Bacterial Counts of Buffalo Milk Preserved with Different Concentrations of Hydrogen Peroxide

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The effect of different concentrations of hydrogen peroxide (0.04, 0.06 and 0.08 per cent) on the standard plate counts of uncooled buffalo milk during summer months was investigated. Counts were also determined for milk preserved in the glass, aluminium, and earthen containers.

The differences between the effects of different concentrations of hydrogen peroxide used were found highly significant. In the untreated samples, there was a direct relationship between the time of storage and standard plate counts ($r = +0.99$). In the treated samples, there was an inverse relationship between the standard plate counts and the amount of hydrogen peroxide used ($r = -0.89$).

The results of these investigations lead to four main conclusions: (*i*) Under a tropical environment, raw buffalo milk can be preserved with 0.06 per cent hydrogen peroxide up to about 21 hours and with 0.08 per cent hydrogen peroxide up to about 36 hours after milking without adverse effects on its wholesomeness. (*ii*) Buffalo milk can be preserved with 0.06 per cent hydrogen peroxide in clean (washed with soap and water) aluminium and unglazed earthen containers up to about 21 hours after milking. For a longer preservation, higher concentrations will be necessary. The overall efficiency of hydrogen peroxide is high in the glass, medium in the aluminium, and low in the unglazed earthen containers. (*iii*) A standard plate count of 1,3000,000 per ml. has been suggested for purposes of fixing minimum microbial standards of clean buffalo milk in Pakistan. (*iv*) Since the production, processing, and sale of buffalo milk is beset with innumerable public health problems, work on milk microbes under tropical environments should be intensified, specially in relation to the dairy practices and equipment in vogue in the different parts.

INTRODUCTION

Of different kinds of milk produced in Pakistan, that of she-buffaloes (*Bos bubalis*) is most important quantitatively as well as qualitatively. Of the total production of 236.3 million maunds during 1965, 133.2 million maunds (56 per cent) represented buffalo milk (Haq and Masud, 1966). Qualitatively, buffalo milk is relished and given preference over milk of other kinds because of its high fat content (6-8 per cent). Even though she-buffaloes have emerged as important dairy animals in this country, very little has so far been done to investigate the problems of production, processing, and consumption of buffalo milk. Since the production of clean milk and its consumption in a wholesome condition depend largely on the extent of its contamination, it is of paramount

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importance to study the microbes invading buffalo milk. As milk is prone to very quick spoilage, this creates many public health problems, specially during the summer months. The daily per capita consumption of milk in the country being small (7.2 oz.), the country can ill-afford the spoilage of milk even in small quantities.

There are several methods of preventing the spoilage of milk, and preserving it in a wholesome condition over prolonged periods, such as, keeping at low temperatures, pasteurization, and addition of preservatives like hydrogen peroxide. The last named agent has been used in other countries for preserving cow milk, but it has not been tried on a large scale in tropical countries, less so for preserving buffalo milk. Occasionally, when hydrogen peroxide was used for preserving buffalo milk, efforts do not appear to have been made to determine the bacterial contents of milk as affected by different concentrations of this preservative in Pakistan or abroad (WHO, 1962; Karim *et al.*, 1962).

The studies reported in this paper were organized to investigate the effects of different concentrations of hydrogen peroxide on the standard plate counts of buffalo milk during the summer season. The counts were determined for milk preserved in the glass, aluminium, and earthen containers. It is hoped that the study will prove helpful in three ways, *viz.*, (*a*) it will help producers and consumers in prolonging the life of fluid milk, (*b*) it will enable the long-distance haulage of milk in a wholesome condition, and (*c*) it will assist in introducing bacterial standards for market milk in Pakistan. In its final analysis, the study will go a long way in developing the dairy industries in the country.

REVIEW OF LITERATURE

As far as ascertained, the literature does not appear to report the effect of different concentrations of hydrogen peroxide on the standard plate counts (SPCs) of buffalo milk during the summer months. Even in those tropical and subtropical countries, where buffalo milk is produced in substantial quantities, no work seems to have been done in this regard (WHO, 1962; Karim *et al.*, 1962).

Considerable work has been done on the preservation of cow milk with hydrogen peroxide, but the major emphasis seems to have been placed on the comparative utility of this preservative as a substitute for pasteurization, or as an agent to improve the keeping quality of milk in cold climates. Babad and Boros (1962) working with raw milk held at 26-28°C, reported reductions of 92.2 ± 2.3 and 93.4 ± 2.1 per cent in bacterial counts, following the addition of 0.2 and 0.3 per cent hydrogen peroxide respectively. Gregory *et al.* (1961) reported that the bacterial counts of clean milk decreased from 1×10^8 to 4 per ml. and those of contaminated milk from 1×10^5 to 1×10^3 per ml. when

milk was treated with 0.05 per cent hydrogen peroxide for 8 hours at 24°C. Roundy (1958) treated milk with 0.06, 0.047, and 0.038 per cent hydrogen peroxide at 127°F for 40 seconds, and found corresponding decreases of 84.04, 82.35, and 78.67 per cent in the total bacterial contents. Satta *et al.* (1943 cited by Luck, 1962) added 0.08, 0.10, and 0.12 per cent of hydrogen peroxide to cow milk at 32°C., and found a reduction in bacteria to the extent of 93.57, 99.53, and 99.96 per cent respectively after 20 hours. Monaci (1949 cited by Luck, 1962) found a reduction varying from 74.3 to 96.3 per cent with 0.08 per cent hydrogen peroxide, acting on milk for 12 to 24 hours at 20-22°C. and at 28-30°C. Nambudripad and Iya (1951 cited by Luck, 1962) found that 0.05 per cent hydrogen peroxide inactivated species like *Streptococcus lactis* and *Escherichia coli* in ½ to 3 hours, those like *S. liquefaciens* and *Bacillus cereus* in 4 to 7 hours, and *B. megatherium* and *B. subtilis* in 16 to 18 hours.

Hydrogen peroxide destroys most of the pathogenic micro-organisms, but *Mycobacterium tuberculosis* is resistant even up to 0.8 per cent concentration. *Brucella abortus* and *B. melitensis* are destroyed within 3 to 24 hours with a concentration of 0.08 per cent at 20-32°C. The same concentration kills *Salmonella typhosa* in 8 to 9 hours at 17-32°C. Consequently, if milk is intended for fluid consumption, it is essential that hydrogen peroxide treatment is followed by standard methods of processing to ensure the destruction of disease-producing microbes.

Depending upon local conditions, different workers have used different concentrations, varying from 0.009 to 0.3 per cent (by weight) of hydrogen peroxide, but FAO (1957) recommends that the quantity of this preservative should in no circumstance exceed 0.8 gm. per litre of milk (*i.e.*, 0.08 per cent), and it should preferably be 0.1-0.4 gm. per litre (*i.e.*, 0.01-0.04 per cent) of milk intended for fluid consumption. Any quantity in excess of 0.1 per cent influences the constituents of milk unfavourably.

Hydrogen peroxide is a strong oxidising agent (oxidation potential $=-1.81 \pm 0.03$ V.) and, when brought into contact with bacteria, exerts a bacteriostatic and bactericidal action. The exact mode of its action in destroying bacteria is not known. While some attribute its effectiveness to oxygen developed in *statu nascendi*, others opine that it is the undecomposed hydrogen peroxide which is so effective (dissociation constant: $H_2O_2 = H + OOH \rightarrow OH^- + O^-$). The bactericidal efficiency of this preservative depends on: (*a*) concentration—concentrations of 0.001—0.1 per cent inhibit and those of a higher order destroy micro-organisms at room temperature (Schumb *et al.*, 1955), (*b*) temperature and period of treatment—the influence of hydrogen peroxide is intensified with an increase in temperature and time of treatment (Luck, 1962), (*c*) initial bacterial contents of milk—the higher the initial count, the lesser

is the bactericidal efficiency, for the catalase in milk decomposes hydrogen peroxide (Nambudripad *et al.*, 1952) (*d*) kind of bacteria—microbes like *M. tuberculosis* and *B. subtilis* are more resistant to the action of this preservative than *E. coli* and *S. lactis* (Nambudripad and Iya, 1951 cited by Luck, 1962) and (*e*) kind of milk containers—materials like aluminium, borosilicate glass, and polyethylene are more highly compatible with this preservative than steel, copper, tin, and soda-glass, which decompose hydrogen peroxide and render it ineffective rapidly (Schumb *et al.*, 1955). In the light of these facts, it is obvious that a maximum bactericidal effect can be obtained if hydrogen peroxide is added to milk soon after milking (when the initial bacterial count is low, and milk is at body temperature), and in sanitized containers made of materials compatible with hydrogen peroxide.

The Expert Group of FAO (1957) has stated that, of the various preservatives available for milk, the only one permissible is pure grade hydrogen peroxide. They have recommended that: (*a*) hydrogen peroxide treatment should be tolerated only in exceptional cases, and in warm or technically less developed countries, where rapid transport of producers' milk to processing centres is not possible, or where effective cooling of milk cannot be carried out, (*b*) the addition of hydrogen peroxide should be carefully controlled and its complete removal ensured before milk is retailed for human consumption, (*c*) since hydrogen peroxide does not destroy all pathogenic microbes, milk treated with it must subsequently be subjected to effective heat treatment, and (*d*) further investigations should be conducted to evaluate more precisely the changes in the quality of milk in relation to human health and nutrition. Kon (1964) has recently stated that, while striving for perfection, people should accept treatment with hydrogen peroxide, intelligently applied when need demands, as a timely and useful measure.

MATERIAL AND METHODS

Material: In all, 15 composite samples (comprising 60 samples and 585 plates) of raw milk from she-buffaloes of *Nili* and *Ravi* breeds, maintained by the West Pakistan Agricultural University, Lyallpur, were collected for investigations spreading over four summer months (July-October, 1965). Every composite sample contained milk from 5 to 9 she-buffaloes, and was taken within 15-20 minutes of milking from the pail used by the University Dairy Farm for routine bulking. Of these, 10 composite samples were used to investigate the effects of different concentrations of hydrogen peroxide on the bacterial counts of milk; while the other 5 composite samples were preserved with hydrogen peroxide to investigate the growth of bacteria as affected by the material of the containers, *viz.*, glass, aluminium, and unglazed earth. These

containers were employed because they are the commonest types of containers in vogue all over the country, and because aluminium is highly compatible with hydrogen peroxide. The glass containers were sterilized, but those of aluminium and earth were not sterilized purposely: they were used after cleaning with soap and hot water, as is the practice in an average home.

Methods: All composite samples were processed in the laboratory within 20-35 minutes of milking. In the first set of experiments, every composite sample was divided into four samples, each measuring 100 ml. One sample was kept aside to serve as control; while the remaining three samples were treated with hydrogen peroxide (30 per cent by weight), to yield final concentrations of 0.04, 0.06, and 0.08 per cent of the preservative. To obtain bacterial counts after 12, 24, and 36 hours, milk was kept in 12 separate flasks (3 for control and 9 for hydrogen peroxide treated samples), so as to minimize contamination from the handling of the same flask every twelve hours. For purpose of determining standard plate counts, milk was diluted in sterilized distilled water, making dilutions varying from 10^{-3} to 10^{-8} with usual precautions (APHA, 1953; Foster *et al.*, 1957). Plating was done on Milk Protein Hydrolysate Medium (BBL, 1964), and the plates were incubated at 32°C. for 48 ± 3 hours. As three or four dilutions were used for each sample, only such readings were accepted as showed proximity to each other in two out of the three or three out of the four dilutions made. The average bacterial counts thus obtained were then subjected to analysis of variance, "t" test, and coefficient of correlation. In the second set of experiments, the same procedure was followed for plating samples preserved in the glass, aluminium, and earthen containers, except that the concentration of hydrogen peroxide was kept constant at 0.06 per cent.

In all cases, milk samples were kept in the laboratory at ordinary room temperature. The laboratory temperatures for the period of experimentation (*i.e.*, 36 hours for every sample) were recorded with the help of a maximum-and-minimum thermometer; while the corresponding daily atmospheric temperatures (*i.e.*, 48 hours for every sample) were obtained from the Physics and Meteorological Department of the West Pakistan Agricultural University, Lyallpur. All investigations were carried out in room temperatures varying between 86° and 98°F (atmospheric temperatures between 70° and 106°F).

RESULTS AND DISCUSSION

(1) Standard Plate Counts Milk Preserved in Glass Flasks

The standard plate counts (SPCs) of the 10 composite samples of buffalo milk stored in sterile glass containers were taken and the average counts of the

samples are summarized in Table 1.

TABLE 1. *Standard Plate Counts of Buffalo Milk Preserved with Different Concentrations of Hydrogen Peroxide.*

Mean temperature (F°)		Time of plating after milking	Average plate counts per ml. (000)			
Atmosphere	Room		Normal milk (control)	Milk preserved with hydrogen peroxide		
				0.04%	0.06%	0.08%
		25 min.	1,446*	1,446*	1,446*	1,446*
87	92	12 hr.	94,703	510	203	148
(70-106)	(86-98)	24 hr.	253,199	16,151	8,565	2,825
		36 hr.	358,650	40,185	20,018	13,370

*Counts before adding hydrogen peroxide.

Table 1 (Fig. 1) reveals that the initial count of 1.4 million per ml. multiplied to 94.7 million after 12 hours, 253.2 million after 24 hours, and 358.7 million per ml. after 36 hours in the case of the control samples, *i.e.*, the initial count increased 65 times after 12 hours, 175 times after 24 hours, and 248 times after 36 hours. In the samples treated with 0.04 per cent hydrogen peroxide, the initial count of 1.4 million dropped down to 0.5 million after 12 hours, and multiplied to 16.2 and 40.2 million per ml. after 24 and 36 hours respectively, *i.e.*, the initial count fell down by two-thirds after 12 hours, and increased 11 and 28 times after 24 and 36 hours respectively. In the samples treated with 0.06 per cent hydrogen peroxide, the initial count dropped down to 0.2 million per ml. after 12 hours, and multiplied to 8.6 and 20.0 million per ml. after 24 and 36 hours respectively, *i.e.*, the initial count fell down by six-sevenths after 12 hours, but increased 6 and 14 times after 24 and 36 hours respectively. In the samples treated with 0.08 per cent hydrogen peroxide, the initial count dropped down to 0.1 million per ml. after 12 hours, and multiplied to 2.8 and 13.4 million per ml. after 24 and 36 hours respectively, *i.e.*, the initial count fell down by nine-tenths after 12 hours and increased 2 and 9 times after 24 and 36 hours respectively.

The analysis of variance showed that the differences between the effects of different concentrations of hydrogen peroxide used were highly significant, *i.e.*, the SPCs of the untreated and treated milk were highly different from one another. The detailed analysis of individual samples showed that the bacterial

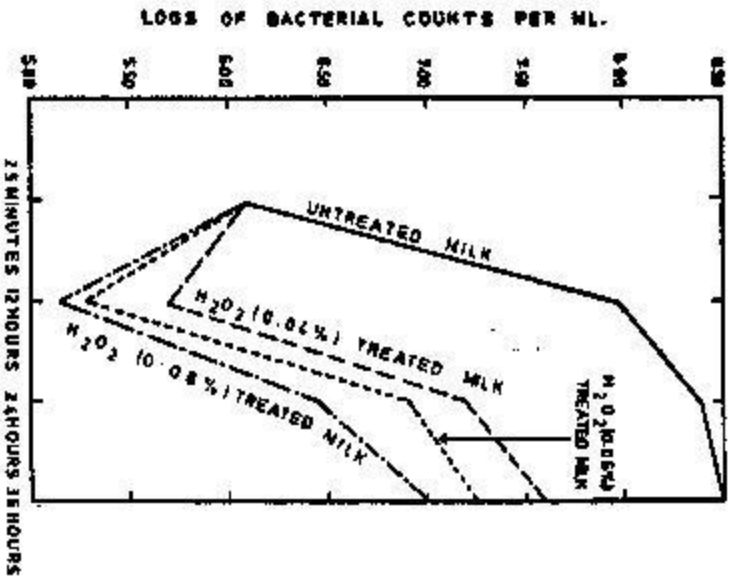

Fig. 1—Effects of hydrogen peroxide on bacterial counts of milk.

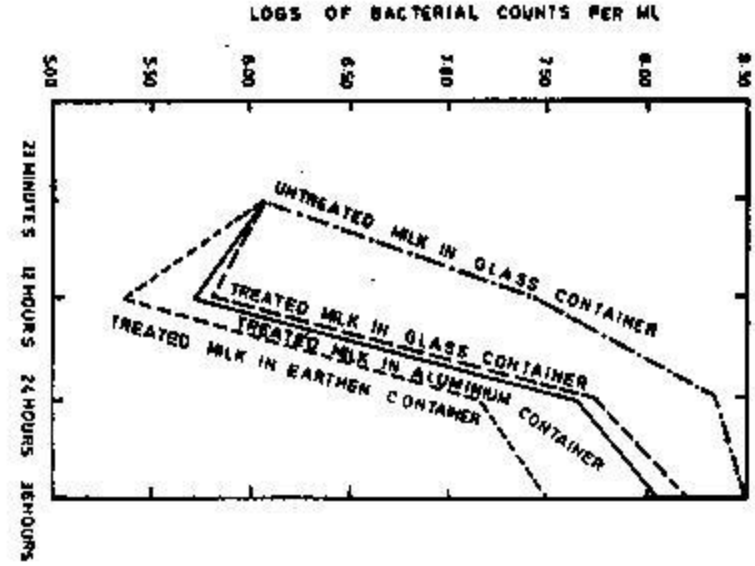

Fig. 2—Effects of 0.06% hydrogen peroxide on bacterial counts of milk kept in different containers.

counts of preserved milk were markedly lower than those of untreated milk. The differences among the bacterial counts of the samples preserved with the three concentrations, however, were not significantly different from one another. The difference between the replications of the experiment was non-significant. Further analysis showed that the differences between the bacterial counts at 12-hourly intervals were highly significant.

The SPCs of untreated samples of the first set of experiments varied from 830,000 to 4,693,000, with an average of 1,446,000 colonies per ml. As regards the effect of atmospheric temperature on bacterial counts, there was no relationship between the temperature and the initial counts, which essentially depend on udder infection, habitational environment, and sanitation of dairy utensils. In so far as the effect of the time of storage on bacterial counts was concerned, the counts of the control samples multiplied progressively after 12, 24, and 36 hours. There was a direct relationship between the time of storage and the SPCs, the coefficient of correlation being +0.99 (significant at 5 per cent level). These figures of SPCs compare favourably with those reported for buffalo milk from other tropical countries, *e.g.*, the counts in some of the private dairies during the summer season in India have been reported to vary from 3,677,000 to 9,795,000 per ml. at 22-45°C., and those of market milk in Egypt from 194,000 to 29,000,000 with an average of 9,000,000 organisms per ml. (WHO, 1962).

In countries like Norway, U.K., and U.S.A., bacterial standards for clean cow milk have been prescribed. Depending upon different types of milk, they have laid down the limit of maximum SPCs at 200,000 per ml., though their counts often reach 1,000,000 per ml. even under sanitary conditions of milk production (Hammer and Babel, 1957; WHO, 1962). Such limits have not been prescribed for tropical countries, while the counts for reasonably clean milk reported from India and Egypt vary widely; from a few thousand to 9 million per ml. Keeping tropical environment and managemental practices in view, it is suggested that the upper limit for SPCs of buffalo milk in Pakistan should be fixed at 1,500,000 per ml. This suggestion is based on three considerations: (*a*) the SPCs under the present investigation averaged 1,307,000 per ml. (including figures of the second set of experiments given later), and milk came from she-buffaloes kept and milked under reasonably clean conditions, (*b*) milk with an initial count of 1.5 million per ml. kept well for about half a day which is the optimum time for unpreserved milk under a tropical environment, and (*c*) tropical environment generally produces milk with high SPCs of 51,000—5,100,000 per ml. (Anderson *et al.* 1961).

The SPCs of buffalo milk treated with hydrogen peroxide fell down considerably after 12 hours, but increased at 24 and 36 hours progressively

with all the three concentrations employed in the present study. There was an inverse relationship between the SPCs and the amount of hydrogen peroxide used, the coefficient of correlation varying from —0.88 to —0.91. These results are in general conformity with those obtained on cow milk (WHO, 1962). Reasonably low counts at the end of 36 hours were observed with the last two concentrations of 0.06 and 0.08 per cent hydrogen peroxide; the counts with the former concentration were nearly half and those of the latter nearly one-third of the corresponding counts with 0.04 per cent concentration. The average counts with the two higher concentrations at the end of 36 hours were only 5.6 and 3.7 per cent of the corresponding counts of the control samples. It was concluded, therefore, that 0.06 per cent hydrogen peroxide should be employed when milk is required to be preserved for about 21 hours, and 0.08 per cent concentration for a longer period up to 36 hours. Three important points should, however, be borne in mind: (*i*) the objective should be to use the smallest concentration of hydrogen peroxide in accordance with the minimum period of preservation desired, (*ii*) hydrogen peroxide in concentrations of 0.1 per cent and above influences the constituents of milk adversely, and (*iii*) milk with high initial counts being prone to quick spoilage, higher concentrations of hydrogen peroxide should be used when the initial counts are high, and *vice versa*.

The concentrations of 0.06 and 0.08 per cent of hydrogen peroxide were very helpful because they killed or inactivated 86-90 per cent of the bacteria present initially within the first 12 hours. Knaut (1960) added 0.04 and 0.08 per cent of hydrogen peroxide to cow milk (inoculated with 250,000 bacteria per ml. of six different species of *Pseudomonas* and *Bacillus* held for 30 minutes at 49°C.), and found that proteolysis was delayed by 24 and 48 hours with the two concentrations respectively. The total counts obtainable after 24 and 36 hours of treatment with 0.06 per cent hydrogen peroxide also fell in line with the safety margins of the bacterial quality of cow milk in cold climates, *e.g.*, Kandler (1964) found that the quality of pasteurized milk and dairy products manufactured therefrom was influenced only to a small degree by SPCs up to 10 million per ml; and that the quality of milk and its products tended to be adversely affected when the counts reached 100 million per ml. mark. Compared with these results, and even without giving a margin for higher counts obtained in a tropical environment (Aijaz-ul-Haq and Majeed, 1965) the counts actually obtained under the present investigation were far lower, *i.e.*, 13-20 million per ml. (min. 0.4, max. 52.0 million) after storage for 36 hours. It is inferred, therefore, that hydrogen peroxide proves as effective in hot climates as in cold climates.

(2) Standard Plate Counts of Milk Preserved in Glass, Aluminium and Earthen Containers

The SPCs of the 5 composite samples preserved in the glass, aluminium and unglazed earthen containers were studied and the average counts of these samples are summarized in Table 2.

TABLE 2. *Standard Plate Counts of Buffalo Milk Preserved with Hydrogen Peroxide in Containers of Different Kinds.*

Mean temperature (F°)		Time of plating after milking	Average plate counts per ml. (000)			
Atmosphere	Room		Normal milk (control)	Milk preserved with 0.06% hydrogen peroxide		
				Glass flask	Aluminium container	Earthen container
86	91	23 min.	1,169*	1,169*	1,169*	1,169*
(70-106)	(86-98)	12 hr.	26,807	236	521	596
		24 hr.	208,467	13,909	43,480	52,960
		36 hr.	297,500	29,020	105,540	149,450

*Counts before adding hydrogen peroxide.

Table 2 (Fig. 2, page 42) reveals that the original count of 1.2 million per ml. multiplied to 26.8 million after 12 hours, 208.5 million after 24 hours, and 297.5 million per ml. after 36 hours in the case of the control samples. In other words, the initial count increased 23 times after 12 hours, 178 times after 24 hours, and 255 times after 36 hours. In the samples in the sterilized glass flasks, the initial count decreased to 0.2 million after 12 hours, and increased to 13.9 and 29.0 million per ml. after 24 and 36 hours respectively, *i.e.*, the initial count fell down by 80 per cent after 12 hours, and increased 12 and 25 times after 24 and 36 hours respectively. In the samples in the aluminium containers, the initial count dropped down to 0.5 million after 12 hours, and multiplied to 43.5 and 105.5 million per ml. after 24 and 36 hours respectively, *i.e.*, the original count fell down by 55 per cent after 12 hours, and multiplied 37 and 90 times after 24 and 36 hours respectively. In the samples preserved in the earthen containers, the initial count dropped down to 0.6 million after 12 hours, and multiplied to 53.0 and 149.5 million per ml. after 24 and 36 hours respectively, *i.e.*, the original count fell down by 49 per cent after 12 hours, and increased 45 and 128 times after 24 and 36 hours respectively.

The analysis of variance showed that the differences between the SPCs of different containers were highly significant, *i.e.*, the SPCs of the untreated and those of the hydrogen peroxide treated milk were highly different from one another. The detailed analysis showed that the bacterial counts of treated milk in the three types of containers were markedly lower than those of untreated milk. The samples in the glass flasks gave lower counts than those in the aluminium and earthen containers. The glass containers were most suitable for preserving milk, followed by the aluminium and the unglazed earthen containers in decreasing order, as was evidenced by the average counts for the three types of containers. The difference between the replications was non-significant. Further analysis showed that the differences between the bacterial counts at 12-hourly intervals were highly significant.

The SPCs of untreated milk varied from 830,000 to 1,946,000 with an average of 1,169,000 per ml. As regards the effect of atmospheric temperature on SPCs, there was no relationship between the temperature and the initial counts. The effects of storage on counts were similar to those given before. The direct relationship between the time of storage and the SPCs gave a coefficient of correlation of +0.97 (significant at 5 per cent level). These results are in line with those on cow milk. As regards the storage of hydrogen peroxide treated milk in different containers, the counts showed a marked decline at 12 hours, but multiplied progressively at 24 and 36 hours. The counts of the preserved samples after 36 hours storage in the glass, aluminium and earthen containers were very low, *viz.*, one-tenth, one-third and one-half of the counts of the control samples respectively. The coefficient of correlation between the bacterial counts and the time of preservation in the glass, aluminium, and earthen containers varied from +0.92 to +0.93. The hydrogen peroxide treatment was most effective in the sterilized glass containers, less so in the unsterilized aluminium containers, and least effective in the unsterilized earthen containers. Of the aluminium and earthen containers, the former proved more effective than the latter, because (*a*) aluminium is more compatible with hydrogen peroxide than unglazed baked earth, (*b*) detergents sanitize the aluminium containers better than the earthen ones, and (*c*) earthen containers are more difficult to clean than the aluminium containers, and their pores harbour microbes and dirt. Although the results show that milk can be preserved with 0.06 per cent hydrogen peroxide in both the aluminium and earthen containers up to 21-23 hours, the aluminium containers should be preferred in view to their far greater durability and possibly lower bacterial contamination. As the literature does not seem to report the SPCs of buffalo milk preserved with hydrogen peroxide in containers made of aluminium and unglazed earth, it is not possible to compare findings with the results obtained elsewhere. It may, however, be

emphasized that the sanitation of milk is extremely important: of nearly 134 diseases of public health importance, many are transmitted through milk and its products (Hull, 1963).

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