A QUANTITATIVE METHOD FOR ESTIMATING BACILLUS ACIDOPHILUS IN SALIVA[1]

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CONTENTS

I. INTRODUCTION

Recent work on the relationship of B. acidophilus to dental caries has developed the need for a special method for quantitative determination of B. acidophilus in the mouth. This has become especially important for further work along two lines; namely, use in comparative studies of the incidence of B. acidophilus in caries-susceptible and caries-free subjects, and in connection with attempts to reduce, by various prophylactic and dietary measures, the aciduric flora of the mouth in extreme cases of caries. Earlier work on these two aspects of dental caries has been carried out solely by qualitative means, the only available criterion for the presence of B. acidophilus in the mouth having been its growth in acid glucose-broth cultures. This test is referred to as qualitative in the sense that either a few organisms or several thousand, in the material inoculated, give the same final reading. This relatively crude method has, however, revealed striking differences between caries-susceptible and caries-free subjects. In cases of active caries, positive cultures of B. acidophilus

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are almost invariably obtained from tooth-scrapings and from saliva, although a majority of subjects continuously free from caries show negative cultures, particularly from tooth-scrapings (1). Our findings have been confirmed by Thompson (9), and Enright, Friesell, and Trescher (2), but were not verified by Tucker (10). It has been our opinion that, in the small number of caries-free cases from which positive broth cultures have been obtained, determinations of the actual number of B. acidophilus in the inocula would have shown a striking contrast to the number present in the inocula from caries-susceptible cases. If this were true, it might explain why some caries-free individuals more or less constantly give positive cultures of B. acidophilus and yet do not develop caries; for, if only a few of these organisms are present in the mouth, the fact indicates that conditions there are not conducive to their growth, and implies that caries will not develop unless a sufficient number of organisms are present to produce the required amount of acid.

With regard to study of the effect of clinical treatment on the aciduric flora of cases of extreme caries, it is obvious that the qualitative broth-method does not reveal gradual numerical decrease of these organisms. A method of quantitative examination is essential for studies of this character.

II. METHODS

Rodriguez (7) recently devised a medium by which, he believed, colonies of B. acidophilus could be recognized and differentiated from those of other mouth organisms. The medium consists of 10 percent horse-serum agar (pH 7.2), which, after inoculation, is incubated anaerobically in an atmosphere containing 10 percent of carbon dioxide. It was stated that by this method B.-acidophilus colonies developed an opaque halo, which the organisms that produced less acid did not exhibit. In our laboratory, however, this test has not given dependable results, since colonies other than those of B. acidophilus frequently showed the halo. Many special media have been recommended for the cultivation of B. acidophilus, from the intestines or from milk. It was considered possible that one of these might give the differential results required for studies on oral acidophilus. The casein-digest agar of Kulp and Rettger, the vegetable-peptone agar of Bachman and Frost, and the tomato-peptone agar of Kulp, are the better known. Any of these may be used for the enumeration of B. acidophilus in commer-

cial-milk preparations, in which the organism is present in pure culture. We have found, however, that none of them permits a satisfactory differentiation of oral B. acidophilus from certain other mouth bacteria since several cultivable mouth species, particularly the streptococci, grew as well as acidophilus on these media.

Howitt and Fleming (4), in their excellent quantitative study of mouth flora in pyorrhea, made use of anaerobic glucose veal-infusion agar-plates (pH 4.8–5.0) for the estimation of gram-positive, aciduric rods. Our attempt to use a glucose beef-infusion agar (pH 5.0, to restrain the growth of non-acid-resisting mouth bacteria) resulted in the difficulty many have encountered (although not mentioned by Howitt and Fleming) when attempting to employ agar media at high alkaline or acid reactions. Under these conditions the jellifying characteristic of agar is destroyed, if heated at high temperatures. However, if the agar is acidified after sterilization and cooling (using aseptic precautions), and plates poured at once, the usual solidification takes place. This method is obviously time-consuming and impracticable for general use. We found that a medium containing 4 percent of agar remains solid after autoclaving, if lactic acid instead of hydrochloric is employed for adjustment. This medium proved unsatisfactory, however, because colonies of B. acidophilus remained too small to permit accurate counts.

A. *Culture medium employed.* We found that Kulp's (6) tomato-peptone agar could be adjusted to pH 5.0 without altering its consistency. The only changes made by us in Kulp's formula were (a) use of 2 percent agar instead of the recommended 1.1 percent, and (b) adjustment of the reaction to pH 5.0, instead of 6.2, by addition of lactic acid. This medium had the following advantages for our purposes: (*a*) The combination of filtered tomato juice, peptonized milk, peptone, agar, and water brings the initial reaction to about pH 5.4. Therefore, very little lactic acid (usually about 5 cc. of normal acid per liter of medium) is required to give a final reaction of pH 5.0. (*b*) Addition of 2 percent agar gives a medium that remains solid after autoclaving at 15-lb. pressure for 15 minutes. (*c*) It is very easily prepared, and gives a clear, yellow product. No addition of carbohydrate is required. (*d*) All the colony phases of B. acidophilus, including smooth, intermediate, and rough, grow well and can be easily recognized. Most oral streptococci are inhibited; occasionally an aciduric strain grows, but its colonies can be differentiated from those of B. acidophilus by their pin-point size. A yeast-like organism, frequently present in the mouth, can tolerate this acidity, but its colonies are easily distinguished by their greater size (*figs. 2* and *8*). Colonies of M. tetragenus and

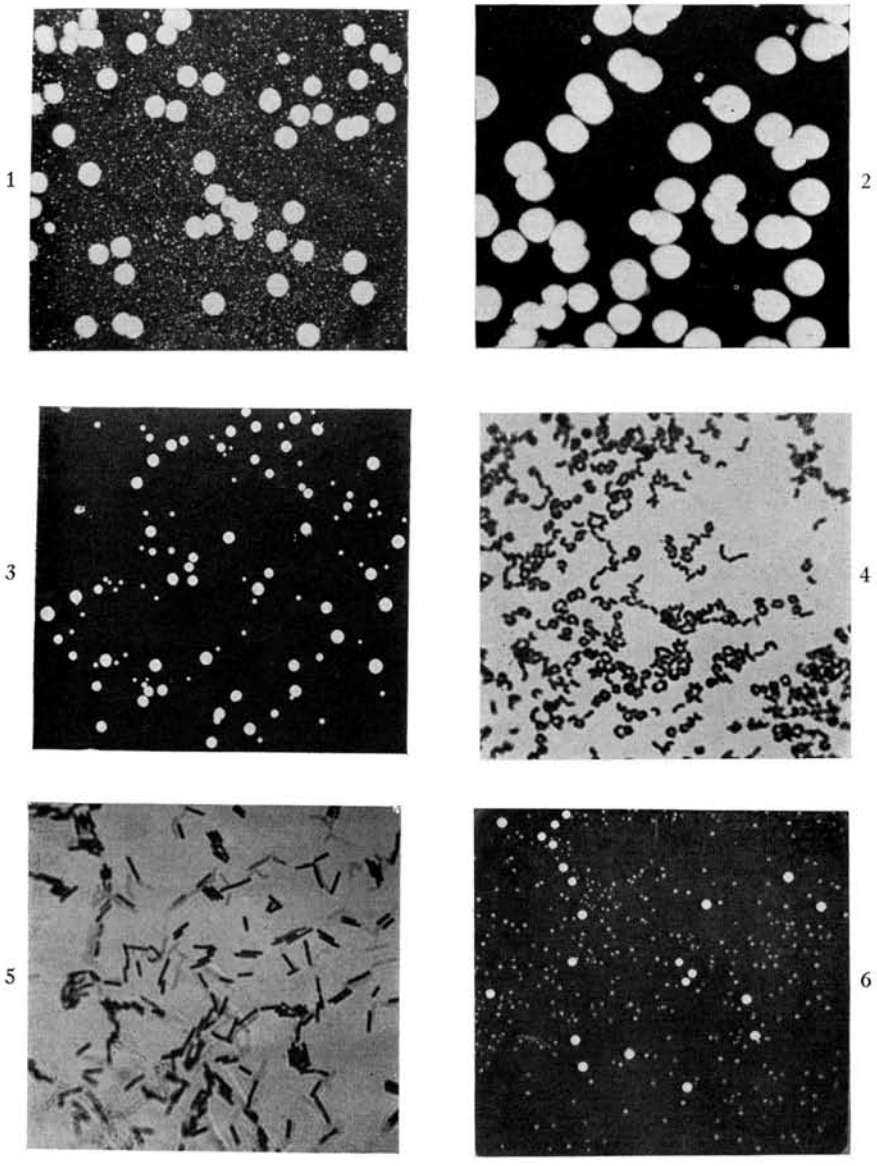

FIG. 1. Undiluted "caries-free" saliva (0.4 cc.) on glucose beef-infusion agar (pH 7.0). Large colonies, yeast; small ones, streptococci, staphylococci, and B. acidophilus. Impossible to count B. acidophilus colonies.

FIG. 2. Undiluted "caries-free" saliva (0.4 cc.)—same sample as in *fig. 1*—on tomato-peptone agar (pH 5.0). Large colonies, yeast. Small colonies, B. acidophilus—25 per cc. of saliva.

FIG. 3. "Caries-susceptible" saliva (0.1 cc. of 1–20 dilution) on tomato-peptone agar (pH 5.0). All colonies, B. acidophilus—23,000 per cc. of saliva. Smaller colonies, B. acidophilus, Type I; larger ones, B. acidophilus, Type II (note darker centers).

FIG. 4. B. acidophilus, Type I; organisms from Type-I colony in *fig. 3*.

FIG. 5. B. acidophilus, Type II; organisms from Type-II colony in *fig. 3*.

FIG. 6. "Caries-susceptible" saliva (0.1 cc. of 1–20 dilution) on tomato-peptone agar (pH 5.0). Larger, white colonies—B. acidophilus, Type I; smaller, gray ones—B. acidophilus, Type III.

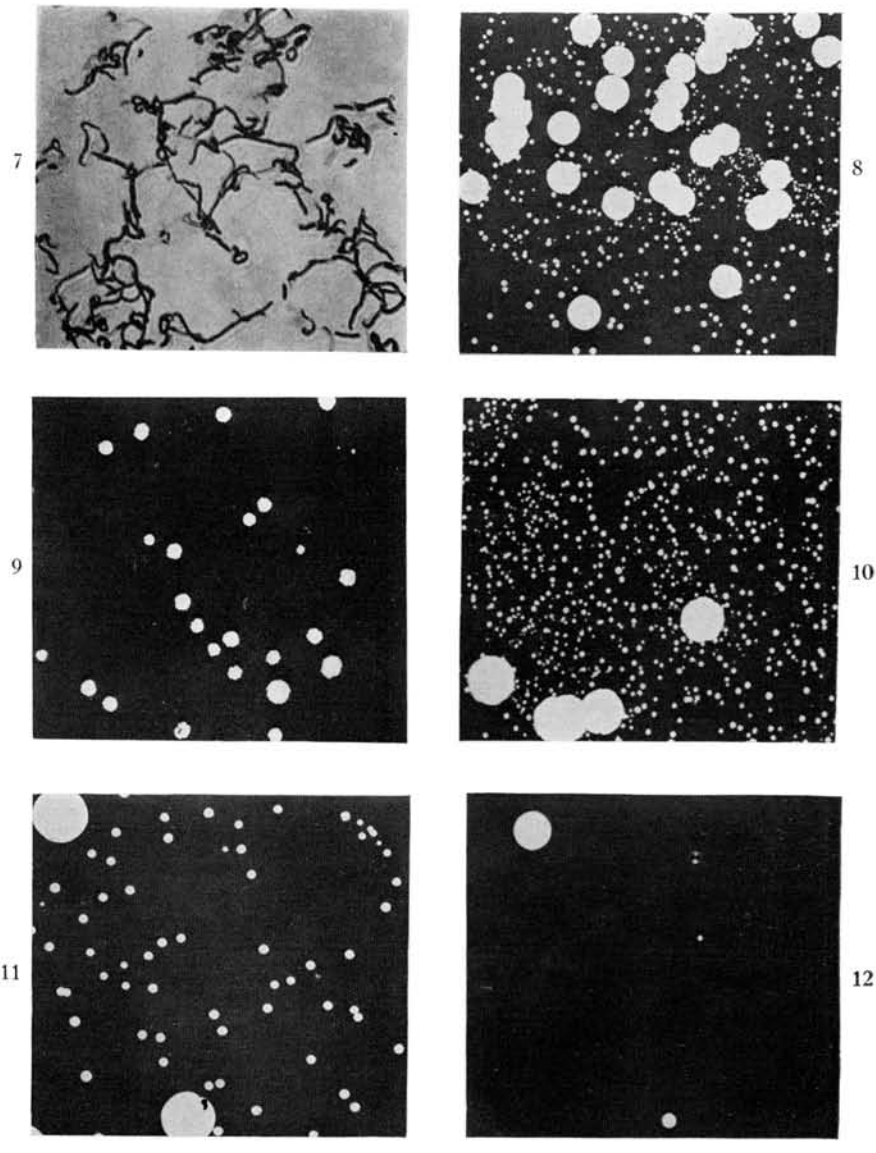

FIG. 7. B. acidophilus, Type III; organisms from Type-III colony in *fig. 6*.

FIG. 8. "Caries-susceptible" saliva (0.1 cc. of 1–10 dilution) on tomato-peptone agar (pH 5.0). Shows yeast and B. acidophilus colonies.

FIG. 9. Undiluted "caries-free" saliva (0.4 cc.) on tomato-peptone agar (pH 5.0). Shows M. tetragenus colonies only. Note irregular borders.

FIG. 10. Patient E. R. S.; caries-susceptible; high-carbohydrate diet. Saliva (0.1 cc. of 1–100 dilution) on tomato-peptone agar (pH 5.0); 1,800,000 B. acidophilus, and 4,000 yeast, colonies per cc. of saliva.

FIG. 11. Patient E. R. S.; low carbohydrate diet for 12 days. Saliva (0.1 cc. of 1–5 dilution) on tomato-peptone agar (pH 5.0); 4,500 B. acidophilus, and 400 yeast, colonies per cc. of saliva.

FIG. 12. Patient E. R. S.; low carbohydrate diet for 18 days. Saliva (0.1 cc. of 1–5 dilution) on tomato-peptone agar (pH 5.0); 100 B. acidophilus, and 50 yeast, colonies per cc. of saliva.

of Staphylococcus albus occasionally appear, and are similar in size to B. acidophilus, but with a little experience one soon finds it possible to differentiate them.

Figs. 1 and *2* show the result of spreading equal amounts of the same sample of saliva on two kinds of medium: glucose beef-infusion agar (pH 7.0) and tomato-peptone agar (pH 5.0). The inhibitory effect exercised on many mouth organisms by the latter medium is well illustrated. Only B. acidophilus and yeast colonies grew on the acid medium, whereas yeast, streptococci, staphylococci, and B. acidophilus were present on the neutral agar. *Figs. 3–7* show the three most common colonial and morphological types of B. acidophilus, from saliva, growing on tomato agar (pH 5.0). These were designated Types I, II, and III in an earlier publication (3). Types I and II form smooth colonies; Type III gives an intermediate type. The cell-forms associated with each colonial type are shown in *figs. 4, 5,* and *7*.

B. *Collection of sample.* For the purpose of enumerating B. acidophilus in mouths of caries-susceptible or caries-free patients, the following procedure is successful: The patient is instructed to chew a small piece of paraffin vigorously for three minutes, moving the paraffin alternately from side to side of the mouth to dislodge from the teeth as much deposit as possible. During this time the sample of saliva, collected in a tube graduated to 10 cc., usually varies in amount from 2 to 8 cc. Its volume is made up to 10 cc. with sterile physiological salt solution. The organisms dislodged by three minutes of chewing are therefore always suspended in the same amount of fluid, so that the error caused by the varying rate of flow of saliva is minimized. The tube is shaken by hand for 30 seconds, and is then ready for dilution.

C. *Dilution of sample.* To facilitate counting, it is necessary to adjust the dilutions of saliva to the relative numbers of organisms in the mouth of the caries-susceptible or the caries-free patient. For *caries-susceptible* cases: to each of two tubes, containing respectively 4 cc. and 19 cc. of sterile physiological salt solution, is added by pipette 1 cc. of saliva sample, thus giving dilutions of 1–5 and 1–20. After these dilutions have been thoroughly shaken, 0.1 cc. from each is placed on the center of a hardened and well-dried tomato-agar (pH 5.0) plate. With a sterile, bent glass-rod, the inoculum is spread evenly over the surface. Plates are incubated, inverted, at 37°C. for three days, and the colonies then counted. The final count from the 1–5 dilution must be multiplied by 50, and that from the 1–20 dilution by 200, to give the number of bacteria per cc. of saliva. After the first test has been made, it is often necessary in subsequent tests to alter

the dilution for a certain patient, if the colonies are found to be either too sparsely distributed or too crowded. It may thus be necessary to spread on the agar 0.2 cc. of the 1–5 dilution of saliva from a patient with a very small amount of caries, while 0.1 cc. of 1–100 or 1–500 dilution may be sufficient for an extremely active case. The same dilution is usually satisfactory for future tests, unless treatment is administered for the purpose of reducing the aciduric flora. Under such circumstances the dilution must be lowered.

In the case of *caries-free* patients, no saline dilution of saliva is as a rule necessary (the volume correction to 10 cc. is made as in susceptible cases). Until a favorable range is determined for a given case, two plates are spread as above with 0.1 cc. and 0.4 cc. of the undiluted but well-shaken saliva sample, the counts being multiplied in such cases by 10 and 2.5 respectively. In a majority of instances, the 0.4-cc. plate remains free from growth of B. acidophilus, and is frequently sterile; larger amounts of saliva cannot satisfactorily be spread over the surface of the agar. The 0.4 cc. plate must be allowed to dry well before being inverted and incubated.

Figs. 8 and *9* demonstrate the distinct and characteristic difference between salivas from caries-susceptible and from caries-free individuals. The caries-susceptible plate (0.1 cc. of 1–10 dilution) shows both yeast and acidophilus colonies; the caries-free plate (0.4 cc. undiluted) shows only colonies of M. tetragenus.

III. QUANTITATIVE DETERMINATION OF B. ACIDOPHILUS IN CARIES-SUSCEPTIBLE AND CARIES-FREE CHILDREN

Using the quantitative method described above, a group of thirty-one children, 9–16 years old, was studied over a period of 18 months. Very careful dental examinations were made at the beginning of this period; also at the end of 12 months, and again at the end of 18 months.[2] Fourteen children during this time developed active caries (thirteen had previously had caries, while one had been free from caries up to this 18-month period). Ten children, who had always been free from caries, had no new lesions at the end of the 18 months. The remaining seven children became "clinically questionable;" that is to say, it was impossible to determine whether new caries had occurred during this time. For this reason the bacteriological findings on the last

[2] The dental examinations were made by Drs. R. W. Bunting, Dorothy G. Hard, and Philip Jay, of the School of Dentistry.

TABLE 1

*Incidence of B. acidophilus in caries-susceptible children during 18 months**

CASE	ACID BROTH		ACID AGAR	AVERAGE	CASE	ACID BROTH		ACID AGAR	AVERAGE
	Tooth-scraping	Saliva	No. per cc. of saliva			Tooth-scraping	Saliva	No. per cc. of saliva	
F. B.	+	+	56,100		G. R.	+	+	120,000	
	+	+	68,100			+	+	7,650	
			2,800					2,500	
		+	14,000				+	950	
		+	9,050				+	41,150	
	+	+	28,400	29,742		+	+	840	28,848
L. F.	+	+	127,000		J. R.	+	+	16,000	
	+	+	448,000			+	+	24,000	
			11,400				+	700	
		+	9,150				+	1,620	
		+	32,000			+	+	1,180	8,700
	+	+	136,400	109,136	W. W.	+	+	50,000	
N. I.	+	+	48,500			+	+	32,500	
	+	+	138,000				+	267,000	
		+	2,600				+	58,600	
			2,750				+	63,800	
		+	4,575			+	+	403,000	145,817
	+	+	116,400	52,137	M. W.	+	+	7,260	
R. L.	+	+	180,000			+	+	23,800	
	+	+	7,800					33,200	
			111,200				+	15,050	
		+	246,200				+	10,250	
		+	28,400			+	+	28,000	19,593
	+	+	36,600	101,700	J. Roy	+	+	24,000	
A. E.	+	+	5,000			+	+	3,300	
		+	4,900				+	41,600	
		+	100					10,000	
		+	5,900			+	+	36,400	23,060
	+	+	42,700	11,720	P. S.	+	+	8,550	
J. P.	+	+	6,000			+	+	100,000	
	+	+	20,000					14,200	
			9,200					5,450	
			3,500					9,150	
		+	164,200				+	34,100	
	+	+	28,600	38,583		+	+	282,000	64,778
A. W.	+	+	151,150		O. L.	+	+	3,200	
		+	303,600			+	+	48,800	
	+	+	276,000	243,583			+	900	
							+	3,150	
							+	16,200	
						+	+	15,800	14,675

* *Qualitative* tests are indicated by the acid-broth cultivations from tooth-scrapings and saliva. *Quantitative* tests are indicated by the number of colonies on acid agar.

TABLE 2

*Incidence of B. acidophilus in caries-free children during 18 months**

CASE	ACID BROTH		ACID AGAR	AVERAGE	CASE	ACID BROTH		ACID AGAR	AVERAGE
	Tooth-scraping	Saliva	No. per cc. of saliva			Tooth-scraping	Saliva	No. per cc. of saliva	
P. B.	−	−	0		M. R.	−	−	0	
	−	−	0			+	+	160	
	−	−	0					2	
		−	0				−	0	
		−	0				−	0	
		−	0				+	0	
	−	−	0	0		+	+	12	25
R. P.	+	+	0		J. E.	−	+	210	
	−	−	0			−	+	50	
	−	+	0			−	+	585	
	−	−	0				+	45	
		−	0				+	117	
		−	0				+	27	
		−	0				+	1020	
		+	0			+	+	0	256
	−	+	20	2					
Jo. E.	−	+	0		V. D.	+	+	0	
	−	+	0			−	−	0	
		−	0				+	45	
		−	2			−	−	0	
		−	0				−	0	
	−	+	7	1		+	+	385	72
W. G.	−	−	0		M. B.	−	−	0	
	−	−	0			−	−	0	
	−	+	30				+	3700	
		−	0				+	905	
	−	+	5				+	70	
		+	0			−	−	0	779
	−	−	5	6					
M. S.	−	−	5		E. B.	+	+	800	
	−	+	15		(Negro)	−	+	7600	
			2				+	19,200	
		−	0			+	+	120	
		−	0				+	1680	
		−	0			−	+	0	4900
	−	−	0	3					

* *Qualitative* tests are indicated by the acid-broth cultivations from tooth-scrapings and saliva. *Quantitative* tests are indicated by the number of colonies on acid agar.

group are omitted from the present report.[3] Cultures were made from the saliva of each child at irregular intervals over the 18-month period. Six months elapsed between some cultivations; only one month, between others. At least six cultures were taken from a majority of cases: the lowest number from any one case was three; the highest, nine. In addition to quantitative acidophilus-counts from saliva, qualitative cultures were made, in the usual 1 percent glucose beef-infusion broth (pH 5.0), from tooth-scrapings and from saliva. For the latter, 0.4 cc., from both caries-susceptible and caries-free cases, was inoculated into broth.

The striking difference, in acidophilus counts, between caries-susceptible and caries-free cases is shown in *tables 1* and *2*. There were, in all, 79 separate quantitative examinations in the susceptible group; 69 in the caries-free group. In the susceptible group, 100 percent of these examinations were positive for B. acidophilus; in the caries-free group, only 42 percent were positive to any degree. The average count for the 14 caries-susceptibles (total of 79 examinations) was approximately 60,000 B. acidophilus per cc. of saliva, whereas that for the 10 caries-free cases (total of 69 examinations) was 600. The range of individual counts for the susceptible group was extremely wide, varying from a few hundred to 500,000, *with a majority over 20,000*. In the caries-free group the range was from 0 to 20,000, *a majority of counts being 0—in 81 percent the counts were less than 100*. It will be noted that the counts for each individual varied considerably, particularly for those in the susceptible group. This may be due to the fact that several months sometimes elapsed between examinations. Besides, it is practically impossible to obtain uniform counts from one individual even from one day to the next, since the paraffin chewing cannot regularly dislodge the same number of organisms from the teeth. Until a method for obtaining more uniform samples is worked out, this method is useful chiefly for comparative purposes.

While only one caries-free case stands out as being completely acidophilus-free during the 18 months, six of the remaining cases show counts so low as to be practically negligible, their average being under 75. The remaining three have somewhat higher averages (256, 779

[3] This same group of children was discussed in a recent paper by Hubbell (5) on chemical studies of saliva in relation to dental caries.

and 4,900, respectively), although in all three cases low or negative counts were frequently obtained. *Case E. B.*, showing the highest figures for the caries-free group, was the only Negro. All the caries-free cases showed one or more negative counts during the period of observation, thus indicating the transient nature of the infection in these cases.

Comparison of the broth cultures from tooth-scrapings and from saliva (our usual method of determining the presence of B. acidophilus in the mouth) shows that all in the susceptible group were positive. In the caries-free cases, however, there were many more positive cultures from saliva than from tooth-scrapings. Of the 38 instances in which broth-cultures were made from tooth-scrapings and from saliva, only 8 cultures from scrapings were positive, while 21 from saliva were positive. An illustration of this appears in *Case J. E.* (*table 2*), in which the first three tooth-scraping cultures were negative, whereas all the saliva broth-cultures, made at the same time, were positive. The saliva counts, however, were very low—210, 50 and 585. It therefore appears that positive broth-cultures from caries-free salivas do not necessarily indicate high acidophilus overgrowths, since frequently the actual number of organisms is low, and tooth-scrapings may be negative. Apparently in such cases the organisms are not localized, or are localized only to a small extent on the teeth, but are free in the saliva. It also should be noted that, in the caries-free cases, broth cultures from saliva were sometimes positive for B. acidophilus although agar plates inoculated with the same quantity of saliva were negative. This may be explained by the fact that acid glucose-broth serves as an enrichment fluid for B. acidophilus, a very small number of these organisms being stimulated to growth in broth, whereas on the surface of an agar plate there is greater initial mortality.

IV. REDUCTION OF B. ACIDOPHILUS BY REGULATION OF CARBOHYDRATE IN DIET

The quantitative method for estimating B. acidophilus in saliva may also be employed advantageously in the evaluation of attempts to control caries by limiting the growth of this organism in the mouth. Howitt and Fleming (4) reported that a diet predominantly carbohy-

drate increased the number of aciduric organisms in the mouth, whereas a diet containing more protein diminished their number. Rodriguez (8) was also able to decrease the number of these organisms by reduction of carbohydrate in the diet. This method was used by us successfully on several cases, one of which will be described.

The patient, age 24, was extremely susceptible to dental caries, several new cavities developing each year in spite of continued dental attention. Before any change in diet was undertaken, quantitative estimations of B. acidophilus were made several times a week for six weeks. During this time the counts varied from a low of 40,000 per cc. of saliva to a high of 300,000, with a usual count of approximately 150,000. The patient was then advised to use a very *low* carbohydrate diet, which was prescribed by Dr. Martha Koehne of our research group. All sweet desserts, candy and sugar were eliminated, as well as starchy foods such as bread, potato, and certain vegetables. After seven days on this diet, the count was reduced to 10,000 B. acidophilus per cc. of saliva; in 4 weeks the count dropped to 900. The patient then returned with much enthusiasm to a carbohydrate diet, again consuming the usual amounts of sugar and starch, whereupon the acidophilus count rose in five days to 1,800,000 per cc. of saliva (*fig. 10*). The count remained above 1,000,000 for several days, during which time large amounts of carbohydrate were consumed. Next, a *moderate* carbohydrate diet was begun (limited quantities of bread and potato allowed, but sugar and sweet desserts eliminated). Since ten days on this diet reduced the count only to 130,000, the very *low* carbohydrate diet was again prescribed, whereupon the count dropped to 4,500 in twelve days (*fig. 11*), and to the extremely low figure of 100 in eighteen days (*fig. 12*). When more carbohydrate was added to the diet, the count again rose and remained around the 150,000 level.

This case illustrates how the quantitative method of estimating B. acidophilus in saliva may be applied to procedures designed to control dental caries. It shows, moreover, what may be accomplished in reducing acidophilus flora by rigid control of carbohydrate in the diet. It was not possible to determine the effect of this carbohydrate reduction on the progress of the disease in this particular case, since long continued adherence to the limited, and rather costly, diet was not advisable. Details regarding other cases, in which carbohydrate in-

take was controlled over longer periods of time, will be reported by Dr. Martha Koehne.

V. SUMMARY

The need for a solid medium for quantitative determinations of oral B. acidophilus in connection with studies on the bacteriology of dental caries has been met by devising a modification of Kulp's tomato-peptone agar.

This modification involved increasing the acidity of the medium to pH 5.0, with lactic acid, and the agar content to 2 percent.

On this medium colonies of B. acidophilus of the smooth, rough, and intermediate phases may be differentiated from each other, and from the other mouth organisms, such as yeast, staphylococcus, M. tetragenus, and occasional streptococci, which tolerate this acidity. Most streptococci and staphylococci do not grow.

Methods for collection of saliva, dilution of the sample, and spreading on the plates are described. The number of B. acidophilus per cc. of saliva can thus be estimated.

In order to secure well-distributed colonies on agar plates, saliva from caries-susceptible patients requires a much higher dilution than does saliva from caries-free patients. In the latter group it is often necessary to employ undiluted saliva.

A group of fourteen caries-susceptible and ten caries-free children was cultured at intervals for 18 months. In the *susceptible* group, qualitative cultures showed B. acidophilus present in all cases from tooth-scrapings and from saliva. Quantitative estimations ranged from a few hundred to 500,000 per cc. of saliva, with a majority over 20,000. The average for 79 examinations was 60,000.

In the *caries-free* group, qualitative cultures showed B. acidophilus present in only 21 percent of tooth-scrapings, but in 51 percent of salivas. Quantitative estimations of B. acidophilus in saliva ranged from 0 to 20,000, with a majority of counts 0—in 81 percent the counts were less than 100. The average for 69 examinations was 600.

While B. acidophilus is invariably present, and usually in great numbers, in the mouths of caries-susceptible subjects, in caries-free individuals its presence is variable and usually marked by few organisms.

By reducing carbohydrate in the diet to the lowest possible minimum, in a patient extremely susceptible to caries, B. acidophilus was decreased in 18 days from the very high figure of 1,800,000 per cc. of saliva to 100 per cc.

The advantage to be gained from applying this quantitative method to comparative studies of the aciduric flora of caries-susceptible and caries-free mouths, and to studies of methods of control of aciduric overgrowths, is indicated.

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