

Original article

## Formula milk increases lactoferrin levels in 7–9 years old children

Luthfiani<sup>1</sup>, Dwi Suryanto<sup>2</sup> and Suzanna Sungkar<sup>3</sup>

<sup>1</sup>Postgraduate student, Faculty of Dentistry, Universitas Sumatera Utara, Medan – Indonesia

<sup>2</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan – Indonesia

<sup>3</sup>Department of Paediatric Dentistry, Faculty of Dentistry, Universitas Syiah Kuala, Banda Aceh – Indonesia

### ABSTRACT

**Background:** Lactoferrin is known to have a bacteriostatic or bactericidal effect by binding ions in saliva to interfere with the survival of bacteria that need such ions, such as *Streptococcus mutans*. Lactoferrin is a whey protein and can be found in formula milk.

**Purpose:** This study aimed to analyse lactoferrin levels before and after consuming formula milk and sterilized milk. **Methods:** This study was conducted on 22 students aged 7–9 years at Public Elementary School (SD Negeri) 060817 using purposive sampling, with 11 students consuming formula milk and 11 students consuming sterilized milk. Saliva was collected by the spitting method before and after consumption on the first and seventh days. Examination of lactoferrin levels was done using the Enzyme-Linked Immunosorbent Assays (ELISA). Differences in lactoferrin levels in each group before treatment on day one and day seven were analysed using the Friedman and analysis of variance (ANOVA) tests. Differences in lactoferrin levels between the formula milk and sterilized milk groups were analysed using the independent sample *t*-test and the Mann-Whitney test ( $p < 0.05$ ). **Results:** The average levels of lactoferrin before consuming formula milk was  $0.212 \pm 0.034$  mg/100ml and increased to  $0.222 \pm 0.036$  mg/100ml and  $0.315 \pm 0.026$  mg/100ml. In the sterilized milk group, lactoferrin levels increased from  $0.216 \pm 0.033$  mg/100ml to  $0.225 \pm 0.032$  mg/100ml and  $0.235 \pm 0.027$  mg/100ml. The increase in lactoferrin levels was more significant in the formula milk group on the seventh day ( $p = 0.001$ ,  $p < 0.05$ ).

**Conclusion:** Formula milk, which contains whey protein, has a high potential in increasing lactoferrin levels.

**Keywords:** dental caries prevention; formula milk; lactoferrin; sterilized milk; whey protein

Correspondence: Luthfiani, Faculty of Dentistry, Universitas Sumatera Utara. Jl. Alumni No. 2, Medan 20155, Indonesia. Email: luthfianismd.dentist@gmail.com

### INTRODUCTION

The growth and development of a child are influenced by several factors, one of which is the health of the oral cavity. Dental caries is a common disease of the oral cavity in childhood.<sup>1</sup> Research in Shimoga City-Karnataka, India, showed the prevalence of caries in children aged 5–6 years, 9–10 years, and 14–15 years was 68.8%, 77.2%, and 48.9% respectively, and the overall prevalence of dental caries was 65.3%.<sup>2</sup> Meanwhile, the national dental health survey conducted by the National Institute of Health Research and Development through Basic Health Research or Riset Kesehatan Dasar (RISKESDAS) 2018 showed that the prevalence of caries in children aged 5–9 years in Indonesia was 92.6%. The 5–9 years group showed the

highest prevalence of caries among other age groups of children.<sup>3</sup>

An early clinical sign of caries is the presence of white spot lesions, which is enamel and dentin areas that are demineralized due to biofilms. Several factors that interact causing the occurrence of caries are a vulnerable host (teeth and saliva), carbohydrate-rich diets that can ferment, the presence of dental plaque, as well as high amounts of cariogenic microorganisms such as *Streptococcus mutans* and *Lactobacillus*.<sup>4,5</sup>

Saliva has many antibodies as a defence mechanism against infection, including antimicrobial proteins.<sup>6–8</sup> Lactoferrin is a non-enzymatic antibacterial protein formerly known as lactotransferrin, which is a transferrin group glycoprotein.<sup>6</sup> It can sequester the iron content of

pathogenic bacteria, inhibiting its growth.<sup>8</sup> Some research has shown that lactoferrin is also capable of eliminating *S. mutans* through an independent mechanism of iron.<sup>8</sup> Iron free lactoferrin, called apolactoferrin, also shows antimicrobial properties through the direct binding of lactoferrin to bacteria and agglutination of *S. mutans*, thereby facilitating the removal of agglutinated bacteria through the mechanical action of salivary ingestion.<sup>9</sup> Previous studies also showed the antimicrobial capacity of iron-free lactoferrin (both in human and bovine lactoferrin) and bovine lactoferrin against the growth of some oral *streptococcus*. Both human and bovine lactoferrin had a bacteriostatic effect that corresponds to the concentration of lactoferrin in the *Streptococcus* species tested in the study.<sup>10</sup>

Lactoferrin is also known to be present in whey, which can be found in milk.<sup>11,12</sup> However, whey protein content in cow's milk and formula milk differs significantly. Cow's milk contains 3.5% proteins, which is 80% casein and 20% whey.<sup>11,13</sup> This milk can be further processed into different types, one of which is sterilized milk that has undergone a heating process of more than 100°C.<sup>14</sup> This heating process can disrupt the stability of protein contents in the milk, including the whey protein, which will be denatured at 70°C, affecting its solubility and functions.<sup>12,14,15</sup> Meanwhile, formula milk manufactured by the industry usually contains more whey protein to mimic human's milk composition, with 40% casein and 60% whey protein.<sup>16</sup> These differences in composition lead to an assumption that formula milk, which contains more whey protein than sterilized milk, will be more effective in increasing the lactoferrin levels in saliva. This study aims to analyse the effectiveness of formula milk consumption in increasing lactoferrin levels in saliva in children aged 7–9 years when compared to sterilized milk consumption.

## MATERIALS AND METHODS

The type of research used in this study is quasi-experimental with pre- and post-test control group design. This study was carried out in Public Elementary School 060817 Jl. Sakti Lubis Workshop No.19, Medan and the Integrated Laboratory of the Faculty of Medicine, Universitas Sumatera Utara, from December 2019 to January 2020. This study was approved by the Research Ethics Commission, Universitas Sumatera Utara (No. 950/TGL/KEPK FK USU-RSUP HAM/2019).

The inclusion criteria of this study were children aged 7–9 years old that have 4–9 carious teeth who were allowed by parents to join the research with written informed consent and are generally healthy both physically and mentally. We only included 7–9 years of age children to reduce the variability of dentition status among those included in the study. The exclusion criteria of this study are children who are currently or have been taking antibiotic drugs in

the last month, have a systemic disease, use mouthwash or are allergic to milk.

The sample size in this study was calculated using the sample size formula for hypothesis testing to detect the mean difference between two paired groups, referred to in a previous study.<sup>17</sup> Subjects in this study consisted of 22 children, which were later divided into two groups: 11 samples consuming formula milk as a test group and 11 samples consuming sterilized milk as a control group.

The study subjects in each group were instructed to consume as much as one glass per day ( $\pm 200$  ml) of formula milk (Blenuten®, Ordesa, Indonesia) and as much as one can per day ( $\pm 189$  ml) of sterilized milk (Bearbrand®, Nestle, Indonesia) at 08.00 am (Western Indonesia Time) for seven days under the supervision of the researchers. The procedures were carried out at this time to make sure that all the children had not had any snacks or beverages beforehand. Subjects were asked not to eat or drink for an hour before consuming the formula or sterilized milk. The subjects were also instructed to brush their teeth at home twice a day, in the morning after breakfast and at night before bed.

Saliva collection was done one hour after consuming formula milk and sterilized milk on the first and seventh day. Saliva collection was done by the spitting method.<sup>18</sup> Before saliva collection began, the subject was instructed to sit in a comfortable position and asked to swallow the saliva present in the oral cavity. Then, the subjects were asked to collect their saliva with their lips closed for one minute and then spit it into the provided container. All containers were labelled and stored in an icebox with an ice pack.

Saliva samples were examined using Human Lactoferrin ELISA Kit (Fine Test, Catalogue No. EH0396, Wuhan Fine Biological Technology Co., Ltd., Wuhan, China) with the sandwich method and then read by the ELISA reader (Thermo Scientific Multiskan GO, 100-240V, Catalogue No. 51119200 and 51119250, Thermo Fischer Scientific Corporation, Japan). The procedures were as follows: the plate was washed two times before adding the standard saliva, the sample and the control into the well. A total of 100 $\mu$ L saliva samples were added to each well and incubated for 90 minutes at a temperature of 37°C. Then, an additional 100 $\mu$ L Biotin-detection antibody solution was added, and the wells were incubated for 60 minutes at a temperature of 37°C. Aspiration and rinsing were performed three times. A total of 100 $\mu$ L HRP-Streptavidin Conjugate (SABC) working solution was then added to each well and incubated for 30 minutes at a temperature of 37°C. Aspiration and flushing were carried out five times. Then, 90 $\mu$ L TMB Substrate was added, and this was incubated for 15–30 minutes at a temperature of 37°C. Finally, 50 $\mu$ L Stop Solution was added and a reading at 450nm wavelength was taken as soon as possible. The calculation of lactoferrin concentration in the sample was done by comparing the value with the standard curve.

Data analysis was performed using IBM SPSS Statistics for Windows, Version 25 (New York, USA). The Shapiro-

Wilk test was used to determine the normality of the data. Baseline data differences between the formula and sterilized milk groups were analysed with the independent sample t-test. Differences in lactoferrin levels in the formula milk group before treatment on days one and seven were analysed first using Friedman’s test, then with the Wilcoxon test. Differences in lactoferrin levels in the sterilized milk group before treatment on days one and seven were analysed using the ANOVA test and then the Bonferroni test. Differences in lactoferrin levels in both groups were analysed using independent sample t-tests and Mann-Whitney tests. A p-value <0.05 was considered statistically significant.

**RESULTS**

Most subjects in both groups were eight years old (68.2%). The least age characteristic in both groups was nine years old (4.5%). The overall characteristics of the study subjects by gender were 12 boys (54.5%) and 10 girls (45.5%). Most subjects had nine carious teeth, which comprised six subjects (27.3%). In the formula milk group, two subjects (9.1%) had four carious teeth, one subject (4.5%) has five carious teeth, one subject (4.5%) had six carious teeth, two subjects (9.1%) had seven carious teeth, one subject (4.5%) had eight carious teeth and the remaining four subjects (18.2%) had nine carious teeth. The subjects in the sterilized milk group were two children with four carious teeth (9.1%), one child with five carious teeth (4.5%), one child with six carious teeth (4.5%), one child with seven carious teeth (4.5%), four children with eight carious teeth (18.2%) and two children with nine carious teeth (9.1%) (Table 1).

The average level of lactoferrin before consuming milk in the formula group was  $0.212 \pm 0.034$  mg/100ml while in the sterilized milk group it was  $0.216 \pm 0.033$  mg/100ml. Normality test results showed  $p > 0.05$ , thus the test was continued using an independent sample t-test.

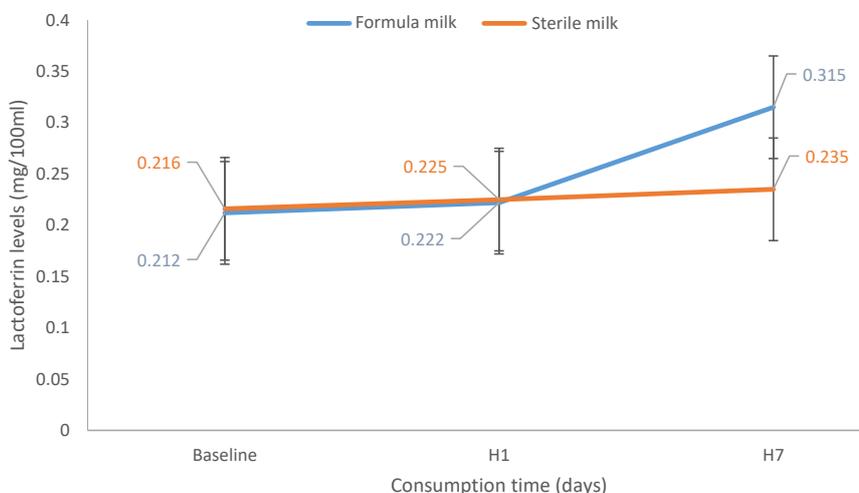
Results showed that there was no significant difference in the lactoferrin levels between either group at baseline with ( $p = 0.782$ ;  $p < 0.05$ ) (Figure 1).

There was an increase in the mean of lactoferrin levels in the formula milk group from  $0.212 \pm 0.034$  mg/100ml before consumption, to  $0.222 \pm 0.036$  mg/100ml on the first day and  $0.315 \pm 0.026$  mg/100ml on the seventh day after consumption. Data on lactoferrin levels in the formula milk group were not normally distributed and were analysed using Friedman’s test and the Wilcoxon test. The Wilcoxon test results showed that the consumption of formula milk was effective in increasing lactoferrin levels on the first and seventh days. Consumption of formula milk increased the lactoferrin levels significantly on the first ( $p = 0.003$ ;  $p < 0.05$ ) and seventh day ( $p = 0.003$ ;  $p < 0.05$ ) compared to the baseline (Figure 1).

Examination of lactoferrin levels in sterilized milk groups showed an increase from  $0.216 \pm 0.033$  mg/100ml before consumption to  $0.225 \pm 0.032$  mg/100ml on the first day and  $0.235 \pm 0.027$  mg/100ml on the seventh day after consumption. The data were normally distributed

**Table 1.** Characteristics of study subjects

Characteristic	Formula Milk		Sterilized Milk		Total	
	N	%	N	%	N	%
Age						
7	2	9.1	4	18.2	6	27.3
8	8	36.4	7	31.8	15	68.2
9	1	4.5	0	0	1	4.5
Gender						
Women	7	31.8	3	13.6	10	45.4
Men	4	18.2	8	36.4	12	54.6
No. of caries						
4	2	9.1	2	9.1	4	18.2
5	1	4.5	1	4.5	2	9.1
6	1	4.5	1	4.5	2	9.1
7	2	9.1	1	4.5	3	13.6
8	1	4.5	4	18.2	5	22.7
9	4	18.2	2	9.1	6	27.3



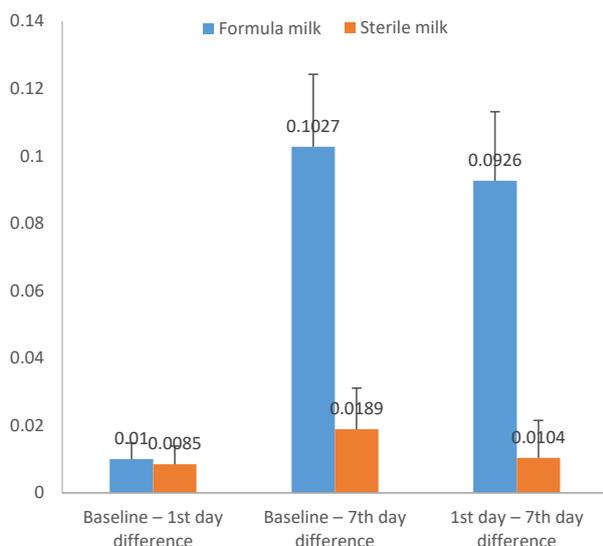
**Figure 1.** Mean lactoferrin levels in formula milk and sterilized milk based on consumption time.

and was analysed using the ANOVA test followed by the Bonferroni test. The results showed a significant increase in lactoferrin levels in the sterilized milk group after the first day of consumption ( $p=0.001$ ;  $p<0.05$ ) and at the seventh day ( $p=0.001$ ;  $p<0.05$ ) after consumption (Figure 1).

An increase in lactoferrin levels from the baseline to the first day was seen, both in formula milk and sterilized milk. However, Figure 2 shows that the mean increase was higher in the formula milk group ( $0.0100 \pm 0.0048$  mg/100ml) compared to the sterilized milk group ( $0.0085 \pm 0.0054$  mg/100ml). The increase in lactoferrin levels from baseline to the seventh day was greater in the formula milk group ( $0.1027 \pm 0.0215$  mg/100ml) compared to the sterilized milk group ( $0.0189 \pm 0.0122$  mg/100ml). The increase in lactoferrin levels from the first day to the seventh day was also higher in the formula milk group ( $0.0926 \pm 0.0205$  mg/100ml) compared to the sterilized milk group ( $0.0104 \pm 0.0111$  mg/100ml).

A normality test showed that data on the increase in lactoferrin levels from baseline to the first day and the baseline to the seventh day in the formula milk group were normally distributed  $p$ -value= $0.847$  and  $p=0.997$ ;  $p>0.05$  respectively, so the data was analysed using an independent samples  $t$ -test. Normality test results on the first to seventh day showed that the data were not normally distributed, so the analysis was done using the Mann-Whitney test.

The results showed that there was a significant difference in the increase of lactoferrin levels from baseline to the first day between the formula and sterilized milk group ( $p=0.496$ ;  $p<0.05$ ). There were also significant differences in the increase in lactoferrin levels from the baseline period to the seventh day ( $p=0.001$ ;  $p<0.05$ ) and the first day to the seventh day ( $p=0.001$ ;  $p<0.05$ ) in the two groups (Figure 2).



**Figure 2.** Comparison of increased lactoferrin levels between formula milk and sterilized milk.

## DISCUSSION

Lactoferrin levels before treatment in the formula milk group and the sterilized milk group show no significant difference. This agrees with the research conducted by Moslemi et al.,<sup>6</sup> which showed no significant differences in lactoferrin levels before treatment in overall samples. Lactoferrin level increases with age, so its concentration is expected to be lower in children than adults.<sup>19</sup> Similar to the research conducted by Moslemi et al.,<sup>6</sup> this study also included samples with a narrow age range, so it can be assumed that lactoferrin levels between the two sample groups before treatment did not have significant differences.

There was a significant increase in the mean lactoferrin levels from the baseline to the first and seventh days in the group consuming formula milk. Lactoferrin levels were also seen to increase significantly in the group consuming sterilized milk. The results of this study showed the effect of milk consumption on increased levels of lactoferrin may occur due to the protein content in the milk used in this study. Protein content in milk consists of various types, one of which is lactoferrin. The increase in lactoferrin levels in this study might be explained by the study conducted by Ye et al.,<sup>20</sup> which stated that after centrifugation in the sediment mixture of lactoferrin and heated saliva, there was an increase in lactoferrin content especially at pH 6.8 and pH 3.6. Whereas in our study, we did not do salivary pH examinations, so the increase in lactoferrin levels in the saliva is assumed to be due to milk consumption. In this study, both formula and sterilized milk consumed by the children contained whey protein. Whey protein in milk includes major and minor proteins, one of which is lactoferrin.<sup>21</sup> Thus, milk consumption is expected to increase lactoferrin levels in saliva.

The immune system is divided into two categories, the innate immune system (natural or non-specific) and the adaptive immune system (specific). The innate immune system is a direct defence against infection when the host is attacked by pathogens (viruses, bacteria, fungi, or parasites). The innate immune system responds before the adaptive one. This type of immunity provides benefits for the body. When pathogens enter the body, the innate immune system controls the development of the incoming pathogens. The antibacterial activity of lactoferrin can be increased by natural factors of immunity.<sup>22</sup> The increase in lactoferrin levels in this study is expected because milk consumption can increase immunity for the subject causing the lactoferrin levels to also increase.

Several studies were conducted to examine lactoferrin levels in saliva. Some studies analysed lactoferrin levels with the decay, missing, and filled teeth index. Lactoferrin levels in caries and caries-free children before and after treatment of their carious teeth showed there was no significant effect of the treatment performed on changes in lactoferrin levels. In contrast, the study conducted by Felizardo et al. showed

significant changes in lactoferrin levels after treatment in the children with caries group.<sup>6,23</sup>

The results showed an increase in lactoferrin levels in both the formula and sterilized milk group, but from the mean value, we can conclude that a greater increase occurred in the formula milk group. The increase in lactoferrin levels in this group was likely due to the higher protein content in the formula milk than sterilized milk. The formula milk used in this study contained 100% whey protein.<sup>21</sup>

Most studies, including our study, only investigate one protein at a time. The antimicrobial proteins in saliva are numerous and interact with each other in various ways. The interaction of these antimicrobial proteins can produce additive, synergistic or inhibitory effects. Low concentration in one of these proteins can be compensated by other proteins with similar functions.<sup>6</sup>

Study results on the sterilized milk group also showed an increased level of lactoferrin, but it was smaller than the formula milk group. The smaller increase of lactoferrin in the sterilized milk group was assumed to be due to the processing of the milk at 115-120°C for 20-30 minutes. Processing milk at high temperatures and long duration can cause denaturation of lactoferrin and reduce its biological activities.<sup>22</sup> This was in line with the previous study done by Lin et al., which showed that whey protein content in milk processed at high temperatures was significantly reduced by more than 85% when compared to raw milk.<sup>24</sup> The formula milk used in this study contained 100% whey protein, so it is reasonable to assume that the higher increases of lactoferrin level in the formula milk group were due to its higher whey protein content.

Dietary patterns and brushing habits in children were uncontrolled variables in this study. The increase in lactoferrin levels was assumed to be not only due to milk consumption but also influenced by other uncontrolled factors. Appropriately consuming formula milk is one alternative to prevent dental caries. Another limitation of this study is that we did not measure salivary pH, which can affect the lactoferrin level in saliva.<sup>20</sup>

Based on the results of this study, it can be concluded that formula milk consumption is effective in increasing lactoferrin levels in saliva when compared to sterilized milk. Formula milk consumption showed higher increases in lactoferrin levels at baseline to the first day and baseline to the seventh day when compared to the control group. It can be suggested to parents and the community to provide additional foods, such as formula milk containing whey protein, to children as an alternative option to prevent dental caries. It should be noted that the milk should be consumed in the proper way and timing, according to recommendations from doctors and dentists. Schools are also expected to educate on the use of formula milk, especially those containing whey protein, as an alternative way to prevent dental caries.

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