The Effect of Mucin on the Survival of Lactobacilli and Streptococci

By W. SIMS

Department of Pathology, Royal Dental Hospital, London, W.C. 2

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SUMMARY

The survival of suspensions of lactobacilli and streptococci incubated at 37° in normal saline and in 0.1% (w/v) and 1.0% (w/v) solutions of dehydrated mucin was studied by counting the number of viable organisms present at regular intervals. The survival time was markedly prolonged in the mucin solutions. In the 1.0% (w/v) mucin solution most of the strains of lactobacilli and all the strains of streptococci were still viable at the end of one year. The mucin solutions appeared to be unchanged when compared with control solutions which had been inoculated with killed bacteria and incubated at 37°.

INTRODUCTION

It has been known for many years that lactobacilli either cannot be isolated at all or are found in very small numbers in the mouths of individuals who are entirely free from dental caries, whereas in mouths where dental caries is active lactobacilli are much more numerous. There is good evidence for believing that both the dental caries and the proliferation of lactobacilli are the result of consuming a diet rich in fermentable carbohydrates. Studies of the bacteriology of the human vagina have also shown that the appearance and persistence of lactobacilli exactly parallels the availability of fermentable carbohydrate in the form of glycogen deposits in the vaginal epithelium (Cruickshank & Sharman, 1934).

It has been assumed that a few lactobacilli are always present in the mouth and vagina and they proliferate when acid conditions develop and afford them a selective advantage. But, since lactobacilli are not known to have exceptional ability to survive adverse conditions it is difficult to account for their persistence, in the absence of acid conditions and for long periods of time, in an environment containing enormous numbers of streptococci which metabolize all the nutrients required by lactobacilli and have a shorter generation time. One possible explanation, speculative, but appealing, is that lactobacilli are in fact acid-resistant mutant forms of streptococci. No experimental support for this suggestion could be obtained.

Dewar (1949) reported that saliva specimens could be kept at room temperature for up to 72 hr without the lactobacillus count falling appreciably, and this has been the experience of many other workers. Both the mouth and the vagina have a mucinous secretion and the possibility that mucin in some way preserves lactobacilli was investigated. In a preliminary experiment it was found that lactobacilli were still viable after 6 weeks incubation at 37° in whole saliva, but in saliva from which the mucin had been removed by precipitation with acetic acid they died

out within 72 hr. Clearly, mucin did prolong the time of survival of lactobacilli. In the present work the survival of strains of lactobacilli and streptococci incubated in mucin solutions was examined by doing periodic viable counts.

METHODS

Mucin. A dehydrated extract of hog stomach was used (Mucin, gastric. L. Light & Co. Ltd., Colnbrook, Bucks.). Two solutions of the mucin powder, 0.1 % (w/v) and 1.0 % (w/v), were prepared by dissolving the powder in sterile distilled water and sufficient -NaOH to give a final pH of 7.2. The solutions were dispensed in 22.0 ml. amounts in sterile Universal bottles. Sterile screw-capped $\frac{1}{4}$ oz. bijou bottles were filled with the 1.0 % mucin solution. The maximum heat treatment these solutions would withstand without a precipitate forming was autoclaving at 10 lb. for 10 min. Samples from the Universal bottles (1.0 ml.) and the bijou bottles (0.5 ml.) were cultured aerobically and anaerobically in glucose broth; all the solutions were found to be sterile. After autoclaving, some bottles were randomly selected and the pH of the mucin solutions measured; it was found to be in the range pH 6.9-7.08.

Bacteria. The mucin solutions were inoculated with either stock strains of lactobacilli and streptococci or strains of these organisms isolated from saliva specimens collected from caries-free subjects. The stock strains of lactobacilli were kindly supplied by Dr M. Elisabeth Sharpe of the National Institute for Research in Dairying, and the stock strains of streptococci were obtained from the National Collection of Type Cultures.

The strains of bacteria which were to be counted at regular intervals during the experiment were subcultured several times on blood agar, then streaked on further plates of blood agar which were incubated for 24 hr for the streptococci and 48 hr for the lactobacilli, except *Lactobacillus acidophilus*, *L. casei* and *L. fermentii* which were incubated for 72 hr. Bacterial growth was scraped from the surface of the agar and suspended in sterile saline to give a turbidity approximately equivalent to Brown's opacity tube no. 2. From each bacterial suspension 1.0 ml. amounts were transferred to each of two Universal bottles containing 1.0 % mucin and to one bottle each of 0.1 % mucin and sterile saline; the remainder of each suspension was heated at 60° for 1 hr and then 1.0 ml. transferred to a further bottle of 1.0 % mucin. All bottles were kept in the incubator at 37° throughout the experiment. Periodic viable counts were made of the bacteria in the saline, 0.1 % mucin and one of the bottles containing 1.0 % mucin; the remaining two bottles of 1.0 % mucin, one of which was inoculated with killed bacteria, were kept in the incubator until the end of the experiment when the pH and viscosity of these two solutions were compared.

Other strains of lactobacilli and streptococci were grown on blood agar and then a small amount of growth was scraped up and suspended in 1.0 % mucin solution in bijou bottles. The bottles were incubated at 37° and every 3 months a 0.3 ml. sample was cultured in either 10.0 ml. of glucose broth or 10.0 ml. of Rogosa broth (Rogosa, Mitchell & Wiseman, 1951) depending on whether the solution contained streptococci or lactobacilli. When growth occurred, a Gram-stained smear was prepared as a check.

Bacterial counts. The number of viable organisms in the solutions was estimated by the method of Miles & Misra (1938). Serial dilutions were prepared in 1.0 % peptone water and from appropriate dilutions eight 0.02 ml. drops were applied to each of three plates of 1.0 % glucose agar for streptococci or to plates of MRS agar (de Man, Rogosa & Sharpe, 1960) for lactobacilli. When the number of organisms remaining became too small for counting by the drop method, plate counts were made by spreading 0.1 ml. of undiluted saline or mucin solution over the surface of each of three plates of medium. If no colonies appeared on any of these plates, 0.5 ml. of saline or mucin solution was inoculated into glucose broth or MRS broth. If growth occurred, then testing for growth in broth was substituted for the bacterial count at the appropriate time intervals. Finally, when no growth occurred in broth, the bacteria were spun-down from the saline or mucin solution and the centrifuged deposit cultured in glucose broth or MRS broth.

At the end of one year the experiment was terminated and the appropriate physiological and serological tests were carried out to confirm that the organisms remaining were those which had been inoculated at the beginning.

RESULTS

The number of weeks survived by the strains of lactobacilli and streptococci, which were incubated at 37° in normal saline and mucin solutions and counted at regular intervals, are shown in Table 1. Two types of viridans streptococci are listed in Table 1; this subdivision is based on other work in which it was found that

Table 1. *The number of weeks lactobacilli and streptococci remained viable when incubated at 37° in saline and in 0.1 and 1.0 % solutions of mucin*

The experiment was continued for 1 year. The percentage of the original inoculum still viable at the end of that time is shown in brackets.

	Saline	0.1 % mucin	1.0 % mucin
<i>L. acidophilus</i> (NCDO 4)	0	2	40
<i>L. casei</i> (NCDO 151)	0	1	52 (0.05 %)
<i>L. casei</i> var. <i>rhamnosus</i> (NCDO 243)	0	6	52 (0.001 %)
<i>L. fermentii</i> (NCDO 215)	0	2	50
<i>L. plantarum</i> (NCDO 352)	0	3	52 (0.0006 %)
<i>L. salivarius</i> (NCDO 929)	0	4	52 (0.01 %)
Oral enterococcus	3	24	53 (2.4 %)
<i>S. faecalis</i> (NCTC 8213)	9	53 (0.001 %)	53 (0.2 %)
<i>S. hominis</i> (NCTC 7366)	0	41	53 (4.2 %)
<i>S. pyogenes</i> (NCTC 8198)	0	7	53 (0.03 %)
Oral viridans streptococcus type 1	0	18	53 (0.06 %)
Oral viridans streptococcus type 2	0	53 (0.0001 %)	53 (0.02 %)

viridans streptococci which ferment raffinose but do not hydrolyse aesculin (type 2 strains) usually produce acid from glucose much more rapidly than viridans streptococci which give other combinations of reactions with these two substrates (type 1 strains). Full details of the viable counts, e.g. dilution, number of drops and number of colonies counted have been published elsewhere (Sims, 1964). In over half the counts 400 or more colonies were counted which should reduce the error to ± 10 % and in very few were less than 100 colonies counted which is the 20 % level of accuracy (Badger & Pankhurst, 1960).

Although the streptococci survived rather better in the mucin solutions than the lactobacilli, the general trend of the viable counts over the 1-year period was similar for both groups of organisms. The results of the counts of *Lactobacillus plantarum* and *Streptococcus hominis* are illustrated in Fig. 1. The high initial death rate of *L. plantarum*, which also occurred with *L. casei* var. *rhamnosus*, *L. fermentii* and *L. salivarius*, was probably a further manifestation of the finding, clearly shown in Table 1, that survival depends on the amount of mucin available to the bacteria. *L. acidophilus* and *L. casei* which were inoculated in much smaller numbers

Fig. 1. Survival of *Lactobacillus plantarum* (○) and *Streptococcus hominis* (●) when incubated at 37° in 1.0 % solutions of mucin.

than the other lactobacilli (about 2×10^6) had initial death rates similar to *S. hominis*. The counts of all the organisms which survived for 1 year in the 1.0 % mucin solution show that survival was distinctly better in the second 6 months than in the first. Of the original inoculum of *L. plantarum* 0.008 % survived the first 6 months, and of these 22 % were still viable after a further 6 months incubation. The corresponding values for *S. hominis* were 15.5 and 27.1 %.

Most of the organisms which were incubated in 1.0 % mucin solution in bijou bottles and tested for viability every 3 months were still viable after 1 year. Three oral strains each of *L. acidophilus*, *L. casei*, *L. casei* var. *rhamnosus*, *L. plantarum* and *L. fermentii* were tested. Of these, one strain each of *L. casei* and *L. casei* var. *rhamnosus* failed to survive 3 months, one strain of *L. fermentii* survived for 3 months but not for 6 months, and two strains of *L. plantarum* survived for 9 months but not for 1 year. The remaining 10 strains all survived the full year. The following streptococci all survived one year: stock strains of Lancefield's group B, C, E, F,

G, H, K, L, M, O, P and Q; stock strains of *S. bovis*, *S. durans*, *S. faecalis* var. *liquefaciens*, *S. faecalis* var. *zymogenes* and *S. faecium*; 4 oral strains each of enterococci and *S. hominis*, and 10 oral strains of viridans streptococci. A stock strain of *S. pneumoniae* failed to survive 3 months.

The pH and viscosity of the control 1.0% mucin solutions were essentially the same whether they had been inoculated with living or killed bacteria. The solutions containing killed bacteria tended to be slightly more viscous and more acid than the solutions inoculated with viable organisms. None of the lactobacilli or streptococci would grow on a medium consisting of the 1.0% mucin solution solidified with agar. Thus, although the viable counts may reflect the balance between growth rate and death rate, there is no direct evidence that mucin is a nutrient for lactobacilli and streptococci, it simply seems to preserve their viability.

DISCUSSION

The results show clearly that mucin preserves the viability of lactobacilli and streptococci. Why it does so is entirely conjectural. Since the manufacturers were unable to supply a chemical analysis of the dehydrated mucin it is possible that the solutions contained some factor other than mucin which enhanced survival.

Tunncliffe (1940) has shown that bacteria acquire 'coats' of mucin when suspended in this substance. The results of the present work could be explained by assuming that the survival of an organism is dependent on its acquisition of an adequate 'coat' of mucin. Survival was better in the more concentrated mucin solution and this could be due to more organisms acquiring satisfactory coverings of mucin. If unlimited amounts of mucin were available, presumably the organisms would survive much longer or all the organisms inoculated would survive for a certain maximum time. In this respect it may be significant that the streptococcus inoculated in smallest numbers, *Streptococcus hominis* (NCTC 7366), had the highest percentage of one year survivals.

The only previous report of mucin prolonging the period of survival of an organism appears to be that of Keefer & Spink (1938) who found that gonococci survived up to 28 hr in mucin. Here again it may have been the acquisition of a mucinous capsule which facilitated survival. In the present study it was found that gonococci would occasionally remain viable for 24 hr in mucin solution at 37°, but frequently did not; the outcome was unpredictable.

There are several reports which suggest that mucin may be a source of nutrition for bacteria (Goldsworthy & Florey, 1930; McLeod, 1941; Rogers, 1948). It may be that the pH and viscosity measurements were too crude to detect small changes in the mucin and perhaps procedures such as glucosamine estimations might have revealed bacterial breakdown of the mucin. On the other hand, there are no reports of lactobacilli and streptococci surviving for such long periods even in ideal nutrient fluids.

The results provide a reasonable explanation for the continual presence of lactobacilli in the mouth and vagina, for one may suggest that if a lactobacillus acquires an adequate covering of mucin it will be able to survive for a long time, even though the competition from other bacteria for the available food is so intense that it receives no nutrients whatever. By similar reasoning it is possible, at least in some

measure, to account for the very large complex streptococcal flora of the mouth, for any streptococcus entering the oral cavity could remain viable for a prolonged period, regardless of its various attributes which would determine its survival or elimination in other non-mucinous environments. Also, any mutant streptococcus which arises may survive even though its characteristics are selectively disadvantageous in comparison with its parent form. In short, all manner of streptococci may persist, whereas in the absence of mucin they would either proliferate or die off.

Present concepts of the bacterial floras of mucous membranes may have to be revised. It seems not unlikely that many of the bacteria in such floras are simply existing rather than actively multiplying and dying. After all, many of them require complex media for their cultivation and certainly very few of them will grow in a culture medium consisting only of the mucinous secretion in which they are found. The findings of studies of the interaction of various groups of bacteria in mixed floras may be inapplicable in mucinous environments. Finally, it is conceivable that the preserving effect of mucin may be of importance in the spread of diseases by droplet infection and in the establishment of the carrier state.

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