

The Effectiveness of Milk Lactoperoxidase in Increasing Salivary Lactoperoxidase Levels in Children with Early Childhood Caries

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Abstract: The high prevalence of early childhood caries (ECC) had always been a prime concern for the pediatrician. A chief causal factor that leads to early dental caries is *Streptococcus mutans*. Saliva has an antimicrobial effect as it contains vast amounts of lactoperoxidase enzyme. Other than the human saliva, lactoperoxidase could also be found in cow milk. Over the years, activation of the lactoperoxidase system had been utilized to preserve milk from microbial contamination. The objective of this study was to analyze the levels of lactoperoxidase count in saliva after consumption of pasteurized and Ultra High Temperature (UHT) milk. This study was conducted on 30 children aged 3-5 years old 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 before consumption, 7 days, and 14 days after consumption. Assessment of lactoperoxidase levels was carried out using an Enzyme-Linked Immunosorbent Assay (ELISA) reader. The results indicated that the average level of lactoperoxidase count in the pasteurized milk group increased to 0.467 ± 0.150 $\mu\text{g/ml}$ on the seventh day and 0.509 ± 0.138 $\mu\text{g/ml}$ on the fourteenth day. In the UHT milk group, there was also a notable increment in the mean level of lactoperoxidase from 0.534 ± 0.256 $\mu\text{g/ml}$ to 0.641 ± 0.276 $\mu\text{g/ml}$ on the seventh day and 0.728 ± 0.280 $\mu\text{g/ml}$ on the fourteenth day. This study had demonstrated that the consumption of milk with high levels of lactoperoxidase has a remarkable potential in increasing levels of lactoperoxidase in saliva.

Keywords: Early childhood caries (ECC), saliva, Lactoperoxidase, pasteurized milk, UHT milk

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I. Introduction

Oral health is a reflection of systemic health and this greatly impacts children's quality of life. Dental caries had always been a nodus for both the parent and clinician involved. It is a disease that attacks the hard tissues of the teeth caused by microbial activity. The occurrence of dental caries requires the interaction between the four causal factors, viz., host, microorganism, substrate, and time. The presence of a substrate attached to the dental surface would provide the necessary nutrients required for bacterial fermentation and this would subsequently lead to acid production which demineralizes the dental enamel.

Early Childhood Caries (ECC) is a term that describes a specific pattern of carious lesions in infants, toddlers, and preschoolers. The high prevalence of ECC and severe ECC (S-ECC) in early childhood had been a prime concern for health workers, particularly the pediatric dentist. Studies had postulated that the occurrence of dental caries in the primary teeth is a risk factor for potential dental caries in the permanent dentition. The etiology of ECC is multifactorial whereby diet or substrate could lead to dental caries along with other factors. The addition of sweeteners to milk or juice increases the risk of dental caries. Children that consume sweet beverages for at least twice at night are susceptible towards ECC compared to children without such dietary habits. In another investigation, the majority of children with ECC consumed more milk at night with excess sugar added compared to caries-free children ($p < 0,001$)

Saliva has a pivotal role in caries prevention (Jayaraj et al, 2015). One of the primary functions of saliva is to defend the oral cavity against the growth and proliferation of excessive microorganisms which promote oral diseases such as dental caries. The saliva protection mechanism includes immune and non-immune antimicrobial factors (lysozyme, lactoferrin, and lactoperoxidase). Lactoperoxidase is a subset of peroxidase, a group of naturally occurring enzymes present in the human saliva. The oxidation reaction between hydrogen peroxide (H_2O_2) and thiocyanate ion (SCN^-) will produce hypothiocyanate ions (OSCN^-). These OSCN compounds are responsible for eradicating bacteria, fungi, and viruses by damaging the sulfhydryl group (S-H group) of the cell membrane, resulting in a bactericidal effect. This bactericidal effect of the lactoperoxidase enzyme had been established in various studies which affirmed a correlation between the lactoperoxidase

enzyme and the *Streptococcus mutans* and *Streptococcus sanguinis* count, as well as the number of *Candida albicans* colonies. Another research predicated that toothpaste with lactoperoxidase was able to substantially reduce bacterial activity in S-ECC cases.

The catalytic activity by the lactoperoxidase enzyme produces hypothiocyanate ions (OSCN⁻) and is part of the lactoperoxidase (LPS) system. Other than the human saliva, the lactoperoxidase system could also be found in large quantities in milk. In the past few decades, the activation of the lactoperoxidase system has been used to protect milk from microbial contamination during collection and storage. Another method often used to preserve the quality of milk is through pasteurization either by high-temperature short time (HTST) or ultra-high temperature (UHT)

Milk is highly effective in the prevention of dental caries. As a complex liquid, milk is an excellent protein source that contains the cariogenic lactose. However, there are other ingredients in milk such as calcium, phosphate, casein, and lipids that are cariostatic in nature. For 50 years, it is a well-established consensus that cow milk contains fluorine which promotes remineralization. Rahardjo et al asserted that topical application using milk has the optimum protective effect against demineralization. The provision of 2-3 cups/day or equal to 480 ml/day could markedly reduce the incidence of ECC.

Hence, it is the prevailing notion of this study that the lactoperoxidase system in milk could potentially reduce the number of *Streptococcus mutans* through its antibacterial effect. This study aimed to better understand and describe the effect of the milk lactoperoxidase system in increasing the lactoperoxidase levels in saliva of children aged 3-5 years with early childhood caries (ECC).

II. Material And Methods

This was a quasi-experiment with a pretest-posttest control group design. Inclusion criteria are 3-5 years old children with dental caries affecting at least two surfaces, physically and mentally healthy, and parents that had agreed to participate in the study by filling the research subjects' consent form. Exclusion criteria included children with ongoing medication and those allergic to milk. The whole saliva and research subjects were utilized as the samples of this study.

The total sample consisted of 30 children divided into 2 groups with each group consisting of 15 children. Group A consisted of children that would consume pasteurized milk whereas group B was comprised of children that would consume UHT milk. Dental examination was first conducted to observe the condition of the child's teeth and to verify if the child had met the ECC criteria. This was carried out using an explorer, dental mirror, and torchlight. This examination was performed with the child held by the parent/guardian. After the dental examination, the whole saliva was collected on the same day. The inspection was carried out at 08.00 am in a closed room with sufficient lighting. The examiners (Z.A.B. and A.P.) were assisted by parents to collect the saliva in children with ECC. The saliva collection method employed in this study was the unstimulated saliva method. The child was instructed to sit in an upright position on the lap of the parent/guardian with his head slightly inclined for 5 minutes. The whole saliva was collected in a cup using the drooling method and if insufficient, then the suction method was employed using a sterile pipette. The saliva obtained was measured in volume and recorded in milliliters. Baseline saliva samples were collected and then stored in an icebox containing icepack. Assessment of the lactoperoxidase levels was conducted using the enzyme-linked immunosorbent assay (ELISA) reader and spectrophotometer at the integrated laboratory of the Medical Faculty of Universitas Sumatera Utara.

Sandwich ELISA was employed to measure the lactoperoxidase levels in the collected saliva samples. 100µL of the saliva sample was first added to each precoated microplate and the microplate was subsequently incubated for 90 minutes at 37°C. Aspiration and rinsing were then conducted thrice to identify the biotin-bound antibodies. 100µL of Avidin-Horseradish Peroxidase (HRP) conjugate was added and incubated for 30 minutes at 37°C. Before detection of the substrate, 5 consecutive aspirations and rinsing were conducted. 90µL of the substrate reagent was later added to allow colour development and incubated for 15 minutes at 37°C. Finally, 90µL of stop solution was added to stop any further change in colour. The colour change was then quantified using the ELISA reader at 450nm.

Statistical analysis

The collected data were processed using the SPSS program and analyzed using the Generalized Linear Model (GLM) Test with Repeated Measured and Simple Linear Regression Test (Univariate and Multivariate). This study was duly approbated by the Health Research Ethical Committee of the Medical Faculty of Universitas Sumatera Utara. Information regarding the objectives and examinations conducted in this study were provided to the subjects' parents and if they provided written informed consent, they would be included in this study.

III. Result

The average level of lactoperoxidase before (Baseline) consumption of pasteurized milk was $0.280 \pm 0.140 \mu\text{g/ml}$ whereas it was $0.534 \pm 0.256 \mu\text{g/ml}$ in the UHT milk group. (Table 1).

Table 1. Average Lactoperoxidase Levels Before Consuming Pasteurized Milk and UHT Milk (n=30)

Group	Average Baseline Lactoperoxidase Level (\pm SD) ($\mu\text{g} / \text{ml}$)
Pasteurized milk	0.280 ± 0.140
UHT milk	0.534 ± 0.256

* $p < 0,05$

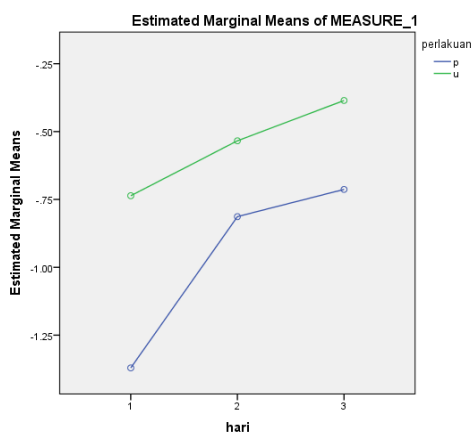
The results showed an increase in the mean levels of lactoperoxidase, on the seventh day, and the fourteenth day after the intervention, from $0.280 \pm 0.140 \mu\text{g/ml}$ to $0.467 \pm 0.150 \mu\text{g/ml}$ on the seventh day and increased to $0.509 \pm 0.138 \mu\text{g/ml}$ on the fourteenth day for the pasteurized milk group. As for the UHT milk group, there was a steady increase in the mean lactoperoxidase levels from $0.534 \pm 0.256 \mu\text{g/ml}$ to $0.641 \pm 0.276 \mu\text{g/ml}$ on the seventh day and increased to $0.728 \pm 0.280 \mu\text{g/ml}$ on the fourteenth day. The results showed that consumption of pasteurized and UHT milk was effective in increasing levels of salivary lactoperoxidase on the seventh day after consumption ($p=0.010$), whereas effectiveness between the seventh day and the fourteenth day was not significant in increasing salivary lactoperoxidase levels ($p=0.178$). (Table 2).

Table 2. The Effectiveness of Consuming Pasteurized and UHT Milk on Salivary Lactoperoxidase Levels Based on Consumption Time

	Observation time	Average Lactoperoxidase (\pm SD) ($\mu\text{g} / \text{ml}$)	Average Lactoperoxidase (\pm SD) ($\mu\text{g} / \text{ml}$)	P
		Pasteurized milk	UHT milk	
I	Before Treatment	0.280 ± 0.140	0.534 ± 0.256	0.010*
	Seven day after treatment	0.467 ± 0.150	0.641 ± 0.276	
II	Seven day after treatment	0.467 ± 0.150	0.641 ± 0.276	0.178
	Fourteen day after treatment	0.509 ± 0.138	0.728 ± 0.280	

* $p < 0,05$

Graphic 1 shows an increase in salivary lactoperoxidase levels in the pasteurized milk group and UHT milk group. The average increase in lactoperoxidase levels in the pasteurized milk group was higher compared to the UHT milk group.



Graphic 1. Average salivary lactoperoxidase levels in the pasteurized and UHT milk groups.

Statistical test for the coefficient relation produces $p=0.072$, which indicates no linear relationship between pasteurized and UHT milk with the increment in lactoperoxidase levels on the seventh day. From the value of $b=0.08$, there is an average 0.08 times increase in lactoperoxidase levels on the seventh day in the pasteurized milk group compared to the UHT group (Table 3).

In both groups, there was a 57.3% increase in lactoperoxidase levels on the seventh day to the fourteenth day and the regression equation $y = 0.087 - 0.045x$. Statistical test for the coefficient relation produces $p=0.001$, which indicates a linear relationship between the pasteurized and UHT milk groups with the increase in lactoperoxidase levels on the fourteenth day. From the value of $b = -0.045$, there is an average 0.045 times reduction in lactoperoxidase levels by the fourteenth day in the pasteurized milk group compared to the UHT group (Table 3).

Table 3. Linear regression analysis after consumption of pasteurized and UHT milk on levels of salivary lactoperoxidase

Lactoperoxidase levels	Group			
	Pasteurized milk and UHT milk			
	A	B	R ²	P
Seventh Day Difference	0,107	0,08x	0,111	0,072
Fourteenth Day Difference	0,087	-0,045x	0,573	0,001

a= constant value (*intercept*); b= regression coefficient; R= coefficient of determination; p<0,05.

IV. Discussion

The results showed that the average level of lactoperoxidase before treatment in the pasteurized milk group was $0.280 \pm 0.140 \mu\text{g/ml}$, whereas in the UHT milk group it was $0.534 \pm 0.256 \mu\text{g/ml}$. Statistical test results showed a significant difference in the initial conditions of lactoperoxidase levels in both groups ($p=0.002$) (Table 1). Various factors could influence the number of lactoperoxidase enzyme in each individual such as dietary habit and the Body Mass Index (BMI) of the child. ECC can have a considerable impact on a child's quality of life. The number of cavities and the pain suffered by the child will result in dysfunction in mastication, speech, and esthetics. Consequently, the child would begin to avoid chewing on the carious teeth, has a preference for a softer diet, consume more milk, or in some cases, refuse to eat altogether. A soft diet results in easier adhesion of bacteria to the dental surface. This could result in low BMI in children with ECC and reduced salivary secretion thus the amount of lactoperoxidase enzyme as an antimicrobial would be reduced as well.

The results showed that the pasteurized milk group showed that the average level of lactoperoxidase before consumption was $0.280 \pm 0.140 \mu\text{g/ml}$ which increased to $0.467 \pm 0.150 \mu\text{g/ml}$ on the seventh day and to $0.509 \pm 0.138 \mu\text{g/ml}$ on the fourteenth day. In the UHT group there was also an increase in the mean level of lactoperoxidase from $0.534 \pm 0.256 \mu\text{g/ml}$ to $0.641 \pm 0.276 \mu\text{g/ml}$ on the seventh day and became $0.728 \pm 0.280 \mu\text{g/ml}$ on the fourteenth day (Table 2). These results indicate that there is a positive effect of pasteurized milk and UHT milk on levels of salivary lactoperoxidase. Pasteurized milk is fresh milk that is subjected through a heating process to prevent milk contamination due to the activity of pathogenic microorganisms whilst preserving the nutritional quality of milk. Pasteurized milk could also extend the shelf life of food by temporarily deactivating pathogenic bacteria and enzymes. The pasteurization process involves heating of fresh milk at 72°C for 15 seconds, whereas UHT milk is heated in two separate heating stages. First, the milk would be heated at 75°C and then heated up to 140°C under pressure for 4 seconds. However, this heating process would alter the natural concentration of enzymes in milk. Lactoperoxidase is a temperature-stable enzyme and could still maintain its activity partially even when heated at low temperatures for a long time (63°C for 30 minutes) or at high temperatures for a short time (72°C for 15 seconds). It is noteworthy to point out that the lactoperoxidase system in milk could also be affected by storage temperature and storage time. Storage for several days at 30°C in a phosphate buffer is sufficient in preserving the amount and activity of the lactoperoxidase system in milk. Hence, on the fourteenth day, there was an insignificant increase in lactoperoxidase levels which could be attributable to the duration of milk storage which was not maintained at 30°C . Furthermore, other uncontrollable factors could have affected the results of this study, for example, the child's compliance with the researchers' instructions.

The simple linear regression equation for lactoperoxidase levels between the initial and the seventh day after the intervention was $y = 0.107 + 0.08x$, whereas the equation for the initial and the fourteenth day after the intervention was $y = 0.087 - 0.045x$. The significance of this regression equation refers to the mean difference in lactoperoxidase levels on the seventh day which was 0.08 times higher in the pasteurization group (Table 3). This could be interpreted that on the seventh day, higher amounts of pasteurized milk increases salivary lactoperoxidase levels. This is consistent with previous studies whereby the lactoperoxidase enzyme is resistant to heating temperatures wherein pasteurized milk, heating was carried out around 72°C . Heating of milk above 80°C can damage the lactoperoxidase enzyme, thus increased levels of lactoperoxidase were higher in the pasteurized milk group compared to the UHT milk group which was subjected to heating at 140°C . The difference in lactoperoxidase levels on the fourteenth day decreased by an average of $0.045 \mu\text{g/ml}$ in the pasteurization group. This was probably a result of diminished enzymatic activity of the lactoperoxidase caused by the overheating of the milk. Marin et al asserted that lactoperoxidase activity was reduced by 10% at a heating temperature of 72°C , decreased by 36% at a heating temperature of 74°C , and 67% at a temperature of 76°C . In UHT milk, heating was carried out up to 140°C and this greatly reduces the enzymatic activity of lactoperoxidase compared to the pasteurized milk. Due to the reduced activity of this enzyme in the UHT milk group, the antibacterial effect was less effective compared to the pasteurized milk group.

The current investigation presented a direct effect of the milk lactoperoxidase system in increasing levels of salivary lactoperoxidase in children aged 3-5 years with ECC. Lactoperoxidase is a subset of peroxidase, a group of naturally occurring enzymes in the human saliva. The lactoperoxidase enzyme functions

as a catalyst for the oxidation reaction between hydrogen peroxide (H₂O₂) and thiocyanate ion (SCN⁻) which produces the hypothiocyanate ions (OSCN⁻). These OSCN compounds are responsible for eradicating bacteria, fungi, and viruses by damaging the sulfhydryl group (S-H group) of the cell membrane, resulting in a bactericidal effect (Al-Baarri et al, 2011; Dwiyanti, 2009). This bactericidal effect of the lactoperoxidase enzyme had been established in various studies which affirmed a correlation between the lactoperoxidase enzyme and the *Streptococcus mutans* count.

V. Conclusion

This study had demonstrated that the consumption of pasteurized milk with high levels of lactoperoxidase has a remarkable potential in increasing levels of salivary lactoperoxidase in children with early childhood caries.

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