that no harmful evidence has proven synthetic antioxidants are toxic or contribute as one of the carcinogenic compounds (Pokorny, 1991; Valenzuela & Nieto, 1996). It may give negative health effects or possibly become toxic if synthetic antioxidants intake is excess the safe limit (Race, 2009). Both natural and synthetic antioxidants play the same major roles in maintaining the homeostasis of the oxidative balance in humans to prevent or treat various human diseases (Mbah et al., 2019).

In fact, until now, there is no rational scientific, or technical argument for the preferences for natural antioxidants except natural antioxidants are more acceptable to

consumers because of personal emotional grounds that natural antioxidants should be safer than synthetic antioxidants especially for consumer who prefer natural organic foods (Pokorny 1991; Valenzuela & Nieto, 1996). Though no harmful effect for the application of these synthetic antioxidants has been shown in human technically, a proper scale of daily intakes for antioxidant should consume had been established by European Food Safety Authority in 2012 for the purpose of food companies to estimate synthetic antioxidants in their products (Shebis et al., 2013).

Table 2.3: Advantages and Disadvantages of Natural and Synthetic Antioxidants

Natural antioxidants	**Synthetic antioxidants**
Expensive	Inexpensive
Restricted to some products	Broad application
Wide range of antioxidant activity	Medium to high antioxidant activity
Perceive as safe substance	Increasing safety concern
Increasing used expanding applications	Use banned for some of them
Broad range of solubilities	Low water solubility
Increasing interest	Decreasing interest

Source: Valenzuela & Nieto (1996); Pokorny (1991)

Numerous research on the natural antioxidants from various sources have been well documented, and, generally, the sources of natural antioxidants were from grains, oil seeds, herbs, spices, fruits, and vegetables (Brewer, 2011; Durazzo et al., 2015; Gacche et al., 2010; Lim et al., 2007; Sarkar & Ghosh, 2016; Shahid et al., 2013; Souri et al., 2008; Yashin et al., 2017). Plant sources have an abundance of natural antioxidants like phenols, phenolic acids, and their derivatives (Sarkar& Ghosh, 2016). It exists in all parts of plants including fruits, vegetables, nuts, seeds, leaves, roots, and barks (Anwar et al., 2018).

The most popular studies on natural antioxidants are polyphenol compounds. For example, polyphenols antioxidant derived from dried green tea extract in a broiler feed against synthetic antioxidants consisting of vitamin E and SELDOX (Ethoxiquine, BHA, BHT and citric acid) found to have almost equal effectiveness of antioxidant activity (Shahid et al., 2013). Antioxidative activity of soybean koji prepared with various filamentous fungi for fermentation was also associated with the phenolic compounds (Lin et al., 2015).

Some colourful fruits, vegetables, herbs, and spices can boost human immunity and it was believed to be associated with certain natural pigments responsible for antioxidant activity (Rodriguez-Amaya, 2016). Natural pigment colours such as carotenoids, anthocyanins, betanin, and chlorophylls are believed to be sources of natural antioxidants. However, the antioxidant activity existed for each coloured food is not only because of its pigment colours but many other compounds such as other phytochemicals, or bioactive peptides involved and contributed to its antioxidant activity (Kaur & Kapoor, 2001; Li et al., 2013; Yashin et al., 2017).

To fight against oxidative stress, antioxidant components as a defense in a biological system can be through enzymatic, non-enzymatic, and repair systems (Lobo et al., 2010; Santos-sánchez et al., 2019; Sharma et al., 2012) (Table 2.4). For example, in enzymatic system, superoxide dismutase (SOD) plays central role in defense against oxidative stress in all aerobic organisms where this enzyme belongs to the group of metalloenzymes that requires metal as cofactor for its activity which catalyses the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (Sharma et al., 2012). For non-enzymatic systems such as vitamin C (ascorbic acid), these antioxidants need to be consumed and available in certain animals and plants because ascorbic acid cannot be synthesised by humans. Ascorbic acid is an antioxidant categorised as a reducing agent which works by

reducing, and neutralising ROS such as H_2O_2 (Lobo et al., 2010). In the repair system, DNA repair enzymes and proteolytic enzymes defense free radicals by repairing or eliminating damaged biomolecules in affected lipids, proteins, and DNA (Santos-sánchez et al., 2019).

Table 2.4: Example of Antioxidants According to Antioxidant Defense System

Enzymatic system Sharma et al. (2012)	**Non-enzymatic system** Lobo et al. (2010)	**Repair system** Santos-sánchez et al. (2019)
Superoxide dismutase (SOD)	Ascobate	DNA repair enzymes (polymerases, glycorases, nucleases)
Catalase (CAT)	Glutathione	Proteolytic enzymes (proteinases, proteases, peptidases)
Guaiacol peroxidase (GPX)	Tocopherols	
Ascorbate glutathione (as-GSH)	Carotenoids	
Cycle ascorbate peroxidase (APX)	Phenolic compounds	
Monodehydroascorbate reductase (MDHAR)	Melatonin	
Dehydroascorbate reductase (DHAR)	Uric acid	
Glutathione reductase		

2.5.1 Mechanisms of Antioxidant Activity

Studying the mechanism of antioxidants is important to understand the determination of antioxidative activity of each possible compound. Understanding the mechanism of antioxidants, additional knowledge of biological meaning of antioxidants, possible uses, production of antioxidants by organic synthesis, or biotechnological methods as well as standardisation for determination of antioxidative activity are important for the application it many industries (Santos-sánchez et al.,

2019). The mechanism of antioxidants has been well explained and discussed in several studies (Table 2.5). Antioxidants deal with oxidative stress in various ways depending on the types of free radicals involved, stages of oxidations occurring, and selection of antioxidant compounds to fight against oxidation because each antioxidant compound plays a role in its unique way.

Table 2.5: Classification of Antioxidant Mechanism's Approaches

Classification of antioxidant mechanism	Reference
Three groups by their mechanisms: 1. Primary antioxidants: free radical terminators (scavengers) 2. Secondary antioxidants: preventive antioxidants (retard chain initiation) 3. Tertiary antioxidants: repair damaged biomolecules	Daramola and Adegoke (2011)
Three different action of antioxidant mechanisms: 1. Inhibition of oxidant enzymes: decrease in the ROS/RNS cellular production 2. Interaction with redox signaling pathways: cellular antioxidant response 3. Direct reaction with ROS/RNS: less toxic/reactive product (mostly as free radical scavenger	Lopez-Alarcon and Denicola (2013)
Through three levels: 1. Prevention: maintain formation of reactive species to a minimum level 2. Interception: antioxidant scavenges reactive species 3. Repair: repair damaged target molecules	Bhattacharya (2015)
Through level of defense antioxidants: 1. First line: suppress or prevent the formation of free radicals or reactive species 2. Second line: scavenge antioxidants 3. Third line: repair damaged biomolecules 4. Fourth line: utilize the signals required for free radicals production and reaction to prevent the formation or reaction of free radicals	Ighodaro and Akinloye (2017)
Through three chemical mechanisms: 1. Hydrogen atom transfer 2. Single electron transfer 3. Ability to chelate transition metals	Santos-sánchez et al. (2019)

One of the common classifications for antioxidant mechanisms was based on three different levels which are before (prevention), present (interception), and after (repair) oxidation occurred (Bhattacharya, 2015). Prevention is an act of antioxidant to maintain formation of reactive species to a minimum level like the action of desferrioxamine as antioxidant to reduce lung contusion (Basaran et al., 2013), and interception is a situation where antioxidant scavenges reactive species either using catalytic and non-catalytic molecules like antioxidant of ascorbic acid, and alpha-tocopherol (Liu et al., 2004). Meanwhile for a repair level, it is an action for antioxidants to repair damaged target molecules like glutathione did as an antiaging (Weschawalit et al., 2017).

2.5.2 Mechanisms of Antioxidant Peptides

Recently bioactive peptides from various sources showed great potential as antioxidative activity (Dávalos et al., 2004; Niu et al., 2013; Sah et al., 2016; Tkaczewska et al., 2019; Xia et al., 2019; Ye et al., 2018). Similar to other antioxidant compounds, peptides with antioxidant properties suggested influencing beneficial effects in promoting human health and in food processing (Esfandi et al., 2019; Sohaib et al., 2016). Antioxidant peptides in foods contained protein have a potential for use as factors to prevent unfavourable food texture development, improve organoleptic properties, and cause functional or nutritional changes in food products especially food prone to be oxidised when extending the storability (Elias et al., 2008; Sohaib et al., 2016; Tkaczewska et al., 2019).

The antioxidant peptides or amino acids of food proteins can inhibit lipid oxidation through multiple pathways including inactivation of ROS, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and

alteration of the physical properties of food systems (Elias et al., 2008). Other than application in food products, these antioxidant peptides can be implemented in the production of pharmaceutical, cosmetic, and in animal nutrition products as one of their bioactive ingredients (Chai et al., 2017; Huo et al., 2017). Like other antioxidant compounds (Table 2.4), antioxidant peptides are also contributed to human health improvement through prevention and treatment of chronic degenerative diseases such as cardiovascular, and cerebrovascular diseases, as well as other autoimmune diseases such as rheumatoid arthritis, and diabetes mellitus (Zou et al., 2016).

Twenty known amino acids found in proteins possibly act as antioxidants because all these amino acids can interact with free radicals if the energy of the radical insult is high (Elias et al., 2008). Antioxidant amino acid residues such as tryptophan, tyrosine, methionine, cysteine, histidine, phenylalanine, and proline may have a potential as antioxidant of food protein hydrolysates (Udenigwe et al., 2011). However, free amino acids without any bonding are not generally found to be effective as antioxidants in food, and biological systems. Regardless, some individual amino acids have little or no antioxidant activity but with some bonding with other amino acids could exhibit antioxidant activity (Liu et al., 2016). Amino acid histidine, and β-alanine found no individual effect towards inhibiting either lipid, or protein oxidation but with carnosine (β-alanyl-L-histidine dipeptide) successfully inhibited lipid peroxidation, and oxidative modification of protein in muscle tissue in rats (Nagasawa et al., 2001).

In fact, antioxidant activities originated from proteins are related to the several factors such as the structure of precursor proteins, and the hydrolytic process on the antioxidant activities (Zou et al., 2016). For example, a proper low α-Helix and high β-Structure (β-sheet and β-turn) might be favourable to yogurt peptides exerting antioxidant activity (Yuan et al., 2018). Furthermore, protein hydrolysis with specific

endopeptidase enzymes, it was obtained that 50 from 54 plant proteins produced 745 bioactive peptides sequences of bioactive fragments have antioxidant activies, and their position were predicted to be on surfaces of plant proteins with hydrophilic surroundings (Dziuba et al., 2004). Other factors include the relationships between peptide structures and antioxidant activity are related to structural characteristic of the peptides such as their molecular mass, amino acid compositions, sequences, and hydrophobicities (Table 2.6) (Sah et al., 2016; Sarmadi & Ismail, 2010; Zou et al., 2016).

Table 2.6: Example for Antioxidant Peptides Affected by Its Structure Matter

Protein source	**Assay**	**Structure of antioxidant peptide**	**Reference**
Fish fillet	DPPH radical scavenging activity assay ABTS radical scavenging activity assay Metal ion chelating activity assay Ferric ion reducing antioxidant power assay Lipid peroxidation assay	Molecular size of peptides ranged from 0.5 to 3.0 kDa	Cheung et al. (2012)
Egg white	Oxygen radical absorbance capacity – fluorescein (ORAC-FL) assay Low density lipoprotein (LDL) oxidation assay	Amino acid composition of Tyr at N terminus and Tyr at intermediate of the amino acid sequence of Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu	Dávalos et al. (2004)
Soybean	Ferric thiocyanate assay	Amino acid composition of His and Pro of the amino acid sequence of Leu-Leu-Pro-His-His	Chen et al. (1996)
Fish skin	ABTS radical scavenging activity assay	Possibly contained hydrophobic amino acid such as Pro, Leu, Ala, Trp, and Phe	Sae-Leaw et al. (2016)

The molecular mass of peptides is one of the crucial roles in depending how powerful the antioxidant peptides may work. For example, using specific protease, the molecular size for peptide fractions with antioxidant activity derived from Pacific hake (*Merluccius productus*) hydrolysates was less than 1.4 kDa and obtained become a major contributor to the ABTS in the linoleic acid peroxidation model system (Cheung et al., 2012). In fact, it was found that the peptide fraction less than 3 kDa exhibited the highest antioxidant activities (Aguilar-Toalá et al., 2017; Cheung et al., 2012; Ngoh et al., 2016).

Amino acid composition and its sequences also affected antioxidant activity of peptides (Elias et al., 2008). Peptides derived from egg white proteins by pepsin enzymes, peptide with the sequence of Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu exhibited synergistic effects for both ACE inhibitory and antioxidant activities (Dávalos et al., 2004). However, it is still difficult for researchers to clarify the structure-activity relationships of antioxidant peptides, as well as the relationships of the peptides which releases antioxidant sequences and these need futher clarification and comprehensive study.

It was also suggested that the existence of a hydroxyl group in aromatic structure of tyrosine makes it act similar to phenolic compounds as a chain-breaking antioxidant (Ou et al., 2001). Moreover, hydrophobic amino acids such as glycine, alanine, valine, proline, and leucine provide antioxidant activity of peptides by gelatin hydrolysate of seabass (*Lates calcarifer*) skin prepared by alcalase enzyme (Sae-Leaw et al., 2016). However, synthetic peptides Leu-Leu-Pro-His-His designated from histidine (containing peptides had a quenching activity on singlet oxygen) do not contribute to the antioxidant activity (Chen et al., 1996).

2.5.3 Antioxidant Activity in Milk and Dairy Products

Bioactive peptides originated from various natural proteins, such as cereals, legumes, milk, meat, egg, fish, and various marine organisms possess antioxidant properties (Carrasco-Castilla et al., 2012; Chai et al., 2017; Coda et al., 2012; Ibrahim et al., 2018; Lin et al., 2014; Liu et al., 2016; Yousr & Howell, 2015). One of the research interests on the natural antioxidant sources are from antioxidative peptides generated by milk and dairy products included various fermented milk (Abubakr et al., 2012a; Ibrahim et al., 2018; Li et al., 2015; Nandhini et al., 2012; Vankudre et al., 2015). Milk fermentation generated peptide antioxidants that mainly derived from 95 % casein (including α_{s1}-, α_{s2}-, β-, and κ-casein) as well as from whey proteins (including α-lactalbumin, β-lactoglobulin, and lactoferrin) (Nielson et al., 2017).

Antioxidant peptides of milk and dairy products including fermented milk can also be divided into lipophilic and hydrophilic antioxidants. Lipophilic antioxidants of milk consist of conjugated linoleic acid, α-tocopherol, β-carotene, vitamins A, vitamin D_3, coenzyme Q10, and phospholipids while hydrophilic antioxidants of milk are proteins, peptides, vitamins, minerals, and trace elements (Grażyna et al., 2017). Regarding to antioxidative peptides derived from milk and dairy products, five to eleven amino acids in the specific sequence included with hydrophobic amino acids such as proline, histidine, tyrosine and tryptophan responsible to act as antioxidant peptides (Nielson et al., 2017; Pihlanto, 2006; Timón et al., 2019). However, antioxidant peptides of milk and dairy products are mainly due to sulphur-containing amino acids such as cysteine, phosphate, vitamin A, vitamin E, carotenoids, zinc, selenium, enzyme systems, superoxide dismutase, catalase, glutathione peroxidase, milk oligosaccharides and peptides that are produced during fermentation (Khan et al., 2019).

There were many in vitro studies that revealed that milk fermentation with proteolytic LAB generated antioxidative peptides (Abubakr et al., 2012a; Aguilar-Toalá et al., 2017; Lim, 2013; Namdari & Nejati, 2016; Nandini et al., 2012; Shu et al., 2018). Milk fermented with *L. plantarum* strains at 37 °C for 48 h produced antioxidative crude extracts and antioxidative peptide fractions. Peptides fraction with the molecular mass less than 3 kDa obtained significantly higher antioxidant value (234.1 µmol of Trolox equivalents) than peptides fraction with molecular mass of 3 to 10 kDa (210.3 µmol of Trolox equivalents).

In fact, fermentation of goat milk by *L. casei* L61 at 41 °C for 16 h produced whey fractions with antioxidant activity. Optimisation composition of nutrients formula (casein, casein peptone, glucose, soybean peptone, inulin, calcium lactate, and cysteine) found that calcium lactate, glucose, and casein peptone had significant effects on the antioxidant activity of fermented goat milk. A mixture of calcium lactate 0.99 % (w/v), glucose 0.21 % (w/v), and casein peptone 0.29 % (w/v) successfully increased hydroxyl radical scavenging rate from 56.50 % to 88.01 % while for the DPPH radical scavenging rate reached up to 63.48 % from 41.97 % under these optimal conditions (Shu et al., 2018).

Fermentation of milk goat by *L. plantarum* at 37°C for 48 was also carried out to produce goat milk hydrolysate or whey with antioxidative activity. The findings obtained that 100 µl whey resulted in DPPH value of 61.7 %, lipid peroxidase activity of 46.0 %, and hydroxyl scavenging activity of 48 %. This study suggested that there are several active components in milk that enable to inhibit lipid peroxidation and peroxyl/superoxide radical generation to maintain milk quality such as lactoferrin and lactoperoxidase. Lactoferrin from whey protein can bind with iron by mode of

transition of metal chelation to inhibit lipid peroxidation while lactoperoxidase acts both antimicrobial and antioxidant activity in maintaining milk quality (Nandini et al., 2012).

Skimmed milk fermentation using seven different LABs isolated from grapes and bananas at 37 ℃ for 24 to 72 h produced antioxidative whey. The two LAB isolates that produce whey skimmed milk with good antioxidant activity were identified as species *L. plantarum* 1 and *Leu. Mesenteroides*. The fermentation of skimmed milk with selected seven proteolytic LAB resulted in DPPH values ranged between 14.7 and 50.8 % while the FCA values ranged between 41.8 and 97.6 %. Increasing fermentation time significantly increased DPPH of the whey skimmed milk. In contrast, the FCA values decreased as fermentation time increased from 48 to 72 h. The highest FCA of the whey skimmed milk was obtained at 24 h fermentation. Fermentation time may not be contributed by generated peptides in whey skimmed milk only but also contributed by other antioxidant compounds in protein such as lactoferrin and serum albumin (Abubakr et al., 2012a).

Making yogurt from fresh milk and soymilk fermented with LAB of *L. casei* PC05, and *L. acidophilus* PC16 isolated from pickle cabbage obtained that at 37 ℃ for 30 h fermentation surrounding condition able to generate antioxidative peptides even after eight days of storage. The antioxidant activity of plain yogurt and soy yogurt fermented with *L. casei* PC05, and *L. acidophilus* PC16 involved termination of free radical reactions, ferrous ion-chelating ability, reducing power, and SOD activity. This indicated that antioxidant activity of soy yogurt was significantly ($P< 0.05$) higher than plain yogurt and were believed to be associated with different LAB strains produced different antioxidant peptides as well as different protein source which are milk (animal protein) and soymilk (vegetable protein) (Lim, 2013).

In the milk industry, three common starter cultures of LABs that are used for milk fermentation are *L. casei*, *L. acidophilus*, and *L. plantarum*. However, several studies were conducted to use non-common starter species of *Lactobacillus helveticus* to produce fermented milk enriched in antioxidant peptides (Griffiths & Tellez, 2013; Namdari & Nejati, 2016). Generally, *L. helveticus* is a versatile bacterium with an excellent proteolytic system consisting of cell-envelope proteinases, transport system, and intracellular peptidases (Griffiths & Tellez, 2013). Skimmed milk fermented with *L. helveticus* at 37 ºC for 24 h released antioxidative peptides possibly related to hydrophobic properties. The antioxidant activity of the hydrolysates (supernatant) was measured using DPPH and ABTS radical scavenging activities during the storage period of 1, 7 and 14 days. Both assays indicated that the supernatant from fermented skimmed milk contains high antioxidant activity with at least 62.32 % (DPPH radical scavenging activity) and 57.64 % (ABTS radical scavenging activity assays).

2.6 Determination of Antioxidant Activity Assays

There are various analytical assays to determine antioxidant activity. A clear understanding underlying the principle for each assay is important to prevent the misled interpretation of assays and could affect inappropriate application (Prior et al., 2005). It is important to understand the principal mechanisms, measurement ruler for each assay, and possible antioxidant compounds in the sample for proper selection of assays in evaluating antioxidant activity (Apak et al., 2016; Shahidi & Zhong, 2015). Each experiment in antioxidant activity should at least have two different antioxidant assays for comparing the different composition, and reactivity of antioxidants in particular assays (Abramovic et al., 2018).

Therefore, a variety of in vitro chemical methods should be critically understood prior to deciding which assay will be used to determine the antioxidant activity of each sample (Moharram & Youssef, 2014). Basically, in vitro assays can be divided into in vitro chemical assays, and in vitro cell structure systems. The common in vitro chemical assays to determine antioxidant activity were DPPH free radical activity, and FCA assays. Through all the examples, the antioxidant activity can be monitored using various assays with different mechanisms.

2.6.1 In vitro Chemical Assays

There are several ways in analysing antioxidant activity by in vitro chemical approach which are DPPH• (1,1-diphenyl-2-picrylhydrazyl) scavenging activity, ferrous chelating activity (FCA), $ABTS^{+}$• [2,2-azinobis (3-ethylbenzothiazoline-6sulphonic acid)] scavenging activity, reducing power, ferric reducing antioxidant power (FRAP) assay, hydroxyl (OH•) radical scavenging, and superoxide anion ($O_2^{\bullet-}$) scavenging activity assays. The two common assays for determining antioxidant activity in milk and dairy products are scavenging DPPH free radical activity, and FCA (Bamdad et al., 2017; Li et al., 2015; Maleki et al., 2015).

2.6.1.1 DPPH• (1,1-diphenyl-2-picrylhydrazyl) Scavenging Activity

One of the preferences in evaluating free radical scavenging activity in various antioxidant substances including peptide proteins is using scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical activity assay (Abubakr et al., 2012a; Namdari & Nejati, 2016; Nandini et al., 2012). This assay is technically simple, rapid, sensitive, and reproducible procedure (Gülçin et al., 2010). A chromogen radical compound of DPPH is deep violet or purple in colour (Gupta, 2015; Prior et al., 2005).

In this assay, antioxidant compound was measured using calorimetry scavenging DPPH radical activity assay using spectrometric technique to determine discolouration of DPPH from deep violet to pale yellow or colourless at it strong absorption band of 515 nm (Gupta, 2015). This method is based on the reduction of alcoholic DPPH solution because of the action of hydrogen donating from the antioxidant compound to form a non-radical DPPH-H by the reaction (Gülçin et al., 2010).

$$DPPH\bullet + AH \longrightarrow DPPH_2 + AH\bullet$$

In other way, this assay is a measurement of loss DPPH colour with absorption band maximum around 520 nm after reaction with tested antioxidant compound (Prior et al., 2005). However, there are several cautions should be implemented when measuring antioxidant activity by DPPH antioxidant assay which are sensitivity range of spectrophotometry, and DPPH is sensitive to light, pH, and solubility of the antioxidant compounds (Moharram & Youssef, 2014).

2.6.1.2 Ferrous chelating activity (FCA)

Metal ions chelation is one of the effective antioxidant actions for the prevention of lipid peroxidation. Among transition metals, iron metal of ferrous ion (Fe^{2+}) is highly reactive and may cause tissue damage by catalysing the formation of ROS and stimulating lipid peroxidation through Fenton reaction (Halliwell & Gutteridge, 1990).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^\bullet \quad \text{(Fenton reaction)}$$

Fenton reaction involves the oxidation of Fe^{2+} to ferric ions (Fe^{3+}) by hydrogen peroxide (H_2O_2) to generate a hydroxyl radical and a hydroxyl anion (Halliwell & Gutteridge, 1992). Chelating agent is needed to protect against oxidative damage by retarding this metal catalysed oxidation (Gupta, 2015). Chelator or chelating agent

captures metal iron and prohibits metal iron from catalysing oxidation (Kim et al., 2005). Antioxidant activity by FCA assay was assessed based on the ability of antioxidant components to interfere with the formation of ferrozine-Fe^{2+} complex in vitro (Chai et al., 2014). In fact, in this assay, ferrozine is a chelator that can quantitatively form complexes with Fe^{2+} at absorbance of 562 nm. However, in the presence of other chelating agents from tested samples, complex formation is disrupted, and this can be observed by decreament the red colour of the complex. Thus, colour reduction can be measured by spectrophotometry at absorbance 562 nm to estimate the chelating activity of the coexisting chelator (Adjimani & Asare, 2015).

2.6.2 In vitro Cell Culture Systems

Instead of in vitro chemical assays, in vitro cell-based assays can determine the antioxidant effect of various compounds at subcellular level. Without experimenting directly with the action of radical scavenging that mainly causes oxidative damage, the in vitro cell culture systems can evaluate compounds that are responsible in enhancing antioxidant pathways (da Silva et al., 2016). The example for this assay is cytotoxicity of antioxidative that measure viability of living cells by spectrophotometer.

2.7 Determination of Antioxidant Peptides Activity Assays

Similar to other antioxidant compounds, antioxidant peptides can be evaluated through several chemical mechanisms include hydrogen atom transfer (HAT), single electron transfer (SET), and chelating transition metals (Esfandi et al., 2019; Santos-sánchez et al., 2019; Zou et al., 2016). Both HAT and SET mechanisms involved with deactivation of free radicals. Even though the mechanism of HAT and SET is different, both mechanisms will produce identical end-products. These two mechanisms may

occur in parallel but depending on the structure of the antioxidant peptide, and the type of assay participating will influence the solubility and partition coefficient (Esfandi et al., 2019).

Usually the HAT-based antioxidant assays associate with competitive reactions in which antioxidant is compared with the substrate for production of peroxyl radicals (Sohaib et al., 2016). The free radical inactivation occurred when one hydrogen atom from an antioxidant was removed and donated to that radical (Liang and Kitts, 2014). The example for antioxidant assays of HAT-dependent includes oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and carotene bleaching assays (Esfandi et al., 2019; Zou et al., 2016; Sohaib et al., 2016).

For SET-based methods, bond dissociation energy and ionisation potential of the reactive functional group affecting electron donation (Esfandi et al., 2019). The lower the ionisation potential of an antioxidant, the easier the electron donation to radical (Liang & Kitts, 2014). By decreasing the ionisation potential values of antioxidants with increasing its pH, causes an increase in electron donation capacity to the free radical and antioxidant itself to become a radical cation (Apak et al., 2016; Esfandi et al., 2019). The most common antioxidant assays of SET-dependent are trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH), and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity assays (Esfandi et al., 2019; Sohaib et al., 2016; Zou et al., 2016).

The other important chemical mechanism is transient metal ion chelation (Zou et al., 2016). This chemical mechanism involved is inhibition of the Fenton reaction to prevent generation of hydroxyl radicals which lead to lipid oxidation (Li et al., 2015; Lim et al 2013). Unlike HAT and SET mechanisms, the metal chelation functionality

of antioxidants neutralise reaction between a Lewis base (antioxidant) and a Lewis acid (metal ion) without involve the donation of hydrogen atoms or, electrons by the antioxidant (Apak et al., 2016). The antioxidant assays related to metal chelation are EDTA Equivalent Iron Chelation Capacity (EECC), and Carnosine Equivalent Iron Chelation Capacity (CECC) (Zou et al., 2016).