Fundamental study on prevention of *Candida albicans* infection in elderly persons using antimicrobial substance in saliva

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Abstract

Background

According to the annual report on the aging society 2018 from the cabinet office of Japan, pneumonia accounts for 95.3 % of deaths in individuals over the age of 65 years. The primary cause of aspiration-related pneumonia is a pneumococcal infection, but it is also caused by fungus and intraoral indigenous, which is formed on the dentures via biofilm. Thus, the biofilms on the teeth, oral mucosa and the dentures have to be removed by various mechanically and chemically methods. Although denture cleaning methods have been promoted in the dental community, the number of patients with aspiration-related pneumonia is still high. Thus, in addition to general cleaning methods, preventive methods to reduce infections by *Candida albicans (C. albicans)* and intraoral indigenous bacteria are in need. From this point of view, this study focused on the host immune factors as the antimicrobial substance, which inhibit the growth of *C.albicans* and intraoral indigenous bacteria. The goal of this study was to establish the test to identify the individual risk of infectious disease, by collecting the saliva, and assessing the individual antimicrobial substance. For this purpose, the reliability of day-to-day and intraday fluctuations of the immune factors and the sustained effect of the antimicrobial substance against *C. albicans* should also be clarified as a fundamental study. Thus, the purpose of research 1 was to investigate the intraday and day-to-day fluctuations to determine the optimal times to collect the saliva sample to assess the individual values of beta-defensin, histatin, and IgA. The research 2 was purposed to investigate the effect of antimicrobial against *C.albicans* in time course.

Materials and methods

Research 1

Twenty volunteers studying or working at the Nihon University School of Dentistry at Matsudo were recruited. Resting saliva was collected twice a day in the morning from 10:00 to 11:00, and in the afternoon from 15:00 to 16:00 for 7 days by the spitting method. Participants were asked not to eat or drink 2 hours before the saliva collection and not to brush their teeth or rinse their mouths 30 min before saliva collection. Beta-defensin 3 and histatin 5 levels in saliva were determined by using a beta-defensin 3 ELISA kit (BD-3, Human, ELISA Mini Development Kit; Peprotech, Rocky Hill, NJ, USA) and the Histatin 5 ELISA Kit (Cusabio, Baltimore, MD, USA) according to the manufacturers' protocols. IgA levels in saliva were determined by using the sandwich method. Two-way analysis of variance (ANOVA) was used to evaluate the effect of intraday and day-to-day fluctuation in beta-defensin 3, histatin 5, and IgA.

Research 2

The antimicrobial effect against *C.albicans* in time course was investigated by evaluating the

fungus count, ATP activity levels and drug susceptibility test. The viable count of yeast was determined by using Brain Heart Infusion (BHI) agar medium, adjusted to 1.0 × 10< CFU/ml suspension, prepared for antimicrobial substance (beta-defensin 3 and Histatin 5) and control (CONT). The concentration of beta-defensin 3 and histatin 5 was prepared with maximum, median and minimum concentration, obtained from research 1. The viable count of yeast was measured at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after mixing and culturing the antimicrobial substances with *C.albicans*. The viable count was calibrated by 10-fold dilution method.

To determine the ATP activity, yeast culture suspensions were prepared at 1.0 × 10< CFU/ml. The ATP activity level was measured at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h by Lucifer HS kit (Kikkoman BioChemifa Co., Ltd., Japan) after mixing and culturing the antimicrobial substances and yeast culture.

The drug susceptibility was tested on the culture BHI agar medium which smeared with *C.albicans*. Antimicrobial substance with different concentrations were immersed (70 μl) on a filter paper (Grade AA DISCS (9 mm), Whatman, Japan) and placed on BHI agar medium. The diameters of inhibition zone were measured after incubation, for 24 and 48 hours at 37°C. Krsukal-Wallis with Bonferroni correction was used to compare the viable count and ATP activity between three different concentrations of antimicrobial substances and CONT at 6 hours. Pearson's correlation analysis was performed to analyze the correlation between viable count and the ATP activity.

Result

Research 1

No significant difference was observed in intraday day beta-defensin 3 (p=0.58) and histatin 5 concentration (p=0.70). IgA was significantly lower in the morning compared to the afternoon (p=0.002). No significant difference was observed in day-to-day fluctuation. No interactions due to intraday fluctuation or day-to-day fluctuation were observed for all antimicrobial substances.

Research 2

Beta-defensin 3 was mixed with the yeast culture medium, the viable count of yeast tended to increase relative to the CONT (p=0.051). Histatin 5 tended to decrease the viable count relative to the CONT (p=0.017). Bonferroni corrected tests demonstrated that Maximum concentration showed a decreasing the viable count of yeast than CONT (p=0.016).

ATP activity of beta-defensin 3 showed significant difference (p=0.050), and Bonferroni corrected tests demonstrated Maximum concentration showed a significant increase of ATP activity than CONT (p=0.039). In contrast, histatin 5 showed no significant difference (p=0.051).

Significant correlation coefficient between viable count of yeast in the culture medium and ATP activity was observed with respective concentrations of beta-defensin 3 (0.999: maximum concentration, 1.000: median concentration, and 1.000: minimum concentration, and 0.998: CONT). The correlation between total viable count and ATP activity was 0.991 (p<.0001), with beta-defensin 3. Also the significant correlation coefficient between viable count of yeast in the culture medium and ATP activity was observed with respective concentrations was observed with histatin 5 (0.913: maximum concentration, 0.907: median concentration, 0.900: minimum concentration, and 0.993: CONT). The correlation between total viable count and ATP activity was 0.975 (p<.0001).

Regardless of the elapsed time, no inhibition zone was observed with the drug susceptibility test with beta-defensin 3 and histatin at all concentrations.

Conclusion

1. This study suggests that saliva sampling in the morning, rather than in the afternoon is optimal to examine the host immune factors: i.e. beta-defensin, histatin, and IgA.

2. Histatin 5 showed significant antimicrobial effect against *C.albicans*, which suggest that the decrease of histatin 5 could lead to an increased risk of *C.albicans* infection.

I. Introduction

According to the annual report on the aging society 2018 from the cabinet office of Japan, the prevalence of 65 years and older accounts 95.3% of all deaths related to pneumonia[1)]. The primary cause of aspiration-related pneumonia is a pneumococcal infection, but it is also caused by fungus; e.g., *Candida albicans (C.albicans)*, and intraoral indigenous bacteria; e.g., *Staphylococcus aureus,* which is formed on the dentures via biofilm[2)]. Thus, the biofilms on the teeth, oral mucosa and the dentures have to be removed by various mechanically and chemically methods[3-6)]. Although denture cleaning methods have been promoted in the dental community, the death rate of elderly people due to pneumonia increasing[7)]. From the above, it is estimated that the death rate due to pneumonia will increase with the progress of the aging in Japan. Thus, in addition to general cleaning, alternative methods to reduce infections by *C.albicans* and intraoral indigenous bacteria are in need. From this point of view, this study focused on the host immune factors.

Antimicrobial factors in saliva include mucin, lactoferrin, lysozyme, peroxidase, histatin, cystatin, secretory leukocyte protease inhibitor, beta-defensin, and secretory IgA. Among these immune factors, this study focused on histatin, beta-defensin, and IgA, which inhibit the growth of *C.albicans* and intraoral indigenous bacteria [8-11)]. Although IgA levels are known to have intraday fluctuation[12, 13)], fluctuations in beta-defensin and histatin, with strong antimicrobial action, are unclear. Furthermore, the effect of concentration and continuous time of antimicrobial substances against *C.albicans* is unclear. Thus, study 1 purposed to sought the influence of intraday and day-to-day fluctuations to determine the optimal times to collect the saliva. The study 2 purposed to examined the antimicrobial effect of beta-defensin 3 and histatin 5 against *C.albicans* under the three concentration of antimicrobial substances, maximum, median and minimum, as presented in the study 1.

II. Methods and Results

1. The intraday and day-to-day fluctuation of the antimicrobial substances in saliva as an indicator of resistance to the oral mucosal disease (Research 1)

1) The participants

The participants were recruited from healthy students or workers at Nihon University School of Dentistry at Matsudo. 10 men (mean ± standard 26.4 ± 1.65, range 25–30 years old) and 10 women (25.0 ± 2.05, range 22–28 years old). The participants were enrolled after providing a written informed consent. Exclusion criteria was those who (i) could not breathe through the nose; (ii) had oral lesions, such as candidiasis, recurrent herpes labialis, recurrent aphthous stomatitis, erythema migrans, hairy tongue, or lichen planus; (iii) had abnormal salivation of <0.1 mL/min; (iv) had been receiving the medication; (v) had an autoimmune disease. This study was approved by the Human Ethics Committee of Nihon University School of Dentistry

at Matsudo (#EC16-019).

2) Protocol for saliva collection

Resting saliva was collected twice a day in the morning from 10:00 to 11:00, and in the afternoon from 15:00 to 16:00 for 7days by the spitting method[13, 14]. The participants were asked not to eat or drink 2 hours before the saliva collection and not to brush their teeth or rinse their mouths 30 min before the saliva collection.

3) Enzyme-Linked Immuno Sorbent Assay (ELISA)

Beta-defensin 3 and histatin 5 levels in saliva were determined by using a beta-defensin 3 ELISA kit (BD-3, Human, ELISA Mini Development Kit; Peprotech, Rocky Hill, NJ, USA) and the Histatin 5 ELISA Kit (Cusabio, Baltimore, MD, USA) according to the manufacturers' protocols.

IgA level was determined by using the sandwich method as following procedures; 96-well Falcon microtest assay plates (BD Biosciences, San Jose, CA) were coated with 100 ng/mL goat anti-human IgA antibody (Southern Biotechnology Associates, Birmingham, AL, USA) in phosphate-bufferd saline (PBS) and incubated overnight at 4°C. then, after the unbound goat anti-human IgA antibody was washed 3 times with 0.05% Tween 20 in PBS (washing buffer), the plates were blocked with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Then standard [human-IgA (Medical & Biological Laboratories Co., Nagoya, Japan)], or 100000-fold dilutions of saliva samples were pipetted in to the plate well and incubated for 24 hours at 4°C, then proteins was washed 3 times with washing buffer. Goat anti human IgA (H+L) HRP (Southern Biotechnology Associates, Birmingham, AL, USA) was added to the wells and incubated for 4 hours at room temperature, then washed 3 times with washing buffer. The color reaction was developed by pipetting 100 µl of 1.1 mM 2, 2'-Azino-bis (3-ethylbenzo-thiazolin-6-sulfonic acid) diammonium salt (EMD Biosciences, La Jolla, CA, USA) to the well and left for 15 minutes at room temperature, then the absorbance was measured using a microplate reader (CORONA ELECTRIC Co., Ibaraki, Japan) at 415 nm.

4) Statistical analysis

Normal distribution of the data was tested with the Kolmogorov–Smirnov test, and parametric statistical analysis was applied. Two-way analysis of variance (ANOVA) was used to evaluate the effect of intraday and day-to-day fluctuation in beta-defensin 3, histatin 5, and IgA. All statistical analyses were performed by using IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA), and $p < 0.05$ was considered statistically significant.

5) Result

Fig.1-A shows levels (mean ± SD µg/mL) of day-to-day and intraday beta-defensin 3 (A), histatin 5 (B) and IgA. No significant difference was observed in day-to-day beta-defensin 3 concentration (Monday: 41.6 ± 35.9, Tuesday: 38.2 ± 39.3, Wednesday: 36.6 ± 31.6, Thursday: 30.2 ± 27.8, Friday: 36.1 ± 36.2, Saturday: 44.22 ± 36.1, and Sunday: 44.1 ± 41.8). No significant difference was observed in intraday day (the morning: 38.0 ± 32.6 and the afternoon:

39.5 ± 38.4) (Fig.1A).

No significant difference was observed in day-to-day histatin 5 concentration (Monday: 25.6 ± 29.0, Tuesday: 22.8 ± 27.7, Wednesday: 15.9 ± 17.7, Thursday: 24.9 ± 23.6, Friday: 21.3 ± 23.4, Saturday: 18.9 ± 18.5, and Sunday: 17.9 ± 20.5). No significant difference was observed in intraday day histatin 5 concentration (in the morning: 20.5 ± 23.0 and in the afternoon: 21.6 ± 22.8) (Fig. 1B).

No significant difference was observed in day-to-day IgA concentration (Monday: 7.96 ± 5.43, Tuesday: 10.4 ± 11.3, Wednesday: 10.5 ± 10.6, Thursday: 6.37 ± 3.74, Friday: 8.46 ± 7.48, Saturday: 8.43 ± 8.73, and Sunday: 8.17 ± 8.32, respectively). IgA in the morning (5.56 ± 3.58) was significantly lower than those in the afternoon (11.6 ± 13.4) (p=0.002) (Fig.1C).

2. Effect of concentration and time on the antimicrobial activity of human saliva against *Candida albicans* (Research 2)

1) Yeast and antimicrobial substances

C.albicans was obtained from the American Type Culture Collection 90028 (Manassas, VA, USA; ATCC 90028), and were cultured at 37 < in Brain Heart Infusion (BHI) liquid medium (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) and BHI agar medium (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). The antimicrobial substances of saliva were assessed by using recombinant mouse beta-defensin 3 protein (R&Dsystems, MN, USA) and histatin 5 (PEPTIDE INSTITUTE, Osaka, Japan) at concentrations equivalent to those in human saliva.

2) Antimicrobial substance concentrations

Since antimicrobial substances concentrations had not been clarified, 3 concentrations (maximum, median, and minimum) were determined by ELISA results of research 1. The maximum, median, and minimum concentrations were 233.8, 24.3, and 2.4 ng/ml for beta-defensin 3, and 114.1, 12.3, and 1.1 μg/ml for histatin 5, respectively.

3) Measurement items and method

(1) The antimicrobial effect against *C.albicans*

The yeast cultures suspension was adjusted 1.0×10^8 CFU/ml with each antimicrobial substance and CONT comprised only the *C.albicans* in the culture. The antimicrobial effect against *C.albicans* over time was investigated using a 10-fold dilution method based on the viable count at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after mixing the antimicrobial solution in the culture medium. The mean viable count from 3-samples was calculated.

(2) Determination of ATP activity

The yeast cultures suspension was adjusted 1.0×10^5 CFU/ml with each antimicrobial substance and CONT comprised only the *C.albicans* in the culture. ATP activity levels was measured for each antimicrobial concentration and CONT by using Lucifer HS kit (Kikkoman BioChemifa Co., Ltd., Japan). Luminescence (RLU: Relative Light Unit) was measured with a

luminescence tester (Kikkoman BioChemifa Co., Ltd.)[15]. The measurement of ATP activity levels was set to 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h culture time point and mean of three samples was taken as the measured value.

(3) The drug susceptibility test

The drug susceptibility was tested on the culture BHI agar medium which smeared with *C.albicans*. Antimicrobial substance with different concentrations were immersed (70 μl) on a filter paper (Grade AA DISCS (9 mm), Whatman, Japan) and placed on the culture BHI agar medium with *C.albicans* smeared. The diameters of inhibition zone were measured at 37°C, at 24 and 48 hours.

4) Statistical analysis

Krsukal-Wallis with Bonferroni correction was used to compare the viable counts and ATP activities between three different concentrations and CONT at 6 hours. Pearson's correlation analysis was performed to analyze the correlation between viable count and the ATP activity. All analysis was performed with IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). A *p*-value less than 0.05 was considered statistically significant.

5) Result

(1) The antimicrobial effect against *C.albicans*

As shown in Table 1, viable count of beta-defensin 3 showed no significant difference (p=0.051). In contrast, histatin 5 showed significant difference (p=0.017), and Bonferroni corrected tests demonstrated Maximum concentration showed a significant decrease of the viable count of yeast than CONT (p=0.003).

(2) Analysis of ATP activity

As shown in Fig.2, beta-defensin 3 at 6 h resulted in 81×10^7 RLU (maximum concentration), 77×10^7 RLU (median concentration) and 76×10^7 RLU (minimum concentration). ATP activity of beta-defensin 3 treated samples showed marginal significance among 4 conditions (p=0.05), and Bonferroni corrected tests demonstrated that the maximum concentration showed a significant increase in ATP activity than CONT (p=0.007). Histatin 5 treatment at 6 h resulted in 19×10^7 RLU (maximum concentration), 20×10^7 RLU (median concentration), and 23×10^7 RLU (minimum concentration), showing a decreasing trend relative to the CONT, and showed no significant difference (p=0.051).

(3) The drug susceptibility

Regardless of the elapsed time, no inhibition zone was observed with the drug susceptibility test with beta-defensin 3 and histatin at all concentrations.

(4) Correlation analysis

Fig.3 shows the correlation between the viable count of yeast and ATP activity. Significant correlation coefficient between viable count of yeast in the culture medium and ATP activity

was observed with respective concentrations of beta-defensin 3 (0.999: maximum concentration, 1.000: median concentration, and 1.000: minimum concentration, and 0.998: CONT). The correlation between total viable count and ATP activity was 0.991 ($p<.0001$), with beta-defensin 3. Also the significant correlation was observed with histatin 5 (0.913: maximum concentration, 0.907: median concentration, 0.900: minimum concentration, and 0.993: CONT). The correlation between total viable count and ATP activity was 0.975 ($p<.0001$), with histatin 5.

III. Discussion

This fundamental study was conducted to clarify the effect of antimicrobial substances in saliva on *C.albicans*. The objective of research 1 was to seek the effect of intraday and day-to-day fluctuations to determine the optimal times to collect the saliva for analysis of beta-defensin, histatin, and IgA. The objective of research 2 was to determine the antimicrobial effect of beta-defensin 3 and histatin 5 against *C.albicans* for the purpose of clarifying the antimicrobial effect against *C.albicans*.

In the research 1, saliva was collected twice a day for one week continuously. Only IgA value showed intraday fluctuation, with lower level in the morning. With intraday fluctuations, Shinada et al[13]. reported that IgA in saliva showed the highest value at immediately after waking and decreased rapidly and became stable after 10:00 a.m. In the afternoon, IgA showed significantly higher values compared to the values in the morning which was comparable to the value of IgA described in the textbook[16]. Shinada et al[13] reported no significant increased IgA value in the afternoon, may be caused from the difference of subjects' background.

The secretory immunoglobulin in saliva is sensitive to psychological variables and psychological stress, which humans are often exposed [17, 18]. The stress may differ between the weekdays and weekends. However, the results indicated no influence of day-to-day effect. To know the ability of individual host immune factors to prevent various oral disease, detecting the low values of immune factors may be important to specify the individual risk. Thus, based on the results, the difference of intraday IgA is to be focused, and saliva sample in the morning, if possible from 10:00 to 11:00, may be preferable to specify the individual risk.

The research 2 revealed that antimicrobial activity against *C.albicans* using three different antimicrobial concentrations in human saliva and also clarified the antimicrobial effect up to 6-hours. The viable count of yeast increased in accordance from lower to high concentrations of beta-defensin 3. The level of viable count was higher than the CONT at 6-hours. Histatin 5 showed decrease in the viable count of yeast according to the increase of the concentrations. In general, beta-defensin 3 adhere to the cell wall[19] and demonstrate antimicrobial effect. The antimicrobial effect of histatin result from its interaction with the fungal plasma membrane [20]. The failure of antimicrobial effect of beta-defensin 3 in this study may have occurred by the experimental conditions. Beta-defensin 3 and histatin 5 did not yield inhibition zone upon

analysis of the drug susceptibility, suggesting that *C.albicans* is not susceptible to salivary antimicrobial substances and that they are naturally occurring proteins of human origin with no pharmacological effects.

Beta-defensin 3 and histatin 5 showed significant positive correlations at all concentrations between the viable count and ATP activity. ATP activity also increased in the yeast with an increase in its viable count. It was suggested that the high correlation was obtained. This may suggest that rapid and simple ATP method could be alternative method of measuring viable count.

These findings indicate that histatin 5 decreases *C.albicans* and ATP activity, thus may become an indicator of resistance or risk against the infections.

There are several limitations to this study. The results of this young age sample is unknown for the application to the elderly persons. Therefore, the sapling of aging population and investigate the optimal concentration of the antimicrobial substance is in need. Also, the in vitro experiment condition of this study may have introduced false results, especially with beta-defensin.

Although within the limitation of the study, the results draw the optimal timing of saliva collection and histatin as potential indicator of individual risk to the infections.

IV. Conclusion

1. Only IgA showed intraday fluctuation with low values in the morning.
2. No significant difference was observed in day-to-day fluctuation with all of antimicrobial substances.
3. To prevent various oral disease, and to know the ability of individual host immune factors, measurement of IgA in the morning, when the concentration of IgA is low, may be optimal.
4. Histatin 5 shows a tendency to decrease the viable count of yeast over time. Therefore, a reduction in histatin 5 may lead to an increased risk of infection.

V. References

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□. Figures and Table

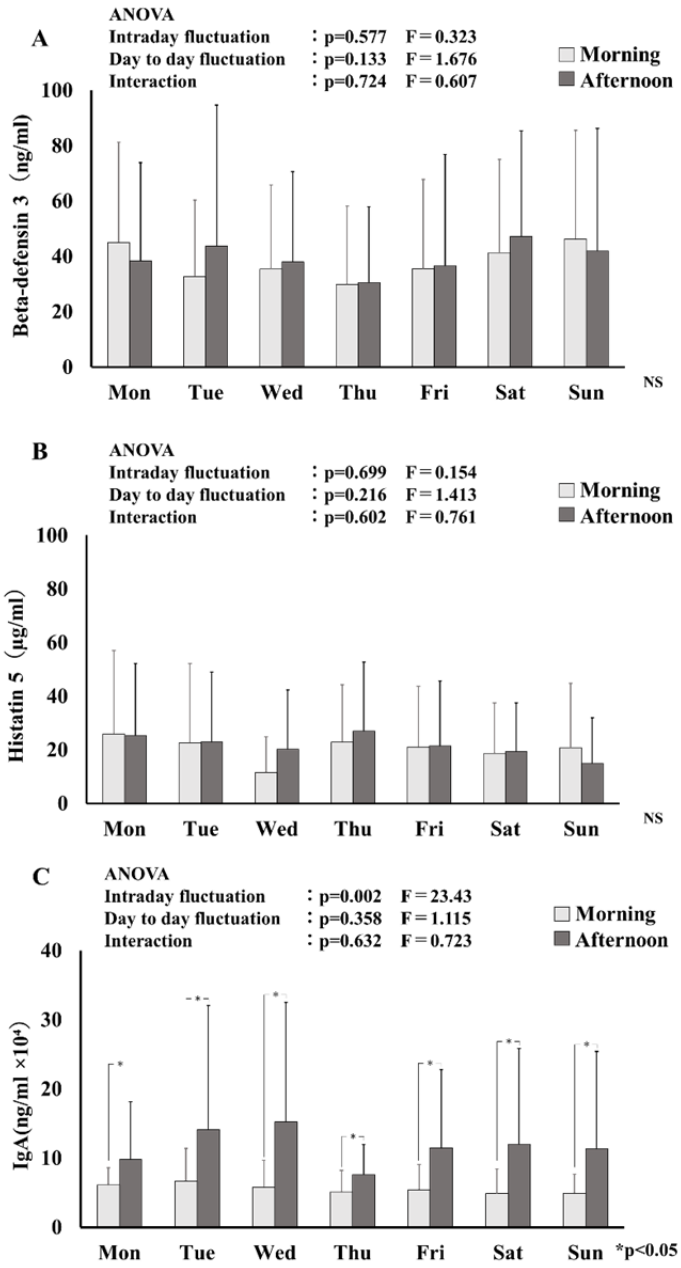

Fig. 1: Influence of saliva sampling time and day of the week on the concentrations of beta-defensin 3, histatin 5, and IgA**.** Two-way ANOVA showed no within-day or day-to-day fluctuations in the beta-defensin 3(A) or histatin 5(B), and no day-to-day fluctuation in IgA but significant differences within a day(C). * $p < 0.05$

No interactions due to intraday fluctuation or day-to-day fluctuation were observed for all antimicrobial substances of saliva.

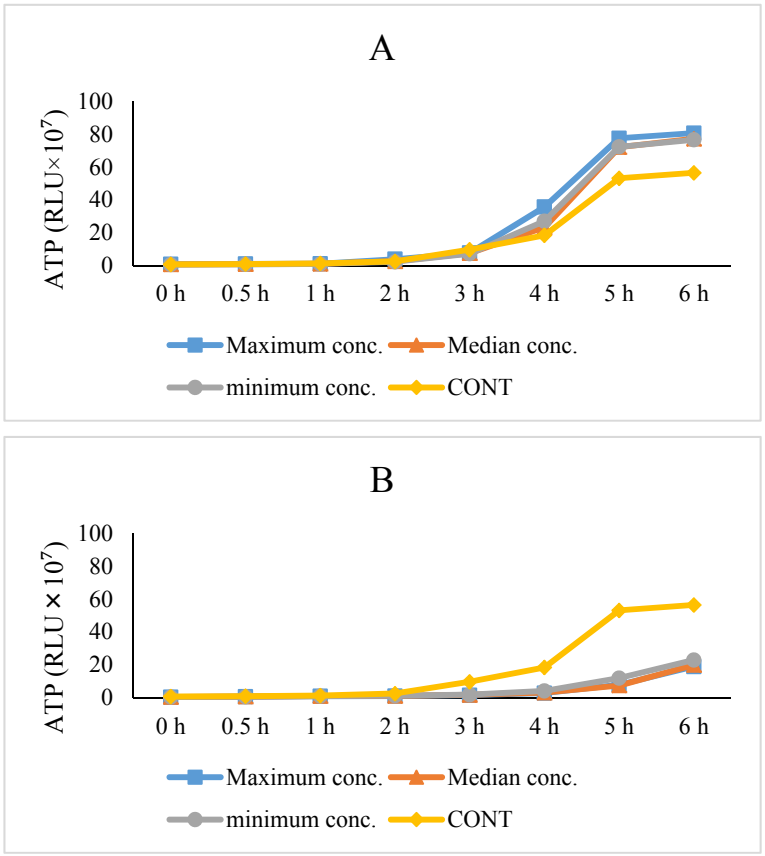

Fig. 2 Changes in antimicrobial concentrations and ATP activity levels over time upon mixing with antimicrobial substances.

A shows changes in the ATP activity levels of beta-defensin 3, B shows changes in the ATP activity levels of histatin 5. ■: Maximum concentration, ▲: Median concentration, ●: Minimum concentration, ♦: CONT. ATP activity of beta-defensin 3 showed marginal significance among 4 conditions (p=0.05), and Bonferroni corrected tests demonstrated that the maximum concentration showed a significant increase of ATP activity than CONT (p=0.007). Yeast cultures were prepared (n=3) for each antimicrobial concentration and CONT.

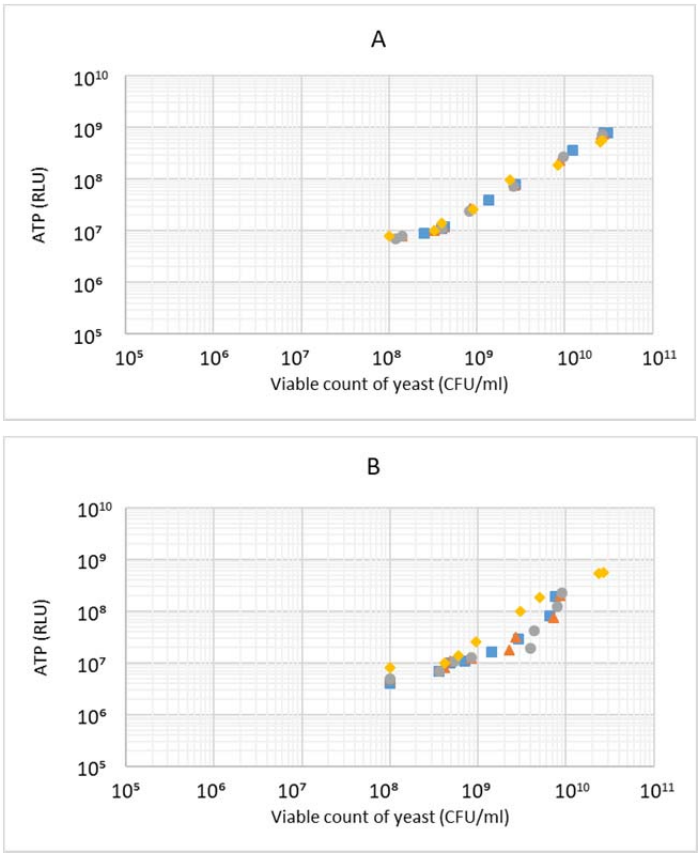

Fig. 3 Relationships between the viable count of yeast and ATP activity levels during mixing with antimicrobial substances.

A shows beta-defensin 3, B shows histatin 5, and the correlation between the viable count of yeast and ATP activity levels is shown. ■: Maximum concentration, ▲: Median concentration, ●: Minimum concentration. ◆CONT

Table 1 Change in the viable count of yeast over time in different antimicrobial substances concentrations.

		BL	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h	6 h
A	Maximum conc.	1.0	2.5	4.0	4.3	13.5	27.3	124.5	283.4	306.2
	Median conc.	1.0	1.4	3.2	4.2	8.4	26.8	88.4	259.2	277.4
	minimum conc.	1.0	1.2	1.4	4.0	8.2	25.9	97.2	264.0	275.2
B	Maximum conc.	1.0	0.6	0.9	3.2	4.0	6.7	9.9	28.8	66.0*
	Median conc.	1.0	0.7	1.0	4.0	4.2	6.8	12.5	26.7	72.0
	minimum conc.	1.0	0.7	0.9	4.0	4.5	6.9	15.8	43.2	79.2
	CONT	1.0	1.0	3.6	4.7	9.2	27.0	63.7	245.2	268.0

Note. BL: Base line. Unit are ×10□ CFU/ml. A, B shows changes in the viable count of yeast over time in different beta-defensin, histatin 5 concentrations. Viable count of beta-defensin 3 showed no significant difference (p=0.051). In contrast, histatin 5 showed significant difference (p=0.017), and Bonferroni corrected tests demonstrated that the maximum concentration showed a significant decrease of the viable count of yeast than CONT (p=0.003). Yeast cultures were prepared (n=3) for each antimicrobial concentration and CONT.