

Establishment of *Streptococcus mutans* in infants induces decrease in the proportion of salivary α -haemolytic bacteria

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Summary

Objective: For paediatric dentists, an indicator to assess caries risk of infants is very important.

Conventionally, the number and/or proportions of *Streptococcus mutans* have been employed as risk indicator. However, since such figures reflect the existing situation, they are not suitable for assessing caries risk of infants that have not yet been infected with *S. mutans*.

Thus, we searched for an indicator for the establishment of *S. mutans*.

Methods: To evaluate the changes caused by the establishment of *S. mutans* in the microbiota of the infant oral cavity, we monitored changes in the oral microbiota of two pre-dentate infants over a 3-year period and in a cross-sectional study of 40 nursery school-aged children by cultivation of saliva on non-selective blood agar, Mitis-Salivarius agar, and Mitis-Salivarius agar supplemented with bacitracin combined with identification of selected isolates.

Results: Two longitudinal observations suggested that the establishment of *S. mutans* would induce a decrease of α -haemolytic bacteria in the microbial population of the oral cavity. This suggestion was compensated with the results of cross-sectional study and it was revealed that the establishment of 10^3 CFU/mL of mutans streptococci in saliva might be predicted by a microbiota comprising less than approximately 55% of α -haemolytic.

Conclusion: Decrease of the proportion of α -haemolytic bacteria in saliva of infant was found to be applicable as an indicator to predict the establishment of *S. mutans* and to assess dental caries risk as a background for planning of dental care and treatment in the infants before infection with *S. mutans*.

Introduction

For dentists, especially paediatric dentists, an indicator that allows assessment of caries risk is very important for planning dental care and prevention of dental caries in infants. As the development of human dental caries and the condition of oral hygiene are associated with social and economical factors such as ethnicity, income, and the application of fluoride, such factors were used as indicators to discuss epidemiologically the timing of oral risk assessment and the establishment of dental care [1-4]. As mutans streptococci, especially *Streptococcus mutans*, are closely related to the development of human dental caries [5] and early colonization of *S. mutans* is a major risk factor for early childhood caries and/or future dental caries [6], the quantity of *S. mutans* in saliva or plaque has been employed as the principal indicator to assess the dental caries risk [7-10]. However, no studies focused on parameters in the oral flora influenced by the establishment of *S. mutans*. Theoretically, phenomena in the oral microbiota occurring before or after infection with *S. mutans* might be a potential predictor of infection with *S. mutans* and/or the occurrence of future caries.

Streptococcal colonization patterns in the oral cavity have been reported in some studies. In pre-dentate infants, *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus oralis* are considered to be the predominant streptococcal species. Soon after the eruption of teeth,

Streptococcus sanguinis and later mutans streptococci become established in the mouth [11-14]. It was also reported that early colonizers such as *S. oralis* play an important role in the establishment of *S. mutans* on tooth surfaces [15]. In addition, a longitudinal study of up to 2 years revealed that streptococci that produce IgA1 protease, including *S. mitis* biovar 1 and *S. oralis* predominate in the mouth and nasopharynx, conceivably because of the presence of secretory immunoglobulin A as the main protective antibody on mucosal surfaces [16]. Accordingly, the initially colonizing streptococci may facilitate establishment of *S. mutans*, which does not possess IgA1 protease. On the other hand, it has also been reported that *S. mutans* produces the anti-bacterial peptide mutacin, which inhibits other Gram-positive bacteria and mainly closely related species [17]. These findings may appear contradictory. However, it was reported that many streptococci use quorum-sensing systems to govern biofilm formation as well as expression of several physiological properties by horizontal gene transfer [18]. In any case, as the establishment of *S. mutans* would affect the other parts of the oral microbiota, we hypothesized that such changes might be a predictor of future caries risks of infants.

In this study, we investigated changes in the oral microbiota that occurred before and after colonization by *S. mutans*. We focused on the relationship between the proportions of

α -haemolytic bacteria in saliva and colonization by *S. mutans*, investigated in a longitudinal study of two pre-dentate infants over a 3-year period and by a cross-sectional study of 40 nursery school-aged children.

Materials and methods

Subjects and saliva sampling

For a longitudinal observation of the oral microbiota, we studied two pre-dentate infants (Subject A, female; Subject B, male) who had been referred to the Osaka University Dental Hospital. For the parents of these infants, we informed the purpose of this study and explained that caries preventive treatments such as application of fluoride and fissure sealant were not carried out to show the natural establishment of *S. mutans* before the experiment. We could obtain only two volunteers and these were selected as the subjects of this study. After informed consent was obtained from the respective parents, saliva samples were collected from the two infants on dentistry cotton rolls each month during the age 4 to 36 months and 3 to 43 months, respectively. The samples were kept on ice and processed within 2 hours of collection.

For the cross-sectional study, saliva samples were collected from 40 nursery school

students (aged 1 to 4 years old) during a routine dental checkup by spitting unstimulated whole saliva into a sterile tube, after receiving informed consent from their parents. The samples were kept on ice and processed within 2 hours of collection.

The study protocols were approved by the Ethics Board of the Institute of Dentistry, Osaka University.

Culture conditions and quantitation of bacteria in saliva samples

The saliva samples were serially diluted with sterilized saline, then inoculated onto Trypticase Soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% defibrinated sheep blood (blood agar) and onto Mitis-Salivarius (MS agar; Difco), or MS agar supplemented with 0.2 U/mL of bacitracin (Difco) (MSB agar). The agar plates were incubated at 37°C for 2 days under a 5% CO₂-enriched atmosphere. Thereafter, the number of colony forming units per milliliter (CFU/mL) was determined by counting the number of colonies growing on each type of culture medium yielding a countable number of colonies. For this study, the number of CFU/mL on blood agar, MS agar, and MSB agar was defined as the number of total bacteria, streptococci, and mutans streptococci, respectively. Further, the number of α -haemolytic bacterial colonies on blood agar was counted and the proportion of the total number of

bacteria in saliva was calculated.

Identification of oral streptococci in saliva samples

In the longitudinal study, we identified selected bacteria growing in the saliva cultures. We isolated at least 10 colonies based on different colony morphologies from the MS agar plate cultures. The isolates were then grown on a blood agar plate for 24 hours at 37°C in a 5% CO₂-enriched atmosphere and checked for purity. These strains were examined for hemolysis and Gram staining reaction, and then characterized biochemically using an API 20 STREP (bioMérieux, Marcy-I'Étoile, France), according to the directions of the manufacturer [19].

Statistical examination

For the cross-sectional examination of the oral microbiota, correlation analysis was performed using the age of the tested nursery school children ([age]) and proportion of α -haemolytic bacteria ([α -ratio]), as well as the common logarithms of total number of bacteria (log [BA]), streptococci (log [MS]), and mutans streptococci (log [MSB]). In addition, regression analysis of any 2 variables that showed a correlation was applied by making one value into a subordinate variable and the other into an independent variable. For these statistical analyses,

statistics processing software, StatView[®] (SAS Institute Inc.) was used.

Results

Longitudinal observation of changes of microbiota in saliva

Both of the two infants became colonized with *S. mutans* during the observation period. The transition of the microbiota in saliva was monitored for approximately 1 year after *S. mutans* was detected to confirm the sustained establishment of mutans streptococci and to study subsequent changes in the microbiota. The transition of the salivary microbiota is shown in Figure 1 and Table 1. During the examination period, the total number of cultivable bacteria and streptococci in saliva ranged from 10^7 to 10^9 CFU/mL and 10^6 to 10^8 CFU/mL, respectively. Thus, no noticeable change in the total number of bacteria occurred, in spite of environmental changes due to tooth eruption and the establishment of *S. mutans* in the oral cavity.

In Subject A, mutans streptococci were first detected at 24 months after birth, which was the time when the first primary molar erupted. On the other hand, in Subject B, mutans streptococci were first detected at 36 months after birth when the second primary molar erupted. In both subjects, the proportion of α -haemolytic bacteria began to decrease rapidly

from the time when mutans streptococci were detected in saliva. However, this result would contain limitation since the subjects were only two.

Cross-sectional study of changes of the microbiota in saliva

To statistically compensate the limitation in the result of the longitudinal observation, the cross-sectional study of changes of the microbiota in saliva was carried out and analysed with correlation analysis. In our correlation analysis of the results of the cross-sectional study of the microbiota in saliva, the following 2 combinations of 2 variables showed a correlation at a p-value of less than 0.01: [age] and [α -ratio], and [α -ratio] and log [MSB] (Table 2). As for the correlation between [age] and [α -ratio], the regression line was expressed as [α -ratio] = $96.989 - 11.082 \times [\text{age}]$; $R^2 = 0.383$ (Fig. 2A). From this expression, it was suggested that the ratio of α -haemolytic bacteria in saliva decreases with increasing age of the children. Regarding the correlation between [α -ratio] and log [MSB], the regression line was expressed as $\log [\text{MSB}] = 4.328 - 0.024 \times [\alpha\text{-ratio}]$; $R^2 = 0.241$ (Fig. 2B). From this, it was revealed that the number of mutans streptococci was high when the ratio of α -haemolytic bacteria in saliva was low.

Discussion

For paediatric dentists, it is very important to know the changes of the oral microbiota relative to pre-dentate to dentate phase, from bottle- or breast-feeding to eating phase. In particular, the transition from before to after the establishment of mutans streptococci has received much focus because this change is considered a predictor of future dental caries. In this respect, the recent comprehensive mapping of the oral microbiota by metagenomic approaches is very interesting [20, 21]. However, due to significant costs, these analyses are not realistic as a tool to assess the caries risk of an individual basis in the clinical setting. The goal of the present study was to develop a inexpensive, easy and effective procedure to monitor changes of the oral microbiota in infants that may predict the future risk of dental caries activity. Although it has been generally assumed that development of a “good oral microbiota” may be conducive to oral health, it is less clear what characterizes such a microbiota.

Our longitudinal observations on bacterial changes in saliva from two infants revealed that the early members of the oral microbiota till 12 months after birth were bacteria acquired from the maternal intestines or vagina, that the succeeding members up till 18 months after birth were early colonizing streptococci such as *S. mitis*, *S. oralis* and *S. salivarius*, and that the establishment of *S. mutans* occurred after the eruption of the primary molars. These

findings are in accordance with previous reports [16, 22, 23]. Although dramatic changes in the oral environment such as the changes of food intake and the eruption of teeth occurred during this period, it had no apparent effect on the total number of bacteria in saliva. This finding suggests a limited capacity of the oral cavity for bacteria. This may explain why introduction of *S. mutans* induced a rapid decrease of α -haemolytic bacteria in saliva.

Cross-sectional observation showed that both increasing age and the increase of mutans streptococci were associated with a decrease of proportions of α -haemolytic bacterial in saliva, in full accord with the result of the longitudinal observations. In the infants, the predominant streptococcal species are the Mitis group streptococci, which are α -haemolytic [11-14]. It was previously reported that an inverse relationship between the occurrence of *S. mutans* and *S. sanguinis* was observed during drastic carbohydrate dietary restriction [24]. With regard to an increase of *S. mutans* associated with decrease of α -haemolytic bacteria including *S. sanguinis*, this previous finding is accordance with ours. It is conceivable that the change from bottle- or breast-feeding to a more varied food intake, which correlates with increasing age, and the change from sucrose-free to sucrose-containing food, which reflects establishment of *S. mutans*, provide the background for the observed increase of *S. mutans* and ensuing decrease of α -haemolytic bacteria.

The present results of both the longitudinal and cross-sectional studies showed that establishment of *S. mutans* was associated with decreasing proportions of α -haemolytic bacteria. We consider this finding important, because the observed decrease in proportions of

α -haemolytic bacteria in saliva may be useful as an indicator of the establishment of mutans streptococci by clinical laboratories. Conventionally, MSB agar, a selective medium for mutans streptococci, has been used to detect mutans streptococci in saliva samples [25]. However, it is difficult to detect fewer than 10^3 CFU of mutans streptococci /mL saliva using that medium. Our findings suggest that the establishment of small numbers of mutans streptococci in saliva can be predicted by the demonstration of proportions of α -haemolytic bacteria less than approximately 55%. Although highly sensitive and reliable detection methods of mutans streptococci by species-specific PCR were reported [26-29], such methods require specialized and expensive equipment. In contrast, we consider the marker demonstrated in this study a convenient method to determine the establishment of mutans streptococci by simple bacteriological procedures. If paediatric dentists can obtain the cooperation of infant patients and their parents, this phenomenon may provide a basis for planning their need for dental prophylaxis and treatment.

In conclusion, our study demonstrates that oral establishment of *S. mutans* is predicted by a decrease of the proportions of α -haemolytic bacteria in saliva. Thus, the relative decrease of α -haemolytic bacteria may be applicable as an indicator to assess dental caries risk and provide a basis of planning dental care and treatment in infants. In addition, that the results

suggest that maintenance and growth of α -haemolytic bacteria in the infant oral cavity may be associated with the prevention of infection with *S. mutans*.

Bullet point:

What this paper adds

- The total number of total bacteria in saliva of infant is not significantly changed as a result of the eruption of primary teeth and the early changes of eating habits.
- Establishment of *S. mutans* in the infant oral cavity induces a decrease in proportions of α -haemolytic bacteria in saliva.
- The establishment of small numbers (10^3 CFU/mL) of mutans streptococci is predicted by proportions of α -haemolytic in infant saliva less than approximately 55%.

Why this paper is important to paediatric dentists

- The result of this paper suggested that the decrease in salivary proportions of α -haemolytic bacteria may be applicable as a predictor of the establishment of *S. mutans* and thereby of dental caries risk, and may be used as a basis for planning of dental care and treatment.

- It was suggested that the maintenance and growth of α -haemolytic bacteria in the infant oral cavity might be associated with the prevention of infection with *S. mutans*.

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Figure legends

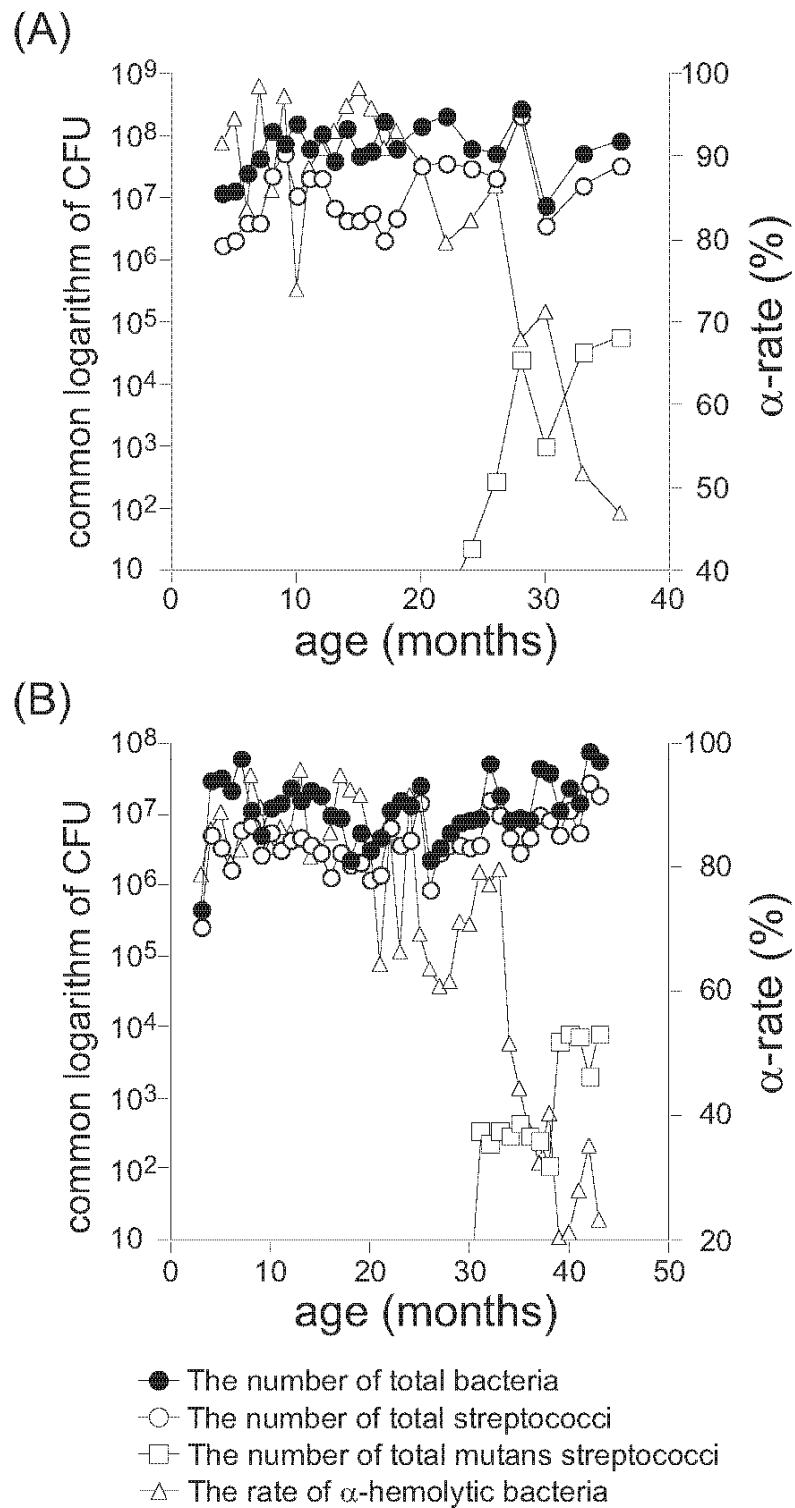


Figure 1.

Changes in number of bacteria during longitudinal observations of the oral microbiota in 2 subjects. (A) Longitudinal results of the number of bacteria in saliva samples from subject A. (B) Longitudinal results of the number of bacteria in saliva samples from subject B.

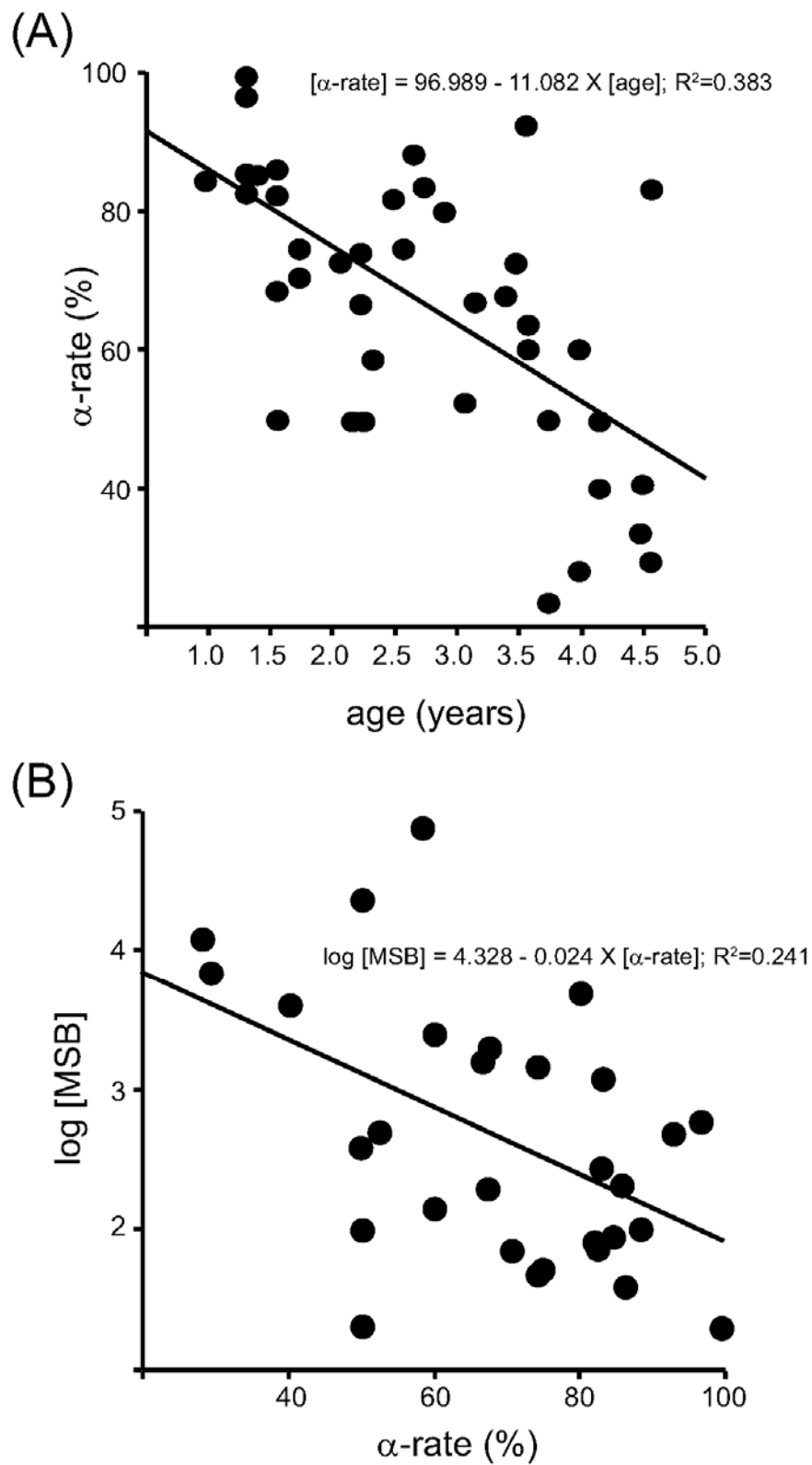


Figure 2.

Regression analyses of variables with a correlation. (A) Correlation between [age] and [α -ratio]. (B) Correlation between [α -ratio] and log [MSB].

Table 1A. The detected bacteria in the saliva of Subject A identified by API 20 Strep

Detected cocci	age (months)						
	4	6	12	18	24	30	36
<i>Aerococcus viridans</i>	3	1					
<i>Gardnerella vaginalis</i>	3	2	1				
<i>Gemella morbillorum</i>		4	1		1		
<i>Gemella haemolysans</i>		1			1		
<i>Enterococcus faecalis</i>		1					1
<i>Enterococcus avium</i>		1					
<i>Lactococcus lactis</i>		1	1				
<i>S. acidominimus</i>		1					
<i>S. mitis</i>		2	1	3	2	3	2
<i>S. sanguinis</i>		2		1		1	1
<i>S. oralis</i>		2	1	1	2	2	2
<i>S. salivarius</i>		1	2	2	1	1	1
<i>S. anginosus</i>							1
<i>S. mutans</i>					1	1	1
<i>S. pneumoniae</i>			1				
Not identified	4	1	2	3	2	2	1
Number tested (n =)	10	20	10	10	10	10	10

Table 1B. The detected bacteria from the saliva of Subject B identified by API 20 Strep

Detected cocci	age (months)						
	4	6	12	18	24	30	36
<i>Gardnerella vaginalis</i>	3						
<i>Gemella morbillorum</i>		3	2				
<i>Gemella haemolysans</i>							
<i>Enterococcus faecalis</i>				1	1		1
<i>Enterococcus faecium</i>			1				
<i>Lactococcus lactis</i>	1	3				1	1
<i>S. acidominimus</i>			4	4	4	3	2
<i>S. mitis</i>						1	1
<i>S. sanguinis</i>	1	1	1	3	2	3	1
<i>S. oralis</i>				2	1	1	1
<i>S. salivarius</i>				1	1		
<i>S. anginosus</i>							1
<i>S. mutans</i>							1
<i>S. canis</i>		1					
Not identified	5	2	2	1	1	1	1
Number tested (n =)	10	10	10	12	10	10	10

Table 2. Correlation analysis in the cross-sectional study of microflora in saliva

	Correlation coefficient	p-value
[age] vs. [a-rate]	-0.619	< 0.0001
[age] vs. log [MSB]	0.334	0.071
[age] vs. log [MS]	-0.354	0.025
[age] vs. log [BA]	-0.174	0.286
[α -rate] vs. log [MSB]	-0.491	0.005
[α -rate] vs. log [MS]	0.300	0.060
[α -rate] vs. log [BA]	0.367	0.019
log [MSB] vs. log [MS]	0.171	0.369
log [MSB] vs. log [BA]	-0.181	0.341
log [MS] vs. log [BA]	0.394	0.011