APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2000, p. 1720–1723 Vol. 66, No. 4
0099-2240/00/$04.00+0

An In Vitro Study of the Effect of Fluoridated Milk on Oral Bacterial Biofilms

J. PRATTEN,[1]* R. BEDI,[2] AND M. WILSON[1]

Departments of Microbiology[1] and Transcultural Oral Health,[2] Eastman Dental Institute for Oral Health Care Sciences, University College London, London WC1X 8LD, United Kingdom

Received 29 September 1999/Accepted 7 January 2000

Microcosmic dental plaques were grown in artificial saliva and supplemented with either milk or fluoridated milk. The presence of fluoride in the milk increased the pH of the biofilms and reduced the proportions of streptococci, demonstrating that in this model, fluoridation of milk produces biofilms with reduced cariogenic potential.

Caries, one of the most prevalent diseases of humans, results from the production of acids in bacterial biofilms (dental plaques) on the tooth surface which causes localized destruction of the teeth (2). The plaque microflora are diverse, and disease is not due to an exogenous species but to changes in the proportions of members of the resident microflora—in particular, an increase in the levels of mutans streptococci (7). Fluoride continues to be the cornerstone of any caries prevention program and is administered, in many regions of the world, by fluoridation of the water supply (11). Milk fluoridation has been suggested as a possible alternative to water fluoridation, as it is an important foodstuff for children, the primary group when considering caries prevention (16). Although several studies have reported on the clinical benefits of fluoridation, (5, 6), no detailed investigation of its effects on biofilms of oral bacteria under controlled conditions in vitro has yet been published.

A considerable amount of information concerning the identity and metabolism of bacteria found in plaque has now been amassed, however, and much in vitro work has involved the use of aqueous suspensions of bacteria rather than the biofilm-grown cells which comprise dental plaque (8). It is well established that many attributes of a bacterium grown in aqueous suspension differ considerably from those found when it is grown in a biofilm (3, 17). The existence of differences between biofilm-grown bacteria and aqueous suspensions argues strongly that studies of plaque bacteria should be biofilm based. The purpose of this study was to use a biofilm-based model to determine the effects of fluoridation of milk on the cariogenic potential of dental plaque microcosms grown under conditions similar to those which would exist in vivo.

Saliva was used as an inoculum to provide a multi-species biofilm consisting of organisms found in the oral cavity. Saliva was collected from 10 healthy individuals, equal amounts from each person were pooled, and 1-ml aliquots were dispensed into cryovials and stored at −70°C for subsequent use.

Biofilms were grown in a constant-depth film fermentor as described previously (13). The nutrient supply was a mucin-containing artificial saliva which was delivered to the biofilms at a rate of 0.72 liters/day (12). In different experiments, the biofilms were supplemented with either ultra-high-temperature milk (containing 0.03 μg of fluoride [Safeway, Hayes, United Kingdom] per ml) or with this milk containing an additional 5.00 μg of fluoride per ml (i.e., a total of 5.03 μg of fluoride per ml). A total of 200 ml of milk was pulsed into the constant-depth film fermentor at the same time each day over a 30-min period after the initial inoculation.

Growth of the biofilms was determined by counting of viable colonies. Selective media were used to culture the following genera: *Actinomyces* spp. were isolated on cadmium fluoride-acriflavin-tellurite agar plates (18), *Veillonella* spp. on Veillonella agar (Difco Laboratories, Detroit, Mich.), streptococci on Mitis Salivarius agar (Difco), and *Lactobacillus* spp. on Rogosa agar (Oxoid, Basingstoke, United Kingdom). *Streptococcus mutans* was distinguished by its colonial morphology on Mitis Salivarius agar. The total anaerobic colony count was performed with Wilkins-Chalgren agar (Oxoid) containing 8% horse blood (Oxoid). All the plates were incubated anaerobically for 4 days at 37°C. The total aerobic viable colony counts were carried out with 8% blood agar (Oxoid) and biofilms were incubated aerobically at 37°C. The pH was determined with a flat electrode (pH-boy; Camlab).

The cryosectioning methodology was carried out as described previously (13) and viability counts from each of the resulting sections were used to determine the number of viable cells of each genus present in each section of the biofilm.

Duplicate runs were carried out, each over approximately a 1-month period. Throughout each run, the proportions of streptococci and *S. mutans* were consistently lower in biofilms grown in the presence of fluoridated milk (Table 1). In climax community biofilms (312 h), the proportion of streptococci in the biofilms grown in the presence of milk was 26.9%, while this figure was only 11.1% when fluoridated milk was used. The proportions of *S. mutans* at the same time point were 5 and 0.04% in the biofilms grown in milk and fluoridated milk, respectively. No significant difference ($P < 0.05$) was seen in the proportions of *Actinomyces* spp. over the course of the experiments, while *Lactobacillus* spp. were only evident in small numbers from around 168 h onwards. The proportions of *Veillonella* spp. present in the biofilms were consistently low. In biofilms grown in the presence of milk, the mean proportion of *Veillonella* spp. comprising the biofilm was 0.64% over the course of the run, while this figure was 2.39% when fluoridated milk was used. The pH data revealed that both of the differ-

* Corresponding author. Mailing address: Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences, University College London, 256 Gray's Inn Road, London, WC1X 8LD, United Kingdom. Phone: 44 (0) 171 915 1050. Fax: 44 (0) 171 915 1127. E-mail: jpratten@eastman.ucl.ac.uk.

TABLE 1. Proportions of organisms in biofilms[a]

Organism(s)	Growth at:					
	24 h		312 h		624 h	
	+	−	+	−	+	−
Actinomyces spp.	90.5 ± 15.8	69.2 ± 17.3	19.1 ± 6.5	27.6 ± 8.9	21.8 ± 7.1	36.5 ± 12.4
Veillonella spp.	0.56 ± 0.12	0	2.8 ± 1.2	0.36 ± 0.21	2.5 ± 0.9	1.1 ± 1.0
Streptococcus spp.	66 ± 8.2	70.2 ± 16.1	11.1 ± 3.7	26.9 ± 5.8	6.12 ± 1.9	18.9 ± 6.4
S. mutans	10.2 ± 2.4	13.8 ± 6.5	0.04 ± 0.01	5 ± 1.6	0.08 ± 0.03	2.13 ± 1.8
Lactobacillus spp.	0	0	0	0.01 ± 0.01	0.06 ± 0.02	4.1 ± 2.4

[a] Values are percentages ± standard deviations, compared to total anaerobic counts of genera and species comprising biofilms grown in artificial saliva supplemented with either milk (−) or fluoridated milk (+).

ently treated biofilms showed similar trends until approximately 168 h; after this time, the results were quite different (Fig. 1). While the pH of the biofilms grown in the presence of milk remained around 4 to 4.5, the pH of biofilms grown in fluoridated milk had risen to over 5. Hence, once the biofilm community had stabilized (after approximately 7 days) there was a substantial difference between the pHs of the biofilms of the communities grown in the presence of milk and fluoridated milk.

The cryosectioning results indicated a change in the proportions of genera present in the differently treated biofilms through the depth of the biofilm (Fig. 2). The bacterial biofilms grown in the presence of milk had higher total aerobic and anaerobic cell counts and larger numbers of streptococci at the biofilm-air interface (240 to 300 μm) than in the rest of the biofilm. In the sectioned fluoridated milk biofilms, there were higher proportions of anaerobic species at the biofilm-air interface, and throughout the biofilm the numbers of streptococci present were reduced compared to the biofilms supplemented with nonfluoridated milk. Indeed, at the biofilm-enamel interface there was a 2 $\log_{10}$ reduction in the number of streptococci present in the biofilms grown in the presence of fluoridated milk compared with those grown in milk.

Several clinical trials have reported the effectiveness of fluoride-containing milk in the reduction of caries (1, 14), although neither has described any microbiological differences between test and control groups. This study mimicked several of the features of the clinical trials, including the amount of milk delivered and the fluoride concentrations used. However, the most obvious difference was the time period over which the studies took place, the clinical trials taking place over several years. In vitro model studies have focused on the availability of fluoride ions available from milk and the action of fluoride on both enamel and dentine (15). Studies investigating experimental caries in rats have shown that milk alone has a caries-preventive role and that the addition of fluoride to the milk

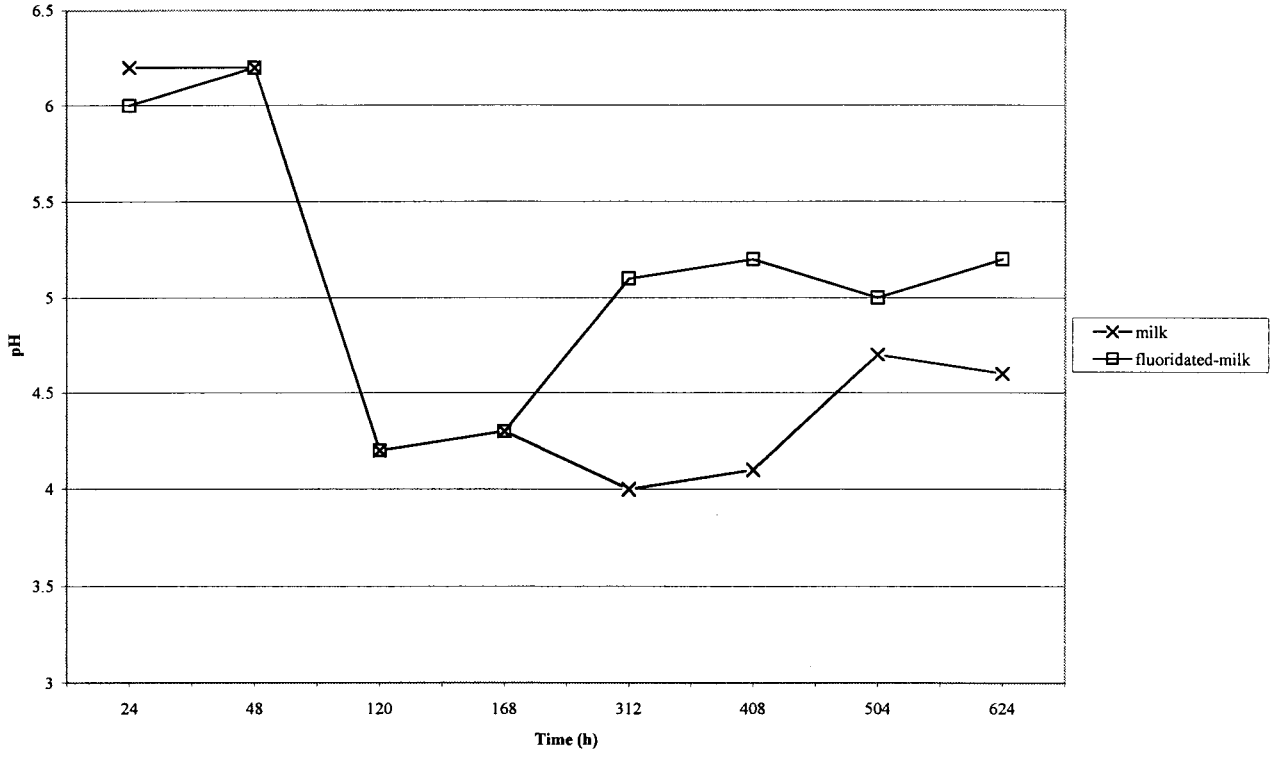

FIG. 1. The pH of bacterial biofilms supplemented with milk or fluoridated milk. pH measurements were taken with pH-boy; the accuracy of the instrument was ±0.1 pH U.

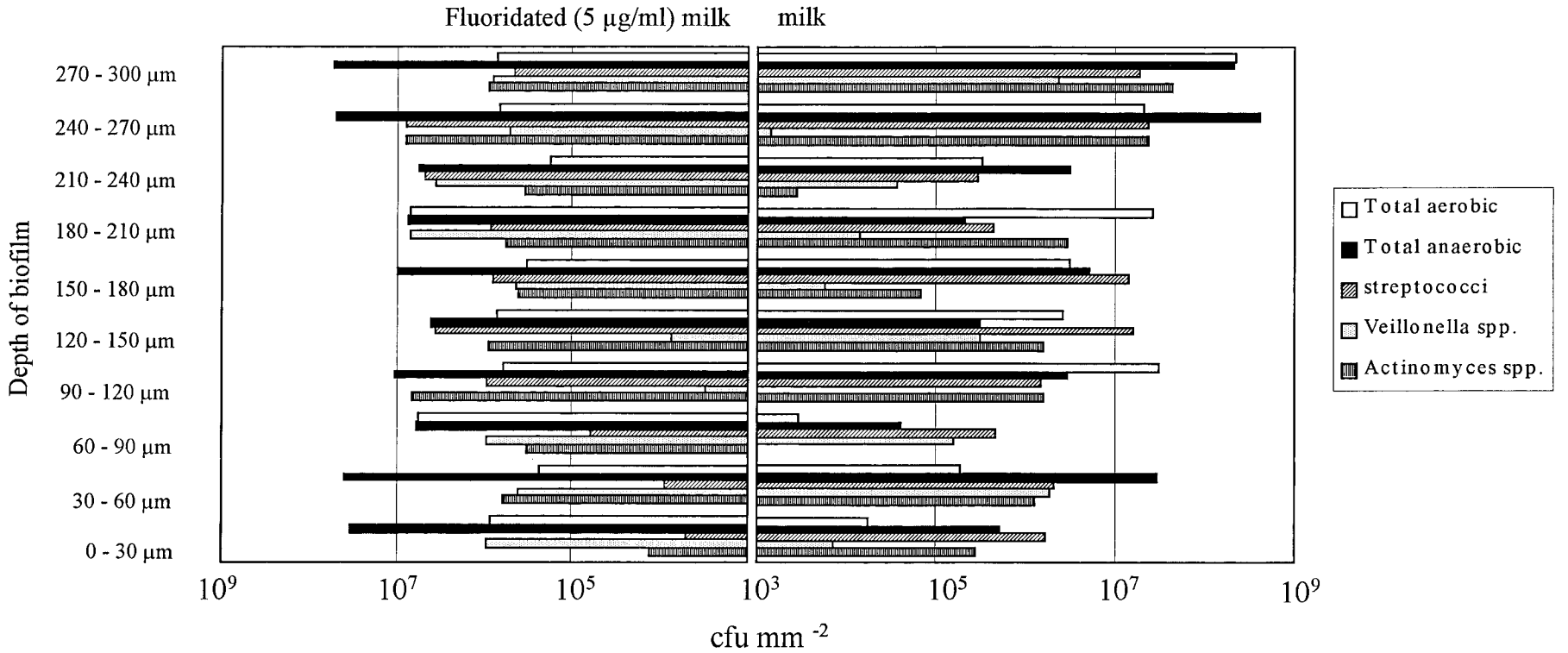

FIG. 2. Viable counts of 30-µm-thick sections through a 624-h bacterial biofilm grown in the presence of milk and fluoridated milk. Bars, means.

increased its efficacy by 40 to 50%. The purpose of this study was to investigate the effect of fluoridation of milk on the composition and pH of microcosmic dental plaques, as these can be related to the cariogenic potential of such plaques.

The caries lesion results from the demineralization of tooth enamel by acids, in particular lactic acid, produced from the microbial fermentation of dietary carbohydrates (4, 9). Hence, the decreased number of streptococci (particularly *S. mutans*) in the biofilms grown in the presence of fluoridated milk implies that they would be less cariogenic than biofilms grown in milk. The presence of higher proportions of *Veillonella* spp. in plaque is also considered to be an indication of a reduced cariogenic potential, as these organisms use lactate as a carbon and energy source and convert it to propionic acid, which is a much weaker acid (10). The sectioning results indicated changes in the proportions of the species through the depth of the biofilm. Of particular interest was the finding that fluoridation of milk resulted in biofilms which had a much lower proportion of streptococci (by a factor of 2 $\log_{10}$) in regions closest to the enamel surface. Although little attention has been paid to the relationship between the location of mutans streptococci in plaque and the induction of the caries lesion, it is likely that the demineralizing potential of these organisms would be greater the nearer they were to the enamel surface. Such close proximity to the enamel would decrease the chances of neutralization (or buffering) by saliva, etc., of the acids produced by these organisms.

The results obtained from these 1-month in vitro studies lend support to the concept of milk fluoridation as an anticaries measure, as they demonstrate that the addition of fluoride to milk (i) increases the pH of the biofilms, (ii) reduces the proportions of streptococci and *S. mutans*, and (iii) increases the proportion of *Veillonella* spp.

We thank P. C. Phillips and N. F. Borrow for their encouragement and support during the study.

This work was supported by the Borrow Dental Milk Foundation.

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