INDIVIDUAL CONSTANCY OF NUMBERS AMONG THE ORAL FLORA[1]

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The internal environment of the human mouth is conditioned by a multitude of factors among which the interactions of bacterial species are particularly accessible to close scrutiny. *In vitro* models of mutual influences among microorganisms (Rosebury *et al.*, 1954) furnish a means by which we can investigate some of the mechanisms which regulate the numbers of bacteria and the proportions among co-existing species.

Appleton (1940) stated that "the qualitative and quantitative characteristics of a microbiota are in a state of dynamic equilibrium, determined by the kinds and relative numbers of the microorganisms already present and of those being introduced and of those being lost, and by the physical or nonorganic factors of the environment." Bibby (1939) observed in bacterial smears from the mouth that "given groups of organisms tended to maintain the same relative frequency of occurrence." Lammers (1953) speculated that "the impression of inertia conveyed by the constancy of the oral flora can result only from a balance of microbiologic forces."

The quoted impressions may be sufficient justification for exploring the basic regulatory mechanisms. However, if "the quantitative interrelationships of the various microorganisms" are truly "characteristic for each person" (Williams *et al.*, 1953), then this individual constancy should be amenable to more exact mathematical definition.

MATERIALS AND METHODS

Collection and dilution of specimens. Twenty samples of unstimulated saliva were obtained from each of 10 female white-collar workers 20 to 30 years of age. The collection took place on, as far as possible, consecutive days 50 to 70 min after the teeth were brushed following breakfast. The 2 to 3 ml of saliva collected in sterile 15-ml bottles were refrigerated immediately at 4 C for approximately 1 hr and then homogenized by mixing 10 times with a pipette. One ml was diluted 10-fold with 9 ml sterile distilled water. The procedure was repeated with fresh pipettes until the desired dilution was reached. The most practical dilution was determined experimentally for each subject and each microbial category.

Plating and media. One ml quantities of the appropriate dilutions were pipetted into sterile petri dishes. Melted agar media were adjusted to pH 7.2, cooled to 45 C, and added to the specimens in approximately 15 ml quantities. The poured plates were incubated at 37 C for 48 hr under aerobic conditions.

All saliva specimens were cultured in duplicate in each of three media. For the purpose of counting the large mucoid colonies of *Streptococcus salivarius* (figure 1) we used the sucrose medium with gelatin and $CaCO_3$ as recommended by Niven *et al.* (1941). Niven mentioned that similar colony forms are produced by *Streptococcus bovis* and possibly by Leuconostoc. Therefore, we picked 200 large mucoid colonies at random and tested them for gram stain, inulin and sucrose fermentation, resistance to 60 C, and growth on 40 per cent bile blood agar. Ninety-seven per cent were typical for *S. salivarius* while the remainder were tentatively classified as *S. bovis*. The latter were encountered predominantly in one subject and, therefore, the application of a general correction factor seemed unwarranted. All colonies of this type are reported as *S. salivarius*.

Packer's (1943) selective medium for streptococci was modified by Rogosa, 1952 (*personal communication*), who prescribed the following formula: brain-heart infusion (Difco), 37.0 g; agar, 15.0 g; sodium azide, 0.2 g; crystal violet, 0.002 g; defibrinated rabbit blood, 50.0 ml per

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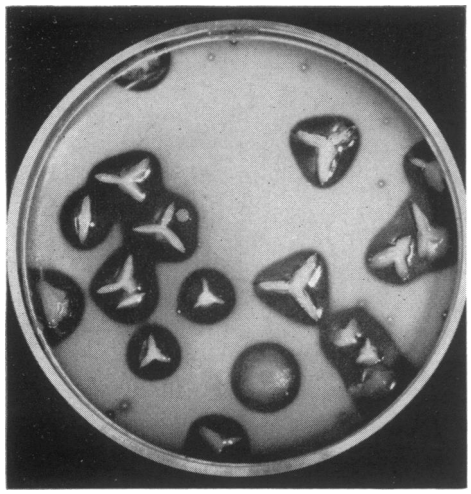

Figure 1. Large mucoid colonies of *Streptococcus salivarius* on Niven's agar.

liter. We substituted 3 per cent defibrinated horse blood for the rabbit blood. A plate of this medium streaked with *Neisseria catarrhalis*, *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, and *S. salivarius* gave growth in 48 hr to *S. salivarius* only. Gram stained slides of 125 colonies picked at random from actual test specimens gave additional evidence for the specificity of this streptococcus medium.

The third set of duplicate plates was poured with 3 per cent defibrinated horse blood in brain-heart infusion agar. It was assumed that the colonies grown in this medium corresponded to the great majority of all species of aerobic microorganisms in saliva and no attempts were made at identification of these colonies.

Estimation of bacterial counts and statistical treatment. The total numbers of colonies in the streptococcus medium and in the brain-heart infusion blood agar and the large polysaccharide producing colonies in Niven's medium were counted by means of a Quebec colony counter. As a rule, the estimates of duplicate plates differed by less than 10 per cent. In order to minimize discrepancies we adhered strictly to the rules set forth by the American Public Health Association (1953) for the countable range, the averaging of plate counts, and the usable number of digits.

The conventional estimates thus obtained were used as raw scores ("counts") for statistical treatment. The individual means were calculated as arithmetic averages of 20 counts and their standard deviations by the formula: $s = \sqrt{Sx^2/(n-1)}$ (Snedecor, 1955*a*). The group means and their standard deviations were obtained in the same way by using the individual means as the basis of calculation. The distributions of ratios were found by the same procedure, i.e., by disregarding the differing bases of the individual ratios. The mathematical methods used to evaluate the significance of our results are described in the following section.

RESULTS

The estimated counts of *S. salivarius*, genus *Streptococcus*, and the aerobic members of the total salivary flora fluctuated within wide extremes from one day to another and from one individual to another. The wide ranges of variation may be appreciated by considering the standard deviations of the means in table 1. An analysis of variance (Snedecor, 1955*b*) performed for each category of microorganisms indicated a probability of less than 1 per cent that the individual trends toward higher or lower counts were due to chance alone (Kraus and Gaston, 1955). Similarly, the variance of proportions was found to be very significantly lower within individual subjects than between them. However, classical analysis of variance is based on the assumption of homogeneity of variance and approximate normality, while our data do not meet either criterion. Accordingly, we re-analyzed our data by means of a statistic known as X_r^2 developed by Friedman (1937). This is a variance analysis based upon ranks and is applicable despite deviations from normality and homogeneity of variance.

The mean values for the individual subjects were based upon 20 observations and, therefore, may be considered reliable. These means (table 1) were made the basis of analysis by the "Method of Ranks" (Friedman, 1937). The mean counts of *S. salivarius*, of genus *Streptococcus*, and of the total salivary aerobes were found to be significantly different beyond the 1 per cent level. It is probable that the true group means are in the neighborhood of 5,000,000 for *S. salivarius*, 10,000,000 for streptococci, and 25,000,000 for all aerobic organisms per ml of unstimulated saliva. A similar analysis indicated that the subjects differed significantly (beyond the 1 per cent level) in their individual mean counts of all the bacteria under consideration.

S. salivarius comprised on an average about 50

TABLE 1

The means and standard deviations of 20 counts each of Streptococcus salivarius, genus Streptococcus, and total aerobic bacteria in millions per ml saliva and the means and standard deviations of ratios of these microbial categories for each of 10 subjects and for the entire group

	Counts			Ratios		
Subject	*Streptococcus salivarius*	Streptococci	Aerobes	*Streptococcus salivarius*/Streptococci	Streptococci/Aerobes	*Streptococcus salivarius*/Aerobes
I	0.7 ± 0.54	4.6 ± 2.61	9.6 ± 4.85	0.1856 ± 0.1126	0.4855 ± 0.1597	0.0855 ± 0.0562
II	0.7 ± 0.64	2.4 ± 1.55	7.7 ± 4.20	0.3240 ± 0.1786	0.2997 ± 0.1022	0.0920 ± 0.0535
III	9.7 ± 4.13	15.0 ± 4.75	39.3 ± 15.23	0.6470 ± 0.1667	0.4245 ± 0.1853	0.2824 ± 0.1679
IV	5.0 ± 2.83	7.3 ± 4.21	14.2 ± 8.31	0.6923 ± 0.2764	0.5364 ± 0.1665	0.4023 ± 0.2243
V	6.3 ± 3.72	16.6 ± 7.92	47.8 ± 25.60	0.4196 ± 0.2476	0.3779 ± 0.1224	0.1470 ± 0.0793
VI	0.5 ± 0.43	0.9 ± 0.69	2.8 ± 2.20	0.6024 ± 0.1350	0.3267 ± 0.1463	0.1944 ± 0.1110
VII	2.0 ± 1.34	5.1 ± 4.05	10.6 ± 6.77	0.4760 ± 0.2796	0.4913 ± 0.1559	0.2057 ± 0.1040
VIII	1.7 ± 1.23	4.5 ± 3.87	20.6 ± 27.20	0.4330 ± 0.2599	0.2678 ± 0.1325	0.1179 ± 0.0939
IX	11.8 ± 7.50	25.8 ± 18.35	52.6 ± 27.02	0.5410 ± 0.2411	0.4910 ± 0.2104	0.2509 ± 0.1617
X	12.4 ± 7.41	20.6 ± 17.67	43.5 ± 25.52	0.7117 ± 0.2344	0.4704 ± 0.0132	0.3090 ± 0.1405
Group means..	5.1 ± 4.74	10.3 ± 8.57	24.9 ± 18.86	0.5033 ± 0.1689	0.4171 ± 0.0934	0.2087 ± 0.1032

per cent of all aerobic oral streptococci (table 1) while streptococci on an average made up about 42 per cent of all aerobic organisms. The difference between these two is more apparent than real as was determined by the X_r^2 analysis. When the mean ratios of all categories were analyzed, they were reliably different ($P < .01$). However, the difference between the first two ratios was not significant while both differed significantly from the ratio of *S. salivarius* to aerobes. Differences among the subjects in average ratios carried significance at the 2 per cent level.

It remained to investigate the absolute and relative interdependence of bacterial categories or as Snedecor (1955*c*) aptly described it, "the tendency of the taller sister to have the taller brother." Such "keeping in step" is commonly measured by correlation techniques. To avoid assumptions of normality and homogeneity of variance we used Spearman's technique (Snedecor 1955*d*) for correlating ranks rather than cardinal values. We obtained an astonishing number of highly significant correlation coefficients with only 3 exceptions out of 30 (table 2). In interpreting these findings we conclude that all three microbial categories are positively related, i. e., fluctuate together within the individual (table 2). Similarly, the three categories vary together within the group (table 3). An increase in the mean *S. salivarius* fraction of the streptococci is accompanied by a proportional increase in the mean *S. salivarius* fraction among the total aerobic organisms in saliva (table 4). Also, a higher mean proportion of *S. salivarius* among the total aerobes is associated with a higher mean proportion of streptococci among the aerobes. On the other hand, a greater average of *S. salivarius* among streptococci does not necessarily go hand in hand with a greater mean proportion of streptococci among the total aerobes.

TABLE 2

Rank correlations between counts of microbial categories for each of 10 subjects

Subject	*Streptococcus salivarius* and Streptococci	Streptococci and Aerobes	*Streptococcus salivarius* and Aerobes
I	.472*	.737†	.399*
II	.738†	.789†	.721†
III	.826†	.038	−.123
IV	.809†	.702†	.365
V	.511*	.778†	.599†
VI	.498*	.711†	.759†
VII	.496*	.790†	.576†
VIII	.721†	.737†	.425*
IX	.709†	.591†	.428*
X	.834†	.772†	.808†

* $P < .05$.
† $P < .01$.

TABLE 3

Rank correlations between mean counts of microbial categories for the group of 10 subjects

Streptococcus salivarius and Streptococci	.891	$P < .01$
Streptococci and Aerobes	.915	$P < .01$
Streptococcus salivarius and Aerobes	.855	$P < .01$

TABLE 4

Rank correlations between mean ratios of microbial categories for the group of 10 subjects

Streptococcus salivarius/Streptococci and *Streptococcus salivarius*/aerobes	.939	$P < .01$
Streptococci/aerobes and *Streptococcus salivarius*/aerobes	.564	$P = .05$
Streptococcus salivarius/Streptococci and Streptococci/aerobes	.345	$P > .05$

DISCUSSION

A variety of sampling techniques are available for studying the relative distribution of microorganisms in the oral cavity and its various parts. Among these techniques are smears, scrapings, rinsing, paraffin stimulation, and the collection of unstimulated saliva. We preferred the latter method since it provided results which were least distorted by the operator's interference. Again, there are a number of ways for handling the specimens. We did not attempt to separate the bacterial cells before plating but agreed with Squires and Fuller (1952) that clumps should be accepted as normal and that it matters little whether single colonies are taken as representative of single cells or rather of "viable units."

We chose to approach our ecological problem by means of counting colonies of *S. salivarius* because these colonies are easily recognizable and because this species was thought to be "the commonest viridans variety found in the human mouth and throat" (Rosebury, 1944). Snyder *et al.* (1955) recently reported that *S. salivarius* occurred "in large numbers in all specimens of saliva" and Krasse (1953, 1954) found in 34 counts that the mean proportion of *S. salivarius* was 45.3 per cent of streptococci. He used stimulated saliva, yet his figures are very close to ours.

Along with others, Bibby (1939), Morris (1954) and Stralfors (1950) shared the view that streptococci are the most numerous aerobic microorganisms in the mouth. Rosebury (1948) cautioned that "the data on relative prominence of indigenous microorganisms on body surfaces . . . are only approximate, since quantitative information on this subject is very incomplete." Lammers (1953) claimed that streptococci and staphylococci are the most numerous oral microorganisms.

The results of the present study show that the average of *S. salivarius* approximates 50 per cent of streptococci present in saliva and that the streptococci on an average amount to little less than half of all aerobes. This leaves us with the possibility that another genus (*Staphylococcus?*) may occasionally be more numerous than the streptococci and another species (*Streptococcus mitis?*) may be equal to or more numerous than *S. salivarius* in human saliva.

We wish to point again to the choice of subjects, lest our results be unduly generalized. We sampled the saliva of a well-defined group of female adults of urban provenience. However, within the scope of our study, the degree of micro-ecological constancy is remarkably high and bears out the contentions of those authors who believe in an ecological balance among the oral flora. The significant contrast between interindividual differences and intra-individual fluctuations adequately confirms the assumption that the average quantitative composition of the salivary flora is distinctly personal.

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SUMMARY

The tendency toward higher or lower counts of aerobic organisms in unstimulated saliva appears to be an individual characteristic. The same holds true for certain genera of these bacteria, such as the streptococci, and again also for certain species of these, such as *Streptococcus salivarius*. Furthermore, an increase of one kind of organism is accompanied by a corresponding increase in the other organisms studied. Therefore, the existence of regulatory mechanisms in the salivary microbiota may justifiably be inferred. This ecological

regulation would affect both the numbers within and the proportions among species and would be a mark of the individual.

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