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ORIGINAL ARTICLE

Relationship between salivary immunoglobulin a, lactoferrin and lysozyme flow rates and lifestyle factors in Japanese children: a cross-sectional study

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ABSTRACT

Objective: The antimicrobial substances in saliva contribute to the maintenance of both oral health and overall health of the body. Therefore, the associations among immunoglobulin A (IgA), lactoferrin and lysozyme flow rates in the saliva of children, and their relationships with the physical attributes and lifestyle factors of children, were examined.

Materials and methods: Saliva was collected from 90 children who visited the Kanagawa Dental University Hospital Pediatric Dentistry, and questionnaires were completed by guardians. IgA, lactoferrin and lysozyme concentrations were measured in the saliva samples using enzyme-linked immunosorbent assays (ELISAs).

Results: The IgA flow rate in saliva increased as age, height and weight increased. A correlation was found between lactoferrin and lysozyme flow rates. When the antimicrobial substance flow rates in the saliva were divided into two groups of 22 children each based on the highest and lowest quartiles, children with either a low or high IgA flow rate also had a high or low lactoferrin flow rate, respectively. The same pattern was observed for lactoferrin and lysozyme flow rates.

Conclusions: There is a high probability that the IgA flow rate in the saliva of children reflects and corresponds to the developmental status of immune function as the child ages and increases in height and weight. The flow rates of lactoferrin and lysozyme were correlated in children. In addition, regarding lifestyle factors, the duration of sleep and lactoferrin flow rate were also related.

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Introduction

Human secretions, which include tears, saliva, breast milk including colostrum, and the mucus in the nasal cavity and intestinal tract, play an important role in protecting the human body from infections. In particular, they inhibit the adhesion of foreign microorganisms to the mucosal epithelium; neutralize viruses, enzymes and toxins; and undergo agglutination with bacteria or viruses. In the oral cavity, saliva has another important role in keeping the oral cavity moist and lubricated for chewing, swallowing and conversing. To achieve this, the major salivary glands in adults secrete approximately 1.0–1.5 L of saliva per day. Saliva also has cleansing, remineralization, buffering, digestive and antimicrobial functions, of which the antimicrobial function is key to oral cavity health. The main antimicrobial substances in saliva are immunoglobulin A (IgA), lactoferrin and lysozyme, each with its own anti-infection reaction mechanism.[1–3]

IgA is the most abundant immunoglobulin in secretions from human mucosal membranes, and approximately, 90% of IgAs exist as multimers (typically a dimer).[1] Multimeric IgA is also known as secretory IgA (sIgA), which is considered the primary detector of foreign antigens at the mucosal surface and is thought to function as a natural immune system. sIgA consists of polymeric immunoglobulin A (pIgA), a J chain and a secretory component (SC), which is necessary for epithelial transport and is resistant to degradation in the oral cavity.[1,2] The high volume of sIgA (50–200 mg) that is secreted into the saliva in adults protects the body against infections of the mucous membrane of the oral cavity, as well as of the pharynx, stomach and intestinal tract.[4] Extreme exercise can suppress sIgA release into saliva and increase the risk of an upper respiratory tract infection.[5,6] Children with an IgA deficiency repeatedly present with bronchial and lung infections, as well as bronchial asthma and gastroenteritis. Therefore, sIgA is considered a vital antibacterial substance for children.[7]

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Lactoferrin, which was originally discovered in milk, is a red glycoprotein with a molecular weight of 76,000 and a strong iron-binding activity. The two domains (N lobe and C lobe) can chelate one molecule of an iron ion. In humans, lactoferrin is widely found in external secretions such as tears, saliva and colostrum [1], where it functions to defend against infections caused by foreign microorganisms. Lactoferrin activates phagocytosis in neutrophils and macrophages and has a bacteriostatic effect through its iron-chelating ability; essentially, it reduces the availability of iron, which is a necessary substance for bacterial survival.[1] Furthermore, lactoferrin has the ability to attach itself to the outer layer of Gram-negative bacteria and weaken the cell membrane.[1] Lactoferrin concentrations in saliva increase with age, resulting in lower concentrations in infants than in adults.[8,9] The antiviral effect of lactoferrin against hepatitis B, hepatitis C and human T-lymphotropic viruses was recently reported.[10–13] Therefore, the mechanisms underlying the antimicrobial activity of lactoferrin are of increasing interest, especially for their ability to combat bacterial and viral infections.[10–13]

In 1922, Fleming observed the antimicrobial properties of the enzyme-like substance lysozyme (mucopeptide-*N*-acetylmuramoyl hydrolase [muramidase]) in secretions from tissues and tears, as well as in nasal mucus.[14] Lysozyme kills bacteria by hydrolysis, breaking the β(1-4) glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell walls.[1,3] Lysozyme can independently lyse Gram-positive bacteria; however, it cooperates with IgA and complement components to lyse Gram-negative bacteria. Hydrogen peroxide, ascorbic acid and sodium lauryl sulphate also help to increase the effectiveness of lysozyme.[1] In addition, bicarbonate promotes the bacteriolytic effect of lysozyme and stabilizes the iron-binding site of lactoferrin. Therefore, the antimicrobial function of lysozyme is boosted in a number of systems.[1]

Despite the involvement of saliva and its substances in oral and overall health, and the immune system [1–3,15,16], there are few reports of these antimicrobial substances, and their associations and dynamics, in the saliva of children. We hypothesized that the antimicrobial substances found in the saliva of children are correlated with each other and are influenced by growth and lifestyle factors.

Materials and methods

Study subjects

This study was conducted in stages from August to December 2013, from September to December 2014 and from June to July 2015. The subjects were paediatric outpatients at the Kanagawa Dental University Hospital Paediatric Dentistry, who visited for a routine or general check-up and were able to provide saliva for examination. Children who were visibly upset or who had a cold were excluded. This study enrolled 90 children (44 boys and 46 girls), with a mean age of 8.6 (2.1) years and an age range of 3–14 years. The subjects and their guardians were provided a written document describing the study, as well as a verbal explanation; saliva and answers to a questionnaire were collected for the subjects for whom written consent was provided. This study was approved by the Kanagawa Dental University's Ethics Committee on Research (approval # 291).

Questionnaire

The guardians were asked to complete a questionnaire, which was collected on the same day as completion. Child characteristics, including birth date, sex, height and weight, were collected. Lifestyle information included the duration of sleep, frequency of exercise per week, frequency of colds per year and frequency of tooth grinding per week. The duration of sleep was defined as the number of hours from the time that the child went to sleep until the time that they woke up. Information about eating habits included the frequency that breakfast and meat were consumed per week, and the frequency that vegetables, milk, fruits and yogurt were consumed per day. In addition, particular food-related likes or dislikes were collated. Each question had four possible answers, and they were instructed to select one response.

Saliva collection

At the outpatient clinic of the Kanagawa Dental University Hospital Pediatric Dentistry, normal business hours are 9 AM to 12 PM and 1 PM to 4:30 PM; children visiting during these hours were seated at the dental unit. After rinsing their oral cavities with tap water 2–3 times, Salivettes® for kids (Sarstedt K. K., Tokyo, Japan) were placed under their tongues for 1–3 min, while the child stayed calm to collect the saliva.

The collected saliva samples were placed in a hybrid high-speed refrigerated centrifuge 6200 (KUBOTA, Tokyo, Japan) and spun at 1712 *g* for 5 min at 4 °C; after the centrifugation, they were stored at −20 °C until the time of measurement.

The saliva flow rate (ml/min) was determined by dividing the weight of the saliva by the sampling time, under the assumption that the specific gravity of saliva was 1.00.

Enzyme-linked immunosorbent assays (ELISAs)

IgA concentrations

Salivary IgA concentrations were measured using a Human IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX). Briefly, goat antihuman IgA antibody (primary antibody), diluted 100 times with a solution of 0.05 M carbonate–bicarbonate (pH 9.6), was added to each well of a 96-well microplate and left at room temperature for 1 h. Excess primary antibody solution was removed by washing five times with a washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween-20, pH 8.0). Blocking was then performed by adding blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, pH 8.0) to each well. After incubating at room temperature for 30 min, the microplate was washed five times with the washing solution. Subsequently, the diluted specimen solution and human IgA standard solution (Bethyl Laboratories, Montgomery, TX) were added to each

well and incubated for 1 h at room temperature, after which the microplates were washed five times with the washing solution. Horseradish peroxidase-conjugated goat anti-human IgA antibody (secondary antibody), diluted 75,000 times, was then added to each well and the microplates were incubated at room temperature for 1 h. After washing five times with the washing solution, a colorimetric substrate containing 3,3′,5,5′-tetramethylbenzidine was added to each well in the dark at room temperature for 15 min; a quenching solution (0.18 M H_2SO_4) was added to each well to terminate the reactions. Absorbance was measured with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) at a wavelength of 450 nm. IgA concentrations were determined by creating a calibration curve (standard curve) and calculating the concentrations based on this curve and the absorbance of the samples. The IgA flow rate (μg/min) was calculated by multiplying the absolute concentration of IgA by the saliva flow rate (ml/min).

Lactoferrin concentrations

Salivary lactoferrin concentrations were measured using a Human Lactoferrin ELISA Kit (Assaypro LLC, St. Charles, MO). Briefly, sample and human lactoferrin standards (Assaypro LLC) were added to each well of a 96-well microplate and incubated at room temperature for 2 h. The excess primary antibody solution was then removed by washing five times with washing solution, followed by the addition of biotinylated anti-human lactoferrin antibody to each well and incubation of the plate for 1 h at room temperature. After washing five times with washing solution, streptavidin-peroxidase conjugate (1:100 dilution) was added to each well. After letting it sit at room temperature for 30 min and washing five times with washing solution, chromogenic substrate was added to each well for 15 min, followed by stop solution. Absorbance was measured with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) at a wavelength of 450 nm. Lactoferrin concentrations were determined by creating a calibration curve (standard curve) and calculating concentrations based on this curve and the absorbance of the samples. The lactoferrin flow rate (μg/min) was calculated by multiplying the absolute concentration of lactoferrin by the saliva flow rate (ml/min).

Lysozyme concentrations

Salivary lysozyme concentrations were measured using a Human Lysozyme ELISA Kit (Assaypro LLC, St. Charles, MO). Briefly, sample and human lysozyme standards (Assaypro LLC) were added to each well of a 96-well microplate and incubated at room temperature for 2 h. The excess primary antibody solution was then removed by washing five times with washing solution, after which biotinylated anti-human lysozyme antibody was added to each well. The plate was incubated for 1 h at room temperature and washed five times with washing solution. Subsequently, streptavidin-peroxidase conjugate (1:100 dilution) was added to each well at room temperature for 30 min, followed by washing five times with washing solution, and addition of chromogenic substrate for 15 min. Stop solution was added to each well to terminate the reactions, after which absorbance was measured with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA) at a wavelength of 450 nm. Concentrations of lysozyme were determined by creating a calibration curve (standard curve) and calculating concentrations based on this curve and the absorbance of the samples. The lysozyme flow rate (μg/min) was calculated by multiplying the absolute concentration of lysozyme by the saliva flow rate (ml/min).

Statistical analysis

The correlations between IgA, lactoferrin and lysozyme flow rates, as well as the correlations between these flow rates and the questionnaire responses, were determined using Spearman's rank correlation coefficient analysis. The flow rate of each antimicrobial substance was divided into quartiles, and the lowest quartiles (lower value group; $n = 22$) were compared with the highest quartiles (higher value group; $n = 22$) using the Wilcoxon rank sum test. In addition, the lactoferrin and lysozyme flow rates were compared between the lower and higher value groups of IgA flow rate; the lactoferrin and IgA flow rates were compared between the lower and higher value groups of lysozyme flow rate; and the lysozyme and IgA flow rates were compared between the lower and higher value groups of lactoferrin flow rate. The questionnaire responses were analysed using the chi-squared (χ^2) test. The significance level was set at 5%. SPSS 22.0 software (IBM Corp., Armonk, NY) was used for the statistical analyses.

Results

Subjects

The mean age of the 90 subjects (44 boys and 46 girls) was 8.6 (2.1) years and the age range was 3–14 years.

Correlations among the antimicrobial substances

IgA and lactoferrin flow rates were not significantly correlated (Figures 1(A,B)). Lactoferrin and lysozyme flow rates were significantly correlated, although this correlation was weak ($r = .229$, $p = .030$; Figure 1(C)).

Differences in the flow rates between the lowest and highest quartiles

The lactoferrin flow rate ($p = .008$; Figure 2(A)), but not the lysozyme flow rate ($p = .814$; Figure 2(B)), was significantly different between the lowest and highest quartiles of IgA flow rate. The IgA flow rate ($p = .016$; Figure 3(A)) and lysozyme flow rate ($p = .0003$; Figure 3(B)) were significantly different between the lowest and highest quartiles of lactoferrin flow rate. The IgA flow rate ($p = .856$; Figure 4(A)) was not, but the lactoferrin flow rate ($p = .0004$; Figure 4(B)) was significantly different between the lowest and highest quartiles of lysozyme flow rate.

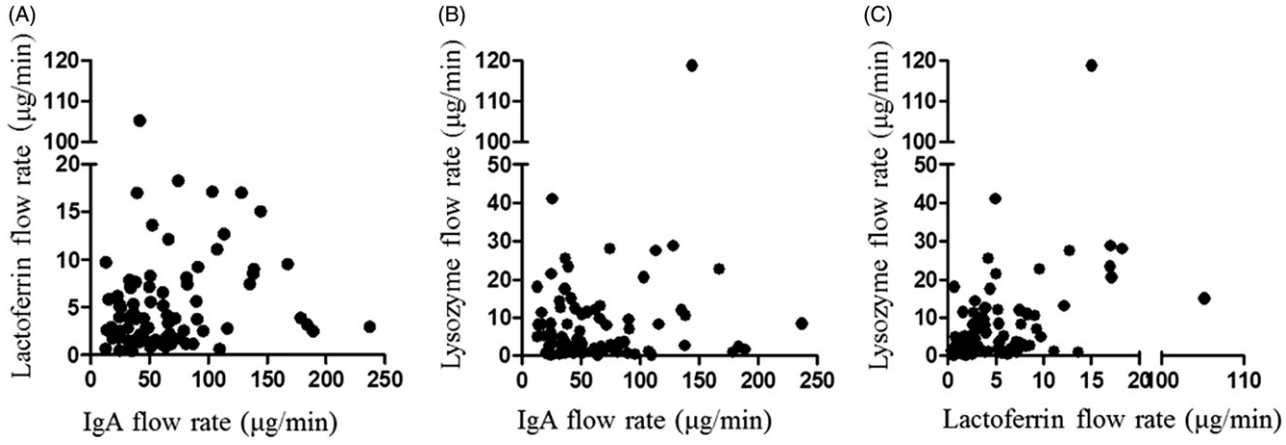

Figure 1. Correlations among the flow rates of antimicrobial substances in the saliva of 90 children, based on Spearman's rank correlation analyses. (A) IgA and lactoferrin flow rates ($r = .202$, $p > .05$), (B) IgA and lysozyme flow rates ($r = .183$, $p > .05$) and (C) lactoferrin and lysozyme flow rates ($r = .229$, $p < .05$).

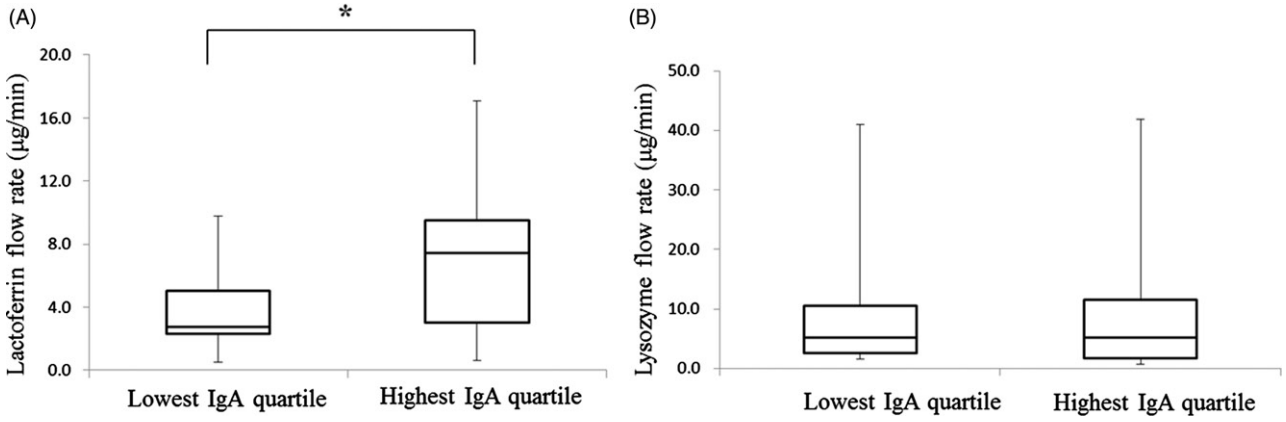

Figure 2. Comparison of (A) lactoferrin flow rate or (B) lysozyme flow rate between the highest ($n = 22$) and lowest ($n = 22$) quartiles of IgA flow rate in children. Box plots represent the smallest observation, lower quartile, median (horizontal bar), upper quartile, and largest observation. $^{*}p < .01$, compared using the Wilcoxon rank sum test.

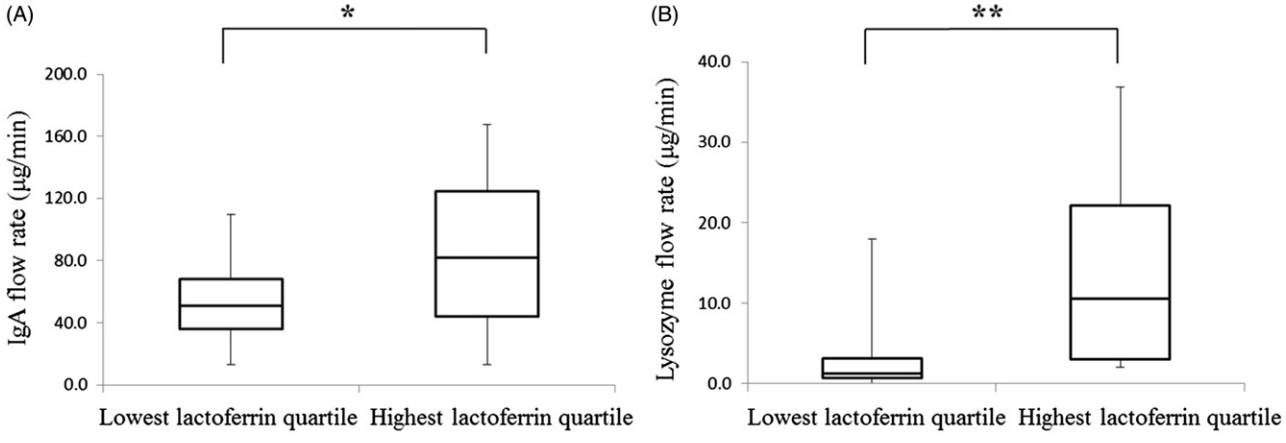

Figure 3. Comparison of (A) IgA flow rate or (B) lysozyme flow rate between the highest ($n = 22$) and lowest ($n = 22$) quartiles of lactoferrin flow rate in children. Box plots represent the smallest observation, lower quartile, median (horizontal bar), upper quartile, and largest observation. $^{*}p < .05$, $^{**}p < .01$, both compared using the Wilcoxon rank sum test.

Correlations among the antimicrobial factors and age, height and weight

A significant but weak correlation was observed between age and IgA flow rate ($r = .247$, $p = .019$; Figure 5(A)). Age was not significantly correlated with the lactoferrin flow rate (Figure 5(B)) or lysozyme flow rate (Figure 5(C)). A significant but weak correlation was observed between height and IgA flow rate ($r = .250$, $p = .017$; Figure 6(A)). Height was not significantly correlated with lactoferrin flow rate (Figure 6(B)) or lysozyme flow rate (Figure 6(C)). A significant but weak correlation was observed between weight and IgA flow rate ($r = .223$, $p = .034$; Figure 7(A)), but not between weight and

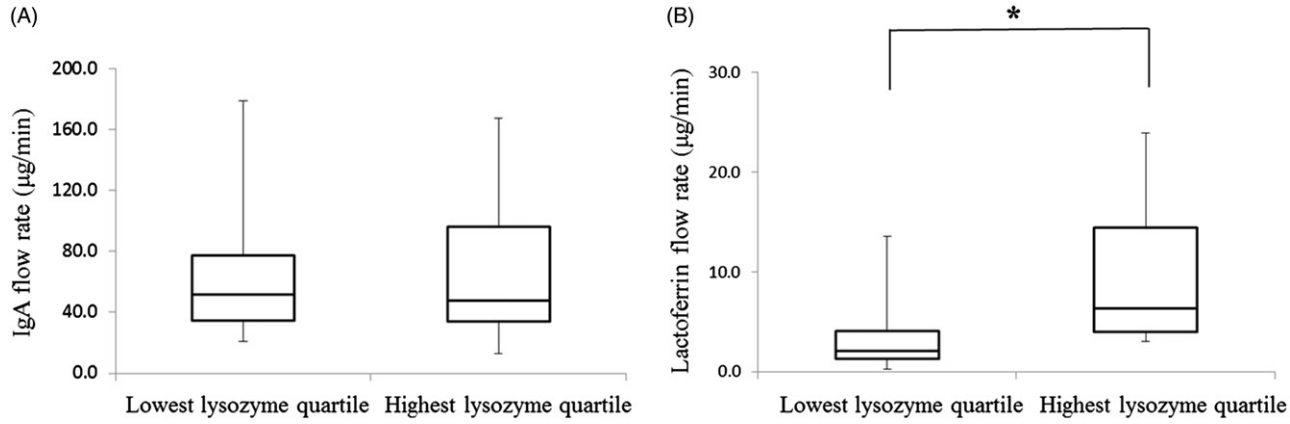

Figure 4. Comparison of (A) IgA flow rate or (B) lactoferrin flow rate between the highest ($n = 22$) and lowest ($n = 22$) quartiles of lysozyme flow rate in children. Box plots represent the smallest observation, lower quartile, median (horizontal bar), upper quartile, and largest observation. $^{*}p < .01$, compared using the Wilcoxon rank sum test.

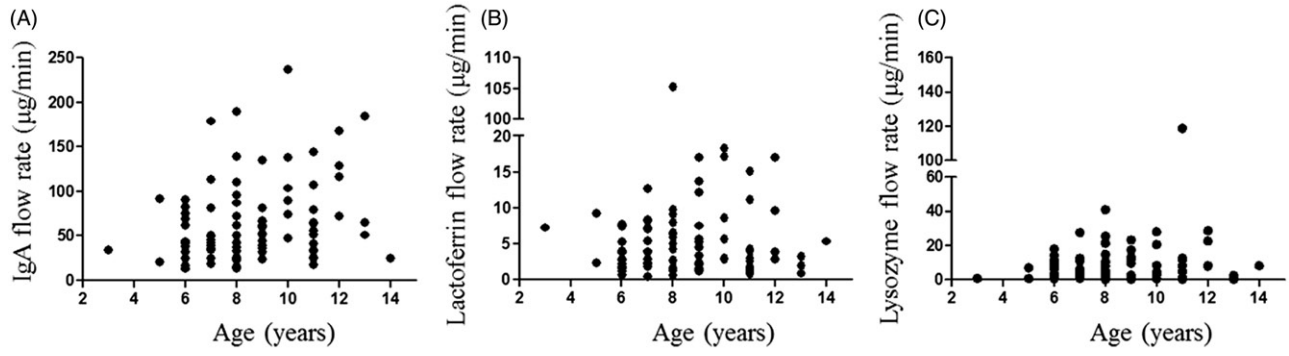

Figure 5. Correlations between age and each antimicrobial substance in saliva in children ($n = 90$), based on Spearman's rank correlation analyses. (A) IgA flow rate and age ($r = .247$, $p < .05$), (B) lactoferrin flow rate and age ($r = -.006$, $p > .05$), and (C) lysozyme flow rate and age ($r = .095$, $p > .05$).

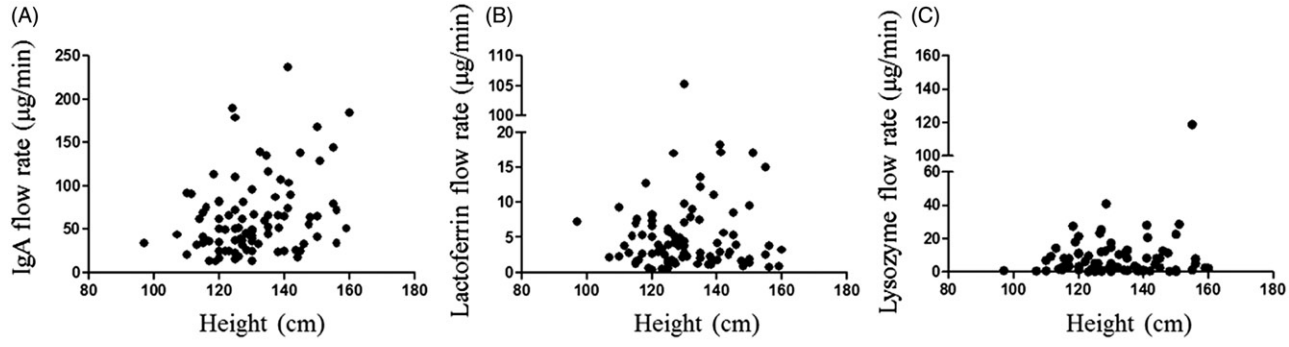

Figure 6. Correlations between height and each antimicrobial substance in saliva in children ($n = 90$), based on Spearman's rank correlation analyses. (A) IgA flow rate and height ($r = .250$, $p < .05$), (B) lactoferrin flow rate and height ($r = .007$, $p > .05$), and (C) lysozyme flow rate and height ($r = .093$, $p > .05$).

lactoferrin flow rate (Figure 7(B)) or lysozyme flow rate (Figure 7(C)).

Association between lifestyle factors and the lowest and highest quartiles of each antimicrobial substance

The Wilcoxon rank sum test for the differences in sleep duration between the highest and lowest quartiles of lactoferrin flow rate showed a significant difference ($p = 0.023$; Figure 8). The chi-square (χ^2) tests for the other lifestyle factors did not show any significant differences (Table 1).

Discussion

In the present study, lactoferrin and lysozyme flow rates in saliva were positively correlated, while the IgA and lactoferrin flow rates were not. Regarding lifestyle factors, only the duration of sleep and lactoferrin flow rates were correlated.

Consistent with the findings of this study, a previous study reported that lactoferrin and lysozyme fluctuate in conjunction with each other.[17] Although not directly associated with health, lactoferrin and lysozyme work together to alter the microbial flora of plaque.[18] In addition, an *in vitro*

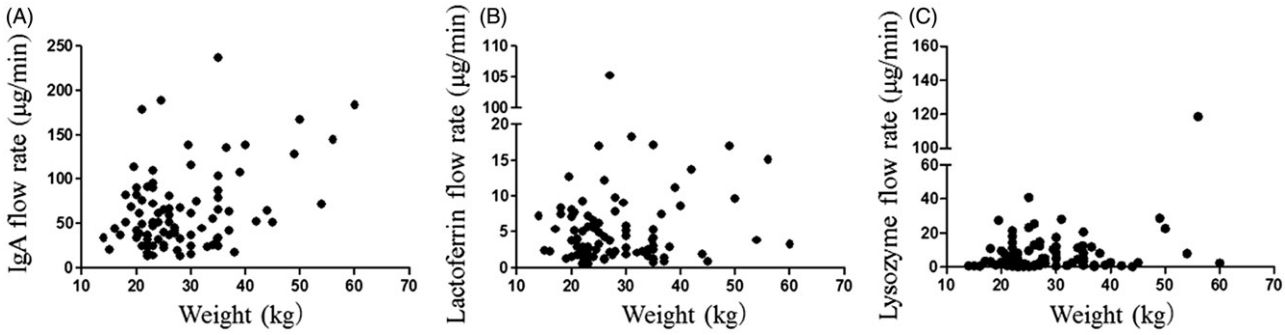

Figure 7. Correlations between weight and each antimicrobial substance in saliva in children ($n = 90$), based on Spearman's rank correlation analyses. (A) IgA flow rate and weight ($r = .223$, $p < .05$), (B) lactoferrin flow rate and weight ($r = .056$, $p > .05$), and (C) lysozyme flow rate and weight ($r = .097$, $p > .05$).

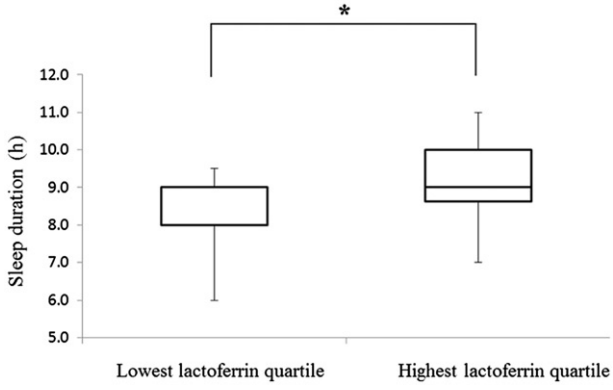

Figure 8. Comparison of sleep duration between the lowest ($n = 22$) and highest ($n = 22$) quartiles of lactoferrin flow rate in the saliva of children. Box plots represent the smallest observation, lower quartile, median (horizontal bar), upper quartile, and largest observation. *$p < .05$, compared using the Wilcoxon rank sum test.

Table 1. Differences in lifestyle factors and eating habits between the lower and higher quartiles of each antimicrobial flow rate in children ($n = 22$ per quartile).

Items	IgA flow rate (μg/min)	Lactoferrin flow rate (μg/min)	Lysozyme flow rate (μg/min)
1. Sleeping duration (h)	0.526	0.023[a]	0.386
2. Exercise (times/wk)	0.940	0.193	0.179
3. Catching a cold (times/yr)	0.623	0.938	0.601
4. Bruxism (times/wk)	0.354	0.826	0.404
5. Breakfast (times/wk)	0.221	0.220	0.157
6. Meat (times/wk)	0.248	0.545	0.672
7. Vegetables (times/d)	0.190	0.926	0.204
8. Milk (times/d)	0.541	0.595	0.328
9. Fruit (times/d)	0.495	0.439	0.144
10. Yogurt (times/d)	0.802	0.739	0.584
11. Food likes and dislikes	1.000	0.220	0.903

Item 1 was evaluated using the Wilcoxon rank sum test.
Items 2 to 11 were evaluated using chi-square (χ^2) tests.
[a]$p < .05$.

study reported that lactoferrin and lysozyme showed a synergistic effect against *Streptococcus pneumoniae*.[19] These findings suggested that lactoferrin and lysozyme are interrelated in adults and children and have anti-infection properties.

Conversely, the results of reports investigating the association between IgA and lactoferrin in saliva have been inconsistent, and a consensus has not been reached.[17,20] Although the present study could not demonstrate a significant correlation between IgA and lactoferrin flow rates in the saliva of children, a higher or lower value of one was associated with a slight tendency for the other to also be higher or lower, respectively. In human immunodeficiency virus carriers, salivary IgA and lactoferrin levels were both elevated, indicating a potential association between the two.[21] A larger sample size and investigation of the dynamics of these substances in the presence of illness are needed in future studies.

Regarding lifestyle factors and eating habits, higher lactoferrin flow rates in the saliva of children were associated with longer sleep durations, and the sleep duration was significantly longer in the highest quartile of lactoferrin flow rates compared with the lowest quartile of lactoferrin flow rates. Lactoferrin reportedly has anti-inflammatory characteristics, antioxidant properties, visceral fat-reducing properties, anti-anxiety properties and an antistress effect.[22,23] When lactoferrin was deposited under the tongue, the blood lactoferrin concentration was increased and lactoferrin was shown to move into the brain.[24] A number of previous studies of lactoferrin were conducted *in vivo* with animals [22–24], while the present study involved human subjects. It is interesting that the highest quartile of lactoferrin flow rate showed longer sleep durations in this study, demonstrating the apparent central actions of lactoferrin.

IgA flow rate was not associated with lifestyle factors in the present study. However, the secretion volume of IgA in saliva is reportedly influenced by stress, exercise and eating habits.[25–27] When indigestible carbohydrates were added to the feed of rats, IgA levels were increased in the cecum, as well as in the salivary glands and the saliva.[28] Therefore, it is thought that activation of the immune system in the intestinal tract following the consumption of particular foods could also influence the salivary glands. Regarding the amount of exercise, too much exercise might burden the body and weaken the immune system.[5,6,26,29] Further studies of a subject group with a different lifestyle are required to evaluate the association between IgA levels and lifestyle factors.

There were no other associations with lifestyle factors in the present study. The study included a limited population of only paediatric patients from a single site. Furthermore, the guardians of the children completed the questionnaire; however, as a child gets older, it becomes harder for a guardian

to know the details of all areas of the child's life, and it might be more accurate for the child to complete the questionnaire. However, the time required to explain the study and complete the questionnaire might make it impractical for a child to complete the questionnaire during the limited time available during a dentist visit.

The IgA flow rate in saliva was significantly correlated with age, height and weight. IgA production is related to the immune system of the intestinal tract and, according to organ growth curves from the research by Scammon [30], the growth of the lymphatic system, which is responsible for the immune system, peaks between the ages of 10 and 12 years. The mean age of the paediatric patients in the present study was at the upper limit of the growth curve and, therefore, the IgA flow rate in saliva might have been associated with the lymph system.[30] Furthermore, the salivary glands grow with age, and the levels of IgA in saliva might increase concurrently.

Inconsistent with previous studies, lactoferrin flow rates were not correlated with age. In previous reports that compared infants and adults [8], or children and adults (including the elderly) [9], lactoferrin values were significantly higher in adults; thus, it is thought that the flow rate of lactoferrin in saliva increases with age. Since children aged 3–14 years were included in the present study, the age range might not have been large enough to show the fluctuation of lactoferrin flow rate in saliva with age. Future studies should consider and investigate when the production capability of lactoferrin in the saliva glands peaks.

In conclusion, based on the examination of antimicrobial substances (IgA, lactoferrin and lysozyme) in the saliva of children, the flow rates of lactoferrin and lysozyme in saliva were correlated. In addition, the IgA flow rate in saliva was correlated with the age, height and weight of paediatric patients. Regarding lifestyle factors, only the lactoferrin flow rate and duration of sleep were associated. Since dental professionals are required to assist with protecting and promoting health from the perspective of oral health, future studies should investigate the characteristics of the antimicrobial substances in saliva further to provide a basis for clinical application.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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