INHIBITORY SUBSTANCES IN MILK

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The potential bacteria growth inhibitors in milk are residues from chemical sanitizers, residues from "sulfa" drugs used therapeutically, residues from antibiotics, bacteriophages and others. The action of each of these inhibitory substances with respect to plate counts and lactic acid development are graphically illustrated and discussed with respect to related work by others, reviewed here in some detail. The presence of various inhibitory substances in milk apparently is sufficiently widespread to warrant considerable concern. It is concluded that every effort should be made to prevent the antibacterial agents from gaining entrance into milk.

Standard Methods for the Examination of Dairy Products (25) lists the potential growth inhibitors in milk as residues from chemical sanitizers, residues from "sulfa" drugs used therapeutically, residues from antibiotics, bacteriophages and other unidentified inhibitors. These growth inhibitors may influence the results obtained in determinations by agar plate methods and the reduction type methods. Also, various inhibitors in milk may adversely affect the rate of lactic acid development essential in the manufacture of various fermented milk products, such as buttermilk and cheese.

The antibiotics and "sulfa" drugs (which for our purpose may be considered synonymously under the term antibiotics) may occur in milk as the result of treatment of udder diseases of dairy cows. Chemical sanitizers, such as chlorine compounds or quaternary ammonium compounds, gain entrance into milk by improper use or possibly by intentional additions. Bacteriophage gains entrance to dairy products as the result of improper sanitation or starter propagation procedures.

The presence of any antibiotic or any sanitizer in milk is objectionable and illegal. Milk containing these inhibitory substances is considered adulterated regardless of whether the materials gained entrance to milk by accidental means or by intentional additions.

When difficulties arise with respect to inhibition of bacterial growth, it is apparent that similar symptoms are caused by several unrelated inhibitors — sanitizers, bacteriophage, antibiotics, or other causes. Without further proof, other than delayed lactic acid formation or failure of colonies to grow on plates, further information should be sought for positive identification of the causative factors.

This paper will be concerned primarily with work done at the Florida Agricultural Experiment Station dealing with the action of certain inhibitory substances in milk as related to plate counts and to lactic acid development by dairy starters. The findings will be discussed with respect to related work by others, which is reviewed here in some detail.

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For complete review of the many problems encountered with respect to the antibiotics in milk the reader is referred to the review articles by Calbert (4), Claybaugh and Nelson (5), Overby (21) and Trout (28).

INCIDENCE OF INHIBITORS

It has been noted that laboratory personnel had observed sudden drops in plate counts or undue lengthening of methylene blue or resazurin reduction times, Calbert (4), Johns & Katznelson (14), with no apparent explanation. In these cases promiscuous use of inhibitory substances, primarily antibiotics might be suspected.

Silverman and Kosikowsky (23) have evaluated and coordinated methods for the systematic analysis of inhibiting substances in milk. Methods are discussed which are suitable for testing large numbers of samples from wide areas for total inhibitory substances, antibiotics, sulfa drugs and quaternary ammonium compounds. These tests are readily adaptable to a systematic laboratory procedure. They point out that no practical method exists for testing for bacteriophage in large numbers of milks over wide areas, so the importance of this particular problem to the dairy industry cannot be fully evaluated until such time when the incidence of such bacteriophage cases can be measured.

The extent of residual antibiotics in milk has been discussed by Henningson (10) for three areas; for the state of New York (16), for Ottawa, Canada (12) and for London, England (27). These three surveys seemed to indicate that there existed no grave problem in the specific areas as far as the manufacture of fermented dairy products was concerned during the years 1951 to 1954. Over 4,000 test samples were involved in these studies. Using an antibiotic assay test, the percentage of samples showing greater than critical levels ranged from 0.3 to 0.8 per cent. These results were specific for penicillin and further starter activity tests showed slightly more inhibition which may have been the result of other antibiotics or some other inhibitory substance. It was generally agreed that no serious problem existed; however, this does not preclude individual cases which may have been quite serious and costly. These troublesome cases should not be minimized. They tend to emphasize the importance of a better understanding of the problems related to inhibitory substances in milk and they demonstrate the need to be on guard against potentially greater difficulties in the future.

Despite the fact that surveys have shown no widespread difficulties due to inhibitory substances in milk, it is believed that the problems may have increased in some sections in recent years. A tabulation of certain data available from one of the Florida State Board of Health laboratories during the first quarter of 1955 (shown in Table 1) reveals that a significant number of standard plate counts were reported “less than 3,000 per ml.” This is indeed desirable and reflects what would be considered excellent care in milk production. However, when almost 10 per cent of the raw samples showed counts of less than 3,000 and 16 per cent of the pasteurized milk samples showed counts of less than 500, either the milk is of unusually good quality, or it may be suspected of containing bacterial growth inhibitors. As no further information concerning these counts is available there may be some cause to suspect inhibitory levels of antibiotics in some of the milk.

NORMAL GROWTH CURVES

An explanation of normal growth curves is necessary in obtaining a better understanding of the problems encountered with respect to the various inhibitory substances in milk.

Figure 1 shows the relationship of plate counts, titratable acidities and bacteriophage concentrations in milk incubated at 21°C. These are typical curves obtained under approximately normal conditions of starter propagation. The data do not represent a single trial but rather a compilation of curves obtained independently. By plotting these on a single graph, an understanding of the relationship is obtained. It should be pointed out that the scale selected for the titratable acidities and the one selected for the logarithm of total plate count are arbitrary and do not necessarily coincide. The plate count illust-
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The relationship of plate counts, titratable acidities and bacteriophage development in milk.

Acidities treated was initially very low as was the initial inoculation of lactic acid organisms. This accounts for the longer period of incubation for maximum development of acidity. The important point to be noted is that the plate counts reach approximately the 10 million range before a measurable amount of acidity is developed. Thus, it can be observed that inhibitory action as measured by plate count procedures would precede by as much as 10 hours the action measured by titratable acidity determinations under these conditions.

ANTIBIOTICS VS. LACTIC ACID DEVELOPMENT

The presence of antibiotics in milk has been found to reduce the numbers of bacteria in milk as measured by standard plating procedures as well as adversely affecting the rate of lactic acid development in the manufacture of fermented milk products. Penicillin, for example, at a concentration of only 0.1 unit, can reduce the rate of lactic acid production to a degree which can be measured by titration methods. Figure 2 shows the influence of penicillin on lactic acid development. It may be noted that as milk was incubated at 30° C. the acidity developed rapidly in the control sample, reaching a maximum in about 8 hours; this is the usual activity shown by a dairy starter with 1 per cent inoculation. In the same period of time, 0.1 unit had retarded the acidity to about one-half that of the control and 0.2 unit or more practically stopped acid production.

ANTIBIOTICS VS. PLATE COUNTS

In studies (29) on the influence of penicillin in milk on total and coliform bacteria plate counts, it was found that a concentration of one unit of penicillin per ml. in raw mixed milk was sufficient to significantly retard growth during a three-day storage period at 10° C. Figure 3 shows the influence of penicillin on the standard plate counts of raw milk held in refrigerated (10° C.) storage over a period of 6 days. It is shown that 0.1 unit or more is sufficient to retard growth for over 3 days below the 200,000 per ml. level. In recent years this has become a more important consideration because many dairy farms are now using refrigerated bulk milk tanks with two-day storage on the farm between pick-ups.

Penicillin and sulfa drugs tend to reduce the numbers of bacteria found in milk by standard bacteriological examinations. The extent of reduction of counts depends upon such factors as concentration, dilution, number and types of organisms and the sensitivities of the various microorganisms to the antibiotics or inhibitory materials under consideration.

Additional research along these lines has confirmed and expanded the observations that plate counts (standard, coliform, psychrophilic) on raw and pasteurized products are reduced by various antibiotics (penicillin, streptomycin, tyrothricin, aureomycin) under various laboratory conditions.

Olson et al. (20) observed that addition of 0.2 microgram of aureomycin per ml. of pasteurized, homogenized milk generally showed slightly lower counts during storage at 45° F. when plated for psychrophilic organisms during a one-week period.
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Stoltz and Hankinson (26) 1953, studied the antibiotics penicillin, streptomycin, aureomycin, tyrothricin, and penicillin and streptomycin in combination at levels of concentration which might be found in market milk supplies of pooled herd milk following antibiotic therapy of a few diseased animals in the herd. At such concentrations all the antibiotics studied were found to inhibit the growth of bacteria in raw milk when stored for 48 hours at 7°C. They concluded that the addition of antibiotics to raw milk would result in receiving poor quality milk as an acceptable grade of milk at the dairy plant, if judged solely by bacteriological standards.

Nisin

Researchers in New Zealand (11) and England (17) have reported that certain milk supplies were inhibitory to lactic cultures. Such milk was referred to as "non-acid" milk. The substance causing this effect is produced by certain selected strains of Streptococcus lactis. It is considered to be an antibiotic and has been named Nisin. This substance has been shown to significantly inhibit the production of lactic acid by other strains of starter bacteria. This is an example of an antagonistic action of one strain of bacteria upon other strains of the same species. Such phenomena are not uncommon in the dairy industry; for frequently when two different commercial starters are mixed together, the rate of acidity development and maximum acidity obtained will be less than when either one of the starter cultures is used separately. With respect to starter activity, the inhibitory action has been found to be associated with unclean milking machines, poor sanitation on farms generally and with high bacterial counts during warm weather. The addition of 5 per cent of "non-acid" milk to an active dairy starter will delay the souring by as much as 15 hours. The presence in milk of nisin-producing organisms might be expected to lower the standard plate counts of milk; however, if the antibiotic was present in sufficient concentration to have a significant effect, the total count probably would be high due to the presence of large numbers of nisin-producing organisms. Such influence, if any, has not been established and must await further research along this line.

QUATERNARY AMMONIUM COMPOUNDS

Mull and Fouts (19) studied the influence of Roccal, a quaternary ammonium compound, on plate counts of raw and pasteurized milk and on the flavor of the milk. Figure 4 shows a portion of the data obtained which illustrates the importance of their findings. They concluded that the affect on plate counts was more noticeable in milk of low initial count (17,500/ml.) than in milk of high initial count (290,000/ml.). Approximately 200 p.p.m. of the Roccal were needed to bring about a significant decrease in bacteria counts, while as little as 10 p.p.m. could be detected by taste in the milk.

Babel (1) has shown that quaternary ammonium compounds inhibited lactic acid production when...
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present in milk to the extent of 3 p.p.m., while generally from 50 to 100 p.p.m. of quaternary in milk were required to prevent acid production entirely. Because the quaternary compounds contribute to slow acid production at relatively low levels, great care should be exercised to prevent their entrance into milk intended for fermented products.

Curry and Barber (7) investigated the "inhibition" of Streptococcus lactis by a quaternary ammonium compound in cheese milks. They found lactic cultures were destroyed within 10 minutes by concentrations of less than 10 p.p.m. active ingredient of a detergent sanitizer containing quaternary ammonium compound. On the other hand, concentrations greater than 600 p.p.m. in milk are required for the same degree of destruction. They further reported inhibition of lactic acid bacteria by concentrations of 2.5 to 10 p.p.m. quaternary in milk to be an effect on acid production and not upon growth.

CHLORINE

In a study made to consider the chlorine contents of waters used in reconstituting nonfat dry milk for manufacture of fermented dairy products, the data shown in Figure 5 were obtained. Chlorinated waters of various concentrations were used in these trials for reconstituting followed by inoculation with commercial lactic acid cultures and incubation at 70° F. Acidity determinations were made at intervals during the incubation period. It may be noted that as much as 100 p.p.m. of chlorine did not adversely affect the growth of organisms after the reconstituting had been completed. The hypochlorite used as the source of chlorine apparently combined with the milk solids during the process of mixing the water and powder and was no longer capable of bactericidal action.

From this study it may be concluded that the very small concentrations of chlorinating normally present in city water supplies (from 0.1 to 0.3 p.p.m. of free residual chlorine up to as much as 1.5 p.p.m. of combined residual chlorine) will not adversely affect the growth of organisms in starters, buttermilk and cottage cheese. The chlorine is bound by the milk solids and does not prevent lactic acid development nor does it impart objectionable off-flavors. Therefore, it appears desirable to use chlorinated waters because of the added advantage that such water will probably be free of undesirable bacteria. It is not only desirable but safe to use sanitizing solutions on equipment and utensils just prior to reconstituting powders with the knowledge that the small amount remaining on the equipment after draining will not reduce starter activity.

Babel (1) observed the changes in the bacterial population of milk due to the addition of some chemical bactericides. He found that 200 p.p.m. available chlorine added as hypochlorite, or 500 p.p.m. added as Chloramine-T were needed to prevent an increase or cause a slight decrease in bacterial population of raw milk for 48 hours at 40° F. Acid production was slightly inhibited by 5 p.p.m. available chlorine and greatly inhibited by 25 p.p.m. added as hypochlorite. An iodine bactericide in concentration of 40 p.p.m. prevented growth for 24 hours and 40-100 p.p.m. prevented growth for 48 hours.

Miller and Elliker (18) showed that it was necessary to add as much as 75 p.p.m. of hypochlorite to milk after it had been reconstituted and sterilized in order to show a significant decrease of acid production in starters. They found that some inhibition apparently occurred between 100 and 200 p. p.m. of sodium hypochlorite but did not always completely stop growth at the normal incubation temperatures of dairy starters.
Menadione

The action of menadione, a vitamin compound, in milk may be selected as a typical example of the antibacterial action by various chemicals which might be added to milk. Since menadione is relatively insoluble in milk, levels from 0.3 to 50 mg. were dissolved in one ml. portions of ethyl alcohol before being added to 100/ml. portions of whole milk. The mixture was heated in flowing steam for one hour followed by cooling to 30°C before inoculation. Controls with and without added alcohol were included. Trials were made using various levels of inoculation with bacteria. A typical result of this study is shown in Figure 6. The menadione could be detected in the milk at concentrations of about 3 to 5 p.p.m. as evidenced by a reduction in the rate of lactic acid development. Similar results were obtained when lower percentages of inoculation and lower incubation temperatures were used except that longer periods of incubation were required for the acid to develop. Similar results also were obtained using skim milk. The direct addition of 10 p.p.m. of menadione to milk extended the length of time to reach 0.25 per cent acidity by approximately 3 hours; 50 p.p.m. extended it about 6 to 8 hours. To preserve milk for several days, levels greater than 100 p.p.m. would be needed. However, such practice would be of no value in the dairy industry, since at levels of over 100 p.p.m. the menadione causes discoloration of milk as well as imparting an undesirable flavor to it.

It has been suggested (15) that the feeding of menadione to dairy cattle had a retarding effect on the souring rate of milk but these findings have not been confirmed (2, 3, 6, 30).

Bacteriophage

Bacteriophage is the general name given to certain viruses capable of destroying bacterial cells. The "phage" multiplies by growing on the living bacterial cells which are inactivated and lysed in the process. The lactic-acid-producing organisms are susceptible to specific strains of bacteriophage; this results in failures in acid production necessary for the successful manufacture of the various fermented dairy products. Unlike the antibiotics which are produced by microorganisms and active against numerous other organisms, the bacteriophage is a living organism which exhibits very specific activity against only certain susceptible species of bacteria and usually only against those strains from which it is produced.

Bacteriophage can be counted by use of special plating procedures or by using limited-dilution methods for determining the concentration, commonly referred to as the titer. Growth curves (Figure 1) of bacteriophage resemble those of other living organisms. The phage can be destroyed by chemical agents (chlorine or quaternary ammonium compounds) and heat very much like bacteria.

Parker and Elliker (22) investigated the destruction of lactic acid streptococcus bacteriophage by hypochlorite and quaternary ammonium compounds. They found that complete inactivation of phage preparations was attained with 200 p.p.m. of either quaternary ammonium or hypochlorite germicides. This concentration was considered sufficient for normal sanitization of dairy equipment for phage destruction.

With the advent of the electron microscope in recent years it has been possible to actually see bacteriophage despite their small size. The larger part of the phage is round with a diameter some-
what less than one-tenth that of the cells of lactic streptococci. A single, short tail on the phage is three or four times the length of the cell body. In addition to being able to see bacteriophage, measurement of its activity against the lactic streptococci has made possible numerous studies, notably at Iowa State College, regarding the nature of phage action. Elliker (8) has reviewed the many problems confronting the dairy industry associated with bacteriophage. Smith et al. (24) further emphasized the importance of bacteriophage as a cause of slow starters.

In commercial practice bacteriophage outbreaks occur suddenly with no apparent warning. As recent as February, 1955, the authors isolated from cottage cheese whey a bacteriophage strain causing difficulty in a Florida commercial dairy plant. At the present time, there are no practical tests which would give suitable advance information that could be used to prevent occasional failures of acid development in cheese and buttermilk manufacture. Therefore, the best practice has been to follow such procedures that are known to reduce the incidence of bacteriophage outbreak. These include rotation of cultures, proper handling and transfer of starters and thorough sanitization of all equipment and buildings.

**Bacterial Inhibitors as Milk Preservatives**

The addition of some kind of preservative to milk which would cause it to be free of all pathogenic organisms, not adversely affect the flavor and not be harmful to the consumer would be of interest. Such practice is permitted in some foreign countries, for example, the use of hydrogen peroxide in Italy. In this country the recommended practice is to not permit the masking of poor quality milk by use of antibacterial agents (4, 9, 23, 29).

None of the inhibitors considered here, and possibly no method of preservation now known, is completely satisfactory. Research results indicate that the numerous studies we hear about cold (radiation) sterilization holds some promise in that regard but the fundamental problem of destroying the bacteria without affecting the flavor still remains.

The various antibiotics and sulfa drugs are limited in range of activity and all exhibit various degrees of specificity against certain microorganisms. While some undesirable bacteria would be destroyed, others would continue to grow. In some instances, this results in a poorer quality product than when no inhibitor is used. Similar results would be obtained in the case of utilizing bacteriophage as preservatives since bacteriophage are known to be highly specific in action. Chemical sanitizers and other chemical compounds thus far have proven of little value. Johns and Berzins (13) tested an iodophor formulation (Iosan) and found that a concentration which would be effective as a preservative in milk could be detected by taste and would be objectionable. They observed that as much as 16 p.p.m. of Iosan influenced resazurin reduction times and plate counts too little to be of practical significance since at the use-dilution of 25 p.p.m. considerably less concentration would accidentally occur in milk due to inadequate drainage of sanitized equipment.

**Summary**

Various bacterial growth inhibitors—sanitizers, antibiotics, bacteriophages, and others—occur in milk. These cause lowered plate counts and retarded lactic acid development. Such difficulties are sufficiently widespread to warrant considerable concern. Very small amounts of certain inhibitory substances are enough to adversely affect growth of microorganisms in milk. However, none are satisfactory as milk preservatives, since none are completely effective against all types of bacteriological spoilage. Also, milk of low bacterial count or activity due to the presence of inhibitory substances might be of poor quality because of non-bacterial defects such as rancid or feed flavors. The use of antibacterial agents as preservatives should not be permitted because such use might result in the masking of poor quality milk and poor production and processing methods.

To avoid starter difficulties due to bacteriophage action, it is important to follow all procedures recommended for proper care and handling of starters. This includes rotation of cultures, proper handling and transferring of cultures, and thorough sanitization of all equipment. Such care will reduce the chances of starter failures due to conditions other than antibiotics.

To obtain reliable plate counts, the products tested must also be free of inhibitory levels of antibiotics and sanitizing agents. The best way to avoid the accidental entry of objectionable levels of antibiotics in milk is to keep producers informed and insist they comply with the recommendations of the Committee on Antibiotics of the American Dairy Science Association and the Federal Food and Drug Administration. These recommendations state that milk from treated segments of udders should be discarded or used for purposes other than human consumption for at least 72 hours after the last treatment.
The tremendous success of antibiotics in treatment of mastitis fully justifies their use; but such products should be used only when needed and not without good reason. The wholesale use of antibiotics in treatment of entire herds when obviously not needed should be discouraged.

Little or no information exists with respect to the long range effects on human beings of small daily intakes of antibiotics or other inhibitory substances in milk over extended periods of time. Further research is needed along these lines. Until such information is available, it seems desirable to keep the milk intended for human consumption as free as practical of trace amounts of any of the bacterial growth inhibitors.

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REFERENCES