*Archs oral Biol.* Vol. 26, pp. 445 to 451, 1981
Printed in Great Britain

0003-9969/81/050445-07$02.00/0
Pergamon Press Ltd

# EFFECT OF MILK ON CARIES INCIDENCE AND BACTERIAL COMPOSITION OF DENTAL PLAQUE IN THE RAT

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**Summary**—Supplementation of a cariogenic diet with pasteurized bovine milk substantially reduced the incidence of dental caries in both male and female Sprague–Dawley rats. The reduction in caries incidence was not associated with the consumption of less of the cariogenic diet and more milk, nor with the animals having a significantly altered bacterial composition of their dental plaque. The results lead to the hypothesis that the anticariogenic effect of milk is attributable to a direct chemical influence on the caries process in the oral environment of the rat.

## INTRODUCTION

Some effects of liquid bovine milk on dental caries incidence in the rat have been reported. Schweigert *et al.* (1946), Anderson *et al.* (1947) and Shaw, Ensfield and Wollman (1959) showed that rats fed only on bovine milk for 14–24 weeks did not develop dental caries. Further, rats maintained for their natural lives on a diet consisting only of bovine milk supplemented with traces of iodine, manganese, copper, iron and cod liver oil had no signs of dental decay at their death (Sperling *et al.*, 1955), milk thus appearing to be non-cariogenic. But as milk contains 4 per cent lactose, a potentially cariogenic sugar, these results suggest rather that milk possesses anticariogenic factors which can interfere with the cariogenicity of the lactose. There is some support for this interpretation: rats fed only milk, in which 10 per cent sucrose was dissolved, did not have dental decay, whereas rats fed milk and a 10 per cent sucrose solution separately did develop moderate decay (Sperling *et al.*, 1955). However, decay was assessed by inspection of whole teeth so that only a crude assessment was obtained, as opposed to an assessment of carious lesions by microscopic examination of stained sections of teeth.

It is important to note the distinction made here between non-cariogenic and anticariogenic diets. Secondly, a distinction must also be made between experiments in which milk contains the experimental cariogenic substances and those in which milk supplements a cariogenic diet. The experimental design of Sperling *et al.* (1955) cannot be used to ascertain the full anticariogenic potential of milk; rather, the alternative of a milk-supplemented diet should be investigated. Shaw *et al.* (1959) showed that replacement by milk of 100, 45 or 33 per cent of the caloric value of a cariogenic diet significantly reduced caries incidence in rats compared with those on the complete diet and water, confirming the findings of Anderson *et al.* (1947) who used a 37 per cent replacement. These workers concluded that milk is anticariogenic. But, because rats fed on milk alone do not develop caries, these results could equally be interpreted as showing the effect of replacing a cariogenic substance with one that is non-cariogenic. Whether milk is in fact anticariogenic cannot be determined because the daily caloric intake of the rats, on supplementation of their diet with milk, was kept constant, at the expense of the cariogenic diet. The caloric content of the cariogenic diet offered to the animals needs to be the same for both groups of rats, i.e. those with access to milk and those with access to water. Stephan (1966) found a reduction in the incidence of dental caries in rats under those conditions. However, the anticariogenic potential of milk could not be properly assessed because the cariogenic diet was poorly defined and contained 32 per cent skim-milk powder. The observed anticariogenic effect of the liquid milk could have been greater if, instead of the milk powder, an alternative protein source was used, which would avoid the possibility of the caries score in that control group being reduced by any anticariogenic activity that the powdered milk may have possessed. Furthermore, no assessment was made of the amount of cariogenic diet consumed by the animals either directly or indirectly via body weight measurements. Part of the reduction in caries observed could have been attributable to the animals having consumed less of the cariogenic diet and more milk.

Thus, the anticariogenic effect of milk in diet supplementation has not yet been firmly established, nor has the nature of the apparent anticariogenicity of milk in the rat been studied. Hence, an investigation of the effect of milk as a diet supplement on caries incidence was undertaken, using a defined cariogenic diet containing a non-milk protein source. The association of observed effects with the growth or dietary intake of the rats and the bacterial composition of their dental plaque was investigated.

## MATERIALS AND METHODS

Weanling Sprague–Dawley rats, 18 days old, bred from the Departmental stock colony were used. The rats were marked for identification and then randomly distributed with respect to diet for both sexes (Table 1). They were housed in raised-bottom stain-

Table 1. Experimental design showing distribution of rats into experimental groups

| Diet type | Sex | Number | Age (days) | Initial weight (g) Mean | SD |
|---|---|---|---|---|---|
| Water | Male | 8 | 18 | 28.9 | 1.4 |
| | Female | 8 | 18 | 27.8 | 2.4 |
| Milk | Male | 8 | 18 | 31.0 | 2.7 |
| | Female | 8 | 18 | 28.0 | 2.5 |

less-steel cages, one group per cage, and fed a powdered cariogenic diet with either deionized water or pasteurized bovine milk *ad libitum*. The water and milk were added aseptically to sterilized conventional ball-valve drinking vessels. The milk was replaced twice daily to avoid its consumption at a pH below 6.0. The cariogenic diet (Table 2) was a modified MIT-200 diet (Navia, Lopez and Harris, 1969). The diet was prepared, mixed, stored and supplied to the animals in containers that restricted scattering and spoiling, as described by Navia (1977). The animals were weighed at weekly intervals and the amounts of powdered diet and liquid consumed over a 24-h period by each group was measured at 15 intervals of time during the experiment. The amounts consumed were assessed by weighing the diets, including scattered food, before and after the 24-h period. Solids were determined to 0.1 g, liquids to 0.1 ml. After 37 days on the diets, the animals were killed by cervical dislocation and treated as described below.

*Caries evaluation*

Fissure caries was assessed using the method of König, Marthaler and Mühlemann (1958). The right half-mandible was removed from each rat and placed in formol saline (10 per cent v/v formalin: 0.1 per cent w/v NaCl). These jaws were then sectioned and stained by the method of König *et al.* (1958), as described by Green and Hartles (1966), to provide series of 100 $\mu$m thick longitudinal mesio-distal sections of the molar teeth. Only the main fissures of the 1st and 2nd molar teeth were assessed for caries. The 3rd fissure in $M_2$ was omitted because its small size led to infrequent appearance in the sections. $M_3$ was not used because of its variable eruption time (Green and Hartles, 1966). The caries incidence for each animal was thus obtained from 5 fissures (3 in $M_1$ and 2 in $M_2$).

*Controls*

To provide controls for carious lesions and animal growth rates, 8 rats were maintained on a standard non-cariogenic diet under the same general conditions and killed 37 days after weaning.

*Plaque collection and culturing*

Five rats were randomly selected from each of the 4 experimental groups for investigation of the fissure plaque flora. For each rat, a sample of dental plaque was taken from the central fissure of the mandibular left 1st molar and added to that removed from the central fissure of the mandibular left 2nd molar using the method described by Huxley (1972). The plaque was placed immediately in a glass–polytetrafluoroethylene tissue homogenizer containing 1 ml of reduced Thioglycollate Medium U.S.P. (Oxoid) and dispersed for 1 min. Serial decimal dilutions in the reduced broth were made from the homogenate and 0.1 ml of appropriate dilutions pipetted on duplicate plates of media and spread with sterile bent glass rods.

Table 3 shows the media used for the isolation of two groups of organisms. Micrococcaceae, neisseriae and streptococci were isolated on AE medium. A few actinobacilli appeared on some plates but their numbers were not included in the total count. Actinobacilli, actinomycetes, streptococci and veillonellae were isolated on medium AN plates, which were stored in an atmosphere composed initially of 90 per cent $H_2$ and 10 per cent $CO_2$ in anaerobe jars fitted with Deoxo catalyst beads (Engelhard) for at least 24 h prior to inoculation. Rogosa SL Agar (Difco, U.S.A.) was selective for lactobacilli, while NCF (Colman *et al.*, 1976) was selective for streptococci.

Table 2. Composition of modified MIT-200 cariogenic diet

| Component | %wt | Manufacturer/Supplier | Relevant specifications |
|---|---|---|---|
| Sucrose* | 67 | J. W. Edwards, Melbourne, Australia | 10–15% $H_2O$, difference sucrose |
| Egg albumen* (spray dried) | 20 | H. J. Langdon and Co., Melbourne | 15–20% $H_2O$, 80–85% protein, less than 0.2% fat |
| Salt mixture† | 3 | Ajax Chemicals, Melbourne | All analytical reagent grade chemicals |
| Cottonseed oil | 3 | Vegetable Oils Pty Ltd, Melbourne | No information available |
| Vitamin mixture *† | 1 | Calbiochem, California, U.S.A. | All vitamins were of A grade quality |
| Cellulose* | 6 | Whatman, Kent, England | Chromatography grade |

* Calcium and phosphate not detectable, fluoride content of complete diet was less than 0.2 $\mu$g per g dry weight.

† Vitamin and salt mixture described in detail by Navia *et al.* (1969).

Table 3. Isolation media for plaque organisms

| Medium | Components | Quantity | Manufacturers |
|---|---|---|---|
| AE | Nutrient broth No. 2 | 2.5% w/v | Oxoid, U.K. |
| | Yeast extract powder | 0.3% w/v | Oxoid, U.K. |
| | Agar | 1.5% w/v | Davis, N.Z. |
| | Sterile horse serum | 5% v/v | C.S.L., Australia |
| AN | Brain heart infusion | 3.7% w/v | Oxoid, U.K. |
| | Yeast extract powder | 0.5% w/v | Oxoid, U.K. |
| | Liver digest powder | 0.5% w/v | Oxoid, U.K. |
| | Polyvinylpyrollidone | 1% w/v | May and Baker, Australia |
| | Cysteine hydrochloride | 0.1% w/v | Calbiochem, U.S.A. |
| | Agar | 1.5% w/v | Davis, N.Z. |
| | Sterile lysed horse blood | 5% v/v | C.S.L., Australia |
| | Sterile ethanolic menadione | 0.5 μg/ml | Calbiochem, U.S.A. |

Each medium base was autoclaved at 116°C for 15 min and sterile components were added aseptically after cooling to 45°C.

The AE, SL and NCF media were incubated in candle jars (Meynell and Meynell, 1970) and the AN medium in anaerobe jars, in an atmosphere composed initially of 90 per cent $H_2$ and 10 per cent $CO_2$. After incubation at 37°C for 3 or 4 days, enumeration, differentiation and maintenance of subcultures of two representative colonies of every type were carried out as described by Johnson, Hayday and Colman (1978). Organisms were subjected to a number of tests for physiological properties to enable identification, based on published schemes: they were streptococci (Colman, 1976); actinomycetes, lactobacilli and *Neisseria* spp. (Cowan, 1974); micrococcaceae (Baird-Parker, 1974); veillonellae (Rogosa, 1974); and *Actinobacillus* species (Phillips, 1974). Appropriate positive and negative controls were employed with all tests.

Counts were expressed as colony-forming units per ml of homogenate.

## RESULTS

### *Caries analysis*

The caries experience and final weights of male and female rats on the two diets are presented in Table 4. No smooth surface lesions were detected and the extent of fissure caries for each rat has been presented as total number of lesions (cumulative-T) and advanced lesions (cumulative-B) as described by Green and Hartles (1966). The caries data (total lesions only) were analysed in a two-way analysis of variance table of Sex by Diet (Table 5). The animals on the milk diet had a substantially lower caries incidence than the animals on the water diet. Males had a somewhat higher caries incidence than females for both diets. Adjustments of the main effect sums of squares for the interactions were not incorporated in the analysis shown because they were small and did not affect the outcome of the significance tests. However, the presence of a very slight interaction could suggest that the anticariogenic effect of milk was different in magnitude for the two sexes.

The correlation of caries incidence with the final weight of the rat was tested for each separate group. No correlation attained significance ($p > 0.1$). Similarly, the initial and final weights showed no correlation, nor were weight gain and caries incidence correlated.

### *Controls*

Study of the sections of the jaws of the control rats enabled identification of the types of artifacts encountered in this system (separation at the dentine-enamel junction, the presence of extraneous organic material). Allowing for these artifacts, no section showed a detectable lesion. Thus the incidence of background counts or interference from hypomineralized areas (Francis and Briner, 1966) was low. This is supported by the large number of zero values for the milk diet group.

### *Diet consumption*

The relative consumption of solid and liquid by male and female rats on the milk and the water diets was tested by an analysis of variance of pair differences (by diet). The overall means were not distinguishable from zero ($p > 0.75$), so that the quantities of both solid and liquid consumed were the same for each diet within each sex. However, the slope of the plots of difference in consumption by males and females for solid and liquid were highly significant ($p < 0.001$) showing that male rats consumed more diet than females. The standard errors of the group differences in daily consumptions were: solid, 0.9 g; liquid, 1.3 ml.

### *Microbiological analysis*

The data on the recovery of the organisms are given in Table 6. Assuming that the total number of organisms collected in a plaque sample has a random error, the data can be analysed through mean counts. Inspection of the data suggested that a logarithmic transformation gave a better approach to normality in the distribution.

The data of Table 6 are presented as $m * f$, where $m$ is the mean and $f$ the confidence interval multiplier, i.e. $f = \exp(s_y)$ with $s_y$ as the standard deviation of the transformed data. The $\pm 1$ SD confidence interval is thus $m \times f$, $m/f$. Two-way analysis of variance tables of Sex by Diet, with 5 degrees of freedom (number of

Table 4. Caries data and final weights of male and female rats on a cariogenic diet supplemented with either milk or water

| | Milk diet | | | | Water diet | | |
|---|---|---|---|---|---|---|---|
| | | Lesions | | | | Lesions | |
| Rat | Weight (g) | Total* (cumulative-T) | Advanced (cumulative-B) | Rat | Weight (g) | Total (cumulative-T) | Advanced (cumulative-B) |
| | | Males | | | | Males | |
| 1 | 180.0 | 0 | 0 | 1 | 140.0 | 3 | 0 |
| 2 | 183.7 | 2 | 0 | 2 | 136.6 | 3 | 0 |
| 3 | 186.6 | 1 | 0 | 3 | 175.7 | 5 | 5 |
| 4 | 162.6 | 1 | 0 | 4 | 144.6 | 1 | 0 |
| 5 | 176.4 | 0 | 0 | 5 | 153.3 | 4 | 2 |
| 6 | 175.1 | 0 | 0 | 6 | 163.7 | 4 | 0 |
| 7 | 159.7 | 1 | 0 | 7 | 124.8 | 5 | 2 |
| 8 | 173.3 | 1 | 0 | 8 | 148.5 | 4 | 2 |
| | | $\Sigma = 6$ | | | | $\Sigma = 29$ | |
| | | Females | | | | Females | |
| 1 | 157.6 | 0 | 0 | 1 | 115.3 | 1 | 0 |
| 2 | 137.0 | 0 | 0 | 2 | 104.2 | 2 | 0 |
| 3 | 140.6 | 0 | 0 | 3 | 101.8 | 2 | 0 |
| 4 | 149.7 | 1 | 0 | 4 | 115.5 | 3 | 2 |
| 5 | 127.0 | 0 | 0 | 5 | 88.6 | 1 | 0 |
| 6 | 154.6 | 0 | 0 | 6 | 118.9 | 3 | 0 |
| 7 | 140.9 | 1 | 0 | 7 | 99.3 | 1 | 0 |
| 8 | 92.8 | 0 | 0 | 8 | 123.5 | 2 | 0 |
| | | $\Sigma = 2$ | | | | $\Sigma = 15$ | |

* Maximum possible 5.

rats) in each cell, for each organism, and of Organism by Diet and Organism by Sex, with nominally 10 degrees of freedom in each cell, were constructed. In the latter two tables, we recognized that, because of competition and interaction, the relative proportions of the organisms are not necessarily independent within a rat, making the total effective independent degrees of freedom less than the tabulated value of 140 (number of organisms 7). Even so, no classification was significant ($p > 0.1$) in any of the above tests, indicating that milk had not detectably altered the composition of the fissure plaque flora.

The most frequently isolated organisms were actinomycetes, lactobacilli and streptococci. By far the most prolific organisms were streptococci and actinomycetes (Table 6). Of the streptococci, *Strep. mutans* formed the largest proportion of the species isolated.

## DISCUSSION

The results on the effect of milk on caries incidence confirm those of Schweigert *et al.* (1946), Anderson *et al.* (1947), Sperling *et al.* (1955) and Shaw *et al.* (1959) in showing that bovine milk is non-cariogenic in the rat. More specifically, the results agree with those of Stephan (1966) in showing that the severity of caries caused by consumption of a cariogenic diet could be reduced by milk supplementation. The anticariogenic effect of milk here was attributable neither to the animals with access to milk consuming less of the cariogenic diet, nor to those animals having a significantly altered bacterial composition of their dental plaque. However, they did have an extra caloric intake as a result of the supplementation of their diet with milk; their body weights were about 20 per cent greater than those of the rats on the diet with water (Table 4).

Table 5. Summary of analysis of variance of sex by diet against caries incidence

| | Degrees of freedom | Mean square | F-ratio | Significance level |
|---|---|---|---|---|
| Sex | 1 | 10.1250 | 13.03 | $0.001 < p < 0.01$ |
| Diet | 1 | 40.5000 | 52.14 | $p \ll 0.001$ |
| Sex by Diet interaction | 1 | 3.1250 | 4.02 | $p > 0.05$ |
| Residual | 28 | 0.7767 | — | — |

Table 6. Bacterial composition of fissure plaque in rats, classified by sex and diet

| Organism | Milk diet | | | | Water diet | | | |
|---|---|---|---|---|---|---|---|---|
| | Female | | Male | | Female | | Male | |
| | *m* | *f* | *m* | *f* | *m* | *f* | *m* | *f* |
| Actinobacilli | 0.06 | *10.5* | 3.07 | *24.9* | 0.08 | *18.2* | 0.10 | *23.0* |
| Actinomycetes | 13.55 | *3.36* | 23.58 | *1.27* | 10.43 | *2.20* | 28.85 | *2.74* |
| Lactobacilli | 0.039 | *3.30* | 0.125 | *3.50* | 0.009 | *4.54* | 0.043 | *4.09* |
| Micrococcaceae | 0.36 | *3.41* | 0.23 | *3.21* | 0.12 | *86.4* | 0.01 | *10.5* |
| Neisseriae | 0.09 | *21.8* | 1.24 | *15.3* | 0.27 | *9.43* | 0.39 | *7.31* |
| Veillonellae | 0.8 | *8.4* | 0.2 | *5.3* | 0.2 | *6.9* | None | — |
| Streptococci | 63.20 | *1.80* | 65.10 | *1.82* | 55.56 | *2.43* | 41.93 | *2.09* |
| (*Strep. mutans*) | (60.49 | *1.85*) | (65.10 | *1.82*) | (53.94 | *2.45*) | (41.93 | *2.09*) |
| Unidentified | 3.38 | *2.55* | 0.61 | *12.9* | 0.89 | *0.45* | 0.53 | *2.68* |
| Gram-negative | 0.84 | *7.68* | 13.04 | *1.99* | 1.85 | *7.76* | 1.84 | *4.19* |
| Gram-positive | 81.09 | *1.88* | 92.07 | *1.58* | 74.81 | *2.09* | 82.96 | *1.69* |
| All organisms recovered | 88.93 | *1.82* | 111.04 | *1.49* | 80.78 | *2.17* | 88.27 | *1.60* |

The data transformation was $y = \ln (x + 1 \text{ LSD})$ where LSD is least significant digit, to include zero counts, and $x$ is colony-forming units $\times 10^5$ per ml of homogenate. The data are presented as means with confidence limit multipliers in italics, where mean $m = \exp(\bar{y})$ and the confidence limit multiplier $f = \exp(s_y)$, $s_y$ is standard deviation of $y$.

Lack of growth has been associated with an increase in caries incidence in rats (Menaker and Navia, 1973). However, in our study the animals on the cariogenic diet and water grew at rates similar to those of the control rats on the standard non-cariogenic diet; therefore there is no reason to believe that the relatively small difference in growth *per se* between the rats consuming milk and those consuming water could be associated with the large difference in caries incidence observed.

The analysis also shows that male rats have a greater susceptibility to dental caries than female, confirming the earlier findings of Keyes (1949), Muhler and Shafer (1952) and Muhler (1959). In an attempt to explain this difference, Muhler and Shafer (1952, 1955) and Bixler, Muhler and Shafer (1955) extensively studied the effect of orchiectomy and ovariectomy with concomitant desalivation and sex hormone replacement therapy on the dental caries experience of the rat. However, their findings did not adequately explain the sex difference. The work of Muhler (1959), who compared the amount of the cariogenic diet eaten and frequency of eating with dental caries incidence in male and female rats, and that of Muhler and Shafer (1952) and Bixler *et al.* (1955), suggest that a sex difference in caries experience is attributable to male rats consuming more cariogenic diet and more frequently, than the females. As feeding frequency could not be assessed in the present work, interpretation of the sex difference observed is difficult, but the consistent consumption by male rats of more diet than females is suggestive of a similar effect.

Few data are available regarding the normal oral flora of laboratory rats. However, the variety of organisms cultivated from the tooth fissure plaque in our experiment is similar to that isolated from the periodontium of the rice rat (McDonald, Socransky and Sawyer, 1959). On the other hand, we isolated few enteric bacteria from the fissures, presumably because coprophagy was minimized by the use of the special feeding devices and cages employed. The predominant organisms cultivated in both milk and water animals were streptococci and actinomycetes. Although it is more desirable to evaluate dental plaque and caries data from identical sites rather than relating data from one side of the mouth to the other, bilateral symmetry in the distribution of certain plaque bacteria and dental caries has been demonstrated in rats (Shaw and Sweeney, 1956; Huxley, 1973, 1978).

Milk contains a number of compounds which could affect the incidence and severity of dental caries. These include lactose, lipids, trace elements, immunoglobulins, vitamins, minerals, proteins, citrate and enzymes. all of which, either through effects on the oral flora or by chemical effects on demineralization and remineralization, could influence dental caries. The lack of association between the bacterial composition of the dental plaque and the experimental diet leads to the hypothesis that the anticariogenic effect of milk is due to a direct chemical influence on the caries process; this is supported by the results of previous *in-vitro* and *in-vivo* studies. Milk has been shown to buffer acid in the oral environment (Jenkins and Ferguson, 1966; Mor and McDougall, 1977), remineralize artificially demineralized teeth *in vitro* (McDougall, 1977) and to coat teeth with a protective film of protein *in vitro* (Weiss and Bibby, 1966). The components of milk mainly responsible for these effects are protein, phosphate and calcium.

Rats receiving milk supplementation were ingesting on average 25 per cent more protein and 40 per cent more calcium and phosphate per day than the rats on the cariogenic diet and water. Bavetta and McClure (1957) showed that increasing the dry milk-protein content of a cariogenic diet, while keeping the sugar content constant, reduced the incidence of dental caries in rats. This effect was not associated with a significantly increased growth rate of the animals and perhaps can be attributed to the ability of protein to buffer acid, form a protective layer on enamel and,

through deamination and decarboxylation of amino acids, maintain a high pH in plaque. Supplementation of calcium and phosphate in solid diets has also been shown to decrease caries incidence in the rat (Nizel and Harris, 1964; Lilienthal, 1977) possibly also by buffering acid, decreasing demineralization and facilitating remineralization. Thus it may seem that the anticariogenic action of liquid milk as a dietary supplement shown here is simply the process of restoring a balance between anticariogenic and cariogenic factors. This balance was disturbed by the inclusion of 67 per cent sucrose in the cariogenic diet. However, the increase in the percentage of protein, calcium and phosphate in the present diet from the liquid-milk supplementation, tending to restore the balance, is small in comparison with that in the studies of Bavetta and McClure (1957) and Nizel and Harris (1964), suggesting that the anticariogenic action is not merely through dietary supplementation of those substances. It is relevant that these components are in solution in milk, and in forms different from those in diet solids.

Approximately 85 per cent of milk protein is casein (phosphoprotein) associated with calcium and existing in micelles. This protein in solution has a high acid-buffering capacity (Hipp, Groves and McMeekin, 1952). It resembles enamel phosphoprotein in amino acid composition (Glimcher and Krane, 1964; McKenzie, 1971), and its high binding affinity for hydroxyapatite (Bernardi, 1971; Donnelly, 1977) may be associated with this. Thus the phosphoprotein in liquid milk may be more anticariogenic than this or other protein types incorporated into experimental diets in solid form. Furthermore, the calcium and phosphate in milk exist in many forms within two main classifications: true solution and colloidal solution. Only a small percentage of the so-called true-solution material exists as free calcium and phosphate ions, the rest is complexed. Of the colloidal form, Rose (1969) and Boulet, Yang and Riel (1970) suggested that there exists an apatite-like structure in the casein micelle. Remineralization could proceed through any of these several species. Further work in these areas is needed to elucidate fully the anticariogenic effect of milk.

*Acknowledgements*—This investigation was supported by the Victorian Dairy Industry Authority. The technical assistance of Mr A. R. Malcolm and Miss J. Cowley is acknowledged.

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