Milk Lactoperoxidase System and Lactoperoxidase Enzyme on the Potential of *Streptococcus Mutans* in Children's Saliva

Literature Review

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ABSTRACT

Lactoperoxidase is a subset of peroxidase, a group of natural enzymes that exist in human saliva. Other than the saliva, these enzymes could also be found in dairy products. The lactoperoxidase enzyme in milk has antibacterial effect, so can greatly reduce the number of viable Streptococcus mutans. Early Childhood Caries (ECC) a unique pattern of caries lesions that occur in infants, toddler, and preschoolers characterized by caries on the surface of primary teeth in children un der 6 years.The purpose of this study was to analyze the levels of lactoperoxidase and Streptococcus mutans count in saliva after consuming pasteurized milk and UHT milk. This study was conducted on 30 children aged 3-5 years in Medan city by applying purposive design, whereby two groups were established with 15 subjects consuming pasteurized milk and 15 subjects consuming UHT milk. Saliva collection was conducted either with passive drooling or spitting method prior to consumption, 7 days, and 14 days after consumption. Examination of lactoperoxidase levels was carried out using an ELISA reader. Acquired salivary Streptococcus mutans were planted in TYCSB media and incubated in an anaerobic jar for 2x24 hours followed by calculation of S.mutans. Data were analyzed using univariate tests, generalized linear repeated measured models, and simple linear regression tests. The statistical results showed a significant increment in mean lactoperoxidase levels before consumption to the seventh day after consumption in both groups (p=0.01), despite a non-significant increment between sevent day and fourteenth day (p=0.178). The results presented a significant decline in the number of S. mutans on the seventh day (p = 0.0001), but not significant on the fourteenth day (p = 0.146). Reduction in the average number of Streptococcus mutans was also observed before consumption, the seventh day, and the fourteenth day after consumption of pasteurized milk from 47.93 ± 8.216 to 31.07 ± 10.152 and 11.60 ± 6.501; whereas for the UHT milk group, from 50.60 ± 7.018 to 42.00 ± 6.665 and 27.07 ± 4.183. The consumption of milk with high levels of lactoperoxidase has a remarkable potential in increasing levels of lactoperoxidase and minimizing the number of Streptococcus mutans in saliva as a risk factor for caries.

Keywords: *Pasteurized milk, Lactoperoxidase, Streptococcus mutans.*

1. LITERATURE REVIEW

1.1 EARLY CHILDHOOD CARIES (ECC)

Dental caries is an infectious disease of the teeth which is one of the health problems of children worldwide. Caries affecting children under the age of 6 years is often referred to as Early Childhood Caries (ECC) [1]. According to the American Academy of Pediatric Dentistry (AAPD), ECC is the presence of one or more caries teeth (non-cavitated lesions or cavitated lesions), missing teeth due to caries, or fillings on any tooth surface in children under the age of 6 years or 71 months [2]. The form of caries that is more severe than ECC is Severe Early Childhood Caries (SECC) [3].

ECC has several unique characteristics in its clinical appearance such as the rapid development of caries, with a large number of teeth exposed as soon as ECC appears in the oral cavity. These lesions involve tooth surfaces that are less prone to caries development [1]. Besides ECC, there are also several terms used to describe this condition, namely, bottle caries, nursing caries, baby bottle tooth decay and bottle rot [2].

2. ETIOLOGY AND RISK FACTORS OF ECC (CHILD LIFESTYLE)

The etiology of ECC is multifactorial. The main factors causing caries are the host (teeth), microflora, and substrate or diet, as well as other factors such as time. The interaction of these four factors is needed for caries to occur [4]. In young children, the bacterial flora and the body's defense system that are still developing, the tooth surface which is in a new state of eruption, and the child's habits of consuming milk or solid food will increase the risk of caries [5; 6]

2.1 Host (teeth)

The crystal density of the enamel greatly determines the solubility of the enamel. The more the enamel contains minerals, the denser the enamel crystals and the more resistant the enamel will be. Primary teeth are more susceptible to caries than permanent teeth because the enamel of primary teeth contains more organic matter and water while the amount of minerals is less than that of permanent teeth. Besides that, there are areas of teeth that are more susceptible to caries, namely the fissure and proximal parts of the tooth [7].

Newly erupted teeth [5] and children who have teeth with hypoplastic enamel will be more prone to caries development [8]. Hypoplastic enamel has low tooth structure integrity, low acid resistance, the presence of pores on the tooth surface, making it easier for plaque to accumulate on it and ultimately increasing the risk of caries [9].

2.1.1 Microorganisms causing Early Childhood Caries (ECC)

The dominant bacteria found in the human oral cavity include *S. sanguis, S. mitis, S. mutans, S. salivarius, L. acidophilus, L. salivarius, L. casei, Staphylococcus spp, Eubacterium spp, Neisseria spp, Actinomyces spp, Peptostreptococcus spp, Micrococcus spp*, etc. *Streptococcus* is the bacteria most commonly associated with the oral cavity [10].

Streptococcus is a gram-positive bacterium and is the predominant bacteria found in the oral cavity. *Streptococcus* can be divided into 4 main species groups based on biochemical, physiological, and DNA analysis namely; mutant group (*S. mutans* and *S. sobrinus*) associated with dental plaque and caries [11], the mitis group (*S. mitis, S. sanguis, S. crista,* and *S. oralis*) were found mostly in dental plaque [12], the salivarius group (*S. salivarius* and *S. vestibularis*) were found mostly on the mucosal surface [13], and the anginosus group (*S.anginosus, S. constellatus,* and *S. intermedius*) could be detected in the gingival sulcus and root canals and were associated with periodontal and pulpal disease [14]. Besides that, there are also 2 other groups, namely the bovis group (*S. bovis* and *S. equinus*) and the pyogenic group (*S. pyogenes* and *S. agalactiae*) [15].

There are 1-5 genotypes of *S. mutans* that colonize the oral cavity of children who have ECC and are caries free. *S. mutans* colonies were more common in ECC children than caries-free children, namely 85.5% compared to 57.9%. In addition, 1-3 genotypes of *S. sobrinus* were also found in SECC children, which was 31.25%, whereas caries-free children only had 1 genotype. Qindkk (2013) reported that children with ECC had higher *S. sobrinus* (18.3%) than caries-free children (3.30%). It was concluded that children with ECC had more genotypes of *S. mutans* and *S. sobrinus* than caries-free children [16].

The study found that there were 379 bacterial species detected in saliva and supragingival plaque from 40 children under 5 years of age who were divided into two groups, namely ECC and caries-free. Microarray results found 13 species (*Streptococcus mutans, Porphyromonas catoniae, Veillonellaceae, Peptostreptococcus stomatis, Streptococcus infantis, Corynebacterium matruchotii, Propionibacterium propionicum, Actinomyces naeslundii, Selemonas, Selemonas flueggei*, TM7, and *Prevotella pallens*) in supragingival plaque and 2 species of bacteria (*Streptococcus mutans* and *Actimomyces)* in saliva which showed a significant difference between the two groups. *Actinomyces* (gram-positive rods) are thought to play a role in the early formation of ECC. It was also found that *Rothia mucilaginosa* was more abundant in the saliva of children with ECC than caries-free children. The study concluded that the microbiota of *Streptococcus, Porphyromonas*, and *Actinomyces* is strongly associated with the incidence of ECC and has the potential to be used as a biomarker of caries disease in primary teeth [17].

2.2 Streptococcus mutans

Streptococcus mutans is one of the normal floras that lives in the oral cavity, but if in excess, it is the main causative agent of dental caries [18]. As it is known that *S. mutans* is a caries-causing pathogenic microorganism [19]. *S. mutans* bacteria can metabolize carbohydrates and produce acids. These bacteria can multiply rapidly in acidic conditions or low pH. Colonies of *S. mutans* are in pairs or chains, immobile and not spore-forming, anaerobic metabolism, but can live as facultative anaerobes. *S. mutans* bacteria perform fermentation in an acidic environment and low pH. *Streptococcus mutans* is a gram-positive, non-motile and facultative anaerobic bacterium. This bacterium has a cocci shape that is round or ovoid and arranged in chains, and grows optimally at a temperature of 18^0C - 40^0C. *S. mutans* bacteria will grow optimally at a saliva pH of 4.5-5. *Streptococcus mutans* (*S. mutans*) is the most common bacterium found in the oral cavity compared to other streptococcal species [19]. *S. mutans* is found in pairs with short or medium chains without a capsule. Under acidic conditions, these bacteria can form short rods with a length of 1.5-3.0 m [20].

S. mutans requires a hard surface (non-desquamating surface) to colonize. Therefore, infants do not have *S. mutans* before tooth eruption [21]. *S. mutans* colonization of the teeth may begin when the baby is 10-14 months old [22].

The main source of *S. mutans* infection in infants is their mothers or can be transmitted by the baby caretakers (vertical transmission) [23; 24]. The incidence of this transmission can be proven through the isolation of *S. mutans* in mothers and their children, and obtained from various studies that *S. mutans* in children are identical to bacteria in their mothers, maintaining identical DNA and plasma chromosomal patterns [25]. Various studies have also found a correlation between the amount of *S. mutans* in the mother's saliva and the amount of *S. mutans* in her child where mothers who have a high number of *S. mutans* tend to have children with a high number of *S. mutans* as well [26]. Berkowitz et al (1981) reported that the risk of babies suffering from caries increased 9 times in children whose mothers had salivary bacteria counts > 10^5 CFU/ml, compared to

mothers with bacteria counts lower or equal to 10^3 CFU/ml.

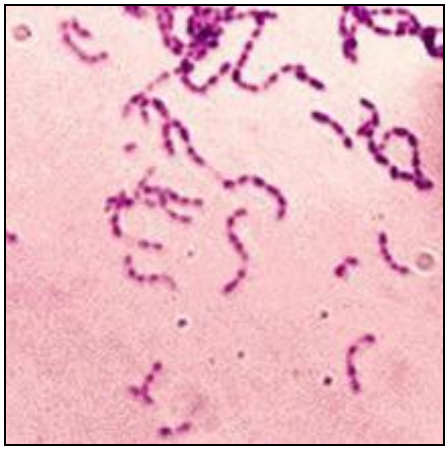

Figure 1 *Streptococcus mutans*.

Sucrose is metabolized faster in *S. mutans* than other bacteria such as *S. mitis, S. sanguis,* and *S. viscous*. The main pathogen in caries involvement is the ability of *S. mutans* bacteria to express various virulence factors. Virulence factors in *S. mutans* function to protect themselves from host defences and maintain the ecology of bacteria in the oral cavity by having the ability to cause damage to the host [27].

2.2.1 Substrate or Diet Pattern

The diet for infants aged 1-3 years recommended by the Ministry of Health (2011) is 3 family meals (main meals), 2 snacks and 2 milk drinking sessions. The American Dental Association (ADA) recommends to avoid feeding at night during sleep after a child's teeth begin to erupt (ADA, 2000). If the child's diet is not good, it could trigger an increased risk of caries.

Study has found that children who often consume sweet food between meals will trigger caries more quickly [28]. It was found that 84% of caries-free children consumed sweet foods less than 2 times a day, compared ECC children who consumed sweet foods more than 2 times which is 59% ($p < 0.001$) [28]. Watanabe et al (2014) reported that from 31,202 children aged 1.5 years who were free of caries and had a lifestyle of eating sweet food 3 times a day, after 21 months of observation, it was found that 24.7% of children suffered from caries, while for children who ate food sweet once a day, only 12.6% children suffered from caries.

Breastfeeding factors have also been identified as a risk factor for caries [29; 30]. The World Health Organization (WHO) recommends that children are breastfeed only up to <3 years of age (WHO, 2003). ECC children consumed more milk at night and added sugar to their milk than caries-free children ($p < 0.001$) [28]. The addition of sweeteners to milk or to juice will increase the risk of caries [31]. Children who consume sugary drinks at night are at least 2 times more likely to get ECC (OR=2.38, Cl=1.34-5.99) than children who never drink sugary drinks at night [32].

20,4% Caries-free children who consume sweet drinks every day would suffer from caries after 21 months of observation; while only 13,2% children who never consumed sugary drinks every day would suffer from caries [33].

2.3 Saliva

Saliva is one of the factors that contribute to the process of dental caries, but its contribution is unique in that it also contributes to caries prevention [34]. Saliva serves a critical function in protecting the oral cavity from microbial growth and excessive microbial production, which can result in oral diseases such as dental caries [35].

2.3.1 Characteristics and Functions

Saliva is produced in the salivary glands by acinar cells, collected in small ducts, and released into the oral cavity. The parotid, submandibular, and sublingual glands are the three major salivary glands. At rest (unstimulated saliva), these three glands account for approximately 90% of total saliva (20% parotid gland, 65-70% submandibular gland, and 7-8% sublingual

gland); whereas minor salivary glands, specifically Von Ebner glands, labial, lingual, and palatal glands, account for approximately 10% of total saliva in unsimulated saliva [36; 37; 38]. The parotid salivary glands produce more than 50% of total salivary secretion in stimulated saliva [37]. Saliva is composed of 99.9% water, 0.3% protein, and 0.2% inorganic substances [36].

Saliva is critical for oral health maintenance [39]. Saliva can help maintain oral health by acting as a buffer against acid formation, protection against demineralization and remineralization, ability to flow clean food (flow-related diet clearance), salivary viscosity, and immunological system (adaptive immunity) [40; 24].

Salivary secretions may be serous, mucous or mixed. Serous secretions produced mainly by the parotid salivary glands are rich in ions and enzymes. Mucus secretions produced mainly in the minor salivary glands are rich in mucin (glycoprotein) and contain little or no enzymes. In submandibular and sublingual salivary glands, content depends on the proportion between serous and mucous cells [37].

Saliva contains components derived from non-glands. Oral fluid produced by salivary glands, oropharyngeal mucosa fluid (transudate oral mucosa cells, bacteria, fungi, viruses, upper respiratory tract secretions, and gastroesophageal reflux), gingival crevicular fluid (produced approximately 2-3 times per hour for each tooth), food debris, and blood components (plasma, erythrocytes, leukocytes [41].

One of the functions of saliva is as a buffer, and those that play a role in this are bicarbonate and phosphate. Urea is another buffer present in the total salivary fluid, urea produces amino acids and protein catabolism which causes an increase in the pH of the biofilm by releasing ammonia and carbon dioxide when hydrolyzed by bacterial urea. The buffer system functions to prevent colonization of pathogenic microorganisms by optimizing environmental conditions, neutralizing and cleaning acids produced by acidogenic microorganisms so as to prevent enamel demineralization. The carbonic-bicarbonate system is the most important buffer in stimulated saliva, whereas phosphate in unstimulated saliva plays a role [37].

Mucin, calcium, and phosphate are the components of saliva that protect against tooth demineralization. Calcium, phosphate, proline-rich proteins (PRPs), statherins, and fluorine are all components of saliva that aid in tooth remineralization. Lubrication serves to flush and clean the mouth of debris and other harmful materials, as well as to eliminate certain bacteria during the swallowing process. Mucin and proline-rich glycoprotein (PRG) are involved [13; 37; 39; 42].

2.3.2 Immune System in Saliva

The immune system is classified into two types: innate (natural or non-specific) and adaptive (specific). When the host is attacked by pathogens, the innate immune system mounts a direct defense against infection (viruses, bacteria, fungi, or parasites). Natural immunity is the precursor to the development of immunity. Immunity of this type benefits the body that has not yet developed an adaptive defense system. When a pathogen enters the body, the innate immune system is in charge of controlling the pathogen's development. The adaptive immune system develops four to seven days after infection, during which the body produces antibodies against previously exposed antigens. Natural factors of immunity, such as the peroxidase system, mucin, lactoferrin, and lysozyme, can enhance IgA's antibacterial activity [43].

The innate immune system consists of anatomical barriers to infection, either physical or chemical, and cellular responses. The main physical barrier (the body's first line of defense) is the epithelial layer of the skin and mucosa and glandular tissue that is connected to the opening of the body, where this epithelial barrier prevents infection by preventing pathogens from

entering the body. Chemical barriers on surfaces include soluble substances, which have antimicrobial activity and an acidic pH. Cellular responses are induced by cell surface or intracellular receptors that recognize molecular pathogen components [44].

Saliva serves an important function in protecting the mouth from microorganisms. Protection against microorganisms in the mouth has two mechanisms of action: the enzymatic rejection system and the non-enzymatic rejection system. The enzymatic rejection system is based on the activity of salivary enzymes that are capable of destroying microorganisms, such as lysozyme (natural immunity) and lacto peroxidase (natural immunity). Lactoferrin (natural immunity), aggregation factors (natural immunity), and immunoglobulins (adaptive immunity) are non-enzymatic rejection systems found in saliva [35]. Additionally, non-enzymatic systems such as histatin, cystatin, secretory leukocyte proteinase inhibitor (SLPI), defensin-2, chromogranin A, catelicidin, mucin, and proline-rich glycoprotein have antibacterial activity (Table 1) [39; 40; 42].

2.3.3 Saliva Flow Rate, pH and Saliva Buffer

There is a balance between the amount of bacteria present in saliva that are free and those that are attached to teeth or oral epithelial cells. Low salivary flow rate is associated with an increased risk of developing caries [39]. Reduced secretion results from changes in salivary flow rate, which can be influenced by drugs, pathological changes in the salivary glands, and age [45]. When the unstimulated salivary flow rate is less than 0.30 ml/min and the stimulated salivary flow rate is less than 0.7 ml/min, a risk factor exists [46].

Saliva's buffering capacity can help protect teeth from caries. Inadequate buffering capacity can result in impaired or absent neutralization of plaque acid, thereby reducing the likelihood of remineralization in early enamel lesions [47]. According to the study, saliva samples from ECC children revealed that their salivary pH and buffers were lower than those from caries-free children. In unstimulated saliva, different results were obtained. Between ECC and caries-free children, there was no difference in pH or salivary buffer.

Table 1. Salivary Glands Antibacterial Proteins

No.	Protein Saliva	Fungsi	Asal jaringan	% Relatif
1.	MUC5B (musin MG1)	Proton-diffusion barrier in the pellicle	All salivary glands mucus	5–20
2.	MUC7 (musin MG2)	Bacterial aggregation	All salivary glands mucus	5–20
3	Immunoglobulin	Bacterial inactivation and aggregation,	B lymphocytes and all salivary glands	5-15
4	Proline-rich Glycoprotein	bacterial aggregation,	parotid	1-10
5	Sistatin	protease inhibitor	submandibular > sublingual	10
6	Histatin	Kills broad spectrum of bacteria	parotid and submandibular	5
7	EP-GP (GFCDP15, SABP, PIP)	not yet known	submandibular, sublingual	1-2
8	Aglutinin ((DMBT1, GP340)	bacterial aggregation	parotid > submandibular > sublingual	1-2
9	Lisozim	kill bacteria	sublingual > submandibular, parotid	1-2
10	Laktoferin	inhibit growth	all salivary glands: mucus > serous	1-2
11	Laktoperoksidase	inhibit growth	parotid > submandibular	<1
12	Katelisidine (Hcap18, LL37)	Kills broad spectrum of bacteria	salivary glands, neutrophils	<1
13	Defensin	Kills broad spectrum of bacteria	salivary glands, epithelial cells, neutrophils	<1

The total pH and buffering capacity of stimulated saliva were greater than those of unstimulated saliva [48]. Salivary flow rate was found to be lower in children with ECC than in children without ECC in 400 children aged 3-6 years [49; 50; 51]. Similarly, caries-free children had higher salivary flow rates than children in the ECC or SECC groups [52]. Preethi et al. (2010) discovered that while there was no significant difference in mean salivary flow between caries-free children and children with active caries, there was a significant decrease in mean salivary flow in children with active caries.

Another study reported the same finding, namely that the pH of saliva without stimulation was higher in caries-free children than in ECC children (p 0.0001). Additionally, salivary pH was found to be negatively correlated between the two groups (r=-0.47, p0.05), indicating that the more severe the caries, the lower the child's salivary pH [40].

3. ANTIBACTERIAL IN SALIVA

Another function of saliva is as an antibacterial agent. Several substances play as antibacterial in saliva, such as histatin, lysozyme, lactoferrin, cystatin, and lactoperoxidase [39]. Histatin in human saliva at least had 12 kinds of identified histatin peptides. Histatin 1, 3, and 5 were commonly found in parotid saliva. Histatin 5 acts to kill S. *mutans* and fungus [53], and commonly contribute to the formation of saliva pellicle, inducing the inhibition of inflammatory cytokine, etc. [40].

Lysozyme is an antimicrobial cationic protein that plays a role in our body's defense. Lysozyme degrades peptidoglycans in the bacterial cell wall [54]. Peptidoglycan degradation will destroy or lysis bacteria cell walls, so bacteria lose their fluid and finally induce the death of bacteria and cause a bactericidal effect on gram-positive microorganisms [39].

Lactoferrin has bacteriostatic and bactericide properties (for example like highly concentrated lactoferrin in dental plaque) [55]. In saliva, lactoferrin binds with sIgA, while sIgA can bind with a specific receptor on *S. mutans* surface. Lactoferrin acts as a secondary rejector system when sIgA isn't present or sIgA could not bind with bacteria or sIgA chains for the most parts are separated because of the proteolytic enzyme [39]. Lactoferrin is a non-enzymatic antibacterial protein and distributes in body fluid, such as saliva, lachrymal, and polymorphonuclear leukocyte (PMN). Lactoferrin was secreted by serous cells from the mayor and minor salivary glands. This protein has an iron-chelating substance that can decrease microorganisms [56].

Cystatin is excreted from the submandibular salivary gland [38]. Cystatin's function is to control mineralization and inhibitor activity of cysteine. Cystatin could inhibit the proteolytic enzyme that is released by *P. gingivalis*, this protein also can inhibit the growth of *P. gingivalis* [57,39]. Cystatin is also related to the formation of pellicles and the balance of hydroxyapatite crystals [37].

Lactoperoxidase is a member of the heme-peroxidase enzyme, which this peroxidase can catalyst the oxidation of one of the electron substrates [58]. Peroxidase activity in the saliva is from two sources, such as; a) human salivary lactoperoxidase (HS-LPO) that are synthesized and excreted from the salivary gland, b) myeloperoxidase (MPO) that found in polymorphonuclear leukocyte (PMN) of gingival sulcus fluids. MPO contribution in total peroxidase in saliva varied firm 30 – 75%, depending on each of individual health [59; 39]. For kids with Down syndrome, the peroxide level of stimulated salivary flow is 1,53 µg/mg, which is lower than normal kids, which is 2,58 µg/mg [60].

Lactoperoxidase can catalyst the oxidation of inorganic and organic substances because of hydrogen

peroxide. This substance includes bromide and iodide and because of its properties, lactoperoxidase could be categorized as halo peroxidase. Another important substance is thiocyanate. Oxidized products from this enzyme action had a strong bactericide property. Lactoperoxidase with inorganic ions, hydrogen peroxide, and oxidated products are known as lactoperoxidase systems (lactoperoxidase-thiocyanate-hydrogen peroxide systems) [61].

A study by Well et al (2011) found that effectivity of *S. mutans* bactericidal level with a combination of the thiocyanate-hydrogen peroxide without the addition of lactoperoxidase increase over time, but only for a small amount. It can be concluded that a combination of thiocyanate-hydrogen peroxide isn't effective as a bactericidal. In contrast with thiocyanate-hydrogen peroxide combination with the addition of lactoperoxidase shows a better reduction amount of S. *mutans* than the control group without the addition of lactoperoxidase [61].

4. LACTOPEROXIDASE AND LACTOPEROXIDASE SYSTEM AS AN ANTIBACTERIAL IN SALIVA

Lactoperoxidase is a member of mammalian heme peroxidase that had wide spectrum activity. Lactoperoxidase has an antimicrobial effect that has been tested using in vitro and in vivo methods [62]. Lactoperoxidase system established from this several processes. First, oxidation reaction with hydrogen peroxide (H2O2) and thiocyanate ions (SCN-) that produce hypothiocyanite ion (OSCN-). And then, OSCN is in charge of killing bacteria, fungus, viruses by destroying sulfhydryl chains (S-H chain) from the cell membrane, which makes vital destruction of membrane cells that could cause bactericidal effects [44; 62].

Overall, the utilization of these three components (H2O¬2, SCN-, dan OSCN-) is known as the lactoperoxidase system (LPS). The antimicrobial effect of LPS could inhibit or kill organisms, depending on the type of organisms [63]. All components of lactoperoxidase are naturally available in humans or animals. For humans, lactoperoxidase could be found in lachrymal or saliva, and for an animal could be found in milk. Lactoperoxidase is mostly found in milk than human body [47]. In cow's milk, the amount of lactoperoxidase is 10-30 μg/ml [64].

Lactoperoxidase enzyme is one of the enzymes that are really stable towards heat and could withstand its activity partially when heated in the low temperature for a long time (63ºC for 30 minutes) or in the high temperature for short time (72ºC for 15 seconds) [65; 66; 67; 68]. Marin et al, in 2003 showed that when lactoperoxidase was heated with 68ºC for 15 minutes or 72ºC for 2 minutes, it could withstand its activity around 90%. But in 74ºC or in 76ºC for 40 seconds, lactoperoxidase activity reduce until 64% and 36% [69].

Classic pasteurization process (72ºC, 15 seconds) didn't make inactive lactoperoxidase system in milk [70], while studies by de Wit and van Hooydonk in 1996, show that full inactivation happened at the temperature of 78ºC for 15 seconds [71]. Commonly said that lactoperoxidase is more sensitive towards increasing temperature rather than treatment duration in lower temperature [72; 73; 74].

$$H_2O_2 + SCN^- \rightarrow OSCN^- + H_2O$$

or

$$2\,SCN^- + H_2O_2 + 2\,H^+ \rightarrow (SCN_2) + 2\,H_2O \rightarrow OSCN^- + SCN^- + 2\,H^+$$

Figure 2 The mechanism action of lactoperoxidase (SCN-: thiocyanate; OSCN-: hypothiocyanate).

4.1 Milk

Cow milk is the best source of nutrition for human and reckoned to be almost perfect because of its complete nutrient. Beside water, milk contains protein, carbohydrate, lipid, mineral, enzymes, gas, as well as vitamin A, C and D in adequate amount [75]. The

benefits of milk are the results of its molecule interactions inside [76; 77; 78].

Cow milk may also contain some microorganisms if the process of milking is unhygienic. The possible microorganisms are bacteria, yeast, and fungi. With the mentioned microorganisms, fresh milk may easily spoil. Only within 4 hours after the milking process, fresh milk will gradually spoil [79; 80]. This is caused by the rapid changes in the health of the animals, milk storage, and contamination from water, air, and et cetera which lead to food poisoning [72].

To maintain the quality of milk, the pasteurization process is carried out. It is the process of heating milk or milk products until certain temperatures during specific period [72]. The aim of pasteurization is to kill the etiology of diseases, or the microorganisms. The combinations of time and temperature, according to Grade A Pasteurized Milk Ordinance from Food and Drug Administration (FDA), are as following [81; 82; 83]:

1. *High Temperature Short Time* (HTST), where the heating process is carried out at a temperature of 161ºF or around 72ºC for 15 seconds and followed by rapid cooling. The HTST method cannot kill all types of microorganisms so milk will remain eventually spoiled, thus it must be stored in a refrigerator to prevent the growth of pathogens [72; 84; 85; 86]. One of the milk brands using this method is Diamond®.

Figure 3 Examples of pasteurized milk sold in the market.

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2. *High Heat Short Time* (HHST), this process is similar to HTST, but uses slightly different equipment and with a higher temperature and shorter time.

3. *Ultra Pasteurized* (UP), where the milk is heated to not less than 280ºF or about 138ºC for 2 second.

4. *Ultra High Temperature* (UHT), where the milk is heated in two heating stages. First, it is heated at 75°C and then at 140°C under pressure for 4 seconds. After cooling, the milk is packaged in an aseptic (sterile) way. In this method, microorganisms can be eliminated more effectively and milk can be stored at room temperature for up to 8 weeks without any changes in taste (FCS, 2010). Several brands of UHT milk available in the market are Ultra Milk®, Greenfields®, Ultra mimi®, Indomilk®, Frisian Flag®, etc.

Figure 4 Several kinds of UHT milk are sold in the market.

In addition to the pasteurization process, the activation of the lactoperoxidase system has been used for many years to protect milk from microbial contamination, both during milk collection and storage [87; 88]. Although the lactoperoxidase enzyme is one of the most heat-stable enzymes, heating process that is too high can cause inactivation of this enzyme, reducing the effect of this enzyme as an antimicrobial in milk [89; 90; 91; 92]. Among all the methods, HTST is the only one that does not cause inactivation of the lactoperoxidase enzyme [93; 94].

4.2 Saliva Collection and Storing Methods

The commonly used salivary collection method are dry method using a Proflow Sialometer, spit method, suction method, swab, or absorbent method and using a salivette [95; 96; 97; 98]. Saliva produced by a single

salivary duct can be collected using a cannula or using a metal or acrylic cup placed in the Stenson's duct to collect pure parotid saliva. Saliva from the submandibular gland can be collected by placing a tip on the orifice of Wharton's canal, after placing a sterile cotton swab on the floor of the mouth and over the buccal mucosal area to close the parotid and sublingual tracts [99, 100].

The salivary flow rate can be measured either stimulated or unstimulated. Whole saliva without stimulation can be collected by using methods such as [101; 102; 103]:

1. Passive drooling (no oral movement), saliva flows through the lower lip into a plastic bottle.

Figure 5 Drooling method.

2. Spitting directly into the collector (spitting), in this way, the specimens contain 14 times more bacteria than drooling methods.

Figure 6 Spitting method.

3. Suction method. A simple way to collect submandibular or sublingual saliva is to cover the Stensen duct with a Lashley cup/cotton roll. Then the saliva is collected on the floor of the mouth which is sucked in by the syringe. The salivary glands were stimulated with citric acid solution (2-6% wt/volume) which was applied with a cotton swab on the lateral side of the tongue at interval of ± 30-60 second.

Figure 7 Suction method (https://www.salimetrics.com/collectionsystem/childrens-swab#)

4. Swab method, by using 3 pieces of cotton roll. 1 piece of cotton roll is placed under the tongue, the remaining 2 pieces are placed on the vestibule of the 2nd molar. After that, saliva was weighed [84].

Total stimulated saliva is obtained by oral movements such as gentle masticatory movements (chewing paraffin wax or using citric acid). The most frequently used tool is a sterile cotton roll such as the Salivette (Sarsted, Newton, NC). The cotton roll is placed in the mouth, then chewed the cotton roll gently for 1-2 minutes, and then put it into a bottle. Saliva was obtained by saturating cotton using a needleless syringe or preferably by centrifugation [104; 105; 106]. The use of cotton rolls can induce variations in several salivary immunoassays such as testosterone, DHEA, estradiol, a possibly higher 17-OH hydroxyprogesterone tests, possibly lower sIgA values. Therefore, it is advisable to use non-cottons such as: polyester swab foam [108], rayon balls such as Orapette (Trinity Biotech, Dublin, Ireland) [105], and polyester Salivette (Sarsted, Newton, NC) [109].

The saliva collection protocol that must be observed is the importance of sample collection time i.e. 8 am, avoid brushing teeth, eating and drinking or chewing gum at least 30 minutes before saliva collection, rinsing with water is recommended (distilled water is recommended) [19]. Saliva collection in children is done by drooling method and assisted by suction method. After saliva collection, saliva specimens should be stored on ice, aliquoted, frozen immediately to maintain sample integrity. Refrigerators prevent the degradation of some saliva molecules, preventing bacterial growth. The study found that sIgA can be degraded at room temperature by bacterial proteases [74]. A 10% decrease in sIgA values was reported after 8 months of storage at -300 C [108].

It is recommended to avoid degradation of salivary components, saliva specimens can be stored as follows [38]:

1. Saliva liquid is stored immediately without processing. Specimens are usually stored at room temperature (when analysis is carried out immediately or within 30-90 minutes of collection) at +4ºC (when analysis is carried out within 3-6 hours after collection), at -20ºC and preferably at -80ºC (when analysis was performed several days to months after collection).
2. Freeze saliva in nitrogen liquid (mixed with saliva liquid equals to the volume of 80% glycerol in H_2O) and then dipping the sample in nitrogen solution. This procedure aims to inhibit bacterial protease activity in degrading some salivary protein components such as sIgA [105].

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