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Journal of Milk and Food Technology

61ST ANNUAL MEETING
ST. PETERSBURG HILTON
August 12, 13, 14, 1974
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(Reservations Page 1)

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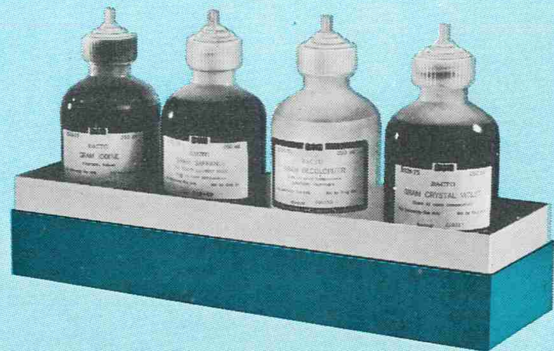

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EDITORIAL OFFICES: Dr. Elmer H. Marth Dept. of Food Science, University of Wisconsin, Madison, Wis. 53706. Earl O. Wright, P. O. Box 701, Ames, Ia. 50010.

Manuscripts: Correspondence regarding manuscripts and other reading material should be addressed to Dr. Elmer H. Marth, Dept. of Food Science, University of Wisconsin, Madison, Wis. 53706.

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J. Milk Food Technol., Vol. 37, No. 6 (1974)

TECHNICAL ASPECTS OF LIQUID COMPOSTING[1]

LOIS S. CRAUER AND BERNARD HOFFMAN
The DeLaval Separator Company
Poughkeepsie, New York 12602

ABSTRACT

As a consequence of stiffening legislation for environmental control, livestock producers are coming to realize that how they handle manure removal, drainage, and odor control (or lack thereof) is no longer their decision alone. While providing a solution to an immediate problem of odor control, the dairy farmer is beginning to become aware that in the future he may be required to produce an effluent which could be discharged to the nearest stream or lake. The De Laval Separator Company has developed a liquid composting system for deodorizing, pasteurizing, biologically decomposing, and chemically purifying dairy cow waste. The theory of liquid composting is discussed and several modes of LICOM™ System operation are described. Particular emphasis is placed on a description of a completely automated manure handling and treatment system operating at an 80-cow, free-stall dairy barn. Data covering 24 months of operation are presented.

Few agricultural issues have received more attention recently by the non-farm sector than have agricultural pollution and solid waste disposal. Dairy farmers, and all livestock producers for that matter, are coming to realize that how they handle manure removal, drainage, and odor control (or lack thereof) is no longer their decision alone. The growing public debate on environmental control, urban migration to rural areas, and stiffening legislation are increasingly affecting all livestock men.

Animal wastes can contribute to water pollution problems in a variety of ways. High nutrient loads resulting from run-off can upset the balance of ecological systems in our water bodies by causing excessive plant growth and depletion of the oxygen supply in water. Additionally, pathogens and chemical additives which may be present in manure can have far-reaching effect on both animals and man through use of manure-contaminated ground waters.

Causes and concerns of animal waste treatment and disposal are analogous to environmental problems caused by people. When both people and animals were fewer in number and better distributed throughout the land, their wastes could be absorbed without adversely affecting the environment. Aggregations of people in cities and development of large scale industrial operations have caused the air and water pollution as well as health problems of which we are increasingly aware. Similarly, it is the development of large scale, confined animal production operations that has caused animal waste pollution problems.

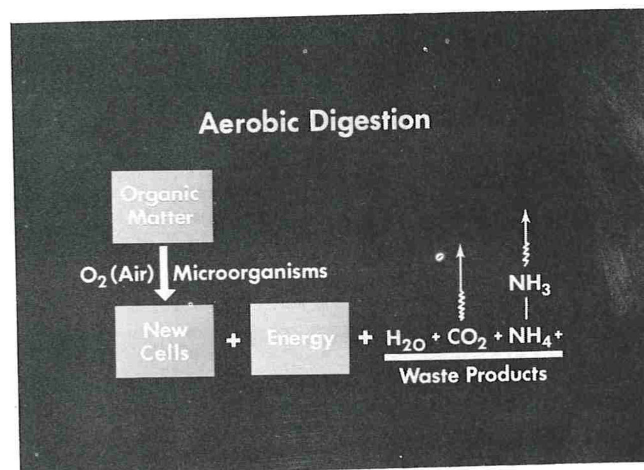

Figure 1. Aerobic digestion process.

AEROBIC AND ANAEROBIC DECOMPOSITION

To understand the reasons for this, consider briefly some basic biological chemistry. When cows are permitted to graze on a grassy range, their feces are scattered in a thin layer on top of the soil.

In this environment, oxygen is readily available and the solids are rapidly metabolized by an aerobic microflora which yields innocuous end products (see Fig. 1) which are produced at the expense of oxygen and include water, carbon dioxide, and certain inorganic ions such as the ammonium ion. Oxygen never becomes limiting and aerobic decomposition proceeds until essentially all the fecal material has been converted to these simple end products or stabilized in highly resistant, humus-like residues.

But, today cows are also confined in a building arranged in such a way that their droppings can periodically be scraped into a storage pit and stored until it is convenient to remove them. Bacterial decomposition of the material in the pit will start immediately but the environment is profoundly different. As the depth of the material in the collection pit increases, oxygen becomes limiting because diffusion of air through the manure slurry will be slow. As oxygen is depleted, a new kind of decomposition begins. This proceeds in the absence of oxygen and is termed anaerobic.

[1]Presented at the 60th Annual Meeting of the International Association of Milk, Food, and Environmental Sanitarians, Rochester, New York, August 13-16, 1973.

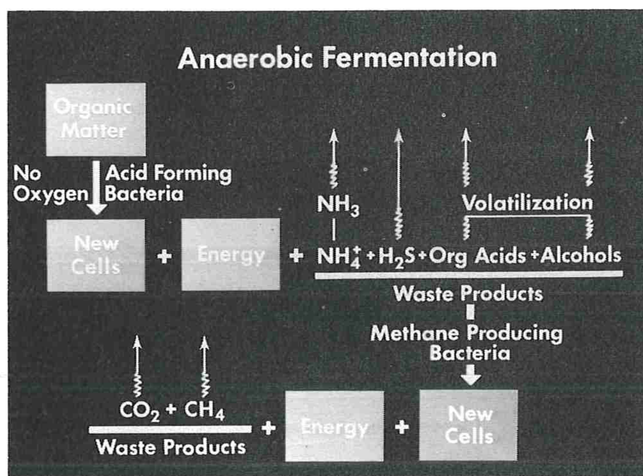

Figure 2. Anaerobic fermentation process.

As shown in Fig. 2, microorganisms responsible for the anaerobic digestion process fall into two classes—the acid-forming bacteria and the methane producers. Acid-forming bacteria convert the complex organic molecules in manure to shorter chain compounds including organic acids and alcohols. The methane forming bacteria utilize these intermediate products to produce methane and carbon dioxide.

The methane-forming bacteria are rather fastidious in their growth requirements and, unless encouraged by careful control of the fermenting mass, they are unlikely to become very active. Therefore, in anaerobically decomposing manure there will be an accumulation of the intermediates produced by the acid-forming bacteria. Accumulations of hydrogen sulfide, ammonia, organic sulfides, amines, methane—all of which are highly malodorous—combine to pollute the environment in and around an animal production unit. They soon reach nuisance levels when manure is agitated before and during disposal on the land.

Problems of Waste Disposal

And therein lies the next major problem of the management of animal wastes—disposal on the land. Surface and subsurface flow of water is the mechanism that transports manure pollutants from the land. Although a watershed may have a high capacity for storing ground water, the infiltration rate may limit the amount which can be absorbed during periods of rain. When such circumstances occur, water drains from the ground surface carrying dissolved chemical elements and suspended solids. Water that is not lost by surface runoff percolates through the soil profile carrying dissolved nutrients to underground aquifers or reappearing downslope as ground water seepage. In either event, groundwater contamination is likely.

Historically, animal wastes have been recycled through the soil environment. However, the change to intensive livestock production facilities has weakened the complementary relationship between crop production and livestock production in which the grain and roughage produced on the land went into livestock production and the manure from the livestock went back on the ground. With increasing concentration of livestock and alternate sources of fertilizers, the practice of distributing the manure on the land has become doubtful from the profit standpoint at many facilities. Furthermore, many production units are situated without sufficient adjacent land on which the manure can be spread. Such producers are being faced with enormous volumes of waste that have low value and a number of social and legal restrictions on their disposal.

Compound these problems with the seasonal changes with which agriculturists have always had to cope, and the situation becomes still worse. Manure cannot be spread on maturing crops, nor can it be spread when the soil is so saturated as to preclude movement on it by heavy equipment. The spreading of manure during periods of rain or when the soil is frozen is to be avoided because of the danger of water pollution when surface run-off occurs.

Livestock producers are interested in waste treatment and disposal methods that have low labor requirements, reduce nuisance conditions, and improve sanitation. But the producers are limited by the lack of viable alternatives and by the premise that treatment and disposal should, if possible, involve no extra cost nor should it increase the cost of the product.

The problem of animal waste management, then, is actually many problems. It is a *technical problem* of finding satisfactory handling, treatment, and disposal methods that can be utilized with these very large volumes of waste. It is the *economic problem* of integrating the technical methods in the animal production scheme and at the same time producing an adequate profit for the producer with a minimum increase to the consumer. It is a *social problem* because of the greater use of our rural resources by urban oriented individuals and by the continuous expansion of suburbia into rural areas. It is an *educational problem* in that the general public requires education regarding the problems of the animal producer and the costs of adequate waste management. And perhaps above all, it is a *communications problem.* Inadequate communication of existing knowledge among various interested groups is a deterrent to effective animal waste management.

The problem appears to be growing faster than are current solutions. The solution to each livestock waste problem will be dictated by the particular circumstances involved. Proximity of urban settlements,

availability of land for ultimate disposal, climate, and profit margin of the livestock being produced are but a few of the variables which combine in specific cases to produce a unique problem requiring a unique solution.

In each case, it must be recognized that objectives change as do circumstances. While providing a solution to the immediate problem of, for example, odor control, it must be realized that in the future it will most probably be necessary to produce an effluent which could be discharged directly to the nearest stream or lake.

THE THEORY OF LICOM WASTE TREATMENT

The De Laval Separator Company has developed a liquid composting (LICOM™) system for deodorizing, pasteurizing, biologically decomposing, and chemically purifying dairy cow waste. Composting usually refers to decomposition of organic matter in a compost heap made from cut grass, leaves, vegetable debris, or manure. Aerobic conditions prevail in the compost pile and, as indicated previously, this is favorable for propogation of aerobic bacteria which convert organic material for building up their body and secreting digestive products.

Great bacterial activity is present in all organic debris whether of animal or vegetable origin. The active bacterial regimes are the psychrotrophic bacteria, active in the temperature range between 42 and 64 F, the mesophilic 64 to 108 F; and the thermophilic, 108 to 149 F. These naturally occuring microorganisms are facultative; that is, they can develop either in the presence or absence of oxygen. The aerobic bacteria will develop and multiply when conditions are favorable.

Optimal environmental conditions are the presence of sufficient digestive material, a constant oxygen supply, and good heat economy.

In a newly started compost pile, the initial temperature is low and only the psychrotrophic bacteria are developed. (See Fig. 3) The psychrotrophic bacteria have a relatively slow metagensis (population growth) rate; and consequently, low activity and little decomposition occur. (See Fig. 4.) From the absorbed organic matter, the psychrotrophic bacteria form bacterial protoplasm and emit a little energy.

After a slow start the temperature in the pile reaches 64 F and the mesophilic bacteria are mobilized. These bacteria develop at a considerably quicker rate and much energy is emitted during the metabolic process.

Then at 108 F, the thermophilic bacteria take over the task of the mesophilic regime. These thermophilic bacteria are highly aggressive compared with the others and decompose protein, carbohydrates, and fats, as well as hemicellulose and lignin. The extent of decomposition of the latter two materials is directly dependent on the composting time.

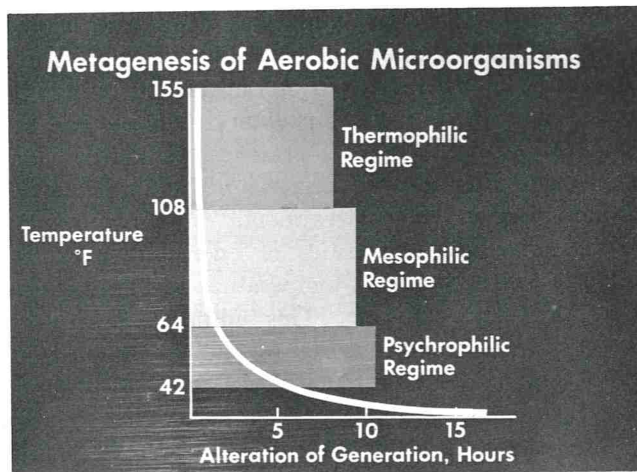

Figure 3. Metagensis of aerobic microorganisms.

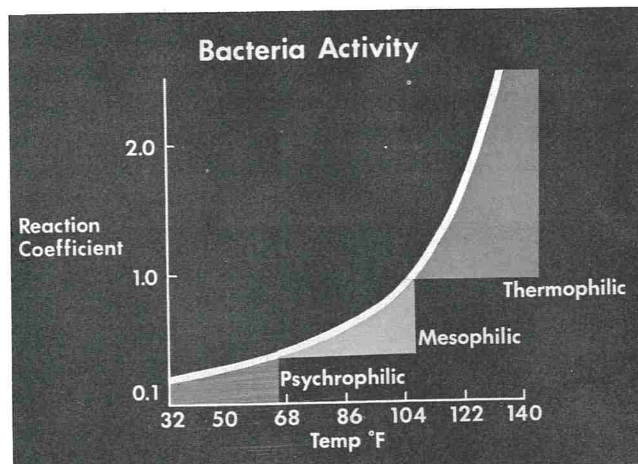

Figure 4. Bacterial activity.

The composting process in a solid medium is easy. The light soil is a good insulator and keeps air (oxygen) evenly distributed. Over an extended time period, composting achieves: high temperature, decomposition to humic substances, oxidation of minerals, disinfection, weed, seed, destruction, deodorization, and neutral pH.

This same composting effect can be achieved in a liquid provided that certain requirements are met. Just as in solids composting, sufficient decomposable organic matter must be present, constant oxygen supply must be provided, and good heat economy must be effected. In addition to satisfying the oxygen transfer requirements, the technique for aeration must provide thorough mixing to induce circulating velocities of sufficient magnitude to maintain the solids in suspension.

Concentrated wastes – such as dairy cow manure –with a high content of oxidizable organic matter and possibly pathogenic bacteria can be safely disposed of without polluting water resources and soil by heating them in the presence of oxygen long enough to degrade the organic matter and destroy the pathogens. Such treatments ordinarily require addition of large quantities of energy to raise the material to the required temperature and to compensate for thermal losses. This addition of external energy can be avoided if the organic matter is digested by aerobic bacteria. The De Laval LICOM System is designed to achieve this effect.

The energy–that is, the carbon content–available in the manure is theoretically sufficient for its spontaneous combustion. The oxidation of carbon to form one mole of carbon-dioxide and hydrogen to form 1 mole of water releases 94,430 calories and 136,600 calories, respectively. This corresponds to 7,870 calories per gram of carbon oxidized and 34, 150 calories per gram of hydrogen oxidized. (A calorie is the quantity of heat necessary to change the temperature of 1 g of water from 58.1 to 59.9 F.) A part of this heat is utilized by the bacteria for the growth of new cell substance. The surplus raises the temperature of the bio-mass up into the thermophilic regime, thus accelerating the biochemical reactions necessary for biological stabilization. In practice, therefore, it is necessary to insulate the reaction tanks to reduce heat losses due to conduction through the walls of the tank, radiation-convection from the surface, and evaporation.

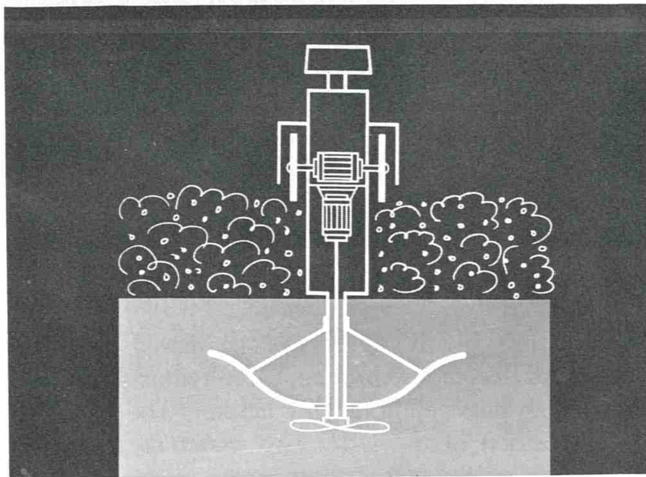

Figure 5. Centri-rator™.

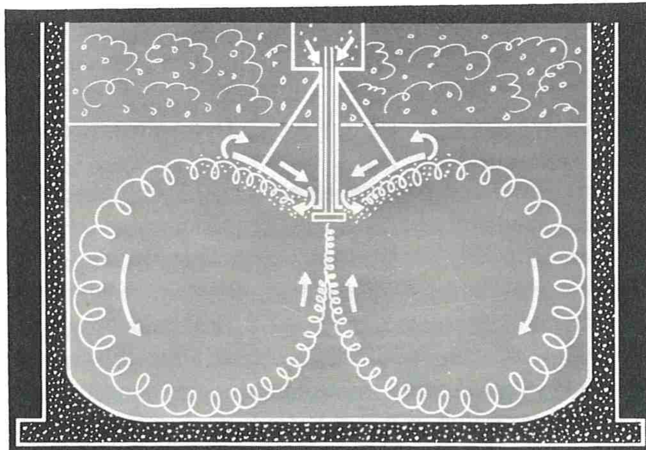

Figure 6. LICOM™ reactor recirculating flow.

Figure 5 depicts the design of the unique aeration device, the Centri-rator, necessary to achieve the LICOM effect. The Centri-rator supplies the air (oxygen) for generation of the aerobic microorganisms. It injects air bubbles of optimum size and mixes air and manure homogeneously by means of a unique impeller designed in accordance with mathematical and physical principles.

Referring to Fig. 6, the Centri-rator installed in an appropriately configured LICOM reaction tank, operates as follows: At the top of the impeller, partial vacuum is created and air is drawn in via the air intake pipe. The bottom portion of the impeller creates an intensive pumping action yielding a homogeneous mixture of the manure slurry. Air and liquid are intimately mixed and ejected radially off the edge of the impeller. To achieve high oxygen utilization efficiency, the air bubbles thus entrained must remain within the bio-mass for a sufficient time in which oxygen diffusion to the reaction site can occur. This necessary residence time is induced by means of a deflector plate, the eddy guide ring, which creates the proper recirculating flow pattern. On aeration of the manure slurry, intensive foam development occurs. Since the solids tend to rise with the foam, the Centri-rators are integrally fitted with foam cutters which control the depth of the foam layer and drive the solids back into the bulk of the bio-mass.

LICOM SYSTEMS

Several modes of LICOM System operation are possible. LICOM I (Fig. 7) consists of a raw manure holding tank, one LICOM reaction tank, a storage tank, and, if necessary, a pump pit. The process is based on a 7-day cycle and once per week manure is pumped from the raw manure holding tank to the LICOM tank, where the composting process takes place. The first batch of a LICOM I process has a longer processing time than the following as the tank itself must be heated. The thermophilic temperature range will be reached in 3-5 days. The maximum temperature will be maintained 1-3 days after which it will gradually and slowly drop off as the available carbonaceous material is exhausted. When the tem-

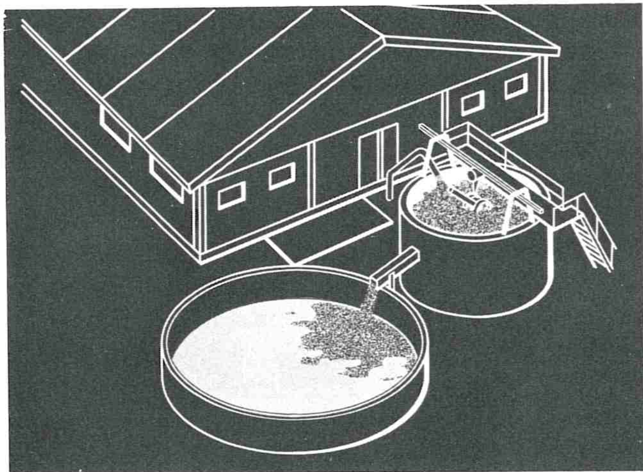

Figure 7. LICOM™ I system.

perature has decreased several degrees, which will normally take 2-3 days, about 7/12's of the LICOM tank volume is discharged into the storage tank. Fresh homogenized raw manure is then pumped to refill the LICOM tank to proper operating level. Provided that the ambient temperature and manure consistency are about the same, processing of the second and succeeding batches will be smoother because the tank is already at the proper operating temperature, only about 60% of "cold" manure is added, and the new batch is immediately innoculated with mesophilic and thermophilic microorganisms.

The effluent from a LICOM I System can be completely stabilized and pasteurized and will remain so over long storage period to await convenient return to the land.

LICOM II (Fig. 8) is a continuously working system and consists of two or, if necessary, more interconnected tanks. In the first tank, the decomposition is initiated and in the second and following tanks undisturbed decomposition takes place. Because of the circulatory flow between tanks, untreated or only briefly treated manure cannot pass directly from the first reaction tank to the final storage tank. A continuous system operates much more efficiently than a batch system. As only small quantities of fresh manure enter the system at frequent intervals, the contents will not be cooled off appreciably by the "cold" fresh manure. The effluent from a LICOM II System is the same as that of a LICOM I System.

LICOM III (Fig. 9) is a completion of LICOM II and can be added to an existing LICOM II System. A third tank, the so called flotation tank, is added for separation of residual undecomposed solids.

The flotation tank is equipped with a Centri-rator without foam cutters. Just as in electroflotation or chemical flotation, the undecomposed solids are lifted by this mechanically generated foam. The higher the foam, the drier the foam at the air-foam interface. The upper foam layers spill into a drying bed to collapse and dehydrate. The residue is an inert mulch resembling tobacco in appearance and texture. It is odorless and pathogen-free and can be used as a soil conditioner or as free-stall bedding.

The liquid phase in the flotation tank discharges continuously to the storage tank via an Imhoff tube. The odor-free, pathogen-free liquid in the storage tank can be decanted from the mineral solids sludge which will precipitate in the tank. Still rich brown in color because of bile substances which are not destroyed during the LICOM treatment, this decanted liquid can be delivered to cropland through pipeline irrigation or clarified by a chemical treatment.

LICOM III Demonstration Plant

To demonstrate the efficacy of the LICOM III Waste Treatment System, The De Laval Separator Company completely automated the waste management at its Research Farm. De Laval owns and operates this modern facility as an adjunct to its

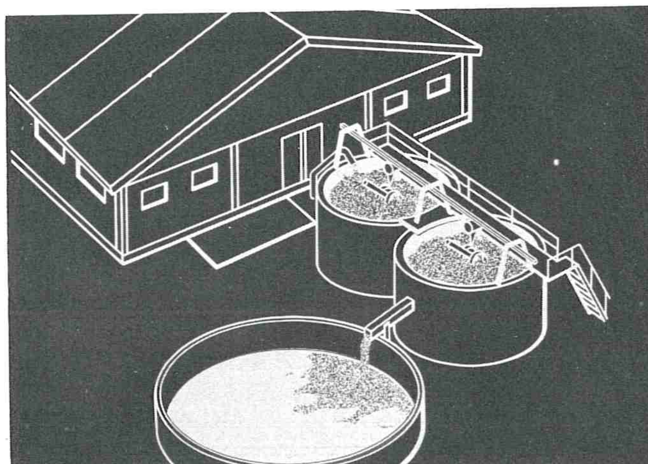

Figure 8. LICOM™ II system.

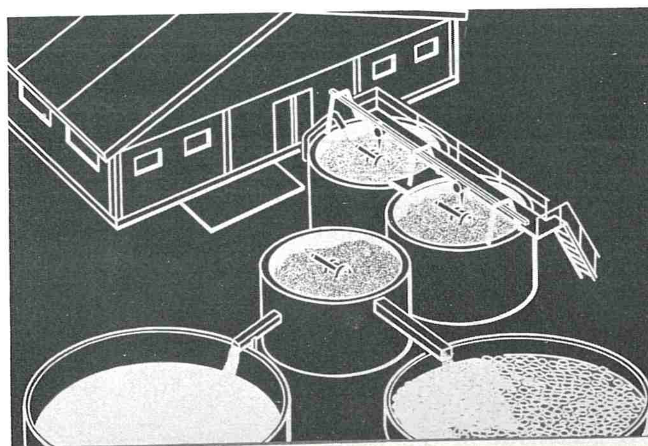

Figure 9. LICOM™ III system.

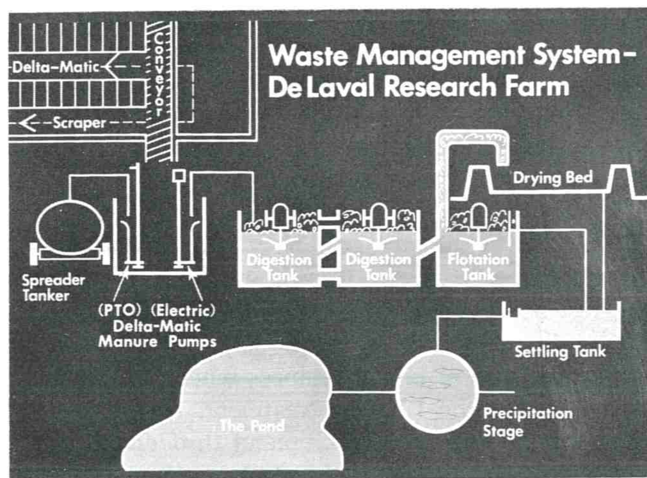

Figure 10. Waste management system - De Laval research farm.

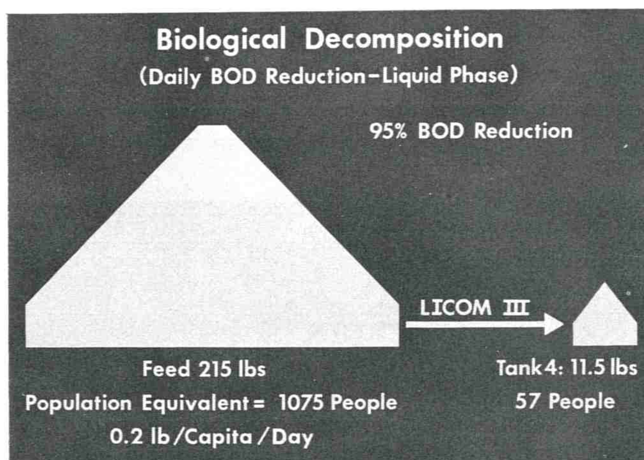

Figure 11. Biological decomposition of dairy cow manure.

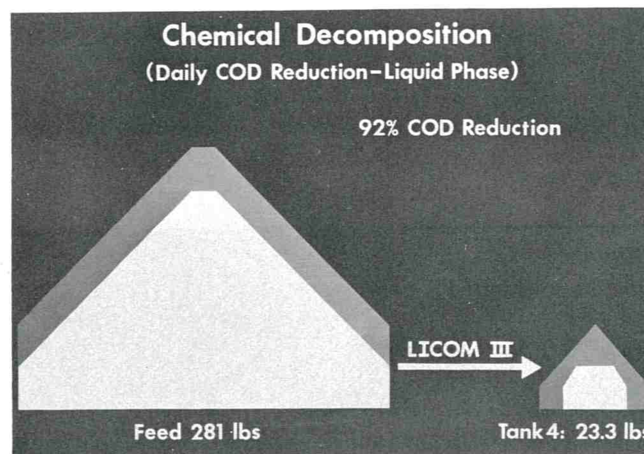

Figure 12. Chemical decomposition of dairy cow manure.

Dairy Division organization for the purpose of testing and evaluating proprietary equipment before general market introductions.

A schematic of the waste management system installed at the De Laval Research Farm is displayed in Fig. 10. The milking herd—about 80 livestock units—is housed in a free-stall barn. Manure is scraped from both the feed alley and the stall alley by a Delta-Matic scraper which operates continuously. Tracking time is 20 min; therefore, fresh feces have a maximum residence time in the alleys of 40 min. The Delta blades discharge at the end of the barn into a cross channel. Manure in the cross-channel is automatically conveyed outside the barn by a Delta-Matic Cross-Channel Conveyor to a manure storage tank.

The conveyor is automatically energized every 2 h for 8 min. This cycle is quite adequate since the total manure load is only about 50 gal per hour based on an 80-cow herd and 2 ft^3 per day per cow.

The manure storage tank, 14 ft in diameter by 12 ft deep provides a maximum of 5-7 days storage capacity for raw manure. Milk house wash waters are also automatically pumped to the manure storage tank—not as a necessary diluent, but rather to handle another dairy farm waste problem. The storage tank is equipped with the Delta-Matic motor-driven manure pump.

From the manure storage tank, the slurry is directed to the LICOM III Waste Treatment System. The concrete reaction tanks, three in number, each 14 ft in diameter and 12 ft deep, were erected below grade for good heat economy. Tanks I and II, the digestion tanks, are interconnected by an underflow, re-inoculation, and overflow conduit. Tank III, the flotation tank, is connected to Tank II by a re-inoculation tube and an overflow channel.

The manure pump is automatically energized every 2 h for 15 sec duration. Approximately 150 gal of slurry are delivered to Tank I.

Although raw manure is added to the system intermittently, foam and treated liquid discharge from the flotation tank almost continuously. The liquid underflow is directed to a 24 ft diameter by 12 ft deep sedimentation tank. Foam discharges to the drying bed—a 60 ft by 25 ft, trickling-type filter. As the foam collapses, it dewaters; and the liquid drainoff flows to the sedimentation tank.

Results of LICOM III Demonstration Plant

The results of the De Laval Research Farm LICOM III Demonstration Plant can be measured against the six basic criteria of an aerobic digestion system: BOD, COD, temperature, pH, nutrients, and bacteria.

Biological oxygen demand (BOD) is a measure of the amount of free oxygen utilized by aerobic organisms in degrading organic matter over a specific time period of 5 days within a specified temperature range. BOD values are measured in milligrams per liter or parts per million. As a measure of degradation, it is often noted as pounds of BOD.

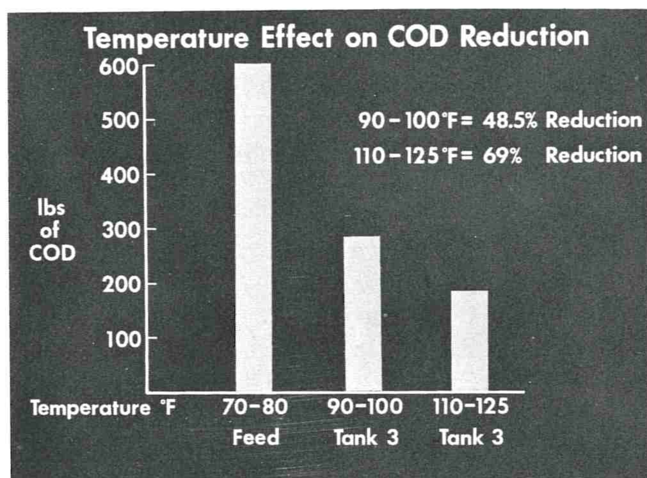

Figure 13. Temperature effect on COD reduction.

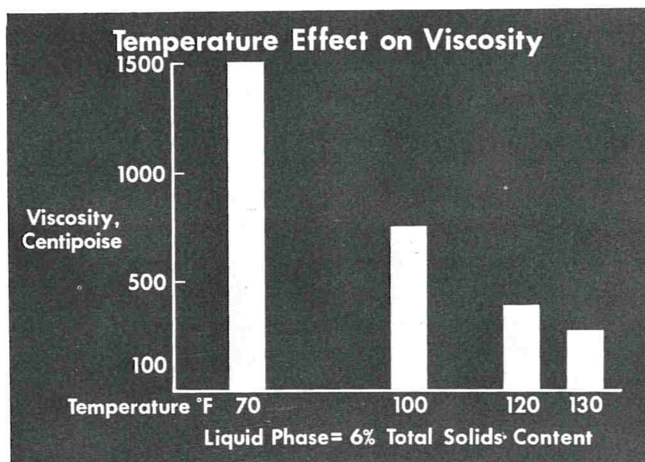

Figure 14. Temperature effect on viscosity.

The daily biological decomposition in the LICOM III System at the De Laval Research Farm is displayed in Fig. 11. The data have been normalized to a 100 cow herd. In moving through the process, 215 lb. of BOD entering the system from the raw manure collection tank is reduced to 11.5 lb. of BOD in Tank 4, the treated manure storage tank. This is equivalent to a 95% reduction in BOD content. In population equivalent, the BOD loading from 1,075 people would be reduced to that of 57 people.

Chemical oxygen demand (COD) is an indirect measure of the biochemical load and is measured in milligrams per liter and also in pounds. The COD of dairy cow waste is always greater than the BOD value due to lignins and hemi-celluloses (woody, fibrous material) in roughage feeds.

This fibrous matter is only slightly digestible by cattle and degrades very slowly by microorganisms. As illustrated in Fig. 12, the daily reduction in COD achieved by the LICOM III process for a 100-cow erd is from 281 lb. in the raw manure feed to 23 lb. in Tank 4; a 92% reduction.

The LICOM System at the De Laval Research Farm operates in the thermophilic bacterial range—typically, at temperatures between 110 and 125 F. This is in contrast to operating in the mesophilic range say 80 to 100 F—which is typical for aerobic systems such as the activated sludge process frequently employed by municipal waste treatment plants.

As the temperature increases, the amount of COD reduction increases. This effect is demonstrated in Fig. 13, by the increased reduction in organic matter occurring at temperatures ranging from 110 to 125 F as the product progresses through the system from the feed tank to Tank 3, the flotation tank.

Another benefit of operating at these higher temperatures results from the decrease in viscosity of the fecal mass as the temperature increases. For example, Fig. 14 notes the decrease in viscosity from 1,500 centipoise at 70 F to only 300 centipoise at 130 F. With this decrease in viscosity, there is improved oxygen transfer and, therefore, increased growth of the bacterial culture.

To be acceptable to the land or water course in the area where the Research Farm is located, the liquid

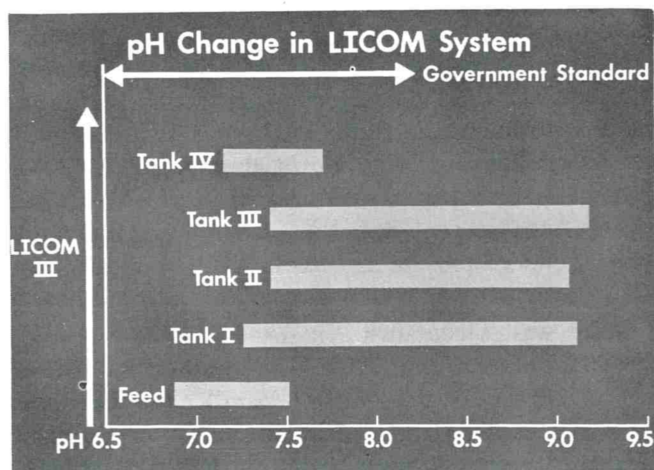

Figure 15. pH Change in LICOM™ system.

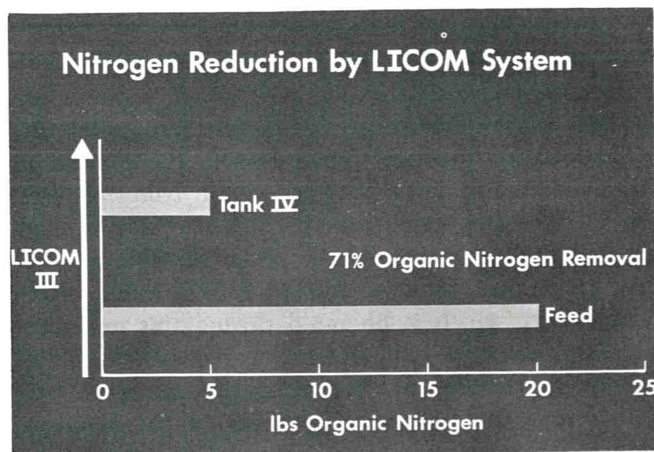

Figure 16. Nitrogen reduction by LICOM™ system.

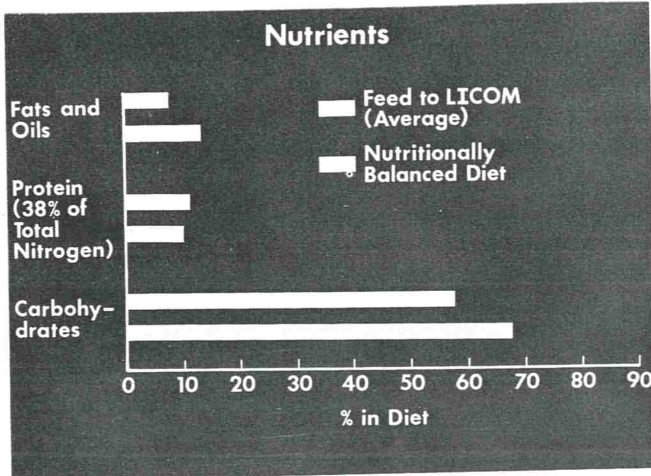

Figure 17. Dairy cow waste nutrients.

effluent must lie in a pH range of 6.5 to 8.0. Fig. 15 indicates that in the LICOM System the pH of the raw manure ranges from 7.0 to 7.5 and increases during treatment in the reaction tanks to a pH range of 8.5 to 9.2. The liquid collected in the treated manure storage tank has a pH of 7.2 to 7.7. This pH change is a result of aerobic biological conversion of organic matter to carbon dioxide CO_2) ammonia NH_3), nitrites (NO_2^-) and nitrates (NO_2^-). The pH is high when the ammonia concentration is high and decreases as ammonia is liberated and nitrification occurs. As shown in Fig. 16, in the typical LICOM System operating at the De Laval Farm, 20 lb. of organic nitrogen entering the system from the feed tank is reduced to 6 lb. in Tank 4. This is better than 70% organic nitrogen removal.

Dairy wastes contain on a dry basis: 7% fats and oils, 11% proteins, and 57% carbohydrates. As shown in Fig. 17, these values closely parallel the standard for a nutritionally balanced human diet. In addition to these three essential basic nutrients, there are many trace nutrient mineral ions present in the raw manure to enhance bacterial growth. Such important minerals include calcium, magnesium, sodium, potassium, phosphorus, and iron.

Bacteria present naturally in the raw manure fed to the LICOM System are the prime movers in the biodegradation process. Their role in the process is displayed in Fig. 18. Feeding on the balanced diet provided by the manure in the presence of dissolved oxygen resulting from aeration, their metabolic products are carbon dioxide, ammonia, and water. The heat energy which is liberated during this oxidation process raises the temperature of the system. The residue of the liquid composting process is an oxidized, stabilized, odor-free product in the flotation tank.

The biodegradation achieved at the De Laval Research Farm by the LICOM process resulted in greatly reduced volume handling requirements. As shown in Fig. 19, the raw manure collection tank received 1,200 gal per day of fecal feed and 670 gal per day of milkhouse waste from a typical herd of 100 cows. The LICOM III System reduced this 1,870 gal to 830 gal per day of treated liquid ready for land irrigation. Only 80 gal per day of fibrous matter passed to the foam bed for air-drying.

As a result of this LICOM III System, the volume of dairy cow waste to be hauled to the fields for disposal has been greatly reduced at our farm. (See Fig. 20) The farmer with a typical 100-cow herd must haul almost 500,000 gal per year of smelly manure to the field for spreading. Using a typical 660 gal manure spreader, this amounts to 720 loads per year or 2 loads per day for 360 days per year. With a LICOM III System, this same farmer would haul only 39 loads per year of dry, odorless, soil conditioner.

If the treated liquid from LICOM III is not to be used for land irrigation and ultimate disposal is to a water course with chemical treatment, some standards

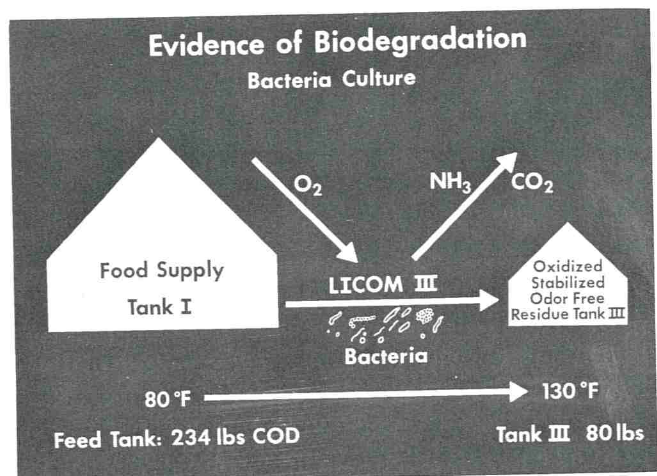

Figure 18. LICOM™ III system - biodegradation.

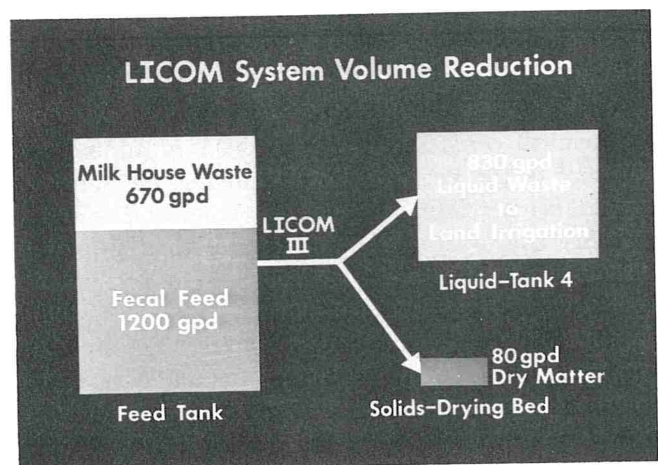

Figure 19. LICOM™ system volume reduction.

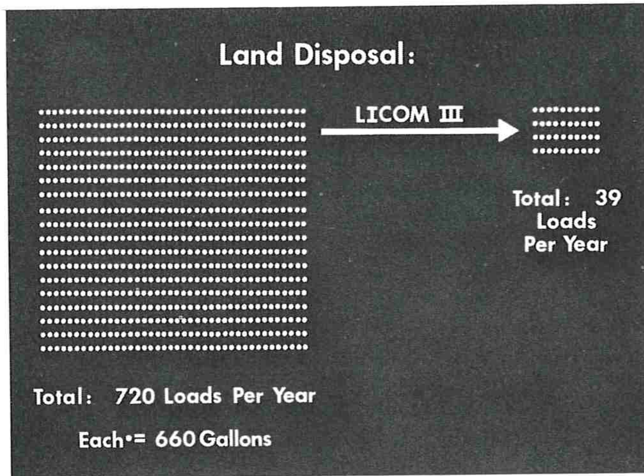

Figure 20. Influence of LICOM™ III system on land disposal of solids.

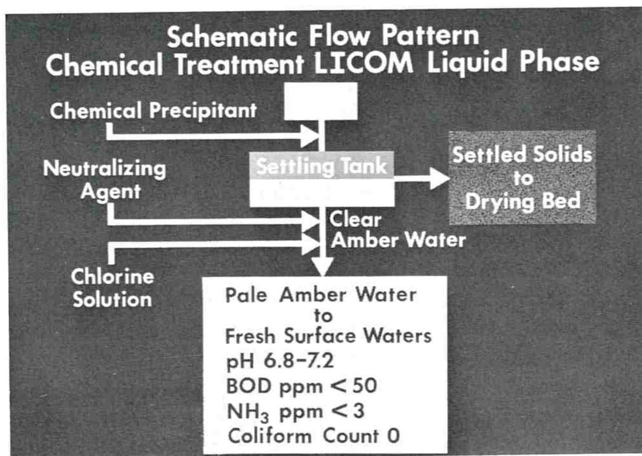

Figure 21. Schematic flow pattern for chemical treatment of LICOM™ liquid phase.

can be met. Figure 21 displays a schematic flow sheet of such a chemical treatment process. The rich-brown liquid in the treated manure storage tank is treated with a chemical reagent and allowed to settle. The precipitated solids, principally soluble lignins and tannins, pass to the foam drying bed for return to the soil as a nutrient. The clear amber liquid phase is neutralized and chlorine treated. The result obtained at our Research Farm was a light amber colored water which analyzed: pH between 6.8 and 7.2, < 50 ppm BOD, < 3 ppm ammonia, and zero coliform count.

Following chemical treatment at the De Laval Research Farm, the BOD dropped from the range of 1,000 ppm in Tank 4 to 25 to 35 ppm in the final treated effluent. Federal and some State regulations require 50 ppm or less of BOD.

Ammonia was liberated in the chemical treatment process with the final effluent having only 2 to 3 ppm at pH 7. New York State regulates ammonia in waste streams to water courses at a value no greater than 2 ppm at pH 8. For comparative purposes, typical data on creeks sampled in New York State range from 0.9 to 1.2 ppm.

The bacteria count was dramatically reduced by chlorine treatment. New York State requires chlorine treatment of all waste waters passing to fresh surface waters. The final effluent from a LICOM III System treating the waste from a 100 cow herd requires a 300 to 400 ppm chlorine dosage to destroy all bacteria. State regulations pertain to zero coliform count only.

The results of operation of a LICOM III System at the De Laval Research Farm have demonstrated that liquid composting of dairy cow waste can provide a pollution-free liquid effluent which may under some circumstances be directed to a nearby stream or lake, and an inert, dry residue which can be used as a soil conditioner or as bedding in the barn. This system will aid the dairy farmer in his efforts to live in a community with increasingly more rigid ecological requirements.

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COMPARISON OF MEDIA FOR ENUMERATING FUNGI IN PRECOOKED FROZEN CONVENIENCE FOODS

W. C. Ladiges, J. F. Foster, and J. J. Jorgensen III
Food Hygiene Division, Letterman Army Institute of Research
Fitzsimons Army Medical Center, Denver, Colorado 80240

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Abstract

Sabouraud dextrose agar (SDA) was compared to potato dextrose agar (PDA) acidified to pH 3.5 and to SDA containing 10 mg kanamycin/100 ml and chloramphenical (SDA+) in enumerating fungi from precooked frozen convenience foods. SDA yielded significantly higher fungal counts in most foods even though it allowed a high degree of bacterial growth. No significant difference was found between the fungal counts of PDA and SDA+.

Until recently the pathogenic significance of fungi in foods has received little attention. However, with the discovery of mycotoxins and the ever-changing technology of food processing and handling, fungal contamination of foodstuffs has been of increasing concern. As a result new techniques have been developed for selective enumeration of yeasts and molds in food products.

The classic studies of White and Hood in 1931 *(6)* suggested that pH 3.5 be used in media to selectively inhibit bacteria when enumerating yeasts and molds. Later studies *(3, 4, 5)* indicated this low pH was inhibitory to a portion of the fungal population in foods while still allowing bacterial growth.

Koburger *(3)* studied the inhibitory effects of potato dextrose agar (PDA) acidified to pH 3.5 ± 0.1 on enumeration of fungi in foods. He compared his results to neutral (pH 7.0) PDA with chloramphenicol and chlorotetracycline, concluding certain antibiotics were superior to acidification for controlling bacterial growth. Yeast and mold counts were higher using PDA containing antibiotics than from the same medium when acidified.

The purpose of the present study was to compare the use of Sabouraud dextrose agar (SDA), with and without the addition of antibiotics, to PDA acidified to 3.5 ± 0.1 for enumerating fungi from precooked frozen convenience foods.

Materials and Methods

Media

Three types of media were tested: potato dextrose agar (BBL No. 11550), acidified to pH 3.5 ± 0.1 using 10% tartaric acid, Sabouraud dextrose agar (BBL No. 11584), and Sabouraud dextrose agar with chloramphenicol sodium succinate (Park Davis and Co.) and kanamycin (Bristol) added at a concentration of 10 mg per 100 ml of media (SDA+).

Procedure

All foods were tested immediately upon receipt by placing 25 g of the test sample into 225 ml of sterile phosphate buffered distilled water *(1)* and blending in a sterile stainless steel Waring blender for 3 min at high speed. One milliliter of the slurry was placed into 100-mm petri dishes (Falcon Plastics) and 13 to 16 ml of one of the media was added. Plating was done in duplicate. All plates were incubated at room temperature (23 C) for 5 days.

Colonies were counted with a Quebec colony counter (American Optical). Any colonies with observable mycelial elements were considered molds. Bacteria were identified by Gram staining.

Experimental Design

Experiment 1. All food preparations except beef stew, chicken pot pie, and chicken chow mein (Table 1), which were obtained at a local retail market, were prepared specifically for the military food chain.* A total of seven single- and multiple-component foods representing 19 individual food types were tested. Five different samples of each food type were tested over a period of 3 months.

Experiment 2. All food samples were obtained at a local retail market. A total of nine single- and multiple-component foods were tested representing 17 individual food types (Table 2). Ten different samples of each food type were tested over a 4-month period.

Data analysis

Analyses of variance were calculated for the mold, yeast, fungal (yeast plus mold), and bacterial counts (3 media by 19 food types for Experiment 1, and 3 media by 17 food types for Experiment 2). Post-hoc Newman-Keuls *(7)* multiple mean comparisons were done, where appropriate, to assess the problem of differences among specific variable means. The 0.05 level of significance was used with all statistical tests.

Results

Results of the analysis of variance (Table 3) showed that a significant effect for media was obtained in Experiment 1 using fungal, bacterial, and yeast counts. In Experiment 2, fungal, mold, bacterial, and yeast counts yielded a significant media effect. Although food effects were observed, further comparisons of the effect of different types of food upon the efficiency of the three media was not studied due to

*Obtained through Col. G. F. Fisher, VC, Chief, Office of Quality Assurance, Defense Personnel Support Center, Subsistence Regional Headquarters, Chicago, Il.

TABLE 1. MEAN FUNGAL COUNTS PER GRAM OF FOOD IN PRECOOKED FROZEN FOODS[1] (EXP. 1)

Food	Sabouraud dextrose agar	Sabouraud dextrose agar with antibiotics[2]	Potato dextrose agar[3]
Beef stew	95	81	67
Chicken pot pie	887	138	76
Chicken chow mein	1582	135	16
rice	77	25	14
egg roll	35	8	2
shrimp	7	0	0
apple roll	13	5	0
Beef burgundy	30	2	3
noodles	113	2	3
carrots	40	5	3
Sliced beef	141	2	3
scalloped potatoes	96	19	9
carrots	115	3	5
Top butt steak	8	3	0
rice	33	6	5
green beans	666	640	627
Waffles	19	1	5
Canadian bacon	7	1	1
peaches	2	1	1

[1]Reported values based upon five different samples of a specific food.
[2]Chloramphenicol and kanamycin.
[3]Acidified to pH 3.5 with tartaric acid.

TABLE 2. MEAN FUNGAL COUNTS PER GRAM OF FOOD IN PRECOOKED FROZEN FOODS[1] (EXP. 2)

Food	Sabouraud dextrose agar	Sabouraud dextrose agar with antibiotics[2]	Potato dextrose agar[3]
Spaghetti and meatballs	406	17	2
peas	141	3	1
apples	20	16	1
Veal parmasan	388	11	1
Turkey pot pie	835	86	46
Beef enchiladas	43	0	5
Cheese enchiladas	1246	1203	78
Turkey dinner	822	6	11
potatoes	88	1	2
peas	686	8	2
Shrimp dinner	1011	408	26
potatoes (French fried)	306	2	4
peas	84	8	7
Enchilada dinner	411	7	3
beans	62	9	1
rice	14	4	1
Sliced turkey with gravy	263	17	2

[1]Reported values based upon ten different samples of a specific food.
[2]Chloramphenicol and kanamycin.
[3]Acidified to pH 3.5 with tartaric acid.

inherent variability factors such as pH, moisture, consistency, etc., associated with the foods.

Post-hoc Newman-Keuls comparisons among the media means indicated SDA yielded significantly higher fungal and yeast counts (Fig. 1 and 2) than PDA or SDA+. No significant media effects were observed between PDA and SDA using fungal, yeast, and mold counts (Fig. 1) in Experiment 1 nor in Experiment 2 using fungal and yeast counts (Fig. 2). Significantly higher mold counts were obtained in Experiment 2 using SDA+ (Fig. 2).

Although SDA+ and PDA allowed some bacterial growth, Newman-Keuls analyses showed there was no significant difference in inhibitory effect between the two media. It can be seen from data in Fig. 1 and 2 that the bacterial population did not affect the efficiency of SDA over SDA+ or PDA in enumerating fungi from the precooked, frozen foods tested.

DISCUSSION

The inferior performance of SDA+ and PDA when compared with SDA in isolating and enumerating fungi from precooked frozen foods indicates that these two media are not selective enough to prevent all bacterial growth and still allow fungi to grow uninhibited. Although acidified PDA allowed little growth of bacteria, it appeared to be much too acidic for adequate fungal growth. SDA+ did not perform better suggesting that kanamycin and/or chloramphenicol, at the concentration used, do not provide the selective inhibition necessary to prevent bacterial

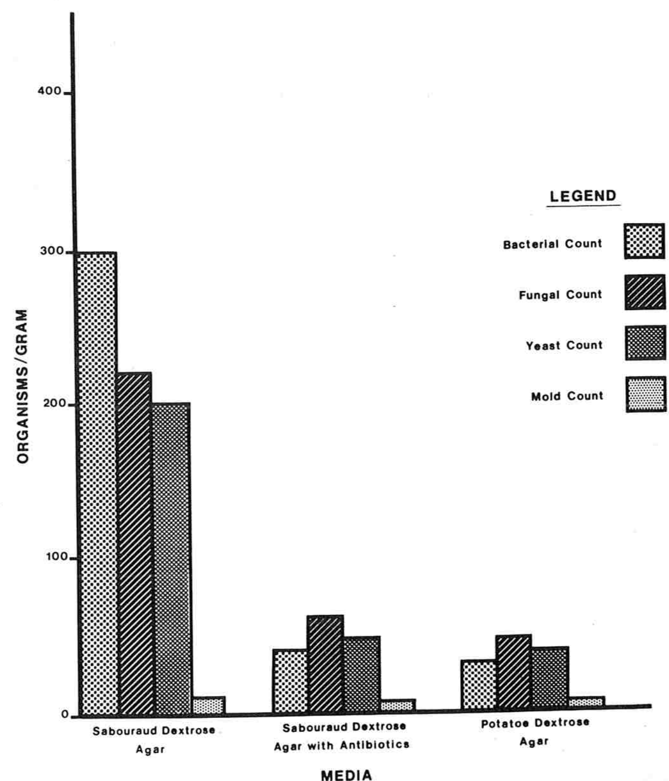

Figure 1. A comparison of mean yeast, mold, total fungal, and bacterial counts obtained with Sabouraud dextrose agar, Sabouraud dextrose agar containing kanamycin and chloramphenicol (10mg/100ml), and potato dextrose agar acidified to pH 3.5; five samples each.

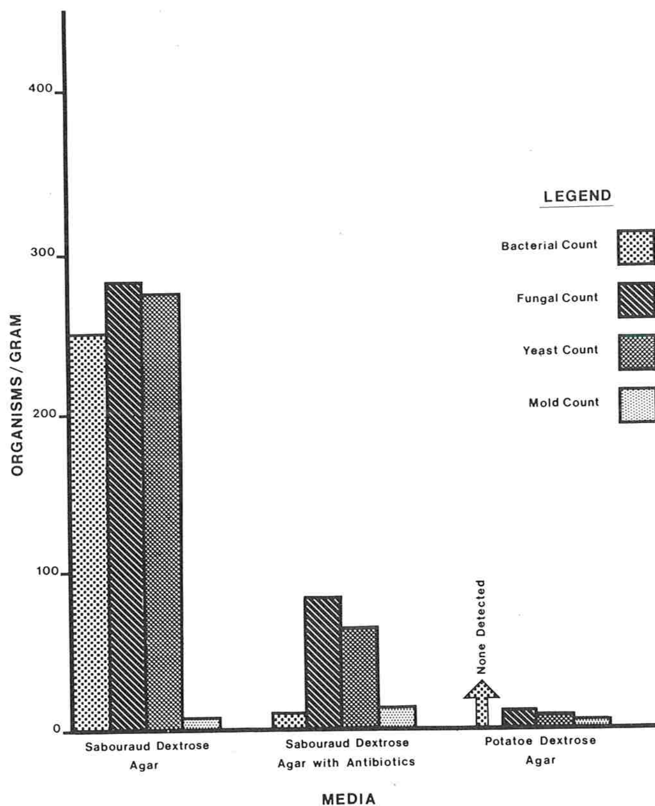

Figure 2. A comparison of mean yeast, mold ,total fungal, and bacterial counts obtained with Sabouraud dextrose agar, Sabouraud dextrose agar with kanamycin and chloramphenicol (10mg/100ml), and potato dextrose agar acidified to pH 3.5; 10 samples each.

TABLE 3. F TABLE SHOWING RESULTS OF ANALYSIS OF VARIANCE FOR ALL MEDIA

	F Value
Experiment 1	
Total fungi	5.6
Yeast	5.3
Mold	1.3[1]
Bacteria	13.1
Experiment 2	
Total fungi	24.4
Yeast	304
Mold	5.1
Bacteria	43.2

[1]No media effect was shown (0.05 level of significance); all other values indicated a significant media effect.

growth while still permitting fungal growth. The fact that Experiment 1 showed no media effect in relation to mold counts suggests that with certain foods any of the media used may be adequate for obtaining mold counts. The significantly higher mold counts obtained with SDA+ in Experiment 2 suggests that SDA+ may be better medium to enumerate molds in certain foods.

We had assumed that if bacteria were allowed to grow, fungal growth would be suppressed. Our data clearly indicate this did not occur when enumerating fungi from the precooked frozen foods tested. SDA+ and PDA appeared to be equally inhibitory to all microflora while SDA was uniformly the least inhibitory medium. The stressed population of organisms within these foods may have been a cause of increased sensitivity to the inhibitory agents in SDA+ and PDA. Perhaps testing of other types of foods with a more vigorous microflora would allow these media to perform better.

The time and labor necessary to do the Gram staining procedure to distinguish yeasts from bacteria points up the need for an inhibitory medium more selective than SDA. Future work with other bacterial inhibitory agents appears justified since a high-acid medium, such as acidified PDA, appears to be quite inhibitory to fungal as well as bacterial populations.

ACKNOWLEDGEMENTS

The assistance of Dr. R. T. Sterner is greatly appreciated in programming the statistical analysis and of Dr. J. L. Fowler for technical advice and support.

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IN-PLANT USE OF AN ATOMIC ABSORPTION SPECTROPHOTOMETER TO MONITOR CALCIUM IN CIP CLEANING SOLUTIONS

M. E. ANDERSON[1], D. B. BROOKER[2], J. R. FISCHER[1],
E. L. RUIZ[3], AND R. T. MARSHALL[4]

(Received for publication February 4, 1974)

ABSTRACT

An atomic absorption spectrophotometer was installed in an automated CIP dairy plant cleaning system to detect the amount of calcium in the cleaning solution as it flowed from milk-processing equipment and storage tanks during each phase of the cleaning cycle. A major portion of the milk residue was removed from the equipment during the first half of the rinse phase. Most of the residue remaining after rinsing was removed during the first 2 min of the alkaline cleaning phase.

In a previous study *(1)* in which an atomic absorption spectrophotometer was used for measurements, it was shown that major ingredients of commercial detergents do not, for practical purposes, interfere with measurements of calcium in cleaning solutions. The feasibility of quantitating the amount of milk (based on calcium detected) flowing in a simulated CIP cleaning system has been shown *(2)*. The literature *(3, 4, 5)* indicated that calcium is present in most residue on milk contact surfaces. The objective of this research was to determine whether an atomic absorption spectrophotometer performs satisfactorily for on-line monitoring of milk in cleaning solutions during different phases of the cleaning cycle.

MATERIALS AND METHODS

Materials

A standard stock solution with 2000 mg calcium/1 was prepared by dissolving 4.9945 g of dried $CaCO_3$ (analytical grade) in 10 ml of 6 N HCl and diluting with distilled water to one liter. Standard solutions for quantitating amounts of calcium in cleaning solutions were prepared by adding stock solution to water used in prerinse and postrinse phases and to solutions of the alkaline (1.0% Klenzade HC-41; Economics Laboratory, Inc., St. Paul, Minnesota) and acid (0.1% Klenzade AC-3) detergents.

An atomic absorption spectrophotometer (Varian Techtron, Model 1000, equipped with a calcium hollow cathode lamp) with a dual-channel strip chart recorder was installed in the dairy plant at the University of Missouri-Columbia to measure the amount of calcium in the cleaning solution as it flowed from the processing equipment. Acetylene was the fuel gas, and nitrous-oxide was the support gas. The lamp current was 6 ma. The dimensions of the nitrous-oxide burner were 6.324 cm × 0.457 mm. The slit width was 0.5 nm.

To remove a small portion of the cleaning solution, a 1/8-inch tube, directed upstream, was installed in the return line. A stainless steel needle valve was attached to the tube to regulate the flow of solution. Solution flowed from the needle valve to a stainless steel channel assembly (Fig. 1). Air bubbles were removed as the thin film of solution flowed between the silicon rubber dikes. The solution was drawn through the aspirator tube into the spectrophotometer.

Methods

Alkaline or acid detergent was added to water in the solution supply tanks to give desired concentrations, and solutions were heated to the desired temperature.

Before each phase of the tank-cleaning cycle or processing-line cleaning cycle was started, standard solutions of calcium that were appropriate for that phase were aspirated into the spectrophotometer, and instrument responses were recorded. These peak responses were used to prepare a stand-

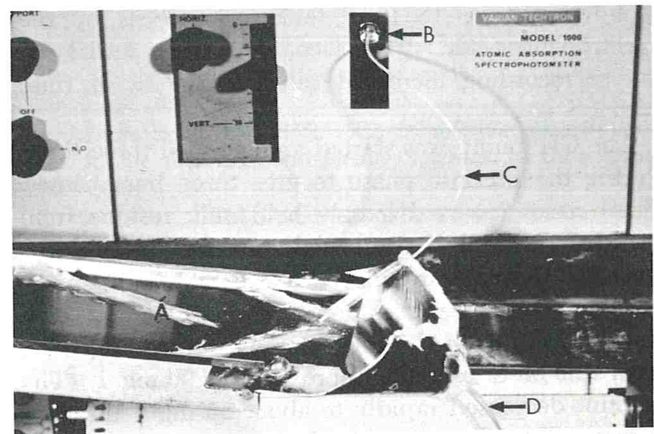

Figure 1. Stainless steel channel assembly with plastic tubing used to aspirate solution during in-plant studies. A—stainless steel channel with silicon rubber dikes, B—nebulizer, C—aspirator tube, and D—solution drain tube.

[1]Agricultural Engineer, U. S. Department of Agriculture, Agricultural Research Service, North Central Region, Columbia, Missouri 65201. Research done in cooperation with University of Missouri, Columbia, Missouri.

[2]Professor, Department of Agricultural Engineering, University of Missouri, Columbia, Missouri 65201.

[3]Assistant Professor, PINFST Philippine Women's University, Manila.

[4]Professor, Department of Food Science and Nutrition, University of Missouri, Columbia, Missouri 65201.

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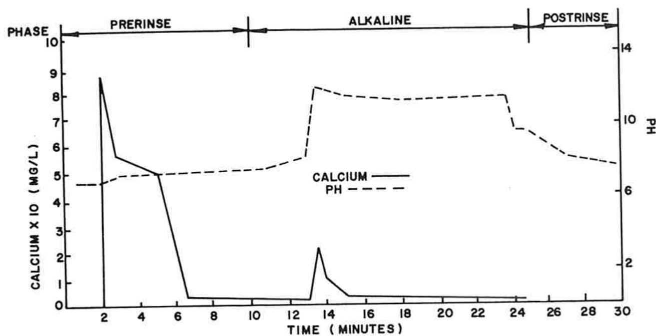

Figure 2. A plot of the calcium content and pH of the cleaning solution versus time as the solution flowed from a 2,500-gal milk storage tank during the cleaning cycle.

ard curve to quantitate the amount of calcium for that phase. Next, the automated CIP system was started, and solution was pumped to the equipment to be cleaned. A CIP return pump forced the solution back to the CIP tank where it was diverted to the drain.

A small portion of the solution was diverted to the spectrophotometer, via the channel assembly, as it flowed back to the CIP tank. The cleaning solution flowed as a thin layer in the channel to remove small, entrained air bubbles. At the end of the respective phases, standard solutions were again aspirated into the instrument for 10 sec to verify standard readings taken at the beginning of the test.

Samples of the solutions were collected intermittently during each phase. These samples were analyzed by aspirating them into the spectrophotometer at the end of the phase to verify readings obtained from the continuous analyses.

RESULTS AND DISCUSSION

Tank cleaning cycle

Figure 2 is a plot showing calcium content of the cleaning solution being removed from a 2,500-gal, raw milk storage tank during the three phases of the cleaning cycle (prerinse, alkaline, and postrinse). After the cleaning cycle was started, approximately 2 min were required for the CIP return pump to remove the rinse solution from the milk storage tank and return it to the CIP tank for analysis by the spectrophotometer. This accounts for the initial delay in recording increased calcium content of rinse water.

The CIP pump was started and stopped three times during the prerinse phase to give three burst rinses. Burst rinses removed loosely held milk residue from walls of the tank and allowed time for the previous rinse solutions to be removed. The first burst sprayed water into the tank for less than 1 min. The first solution from the milk storage tank contained a high amount of calcium, approximately 90 mg/1. The amount decreased rapidly to about 55 mg/1 in about 1 min. Therefore, the initial rinse water removed a large portion of the milk residue from the walls and floor of the tank.

Return of the second burst began at about 3 min (Fig. 2) and continued for about 2 min. Mixing of solutions from the first two burst rinses in the bottom of the tank produced a gradual dilution of calcium to 50 mg/1.

The third burst rinse lasted for about 2 min with calcium concentration decreasing to less than 4 mg/1. Most residual milk was rinsed from the walls and floor during the rinse cycle. The phase should have been stopped and the alkaline phase started after about 8 min; the next 5 min accomplished little further cleaning.

A plot of the pH (Fig. 2) of the returning solution indicated that the milk did not materially affect the pH of the solution. There was an unexplained increase in the pH of the rinse solution with time.

During the alkaline phase of the cleaning cycle, the CIP pump operated continuously, and the returning solution was diverted to the drain. The initial returning alkaline solution contained about 22 mg calcium/1. This amount decreased to about 12 mg/1 after 1 min and more slowly to about 5 mg/1 after approximately 2 min. During the remaining 10 min of the alkaline phase, a small decrease in calcium concentration was noted. During the postrinse phase, no calcium was detected in the rinse water.

Processing line cleaning cycle

Cleaning solutions were initially pumped from the solution makeup tank (CIP tank) into a 110-gal batching tank, through the processing line (approxi-

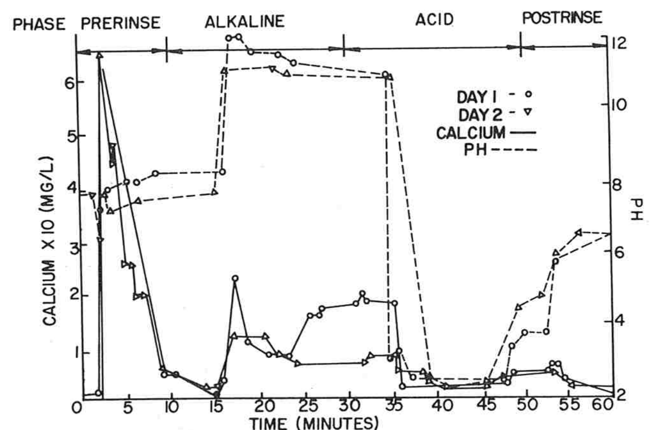

Figure 3. Plots of the calcium content and pH of the cleaning solution versus time as the solution flowed from the milk processing line on two different days. Note that time when the cycle started, indicated by the horizontal line with arrows at top of figure, does not coincide with time when the spectrophotometer detected changes in calcium concentration. This occurred because it took approximately 4 min for solutions to pass from the CIP storage tank through the system to the spectrophotometer. Initial lag time was only 2 min because part of the rinse solution originated in the constant level tank.

mate volume 40 gal), and back to respective compartments of the CIP tank for recirculation.

Rinse water initially picked up more than 60 mg calcium/1 in flowing through the processing line (Fig. 3). Concentration decreased rapidly during the first part of the phase (4-9 min), and then more slowly at the end (9-14 min), to approximately 2 mg/1. The irregular tracing observed on day two may have been caused by the recirculation (diversion to the constant level tank) of approximately 10% of the solution from the system each time the flow-diversion valve pulsed to clean the leak detector line. This was programmed into the system. However, pulsing of the flow-diversion valve and control of the CIP cycle were independent. This allowed pulsing of the flow-diversion valve to occur at different times in the cleaning cycle and account for minor differences in tracings (Fig. 3) on the two days.

Next, alkaline solution was pumped through the processing line and recirculated (14-37 min). A high peak was noted on day one at 18 min. This peak was lower and wider on day two. The difference was attributed to pulsing of the flow-diversion valve with consequent recirculation of solution containing the high concentration of calcium.

The increase in calcium content after 24 min (Fig. 3) for day one was caused by the recirculation of the alkaline solution, with its higher calcium content, through the processing line. Also, additional calcium may have been removed.

The start of the acid phase, as indicated by the change in pH, occurred 34 min after the start of the cycle. The leading boundary of the acid solution was mixed with alkaline solution remaining in the line. This mixing is indicated by the slope of the pH plot that ends at 35-38 min. The quantity of calcium in the acid water was about 2 mg/1. This indicated that very little calcium remained in the processing line after the alkaline phase. If small quantities of calcium and traces of other constituents of milk are not removed, they will tend to build up and may form a visible residue after 3 or 4 days. Previously, the foreman of the University plant had noted that when the processing line was not routinely washed with acid, a residual film built up after several days.

A small amount of calcium (4 mg/1) was dissolved in the acid solution remaining in the system after the acid phase was stopped. Some of this was removed during the final rinse phase as indicated by the plot of the last 15 min of the cycle. Final rinse water flowing through the system picked up about 1 mg calcium/1.

During the collection of these data, it was observed that fat globules in the returning rinse solution adhered to the inside of the aspirator tube of the spectrophotometer, causing a decrease in the amount of solution aspirated into the nebulizer. To determine whether the aspirator tube or nebulizer was restricted, a solution of known concentration was aspirated, and the instrument response was observed. If the response was not equivalent to earlier readings of the same solution, the operation was interrupted, and the aspirator tube and nebulizer were cleaned.

CONCLUSION

This research demonstrated that an atomic absorption spectrophotometer can be successfully used, with calcium as an indicator, for on-line monitoring of milk in a cleaning solution during the different phases of a cleaning cycle used by a milk processing plant.

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EXPERIMENTAL PRODUCTION OF AFLATOXIN IN CITRUS JUICE AND PEEL[1]

G. G. Alderman and E. H. Marth

Department of Food Science and the Food Research Institute
University of Wisconsin-Madison, Madison, Wisconsin 53706

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Abstract

Grapefruit juice and grapefruit peel each supported growth of and aflatoxin formation by *Aspergillus parasiticus* and *Aspergillus flavus*. Grapefruit peel was a better substrate than grapefruit juice for toxin production; amounts of aflatoxin B_1 and G_1 were 5-10 times higher in the peel when the same mold strain grew at 28 C in both substrates for up to 62 days. In juice, amounts of aflatoxins B_1 and G_1 produced by *A. flavus* markedly decreased after 18 days and then became stabilized, whereas amounts of aflatoxin G_1 formed by *A. parasiticus* increased to 34 days then declined and stabilized. When the molds grew on grapefruit peel, amounts of aflatoxins B_1 and G_1 produced increased for 38 days (*A. flavus*) and 26 days (*A. flavus* and *A. parasiticus*), and then declined and became stabilized. Amounts of aflatoxin B_1 (*A. parasiticus*) increased rapidly early during incubation and then remained relatively constant through an extended holding time. Concentrations of aflatoxins B_2 and G_2 formed by both molds in each substrate did not significantly change during extended incubation. *A. parasiticus* grew in grapefruit juice for 14 days at 28 C after which the juice was separated into three fractions. The solids fraction contained 72.8%, mold 16.9%, and filtrate 10.3% of the total amount of aflatoxin produced. Similar results were obtained with lemon and orange juice. Frozen storage (−18 C) of grapefruit juice samples containing aflatoxin did not significantly affect the amount of aflatoxin recovered until after 18 weeks.

Production of aflatoxin in or on foods has been studied by several investigators. Lie and Marth *(20, 21)* reported that aflatoxin was produced on Cheddar cheese and a casein substrate that were inoculated with *Aspergillus flavus* and *Aspergillus parasiticus*. Frank *(11, 12)* recovered aflatoxins from numerous foods that were experimentally inoculated with toxigenic *A. flavus*. These foods included apple juice, bacon, condensed and dried milk, egg noodles, and rye and wheat breads. Numerous sterilized and nonsterilized solid foods and fruit juices were inoculated with *A. flavus* by Wildman et al. *(35)* with resulting production of aflatoxin. Hanssen *(15)* reported that a variety of foods, including peaches, lemons, and oranges, contained aflatoxigenic molds and aflatoxin B_1.

Citrus products are very prone to mold spoilage in the field, after harvest, in transit and storage, during processing and marketing, and in the home kitchen *(13)*. Aflatoxigenic fungi, such as *A. flavus (22, 26)* and *A. parasiticus*, are ubiquitous in nature and *A. flavus* and other aspergilli have been isolated from citrus fruit *(24, 32)*. The microflora of the citrus peel is abundant and some fraction of this microflora inevitably finds its way into the juice *(5)*. Because of the high acid and sugar content of citrus juices and concentrates, yeasts and molds are the organisms most apt to grow *(25)*.

Since aflatoxins are hazardous to animal and human health *(4, 14, 29)*, aflatoxigenic aspergilli are ubiquitous in nature *(16, 36)*, and citrus fruits are commonly infected with molds, it was believed useful to study aflatoxin formation in this commodity. Experiments were undertaken to determine: *(a)* if, under ideal conditions, growth of known toxigenic aspergilli on citrus products is accompanied by aflatoxin formation, *(b)* levels of aflatoxins present in such substrates during an extended incubation, and *(c)* which fraction of citrus juices (solids, filtrate, mold) contained the highest level of aflatoxins.

Materials and Methods

Organisms

A. flavus CMI 89717 and *A. parasiticus* CMI 15957 were used in this study. They are known toxigenic strains that were obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. Also employed in this study was *A. parasiticus* NRRL 2999, a toxigenic strain obtained from the Northern Regional Research Laboratory, Peoria, Illinois. Stock cultures were maintained at 5 C on slants of mycological agar (Difco).

Preparation of spore suspensions

Molds were grown on slants of mycological agar (Difco) for 7-10 days at 28 C. Spores were harvested by adding sterile distilled water and a drop of Leconal wetting agent (Laboratory Equipment Co., St. Joseph, Michigan) to the slants. An inoculum of 0.2 ml of the heavy spore suspension was added to all solid and liquid substrates.

Preparation and incubation of fruit

Grapefruits, lemons, and oranges were purchased from a local supermarket and juices were extracted with a home-type juice extractor. Cans of frozen concentrated grapefruit

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juice (unsweetened) were also purchased locally and the contents were diluted with three volumes of sterile tap water to obtain approximately the same percentage of soluble solids as in freshly squeezed juice. One-hundred thirty milliliters of juice were added to 300-ml Erlenmeyer flasks, whereas pieces of fruit peels (grapefruit, 15-25 g; lemon, 9-14 g) were placed onto moistened Whatman No. 1 filter paper in sterile petri dishes. All substrates were placed under flowing steam for 10 min, cooled, and inoculated. Incubation was stationary and at 28 C. Sufficient samples were prepared so a fresh sample could be used for analysis at each time interval.

Fractionating the juice

Grapefruit juice was inoculated with *A. parasiticus* and incubated at 28 C. The juice was then filtered through Whatman No. 1 filter paper in a Büchner funnel and the filtrate was collected. The solids was considered as the material that remained on the filter paper, whereas the intact mold mycelium remained in the original flask. The mold was washed twice with sterile water before extraction.

Frozen storage of grapefruit juice

A. parasiticus was grown for 18 days in freshly squeezed grapefruit juice. Flasks of juice were then frozen at −18 C and tested for aflatoxin content at 2-week intervals for 20 weeks.

Aflatoxin analysis

The sample (50 ml of juice or entire fruit peel sample) was mixed with 100 ml of hexane (Skelly B) and a mixture of 250 ml methanol: water (55:45, vol/vol) in a Waring blender operating at high speed for 3 min. The contents of the blender were dispensed into 250-ml centrifuge bottles and then were centrifuged at 1000 rpm in an International Centrifuge, model K (International Equipment Company, Needham Heights, Mass.). Fifty milliliters from the methanol phase were mixed in a beaker with 45 g of Celite 545 (Sargent-Welch, Skokie, Ill.).

A chromatographic column (38 × 330 mm) was packed with the Celite-sample mixture to a final column height of 170 mm. The column was washed with 400 ml of hexane and aflatoxin was eluted with 500 ml of a chloroform : hexane (50:50, vol/vol) solvent mixture. The eluate was evaporated on a steam bath and the residue in the boiling flask (about 2 ml) diluted to 10 ml with chloroform. Thin-layer chromatoplates were prepared with Adsorbosil-5 (0.25 mm thick) (Applied Science Laboratories, Inc., State College, Pa.). Plates were air dried and then heat-activated for 2 h at 110 C. Aflatoxins were resolved by thin-layer chromatography, the chromatograms being developed with a solvent system comprised of chloroform : methanol : water (98:1:1, vol/vol/vol) or benzene saturated with formamide (1). Aflatoxins were quantitated by fluorometry *(30)* on a Turner fluorometer (G. K. Turner Associates, Palo Alto, Calif.) equipped with a plate scanner stage and a recorder (Leeds and Northrup Speedomax-H, Philadelphia, Pa.). Aflatoxin concentration was measured as recorder response and was calculated from a standard curve, taking into account the dilutions involved.

RESULTS

Alfatoxin formation on grapefruit peel

Data in Table 1 show that when *A. flavus* grew on grapefruit peel, the amount of alfatoxin B_1 increased for 38 days and then it decreased and tended to

TABLE 1. AFLATOXIN FORMED ON GRAPEFRUIT PEEL INOCULATED WITH *A. flavus* AND HELD AT 28 C[1,2]

Days	Aflatoxin B_1 (μg/g)	B_2	G_1	G_2
4	1.1	0.25	1.3	0.14
6	1.8	0.30	3.5	0.23
8	1.4	0.12	2.1	0.10
14	2.4	0.14	3.5	0.12
18	5.9	0.05	2.4	0.06
26	6.6	0.07	4.0	0.04
38	8.8	0.04	3.1	0.02
46	4.3	0.03	1.9	0.04
54	6.5	0.03	2.3	0.02
62	5.0	0.03	2.6	0.02

[1]Averages of three samples.
[2]Benzine-formamide development system.

TABLE 2. AFLATOXIN FORMED ON GRAPEFRUIT PEEL INOCULATED WITH *A. parasiticus* AND HELD AT 28 C[1,2]

Days	Aflatoxin B_1 (μg/g)	B_2	G_1	G_2
4	1.2	0.42	0.42	0.06
6	3.3	0.31	0.85	0.06
8	1.2	0.26	0.25	0.04
14	0.97	0.10	0.57	0.41
18	0.68	0.05	0.62	0.03
26	1.6	0.11	1.5	0.31
38	1.7	0.06	1.3	0.05
50	1.5	0.03	0.81	0.07
54	1.2	0.03	0.88	0.04
62	2.0	0.04	0.92	0.05

[1]Averages of three samples.
[2]Benzine-formamide development system.

TABLE 3. AFLATOXIN FORMED IN FRESH GRAPEFRUIT JUICE INOCULATED WITH *A. flavus* AND HELD AT 28 C[1,2]

Days	Aflatoxin B_1 (μg/ml)	B_2	G_1	G_2	pH[3]
4	1.1	0.03	0.67	0.02	3.42
6	1.2	0.04	0.73	0.03	3.60
8	1.2	0.03	0.70	0.01	3.82
14	0.90	0.02	0.61	0.01	5.65
18	0.90	0.01	0.88	0.01	6.60
26	0.30	0.01	0.28	0.01	7.83
38	0.08	0.03	0.17	0.01	8.40
46	0.04	0.04	0.06	0.01	8.33
50	0.02	0.03	0.04	ND[4]	8.25
62	0.07	0.07	0.07	ND	9.06

[1]Averages of two samples.
[2]Benzine-formamide development system.
[3]Initial pH = 3.0.
[4]ND = Not detectable.

stabilize. The amount of aflatoxin G_1 was maximal after 4 days and after 26 days it decreased and also tended to stabilize. There was no apparent increase in concentration of aflatoxins B_2 and G_2 after 6 days. A slight decrease in amounts of these toxins also occurred during extended incubation. When *A. parasiticus* grew on grapefruit peel (Table 2), amounts of aflatoxin B_1 appeared to neither increase nor decrease substantially after 6 days and up to 26 days, whereas the amount of aflatoxin G_1 gradually increased to 26 days, then decreased and stabilized. Aflatoxin B_2 concentrations declined after 4 days and remained constant toward the end of incubation while amounts of aflatoxin G_2 remained fairly constant for 62 days. Detectable levels of all four aflatoxins were present after 4 days of incubation.

Aflatoxin formation in grapefruit juice

When *A. flavus* grew in juice (Table 3), maximum amounts of aflatoxins B_1 and G_1 developed in 4-6 days and then the amounts declined markedly after 18 days and stabilized after 38 days of incubation. No apparent increase or decrease was observed in 62 days for aflatoxins B_2 and G_2. Table 4 summarizes results obtained when *A. parasiticus* grew in grapefruit juice. The amount of aflatoxin G_1 present increased to 34 days, then declined and stabilized after 46 days. Amounts of aflatoxins B_2 and G_2 did not increase or decrease appreciably during the 58 days of incubation. This strain of *A. parasiticus*, in this trial, did not produce aflatoxin B_1.

TABLE 4. AFLATOXIN FORMED IN FRESH GRAPEFRUIT JUICE INOCULATED WITH *A. parasiticus* AND HELD AT 28 C[1,2,3]

Days	Aflatoxin B_2	G_1	G_2	pH[4]
	(µg/ml)			
6	0.01	0.23	0.01	3.0
14	0.01	0.48	0.01	3.65
18	<0.01	0.62	0.01	3.85
22	0.01	1.04	0.01	7.03
34	0.01	1.65	0.01	8.15
46	0.01	1.30	0.01	8.35
58	<0.01	1.36	0.01	8.45

[1]Averages of two samples.
[2]Benzine-formamide development system.
[3]Aflatoxin B_1 was not produced.
[4]Initial pH = 3.10.

Aflatoxin formation on lemon peel and in lemon juice

Data in Table 5 show that *A. flavus* produced only aflatoxins B_1 and G_1 on lemon peel. The amount of the two toxins in lemon peel was nearly equal and was maximal at 14 days. Markedly less toxin was present on lemon peel than when the same strain of mold grew on grapefruit peel. *A. parasiticus* produced all four aflatoxins in lemon juice and highest concentrations were observed after 10 days of incubation.

Aflatoxin formation in orange juice and on orange peel

A. parasiticus NRRL 2999, a potent producer of aflatoxin, was inoculated into freshly squeezed orange juice and incubated quiescently for 14 days at 28 C. At that time the concentrations of aflatoxins B_1, B_2, G_1, and G_2 were 5.5, 0.013, 2.95, and 0.05, respectively. The mold was inoculated on orange peel but amounts of aflatoxin could not be measured because naturally occurring fluorescent substances had R_f values similar to aflatoxins and thus interfered with evaluation of thin-layer chromatographic plates.

Aflatoxins recovered from fractions of grapefruit juice

Analysis (wet weight basis) of each fraction (Table 6) obtained from grapefruit juice, usually revealed a greater amount of aflatoxin in the solids fraction than in the filtrate. Generally, the highest concentration of aflatoxins B_1 and G_1 was recovered from the solids, the next highest from the mold, and least from the filtrate. Data in Table 7 list the percentage of aflatoxin recovered in the solids fraction. With the exception of aflatoxin G_1, nearly three-fourths of the total aflatoxin was recovered from grapefruit juice solids. Similar results (data not presented) were obtained with lemon and orange juices.

Stability of aflatoxin in frozen grapefruit juice

A. parasiticus was grown for 18 days in freshly

TABLE 5. AFLATOXIN FORMED ON LEMON PEEL AND IN FRESH LEMON JUICE INOCULATED WITH *A. flavus* OR *A. parasiticus* AND HELD AT 28 C FOR 10, 14 AND 18 DAYS[1,2,3]

Days	Aflatoxin B_1 juice	B_1 peel	B_2 juice	G_1 juice	G_1 peel	G_2 juice	pH of juice[4]
	(µg/ml or g)						
10	0.09	0.035	0.03	0.063	0.03	0.01	2.19
14	0.05	0.054	0.014	0.031	0.067	0.005	2.1
18	0.055	0.045	0.023	0.03	0.056	0.011	2.3

[1]Averages of two samples.
[2]*A. flavus* inoculated on peels; benzene-formamide development system; aflatoxins B_2 and G_2 not produced.
[3]*A. parasiticus* inoculated in juice; benzene-formamide development system.
[4]Initial pH = 1.85.

TABLE 6. AFLATOXIN RECOVERED FROM FRACTIONS OF GRAPEFRUIT JUICE INOCULATED WITH TWO STRAINS OF *A. parasiticus* AND HELD FOR 14 DAYS AT 28 C[1]

	Aflatoxin							
	B_1		B_2		G_1		G_2	
Fraction	T-1[2]	T-2[3]	T-1	T-2	T-1	T-2	T-1	T-2
	(μg/ml or g)							
Filtrate	4.0	0.17	0.11	0.03	1.7	0.29	0.06	0.05
Solids	38.3	2.7	0.40	0.38	4.9	0.25	0.48	0.21
Mold	6.7	0.38	0.05	0.04	3.1	0.43	0.05	0.02

[1]Averages of two or three samples.
[2]T-1 = Trial 1, thawed frozen concentrated juice diluted with an equal volume of water, chloroform : methanol : water (98:1:1, vol/vol/vol) development system.
[3]T-2 = Trial 2, undiluted thawed frozen concentrated juice, benzene-formamide development system.

squeezed grapefruit juice that was then frozen at −18 C. Data in Table 8 for zero-time indicate aflatoxin levels before freezing. The data show that only after 18 weeks did frozen storage appreciably affect aflatoxin B_1 and G_1 concentrations, which increased after this time.

DISCUSSION

Citrus juices, although able to support mold growth, are not an ideal medium for aflatoxin production as shown by the low yields reported in Tables 3, 4, and 5. Fresh grapefruit and lemon juice contain 0.092 and 0.08 g carbohydrate/ml, respectively *(34)*. The major carbohydrates are sucrose, glucose, and fructose (in a ratio of 2:1:1) and they comprise a large percentage of the soluble solids *(18)*. Mateles and Adye *(23)* demonstrated that a 1% concentration of each of these sugars supported mold growth but not aflatoxin formation. The YES medium of Davis et al. *(8)* contains 20% sucrose and thus is very satisfactory for aflatoxin production. Grapefruit and lemon juices contain 1.5-2.5% and 5-6% acid, respectively, with citric acid being dominant *(19)*. Davis and Diener *(7)* observed that substrates containing high amounts of carbohydrate generally supported large yields of aflatoxin and that citric acid supported growth of but not toxin production by *A. parasiticus*. Grapefruit peel, under ideal conditions, is a better substrate than juice for production of aflatoxin (Tables 1-4), probably because the peel contains more carbohydrate than the juice. Thirty to fifty percent of the dry weight of grapefruit peel is comprised of alcohol-soluble solids, of which about 80% are glucose, sucrose, and fructose *(19)*. Traces of xylose are present in grapefruit peel *(19)* and this sugar has been reported to stimulate aflatoxin production *(7)*. Citrus juices are low in nitrogenous compounds and this also may have served to retard production of aflatoxin.

Several investigators *(10, 17)* have reported the time required for maximal production of aflatoxin varies according to the mold strain, substrate, and cultural conditions. When *A. flavus* grew on grapefruit peel (Table 1), maximal amounts of aflatoxins B_1 and G_1 occurred at 38 and 26 days, respectively, and then declined. Data in Table 2 (*A. parasiticus*) show that the amount of aflatoxin B_1 was maximal at 6 days and of aflatoxin G_1 at 26 days. Diener and Davis *(9)* reported that the highest amount of aflatoxin B_1 occurred between 5-13 days when *A. flavus* grew on peanuts. A second peak in aflatoxin production was observed at 21 days. The same workers found that *A. parasiticus* produced the highest concentration of aflatoxin B_1 on peanuts at 7-15 days. Stubblefield et al. *(33)* grew *A. flavus* on a solid wheat substrate and noted maximal production of aflatoxins B_1 and G_1 at 4-5 and 3-4 days, respectively, and then the amounts

TABLE 7. PERCENTAGE OF AFLATOXIN RECOVERED FROM SOLIDS FRACTION OF GRAPEFRUIT JUICE INOCULATED WITH TWO STRAINS OF *A. parasiticus* AND HELD FOR 14 DAYS AT 28 C[1]

	Percent aflatoxin recovered in solids fraction	
Aflatoxin	Trial 1[2]	Trial 2[3]
B_1	78.2	83.1
B_2	71.4	84.4
G_1	50.5	25.8
G_2	81.4	75.0

[1]Averages of two or three samples.
[2]Thawed frozen concentrated juice diluted with an equal volume of water; chloroform : methanol : water (98:1:1, vol/vol/vol) development system.
[3]Undiluted thawed frozen concentrated juice; benzene-formamide development system.

TABLE 8. STABILITY OF AFLATOXIN IN FROZEN GRAPEFRUIT JUICE PREVIOUSLY CULTURED WITH *A. parasiticus*[1,2]

	Aflatoxin				
Weeks	B_1	B_2	G_1	G_2	pH
	(μg/ml)				
0	0.20	0.02	0.46	0.01	6.38
2	0.24	0.07	0.05	<0.01	6.43
6	0.32	0.02	1.01	0.01	6.87
10	0.40	0.02	0.58	0.01	6.40
16	0.39	0.03	0.57	0.01	6.58
18	0.43	0.02	0.34	0.01	6.48
20	0.55	0.02	0.87	0.01	6.72

[1]Incubated at 28 C for 18 days, then frozen.
[2]Benzine-formamide development system.

of the toxins declined. Arseculeratne and Bandunatha *(2)* observed several phases of increased aflatoxin production when *A. flavus* grew on grated coconut. Peaks in aflatoxin production occurred at 7 and 14 days while the greatest amounts of aflatoxin B_1 were recovered at 4-5 days and 12-13 days.

Data in Table 3 show that *A. flavus* produced the greatest amount of aflatoxin B_1 in grapefruit juice at 4-6 days and of G_1 in 18 days, whereas *A. parasiticus* produced the maximal concentration of aflatoxin G_1 at 34 days (Table 4). Data obtained with *A. flavus* more closely correspond to those of other workers when a liquid medium was the substrate. When *A. parasiticus* and *A. flavus* grew in a semisynthetic medium *(9)*, highest concentrations of aflatoxin B_1 and G_1 were recovered at 13 and 9-17 days, respectively. After these times, the toxin levels decreased. Similarly, when *A. flavus* grew in YES medium *(8)*, maximum amounts of aflatoxin B_1 and G_1 appeared at 5-12 days and then decreased. Schroeder *(27)* observed a peak in total aflatoxin production at 4 days followed by a rapid decline of toxin when *A. parasiticus* grew in Czapek-corn steep medium. These workers suggested that after the nutrients are exhausted, the mold may utilize aflatoxin in its metabolic pathways or alter the toxins so they can no longer be detected. It has been reported *(3)* that *A. flavus* can metabolize its own toxin although Ciegler et al. *(6)* suggested that the mold does not utilize its own toxin even after available carbohydrates are exhausted. When excess carbohydrates were added after maximal toxin yields were achieved, degradation of aflatoxin was not prevented. Furthermore, these same workers indicated that mycelial lysis appeared to be necessary for aflatoxin degradation and the percentage and rate of degradation seemed to be nonenzymatic and nonspecific.

Data in Tables 6 and 7 show that when *A. parasiticus* was incubated at 28 C for 14 days in grapefruit juice, the highest concentration of aflatoxins appeared in the solids fraction. The mold fraction contained 1.7 times the concentration of total aflatoxin that was in the filtrate. It is not susprising to find such a high concentration of aflatoxin in the mold, for Schroeder and Ashworth *(28)* noted that after 18 days of growth on a shredded wheat substrate, 50-80% of the toxin appeared in the mycelium rather than in the substrate. Shih and Marth *(31)*, working with a liquid glucose-salts medium, observed that the distribution of toxin between broth and mycelium varied with incubation time, temperature, and glucose concentration. In general, toxin was gradually released from the mycelium into the medium, the amount being a function of certain environmental factors and time.

Although it is commonly accepted that freezing is not deleterious to aflatoxin in many substrates, such information was lacking for the fate of aflatoxin in frozen grapefruit juice. Since the analysis for aflatoxin is quite time consuming, samples often have to be frozen for several weeks and hence one must be assured that the toxin content of a sample does not change during such storage. From data in Table 7 it appears that storage at −18 C did not appreciably affect amounts of aflatoxin in grapefruit juice until 18 weeks and then a slight increase was noted. One factor contributing to the increase in amount of aflatoxin B_1 and G_1 after 18 weeks could be the release of toxin from the mold mycelium.

Evidence from this study and the literature indicates that known aflatoxigenic molds can grow and form toxin on citrus peel and in juice. The aflatoxins do not parallel each other in time required to attain maximal concentrations and in the decline of these concentrations. It follows that testing after a single period of incubation may not provide a true estimate of the aflaxtoxigenic capacity of a particular strain of *Aspergillus* unless such testing is done when each toxin is at its maximal concentration. Also, the data indicate that determination of aflatoxin in only the substrate can lead to erroneous conclusions when aflatoxigenesis by certain molds is studied under specifically defined conditions.

Results of these experiments indicate that citrus products can support production of aflatoxin, albeit they are not an optimal substrate for this purpose. This possibility must be borne in mind by workers in the citrus industry when fruit is handled and processed for human consumption and when wastes from citrus fruit are processed as animal feeds.

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CAPSULATION OF *PROPIONIBACTERIUM*[1]

L. O. Skogen[2], G. W. Reinbold, and E. R. Vedamuthu[3]
Department of Food Technology
Iowa State University, Ames, Iowa 50010

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Abstract

Strains of nine species of *Propionibacterium* were examined for slime and/or capsule formation. Direct evidence of capsules was demonstrated by negative staining with India ink. Changes in viscosity of growth media, estimated using an Ostwald viscosimeter, were related to capsule or slime production. Mucoid colony production provided further evidence of slime formation. Under the criteria and experimental conditions adopted, at least one strain of each species and 34 of 82 strains formed slime. Incubation temperatures of 15 and 21 C which are less than optimal for growth, adjustment of media pH above neutrality, addition of sodium chloride, and as many as 11 different carbohydrates were conducive to slime formation. Paper and thin-layer chromatographic analyses of acid-hydrolyzed capsular material indicated that it contained mannose and lesser amounts of glucose and galactose.

A recent study of members of the genus *Propionibacterium (13)* indicated a need for reevaluation of the taxonomy of this genus based on additional investigations of cultural, physiological, serological, genetic, and phage host-range characteristics. Since then it has been shown *(16)* that slime from three different species of *Propionibacterium* did not produce detectable precipitating antibodies in rabbits.

In 1921, Sherman *(20)* noted that propionibacteria were able to produce slimy growth in broth. Breed et al. *(2)*, described the appearance of three species of *Propionibacterium* in liquid culture, and used the terms "ropy sediment," "ropy," and "very ropy." Malik et al. *(13)* mentioned "viscid" and "filamentous" sediment in broth cultures of propionibacteria.

This study was undertaken to first demonstrate and then study capsule and loose slime formation by propionibacteria. Such an investigation may provide an additional criterion for grouping or clustering members of the genus.

Materials and Methods

Cultures and culture maintenance

A total of 82 strains of nine different *Propionibacterium* species, as recognized by Breed et al. *(2)*, were investigated for slime-producing ability (see Table 1). Strains were selected for further study on the basis of their ability or inability to increase the viscosity of liquid media. They were maintained in Yeast extract-sodium lactate (YEL) broth *(15)* from which $MnSO_4$ had been deleted. Inocula for viscosity studies were taken from cultures incubated for 3 days at 32 C. The usual inoculation rate was 1%.

Viscosity measurement

The term "slime" is used to collectively refer to loose slime and capsule formation. Increase in viscosity of broth cultures relative to comparable sterile media was used as an index of slime production. Viscosity was measured with an Ostwald viscosimeter, according to the procedure described in *Laboratory Manual of Physical Chemistry (12)*. The viscosimeter was submerged in a water bath at 25 ± 0.5 C. Cultures were mixed on a Vortex Genie mixer (Scientific Industries, Springfield, Mass.). After 10-min temperature equilibration in the water bath, the flow rate of a 6-ml aliquot from each broth culture was measured four times. Detailed descriptions of the procedures used are reported elsewhere *(21)*. Absolute viscosity (AV) values were determined by the formula

$$AV = \frac{\text{average culture flow rate in sec}}{\text{average sterile broth flow rate in sec}} - 1.00$$

Because of the deduction of 1.00, the reported AV values reflect only the increase in culture viscosity.

Microscopic and macroscopic examination

Capsule formation was demonstrated by the wet-film, India-ink method of Duguid *(4)*. Details of the photographic procedures and equipment are reported elsewhere *(21)*. For macroscopic demonstration of slime formation, cultures were streaked on both YEL and YES agar. In the YES agar, 2.0% sucrose was substituted for 1% sodium lactate. After 7-day incubation in a candle oats jar *(23)* at 32 C, colonies that had developed were examined for appearance and consistency.

Slime production as measured by broth viscosity increase

Because all species of *Propionibacterium* utilize glucose but not necessarily sucrose *(2)*, Yeast extract–2.0% glucose (YEG) broth was used. Each of 82 strains of propionibacteria was inoculated into a separate flask containing 50 ml of YEG broth, incubated for 21 days at 21 C, and tested for AV value.

Absolute viscosity as related to dry cell weight

Selected strains were inoculated into 1-liter portions of YES broth and incubated for 21 days at 21 C. Cells were harvested by centrifugation (35,000 × *g* for 30 min), washed once in isotonic saline solution (0.85% NaCl), and resuspended in saline solution at 40-fold concentration. Cell concentrates were then further diluted with saline solution for viscosity measurements. Viscosities of various dilutions of

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[2]Present address: Kraft Foods, 419 S. Center Street, Beaver Dam, Wisconsin 53916.

[3]Present address: Microlife Technics, Sarasota, Florida 33580.

TABLE 1. *Propionibacterium* CULTURES USED IN SPECIFIC EXPERIMENTS

Experiment	Strain used for experiment		
	Species	Strain designation	Absolute viscosities[a, b]
Microscopic demonstration of capsule formation	*P. freudenreichii*	P39	0.06
	P. jensenii	P69	0.57
	P. rubrum	P21	20.41
Macroscopic colony appearance	*P. freudenreichii*	P30	0.00
	P. peterssonii	P38	0.02
	P. shermanii	P33	0.07
	P. zeae	P74	1.46
Survey of slime production by viscosity measurements	*P. arabinosum*	4 strains	0.02 - 0.11[c]
	P. freudenreichii	12 strains	0.00 - 0.38
	P. jensenii	9 strains	0.00 - 1.25
	P. pentosaceum	5 strains	0.01 - 0.23
	P. peterssonii	5 strains	0.00 - 0.10
	P. rubrum	5 strains	0.03 -20.41
	P. shermanii	35 strains	0.00 - 0.93
	P. thoenii	5 strains	0.00 - 1.32
	P. zeae	2 strains	0.02 - 1.46
Absolute viscosity as related to dry cell weight	*P. arabinosum*	P9	0.11
	P. shermanii	P19	0.03
	P. zeae	P74	1.46
Incubation temperature effect	*P. arabinosum*	P9	0.11
	P. zeae	P74	1.46
Incubation time effect	*P. shermanii*	P19	0.03
	P. zeae	P74	1.46
Carbon source effect	*P. arabinosum*	P9	0.11
	P. arabinosum	P42	0.02
	P. freudenreichii	P6	0.38
	P. zeae	P74	1.46
Initial pH effect	*P. zeae*	P74	1.46
Sodium chloride concentration effect	*P. freudenreichii*	P1, P56	0.36, 0.19
	P. rubrum	P26	1.08
	P. shermanii	P7, P12, P24	0.21, 0.02, 0.62
	P. thoenii	P4	1.32
	P. zeae	P74	1.46
Isolation and characterization of slime	*P. zeae*	P74	1.46

[a]Cultures were incubated for 21 day at 21 C in 2% glucose-substituted YEL broth.

[b]Capsules were not microscopically visible when absolute viscosities were less than 0.10.

[c]Extreme range of absolute viscosities produced by the various strains.

each cell suspension as well as the viscosity of the suspending saline solution were measured.

Cell weights were obtained by drying thinly spread layers of concentrated cell suspensions on tared aluminum weighing dishes for 24 h at 110 C in a drying oven. After cooling in a desiccator for 30 min, the dishes were weighed. Dry cell weights were caculated, compensating for the Nacl in the suspensory medium.

Incubation temperature effect

Inoculated tubes of YES broth were incubated at 7, 15, 21, 32, and 37 C. At 32 days, AV and broth turbidity were measured. Turbidity determinations were made with a Bausch and Lomb 340 spectrophotometer (Chicao Apparatus, Chicago 22, Ill.) at 540 nm.

Incubation time effect

Inoculated tubes of YES broth were incubated for 7, 14, 21, 28, 35, 42, 90, and 180 days at 15 C. This temperature was chosen on the basis of maximum response obtained with highly viscous strain *P. zeae* P74. Immediately after inoculation and at designated times, AV and viable cell numbers were determined *(1, 8)*.

Carbon source effect

Different carbohydrates (2.0% w/v) were substituted for sodium lactate in YEL broth. Twenty percent carbohydrate solutions were sterilized by passage through Millipore filters (0.45 μ pore size) before addition to carbohydrate deficient basal medium. Inoculated media were incubated for 21 days at 21 C, a time-temperature combination producing

abundant growth for all strains examined.

Initial pH effect

Sterile NaOH or HCl was used to adjust the pH of sterile YEG broth to 4.0, 5.0, 6.0, 7.0, and 8.0 (all ± 0.1). After pH adjustment, media were inoculated, incubated for 14 days at 21 C, and AV measurements made.

Sodium chloride concentration effect

Tubes of YEG broth containing 1, 2, 3, and 4% NaCl (w/v) were inoculated at a 5% rate with culture. After incubation for 7 days at 21 C, AV measurements were made.

Isolation and characterization of slime

Lindeberg's procedure *(11)* was used to obtain loose slime produced by *P. zeae* P74 in YEG broth after 21-day incubation at 21 C. Cells were removed by centrifugation (35,000 × *g* for 15 min) and the supernatant liquid was collected. Two volumes of 95% ethanol were gradually added to the supernatant fluid. The stringy white mass that formed was spooled on a glass rod, dried on a watch glass for 24 h at 45 C, weighed, and resuspended in distilled water at 10 mg/ml. The resuspended slime was dialyzed for 24 h with cold tap water and was stored at 4 C.

Intact capsular material was extrated from *P. zeae* P74 using the methods of Webb *(25)* and Guex-Holzer and Tomcsik *(6)*. Cells from 1 liter of YEG broth were resuspended in 100 ml of tap water, shaken with 5 ml of chloroform, and held overnight at 23 C ± 1 C. The cell suspension was then heated in flowing steam for 6 h, centrifuged at 35,000 × *g* for 30 min, and washed twice in tap water. The yellow-brown supernatant fluid was concentrated to 20 ml by evaporation in a dialysis tube exposed to a stream of air. The white mass formed by addition of two volumes of 95% ethanol was treated as described previously.

Equal quantities of loose slime or capsular material and 4 N HCl were combined and heated for 4 h in a boiling water bath. Resulting hydrolyzates were stored without neutralization at 4 C.

Multiple-ascent paper chromatography using water in n-propanol (1:9) and 1-butanol, pyridine, and water (6:4:3) was used to analyze the hydrolyzates. Because this system did not give adequate separation of the leading components, the thin-layer method described by Lato et al. *(10)* was used to identify these compounds. Both procedures are described in detail elsewhere *(17, 21)*.

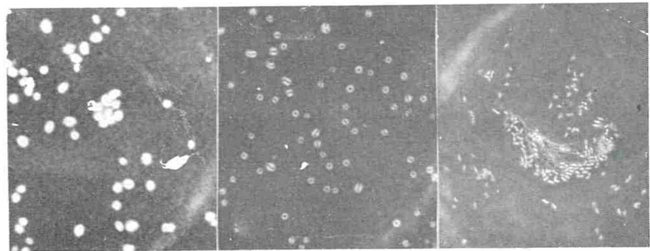

Figure 1. Photomicrographs of wet mount-India ink negative stain preparations of *P. rubrum* P21 (left), *P. jenseii* P69 (center), and *P. freudenreichii* P39 (right). Magnification 970X.

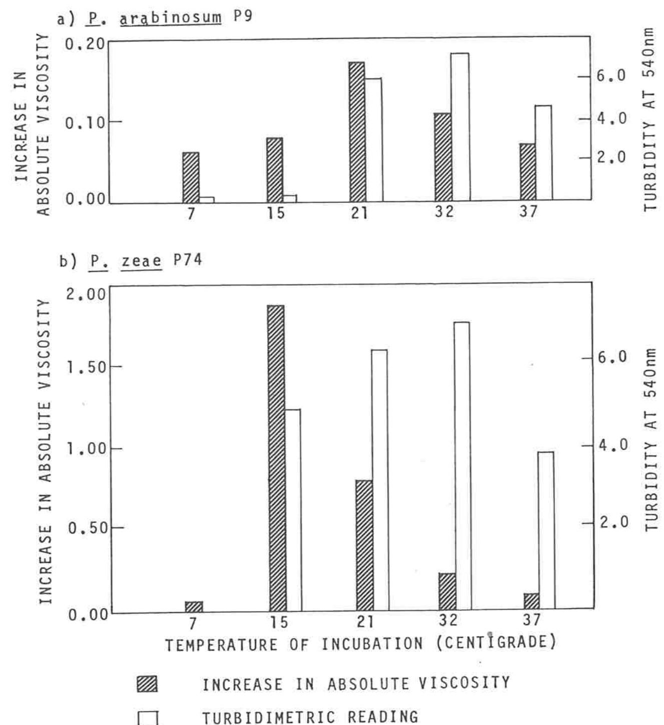

Figure 2. Effect of incubation temperature on absolute viscosity and turbidity of broth cultures of two *Propionibacterium* species.

RESULTS AND DISCUSSION

Microscopic and macroscopic demonstration of capsule formation

Figure 1 provides visual proof of capsule formation by two strains of *Propionibacterium*. Absolute viscosities were 20.41 and 0.57 for strains P21 and P69, respectively. Also shown is strain P39 which, at AV 0.06, was not capsulated; if microcapsules were present they were below the resolution of the light microscope. Capsule size and AV values seem to be correlated. Further microscopic observations using other strains provided additional direct evidence of propionibacterial capsule formation. Strain differences and nutritional effects on slime production were demonstrated by colony consistency when grown on YEL and YES agars. Strains viscous in broth were very mucoid on YES agar and less mucoid on YEL agar. Strains nonviscous in broth were not mucoid on either agar.

Survey of slime production by viscosity measurements

To determine if slime production could be used as a taxonomic aid, AV values of 82 strains of nine *Propionibacterium* species grown in YEG broth were determined. These values are summarized in Table 2. Strains of *Propionibacterium* species with AV values over 0.10 usually showed visible capsules when viewed microscopically and were considered as slime producers. Therefore, at least one strain of each of the nine species produced slime. A total of 34 of the 82 strains (41%) produced AV values ≥AV 0.1. Thus, slime formation by propionibacteria was shown to be a common occurrence not related to strain specificity. Data obtained subsequent to this survey

TABLE 2. SURVEY OF NINE *Propionibacterium* SPECIES FOR SLIME PRODUCTION BY ABSOLUTE VISCOSITY MEASUREMENTS

Species	No. of strains examined	No. of strains showing AV range of:					Percentage of slime producers (AV > 0.10)
		<0.10	0.10-0.50	0.51-1.00	1.01-5.00	>5.00	
P. arabinosum	4	3	1	0	0	0	25
P. freudenreichii	12	6	6	0	0	0	50
P. jensenii	9	6	0	1	2	0	33
P. pentosaceum	5	3	2	0	0	0	40
P. peterssonii	5	4	1	0	0	0	20
P. rubrum	5	1	0	0	1	3	80
P. shermanii	35	20	13	2	0	0	43
P. thoenii	5	4	0	0	1	0	20
P. zeae	2	1	0	0	1	0	50
TOTAL	82	48	23	3	5	3	41

suggest that incorporation of other carbohydrates in the growth medium (such as sucrose, galactose, or raffinose) and manipulation of other cultural conditions may have greatly increased the 41% incidence of slime formation. For example, data in Table 3 show that replacement of glucose with galactose raised the AV value of *P. arabinosum* P9 from 0.08 to 0.57. Similarly, data in Fig. 2 show an increase in AV value of over 100% when *P. zeae* P74 was grown at 15 C rather than 21 C. Determination of specific cultural conditions for individual strain slime formation enhancement, however, was not the purpose of this experiment. It would be unwise to attempt to draw conclusions from the data as to the proclivity of each species toward slime formation. Reasons precluding against this approach include *(a)* use of only one combination of cultural conditions to assess reaction of a great number of strains, *(b)* disparity between the number of strains in each species studied (from 2 for *P. zeae* to 35 for *P. shermanii*), and *(c)* the great dissimilarity shown within species as to number of strains producing slime and strain variation in amount of slime produced. With *P. freudenreichii*, 6 of 12 strains produced less than the reference AV value of 0.10; the remaining 6 strains produced AV values of 0.10 to 0.50. Testing 35 strains of *P. shermanii* showed a somewhat comparable ratio of 20 "negative" strains to 15 strains with AV values ≥ 0.10. With *P. zeae* only two strains were tested; one strain had less than the AV 0.10 reference value, the second strain was quite viscous at AV 1.46. It is difficult to predict if other strains of *P. zeae* would be viscous or nonviscous. *P. rubrum*, however, seems predisposed toward slime formation. Four of five strains tested were very viscous with one strain showing an extremely high AV value of 20.41. Overall, therefore, it seems reasonable to conclude that slime formation by propionibacteria can not be used as an aid to species classification.

Absolute viscosity as related to dry cell weight

To establish that AV values were not merely reflecting a direct relationship between cell numbers and viscosity increase, the relationship of dry cell weight to AV was determined. Slopes of the curves shown in Fig. 3 denote the extent of slime and (or) capsule formation by three propionibacteria strains. Microscopic examination confirmed that *P. shermanii* P19 was not capsulated at AV 0.03 but that *P. arabinosum* P9 (AV 0.11) and *P. zeae* P74 (AV 1.46) possessed capsules of different size in direct proportion to their AV values.

Incubation temperature effect

Examples of the effect of incubation temperature on slime production are presented in Fig. 2a and 2b. A 32-day incubation period was used. For viscous *P. zeae* P74 (AV 1.46) the optimum temperature for slime production was 15 C (Fig. 2b). For slightly viscous *P. arabinosum* P9 (AV 0.11), however, the optimum temperature was 21 C. Since viscosity measurements were made after equilibratin broth temperature for 10 min at 25 C, the AV differences indicated the effect of incubation temp-

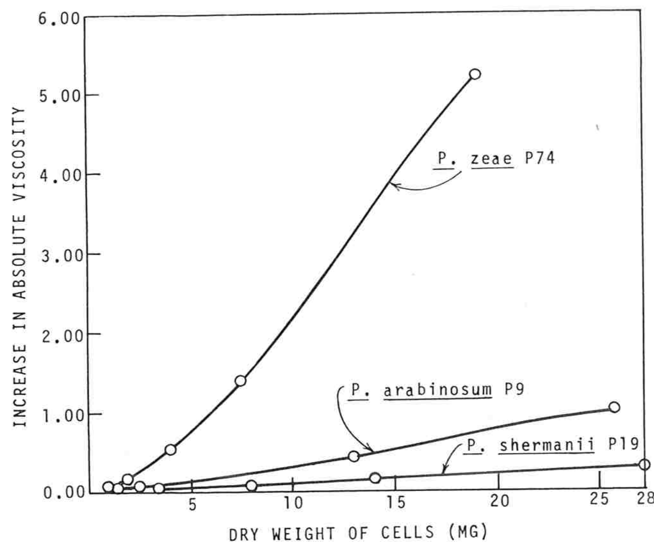

Figure 3. Relationship between absolute viscosity and dry cell weight of three *Propionibacterium* species.

erature on slime production and not on broth viscosity per se. Cell growth (Fig. 2a and 2b), as evidenced by turbidity measurements of the 32-day-old cultures, showed that maximum growth had occurred at 32 C. The AV values obtained for the two strains at the five incubation temperatures, therefore, were not direct functions of cell numbers.

Morgan and Beckwith *(14)* reported that low incubation temperatures (10 to 20 C) increased the mucosity of colonies of *Salmonella and Escherichia.* Wilkinson et al. *(27)* noted that temperatures of 15 to 20 C, rather than 35 C, greatly increased polysaccharide production by *Escherichia coli,* but decreased polysaccharide production by *Aerobacter cloacae.*

Explanations for this phenomenon of low-temperature stimulation of capsule production can be found in the literature. For instance, Rogers *(18)* believes that the intracellular nucleotide pool exerts an indirect influence on capsular polymer and cell wall synthesis. Because cell growth and division compete for nucleotide pool constituents, more rapid growth at higher temperatures would preferentially consume available nucleotides, thereby preventing or retarding capsular synthesizing activity.

Slime production at higher temperatures, however, could have been greater than that shown in Fig. 2. Degradation of the polymers may have occurred at a greater rate at higher temperatures. Wilkinson *(26)* stated that minimum degradation of extracellular polysaccharides occurred when lytic enzymes were absent in the medium (minimum cell autolysis) and when a low temperature and a pH near neutrality were maintained. Any one, or combination of, these factors may have been involved.

Incubation time effect

Although other experiments indicated that viscosity was not directly related to cell numbers, it was desirable to further investigate this possibility. Changes in viscosity (or production and degradation of the slime) at a low temperature over a long incubation period also required further investigation. Two strains, one viscous and one not viscous, were

TABLE 3. EFFECT OF DIFFERENT CARBON SOURCES ON ABSOLUTE VISCOSITY OF BROTH CULTURES OF SELECTED STRAINS OF *Propionibacterium* SPECIES

Carbon source	*P. arabinosum* P42			*P. arabinosum* P9			*P. zeae* P74			*P. freudenreichii* P6		
	Viscosity[a]	A[b]	B[c]	Viscosity	A	B	Viscosity	A	B	Viscosity	A	B
Sucrose	0.01	+	+	0.07	+	+	0.78	+	+	0.00	–	–
Galactose	0.01	+	+	0.57	+	+	2.37	+	+	0.08	+	+
Arabinose	0.01	+	+	0.09	+	+	0.16	+	+	0.03	–	+
Mannose	0.00	+	+	0.05	+	+	0.71	+	+	0.06	+	+
Mannitol	0.02	+	+	0.06	?	+	0.61	+	+	0.00	–	–
Maltose	0.02	+	+	0.19	+	+	3.89	+	+	0.02	–	–
Lactose	0.05	?	+	0.08	?	+	0.04	+	–	0.00	–	–
Sorbitol	0.04	+	+	0.06	+	+	0.06	?	+	0.00	?	–
Starch	0.00	?	+	0.13	?	+	0.40	–	+	0.00	?	–
Sodium lactate	0.05	+	–	0.08	+	–	0.11	+	–	0.05	+	–
Rhamnose	0.02	–	+	0.03	–	+	0.05	+	–	0.00	–	–
Glucose	0.03	+	+	0.08	+	+	1.01	+	+	0.44	+	+
Fructose	0.02	+	+	0.06	+	+	0.69	+	+	0.12	+	+
Raffinose	0.03	+	+	0.15	+	+	8.05	?	+	0.03	–	–
Xylose	0.02	–	+	0.00	–	–	0.04	–	–	0.00	–	–
Yeast ext.	0.01		–	0.01		–	0.04		–	0.00		–

[a]Increase in absolute viscosity.
[b]Fermentation according to *Bergey's Manual of Determinative Bacteriology.*
[c]Fermentation measured by pH change of the medium.
Column A: + = fermentation, – = no fermentation, ? = not specified.
Column B: + = pH lowered from 7.0 to 6.0 or less, – = pH not lowered to 6.0 or less.

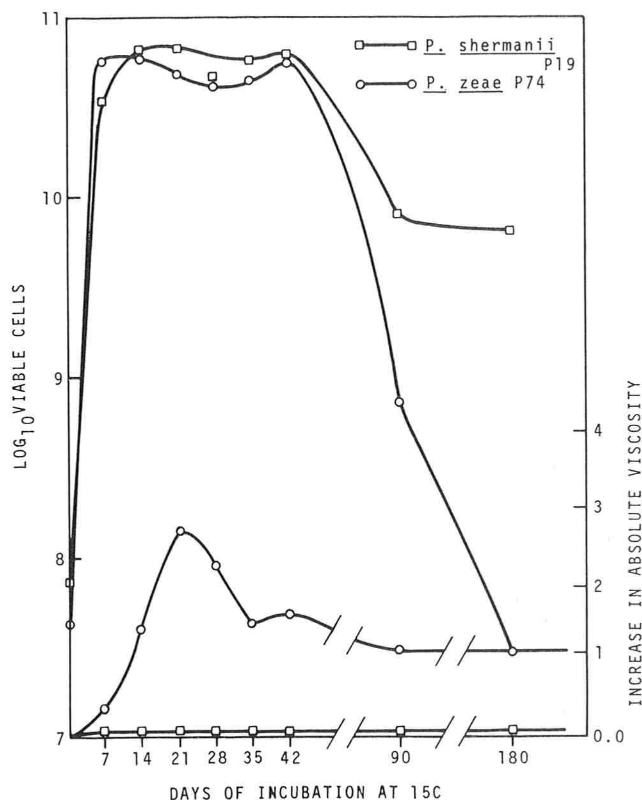

Figure 4. Viable cell numbers vs. absolute viscosity of broth cultures of *P. zeae* P74 and *P. shermanii* P19 measured at comparable incubation periods.

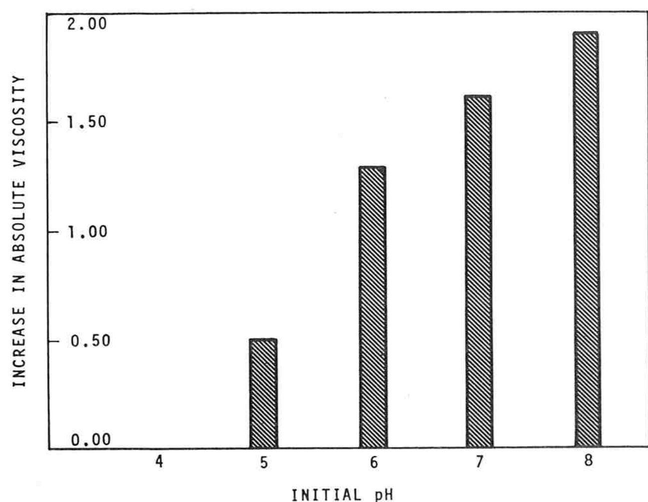

Figure 5. Effect of initial pH of medium on slime production by *P. zeae* P74.

grown at 15 C for 180 days in YES broth. Changes in viable cell numbers and AV values are presented in Fig. 4. Even after 180 days, the nonviscous strain exhibited no appreciable viscosity. Periodic microscopic examination of the cultures revealed no tendency toward clumping. Dissimilar to results obtained with some chain-forming and clump-forming microorganisms other than propionibacteria, no difficulty was experienced in obtaining accurate viable cell counts by the plating method.

When incubated at 15 C, *P. zeae* P74 broth culture reached maximum viscosity in about 21 days, followed by an equally rapid decrease in viscosity until the 35th to 42nd day, and then a gradual decrease until the 180th day (Fig. 4). This indicates that some degradation of the extracellular material had taken place after maximum viscosity had been reached. It is probable that the rate of slime breakdown exceeded that of synthesis (or synthesis had ceased) after the 21st day, resulting in a decrease in AV.

Both strains reached the stationary growth phase after 14 days when incubated at 15 C (Fig. 4). The coincident onset of the death phase for these cultures indicates that slime production offered no advantages for survival under the cultural conditions maintained in this study. Indeed, the number of viable cells of viscous P74 decreased more rapidly than did those of the control strain. The actual reason is unknown. Under some adverse conditions, however, capsules may be advantageous for propionibacteria; i.e., to protect against possible attack from bacteriophage by covering receptor sites, to prevent cellular dehydration, or to aid dispersal *(26)*.

Carbon source effect

The effect of different carbon sources on slime production is recorded in Table 3. Table 3 lists changes in AV induced by 15 carbon sources as compared with a carbohydrate-free control medium. *P. arabinosum* P42 produced no appreciable AV increase when grown with any of the 15 carbon sources or in the control basal medium. On the other hand, *P. zeae* P74 showed increases in AV with all "fermentable" carbon sources (with lowering of pH as the criterion for fermentative utilization) except sorbitol. Because of smaller AV increases with *P. freudenreichii* P6 and *P. arabinosum* P9, it is difficult to form any definite conclusions about their carbon utilization pattern for synthesis of slime. In all three slime-producing strains there was great variability in effectiveness of the various carbon sources to stimulate slime production. The exact reason for this variability is not understood. There are, however, reports in the literature of similar patterns of variability in carbon utilization with regard to synthesis of capsular material and extracellular slime. For example, Wilkinson *(26)* described two types of extracellular polysaccharides: first, those requiring specific carbon sources for formation and, second, those synthesized to various degrees from any utilizable carbon source. In the first type, the products are all homopolysaccharides formed by the action of a single enzyme on a specific oligosaccharide. In the

second type, there is considerable variation in the amount of assimilation from various carbon sources. Extracellular material was produced from many different carbon sources by *P. zeae* P74 and from a fewer number of carbon sources in *P. arabinosum* P9 and *P. freudenreichii* P6. Slime production in *Propionibacterium*, therefore, more closely resembles the second type of extracellular polysaccharide formation described by Wilkinson *(26)*.

Initial pH effect

Figure 5 graphically illustrates the effect of initial pH on slime production by *P. zeae* P74. *P. arabinosum* P42 is not represented because it did not produce an AV increase greater than 0.03. *P. zeae* P74 showed increasing AV as pH increased. At a lower initial pH, a self-limiting acidic environment is reached more quickly, resulting in less slime formation as well as less growth. Adjustment of the pH of the sterile broth to the values used in this experiment did not alter the viscosity of the sterile medium. As another control, a broth culture was grown in the same broth without initial pH adjustment. After incubation, the culture was divided into five portions, and these aliquots were treated with acid or alkali to attain the initial pH levels used in the experimental tubes; namely pH 4.0, 5.0, 6.0, 7.0, and 8.0. The AV for each of these portions was determined after holding for 1 h. There were no significant differences between the AV of these aliquots, indicating that the differences in AV in the experimental cultures were not because of the effect of acid or alkaline pH on the viscosity of the slime per se, but because of differences in the amounts of slime produced by the cultures at the various pH levels.

Sodium chloride concentration effect

The effect of various concentrations of NaCl on slime production is graphically represented in Fig. 6. There was considerable variation in strain response to NaCl concentrations. Greatest AV indices were obtained when NaCl was not added to the medium with *P. zeae* P74 and *P. rubrum* P26. *P. freudenreichii* P56 yielded its maximum AV at 3% NaCl concentration. The remaining strains produced maximum amounts of slime at 1% and (or) 2% NaCl. Some strains were affected dramatically (for example, *P. zeae* P74 and *P. rubrum* P26) when AV values were greatly reduced with increasing salt concentrations. Other strains, such as *P. thoenii* P4 and *P. freudenreichii* P56, exhibited relatively small differences in amount of slime produced at various NaCl concentrations.

Isolation and characterization of slime

A chromatogram developed with l-butanol, pyridine, and water (6:4:3) is reproduced in Fig. 7. Hydrolyzate A was prepared from capsular material and hydrolyzate B from loose slime. The hydrolyzed capsular material and slime seem to contain at least three distinct components. They were clearly glucose and galactose. The exact identity of the leading spot in the hydrolyzates was not established in this system because of similar migrations of fructose, mannose, and arabinose.

Thin-layer chromatography was then used to identify the leading spot. Plates were spotted with the respective hydrolyzates alongside authentic solutions of the three sugars exhibiting overlapping migrations in paper chromatographic systems. After chromatographed plates were sprayed with naphthoresorcinol, colors of the individual spots were compared. The leading spot had the characteristic violet color of mannose and a R_f value similar to that of mannose. Known solutions of fructose and arabinose produced, respectively, dark-red and blue-green spots.

The composite results of investigations using thin-layer and paper chromatography indicate that a large amount of mannose and lesser amounts of glucose and galactose are present in loose slime and capsular ma-

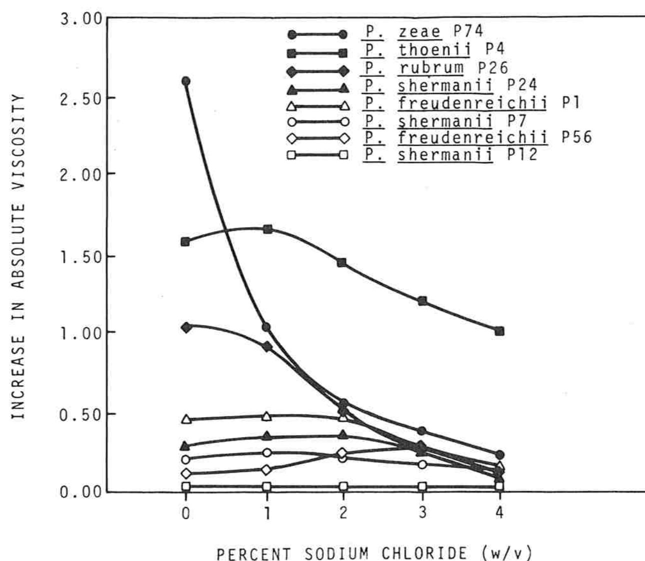

Figure 6. Effect of NaCl concentration on slime production by eight strains of *Propionibacterium*.

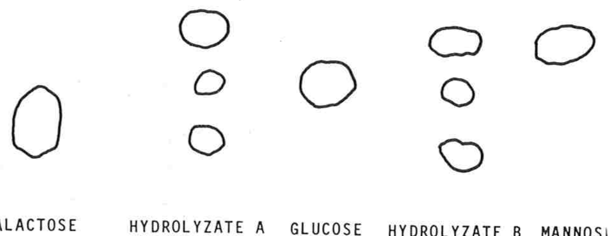

Figure 7. Sketch of a paper chromatogram of capsular material (hydrolyzate A) and loose slime (hydrolyzate B) of *P. zeae* P74 [solvent system: 1-butanol, pyridine, and water (6:4:3)].

terial produced by *P. zeae* P74 when grown in YEG broth.

The presence of mannose, glucose, and galactose in extracellular hetero-polysaccharides is widespread *(3, 5, 7, 9, 22, 24).* The presence of these sugars in the capsular material of *Propionibacterium* is entirely feasible.

Under the conditions used for the preparation of the hydrolyzates, the presence of cell-wall sugars in the loose slime and capsules was unlikely. Rogers and Perkins *(19)* reported that glucose and galactose are present in the cell walls of *P. technicum, jensenii, pentosaceum,* and *shermanii,* but that mannose was absent in all four species. *P. jensenii, shermanii,* and *technicum* also contained arabinose which was absent in the loose slime and capsules of *P. zeae* P74.

Use of capsule formation and loose slime production as a criterion for the separation of the members of the genus *Propionibacterium* does not seem promising, because these characteristics are widespread in the genus and show no obvious pattern of occurrence.

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SPLIT DEFECT OF SWISS CHEESE

I. EFFECT OF STRAIN OF PROPIONIBACTERIUM AND WRAPPING MATERIAL[1]

D. H. Hettinga[2], G. W. Reinbold, and E. R. Vedamuthu[3]

Department of Food Technology
Iowa State University, Ames, Iowa 50010

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Abstract

When strains of propionibacteria able to grow at 3.8 C were used in Swiss cheese manufacture, resulting 3- and 6-month-old cheeses had a high incidence of splits (6 of 13 and 10 of 13 lots, respectively). With strains lacking this low-temperature growth ability, only 1 of 10 and 2 of 10 lots of cheese split after comparable curing periods. Degree of cheese proteolysis, moisture, and sugar content could not be related to *Propionibacterium* strain used nor to split incidence. Carbon dioxide production in the warm (21 C) room by strains able to grow at low temperatures was essentially twice that of strains lacking this ability. This CO_2 production difference between strains was maintained during subsequent cold-room curing. Type of cheese-wrapping film also affected split incidence. Commercially manufactured cheeses wrapped in four films of differing gas permeability showed a distinct tendency toward splitting in the films impermeable to oxygen.

The increasing popularity of small consumer packages and presliced portions of Swiss cheese continues to make the defect known as splitting an important economic problem. Many theories have been advanced to explain the spasmodic occurrence of this defect, but none has been truly acceptable. Statements to the effect that splits are caused by too-acid and too-high-fat milk, or poor whey drainage inducing a brittle condition, account for only a minor portion of the defect. Studies have shown that no differences in the extent of proteolysis, salt distribution, pH, moisture levels, and production of excessive amounts of CO_2 could be found between split and unsplit Swiss cheese *(3, 11, 13)*. Continuing studies of this defect, as conducted in our laboratory, have caused us to question some of these findings.

Propionibacteria have been suspected as having a role in the split defect of Swiss cheese *(13)*. Implicated strains usually can grow at low temperatures *(14)*. Propionibacteria are considered to be heat resistant and able to survive pasteurization temperatures *(17)*. Some of the propionibacteria that survive pasteurization also may be able to grow at low temperatures. Such strains, when present as a natural inoculum in the cheese milk, would provide an inherent *potential* for split formation. Justification for screening propionibacterial strains intended for Swiss cheese manufacture for their inability to grow at low temperature was based primarily on the observation that the split defect in Swiss cheese is a cold-room phenomenon. If a *Propionibacterium* strain was able to metabolize lactate and release substantial amounts of CO_2 at low temperatures encountered in the finish curing room, with concomitant loss of elasticity in the body of the cheese, conditions would be conducive for a possible disposition towards splitting. Many factors could influence this tendency, but a thorough investigation of this hypothesis may provide an understanding of the causes for this defect.

Although often present, splitting has not been a widespread and recurring problem in conventional Swiss wheels. Because the cheese rind acted as the natural barrier against CO_2 diffusion in conventional Swiss wheels, studies were directed at evaluating relative gas diffusion rates in conventional wheels and rindless blocks wrapped in various protective coverings and films. In previous studies, reduced diffusion of gas resulted in swelling and splitting of the cheese *(2, 9)*. Also, when the cheese surfaces were permitted to dry, gas permeability decreased, and the split defect increased dramatically *(9)*.

Materials and Methods

Swiss cheese manufacture

Rindless-block, Iowa-style, and conventional, commercial Swiss cheese were made according to procedures described by Reinbold *(15)*.

Source of propionibacteria

Propionibacterium strains used in this investigation were obtained from the culture collection of the Department of Food Technology, Iowa State University.

Low-temperature growth of propionibacteria

Low-temperature growth of various propionibacteria was determined at 3.8, 6.8, 10, and 15 C. The range of temperature normally encountered in Swiss cheese during finish curing is between 3.8 and 15 C, and these two extremes were included in this screening. To determine growth of propionibacteria, tubes containing 10 ml of sterile Sodium lactate broth *(14)* were adjusted to 3.8, 6.8, 10, and 15 C, respectively, before inoculation. Tubes were inoculated at a level of

[1]Journal Paper No. J-7771 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa 50010. Project No. 1839.

[2]Present address: Research and Development Div., Kraftco Corp., 801 Waukegan Road, Glenview, Illinois 60025.

[3]Present address: Microlife Technics, Sarasota, Florida 33580.

0.1%, sealed with sterile paraffin wax, and returned to their respective incubators. A sterile control was included for each series of tubes. Tubes of broth were examined for turbidity at 4, 7, 11, 17, 21, 28, 37, and 78 days. Microscopic smears from randomly selected tubes exhibiting turbidity were examined to confirm purity.

Cheese analysis

Enumeration of propionibacteria in Swiss cheese was accomplished by the pouch technique of Hettinga et al. *(7)*. The method of Harper and Randolph *(5)* was used to determine lactic acid. An index of proteolysis within the cheese during curing was determined by measuring the total protein by the dye-binding technique of Hammond et al. *(4)*. The enzymatic method of Hettinga et al. *(6)* to determine glucose, galactose, and lactose in Cheddar cheese was applied to Swiss cheese.

Carbon dioxide diffusing out of Swiss cheese blocks was estimated by use of the following assembly: Wooden boxes containing Swiss cheese blocks weighing 46 kg were stacked one over the other in a molded polyethylene barrel. The barrel was provided with an air-tight lid and sealing system. Two outlets were provided in the lid for external connections. One of the outlets was connected to a stream of air from a simple aquarium pump, with the flow regulated at 1 cm^3 min by a needle-valve meter (Brooks Instrument Division, Emerson Electric Co., Hatfield, Pa). The air, before entry into the barrel, was bubbled through a series of CO_2 traps containing 1.0 N NaOH and soda lime to remove atmospheric CO_2, and then through distilled water to replace the humidity that might have been lost during passage through the CO_2-scrubbing system. The incoming stream of CO_2-free air constantly swept the CO_2 released from the cheese through the second outlet into a trap containing 100 ml 1.0 N NaOH. The NaOH trap was connected to a water seal to prevent contamination by atmospheric CO_2 at the open end.

A 25-ml aliquot from the CO_2 trap connected to the outcoming air from the barrel, was pipetted into a 100-ml beaker. To this, 10 ml 1 M $BaCl_2$ were added, and the sample was titrated against 1.0 N HCl to the phenolphthalein end point. Moles of CO_2 produced for a 24-h period were calculated by the equation

$$\text{Moles } CO_2/\text{day/kg cheese} = \frac{(\text{meq NaOH/day/kg cheese})}{(\text{eq } CO_2)\ (1/1000)}$$

RESULTS AND DISCUSSION

Low-temperature growth of propionibacteria in broth

Results for the growth response of different strains, representing 9 of the 11 recognized species of the genus *Propionibacterium (1)* at the various temperatures tested, are shown in Table 1. The data revealed that 8 of the 32 strains tested grew at 3.8 C, 17 strains grew at 6.8 C, 29 grew at 10 C, and all strains grew at 15 C. Among the species tested, *Propionibacterium shermanii* and *Propionibacterium freudenreichii* exhibited a wider range of growth at low temperatures. Several other strains not shown in Table 1 also were tested for low-temperature growth. Some of these strains also were used for cheese manufacture.

Production of splits and checks in cheese by selected strains of propionibacteria

Parallel vats of cheese were manufactured under identical conditions, with *P. shermanii* 59, a known splitter in one vat, and *P. arabinosum* 129, a nonsplitter in the other. These two cultures were selected for their innate tendency to produce defective (split and checked) and normal cheeses in our earlier investigations *(13)*. Cheeses also were manufactured with other strains of propionibacteria selected on the basis of preliminary work pertaining to low-temperature growth.

Samples from each lot of cheese manufactured were observed visually for body defects at 3 and 6 months of age, and the results are shown in Table 2. The data provide information on the incidence of splits and checks in the experimental Swiss cheese. After 3 months, of the 9 lots made with strain P-59, 4 (44%) were split but checks were not present. After 6 months, 5 of the same 9 lots (56%) showed splits, and 8 lots (89%) contained checks. Splits or checks were not observed in 3-month-old cheese made with strain P-129, but after 6 months, 1 of the 7 lots made (14%) was scored for splits and checks. These results support the hypothesis that certain propionibacteria that grow well at low temperatures have a greater dis-

TABLE 1. ESTIMATION OF GROWTH OF *Propionibacterium* AT LOW TEMPERATURES

Species	No. strains	Incubation temperature and time[a] 3.8 C 80 days	6.8 C 80 days	10 C 20 days	15 C 4 days
P. arabinosum	3	0/3[b]	0/3	1/3	3/3
P. freudenreichii	5	2/5	3/5	5/5	5/5
P. jensenii	2	0/2	0/2	2/2	2/2
P. pentosaceum	3	0/3	0/3	2/3	3/3
P. peterssonii	2	0/2	1/2	2/2	2/2
P. rubrum	1	0/1	0/1	1/1	1/1
P. shermanii	13	6/13	12/13	13/13	13/13
P. thoenii	2	0/2	1/2	2/2	2/2
P. zeae	1	0/1	0/1	1/1	1/1

[a]Trials done in duplicate in Sodium lactate broth.
[b]0/3 = no growth by three strains tested.

TABLE 2. OCCURRENCE OF SPLITS AND CHECKS IN SWISS CHEESE MADE WITH VARIOUS STRAINS OF PROPIONIBACTERIA

Strain no.	No. cheese lots	Age of cheese: 3 months Splits	3 months Checks	6 months Splits	6 months Checks
P. freudenreichii 1	1	0/1[a]	1/1	1/1	1/1
P. pentosaceum 5	1	0/1	0/1	0/1	0/1
P. peterssonii 35	1	0/1	0/1	0/1	0/1
P. shermanii 47	1	0/1	0/1	1/1	1/1
P. shermanii 83	3	1/3	2/3	3/3	3/3
P. shermanii 109	1	0/1	0/1	0/1	0/1
P. shermanii 59	9	0/9	4/9	5/9	8/9
P. arabinosum 129	7	0/7	0/7	1/7	1/7

[a]0/1 = no splits in one cheese lot.

position to produce cheese with splits and (or) checks.

Growth and metabolic activity of propionibacteria in cheese

Growth patterns in cheese. Growth of lactic starters during the early stages of cheese manufacture precedes the initiation of propionibacterial growth in Swiss cheese. The rapid growth of propionibacteria does not begin until the cheese enters the warm (21 C) room.

Figure 1 illustrates the relative growth patterns of strains P-59, and P-129 in Swiss cheese made in parallel vats under identical conditions on the same day. Strain P-59 increased in numbers more rapidly, and attained greater numbers than strain P-129. This observation was substantiated in every lot of Swiss cheese made with P-59 and P-129 under identical conditions in two parallel vats. In relating this observation to other analyses made on the cheese, there was a good agreement in the data with respect to cell numbers (Fig. 1), lactate disappearance (Fig. 2), and CO_2 production in the cheese (Fig. 3). Oehen et al. *(12)* have related increases in numbers of propionibacteria in Emmental cheese to parallel increases in volatile fatty acids and CO_2. The growth patterns shown in Fig. 1 indicate that propionibacterial numbers increased rapidly during the warm-room curing and gradually decreased during the finish curing. Cheeses were transferred to the cold room after sufficient eye development had occurred.

In a similar experiment, cheeses were made in parallel vats, with strains that grew at the low temperatures tested (P-35, P-47, P-59, P-83, and P-109) in one vat and strains that failed to grow at these temperatures (P-1, P-5, and P-129) in the other vat. (Some of the strains mentioned here are not included in Table 1.) Counts of propionibacteria in cheeses made with the first group of strains numerically exceeded similar counts on cheeses made with the latter group. Several of the low-temperature strains attained exceptionally great numbers in cheese and persisted at these levels even during the later stages of curing (Table 3).

Lactic acid utilization. Lactic acid is the primary fermentable carbon source for propionibacteria in Swiss cheese during curing. Hence, lactic acid disappearance in experimental cheeses was determined to obtain a relative measure of the metabolic activity of the cheese flora. The lactic acid concentration in cheeses made with P-59 and P-129 during curing is shown in Fig. 2. In these studies, it was assumed: *(a)* that the predominant propionibacteria in these cheeses were the ones added to the vat as starters, and *(b)* that, because the predominant flora of Swiss cheese during and after warm-room cure are propionibacteria, the various biochemical changes within the cheese could be attributed to these microorganisms. Strain P-59 rapidly fermented the lactic acid in the cheese, whereas P-129 utilized this acid at a much slower rate. This trend was in agreement with the relative magnitude of increases in cell numbers and production of CO_2 in the cheese made with the respective strain. At the end of 3 months of curing, P-59 had utilized virtually all available lactic acid. Because gradual disappearance of lactic acid was observed during cold-room curing, bacterial metabolism probably continued during this period. The amount and rate of utilization of lactic acid by the P-59 strain during the cold-room cure was much

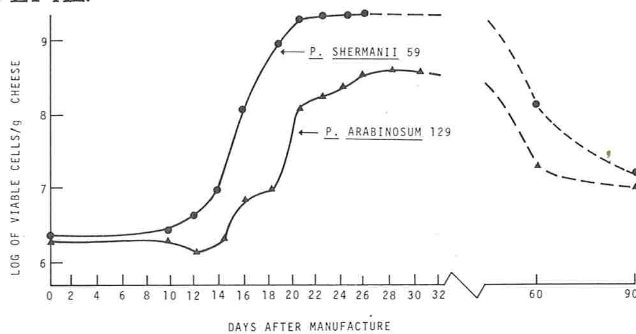

Figure 1. Growth of *P. shermanii* 59 and *P. arabinosum* 129 during curing of experimental Swiss cheese.

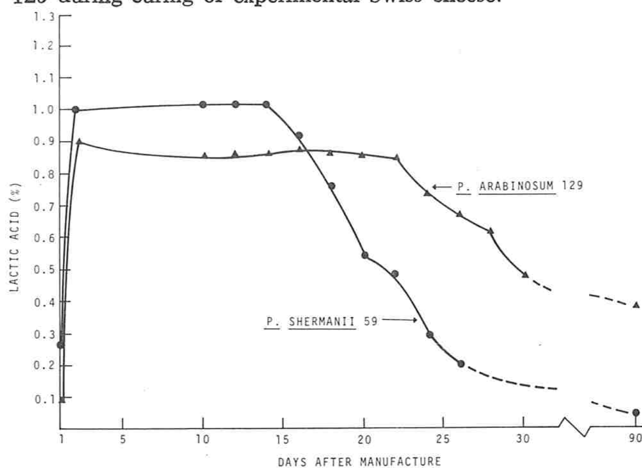

Figure 2. Levels of lactic acid in Swiss cheese made with *P. shermanii* 59 and *P. arabinosum* 129.

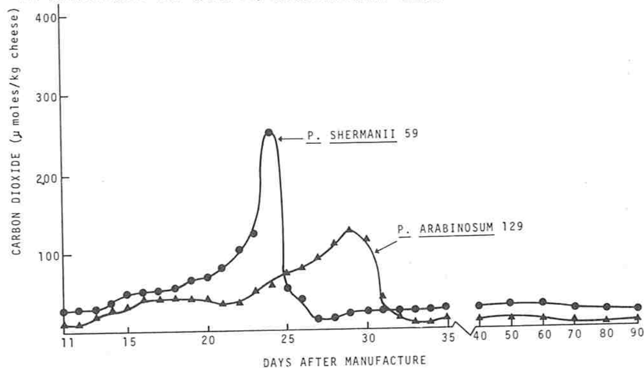

Figure 3. Carbon dioxide production in single lots of Swiss cheese made with *P. shermanii* 59 and *P. arabinosum* 129.

TABLE 3. NUMBERS OF PROPIONIBACTERIA PER GRAM OF SWISS CHEESE AT VARIOUS STAGES OF MANUFACTURE AND CURING

		Stage of manufacture and curing								
Strain no.	No. cheese lots	In press	Into warm room	Out of warm room	3-months cure			6-months cure		
		Avg count/g cheese × 10^{-7})								
P. freudenreichii 1	1	.35[a]	.80	44.	50.			8.5		
P. pentosaceum 5	1	5.1	4.6	24.	.73			.28		
P. peterssonii 35	1	.63	.24	250.	38.			9.2		
P. shermanii 47	1	.50	.50	560.	310.			160.		
P. shermanii 83	1	.41	.89	110.	87.			6.5		
P. shermanii 109	1	.98	1.1	100.	190.			85.		
						S[c]	C		S	C
P. shermanii 59	5[b]	.49	.93	180.	32.	0/5	3/5	5.1	3/5	5/5
P. arabinosum 129	5	.98	.60	48.	16.	0/5	0/5	4.5	1/5	1/5

[a]Figures for first six strains reported represent averages of duplicate determinations per test period.
[b]Strain no. 59 and 129 cheese lots were made, paired, from the same milk, at the same time under similar conditions. Duplicate platings were made for each lot.
[c]S = splits; C = checks.

TABLE 4. PROTEOLYSIS VALUES OF SWISS CHEESE DETERMINED AT VARIOUS CURING PROCESS STAGES

		Stage of cure						
		Into warm room		Out of warm room		3-months cure		6-months cure
Strain no.	No. cheese lots[a]	PI[b]	%[c]	PI	%	PI	%	PI
P. shermanii 59	4	11.106	8.89	10.118	14.18	8.680	2.75	8.438
P. arabinosum 129	4	10.740	11.41	9.514	11.66	8.404	2.96	8.157

[a]Cheese lots were made, paired, from the same milk, at the same time under similar conditions.
[b]PI = Proteolysis index (absorbency units of orange G bound/g cheese. Duplicate determinations were made for each cheese lot.
[c]Per cent change between proteolysis indexes.

greater than that fermented by P-129, reflecting a definite difference in their rate of metabolic activity at low temperatures.

Eight lots of cheese were analyzed for lactic acid at the end of 6 months of curing, and no detectable amounts of lactic acid were found in cheese made with P-59. Significant amounts (up to a maximum of 0.2%) of the acid were present in cheese made with P-129.

Proteolysis. The extent of proteolysis was determined in eight lots of Swiss cheese. These lots consisted of four pairs of cheeses. Each pair was manufactured at the same time and under similar conditions, varying only in the strain of propionibacteria used as starter. In all cheeses, the amount of dye bound/g of cheese decreased, indicating a breakdown of the protein as the cheese aged. No significant differences in the degree of proteolysis (calculated as percentage change) were observed in each lot between parallel vats of cheeses made with P-59 and P-129 (Table 4). Chi-square statistics were computed by comparing the proteolysis indexes, taken at four different periods during curing, of P-59 cheese with those of P-129 cheese. No correlation was found between degree of proteolysis, strain of propionibacteria used, or incidence of splits. Results support previous findings that proteolysis of Swiss cheese could not be correlated with splitting. The propionibacteria used did not seem to affect proteolysis as measured.

Fermentation of residual carbohydrate. Because starter cultures and many adventitious microorganisms can utilize lactose, glucose, and galactose, it was of interest to determine the amount of residual sugars in the cheeses after manufacture. These sugars provide fermentable carbohydrate substrates and could possibly play a role in production of "unwanted" CO_2 during cold-room curing. Hettinga et al. *(6)* were able to demonstrate that detectable amounts of these sugars were present in 1-year-old Cheddar cheese. Analysis of 10 lots of Swiss cheese manufactured in this investigation, indicated that low levels of glucose and lactose and relatively large amounts of galactose were present when the cheese went into the press. Glucose was approximately 0.1 of the amount of galactose recovered. Analysis of the cheese for sugars after removal from the press revealed the absence of glucose and the presence of only small amounts of galactose (0.1-0.3 mg/g cheese). This indicated that all the lactose, glucose, and galactose had been utilized in the press by the microorganisms present. This observation was indirectly confirmed when it was noted that there was an increase in the amount of lactic acid and a decrease in the pH of the cheese during pressing.

Production of carbon dioxide. Production of CO_2 by propionibacteria is responsible for development of eyes in normal Swiss cheese. Overproduction or underproduction of this gas during curing may produce a variety of defects in the cheese. Given the proper conditions, any microorganism that can produce sufficient gas could form eyes in Swiss curd. The production of gas does not necessarily terminate, however, once eye formation is complete. Because certain strains of bacteria are able to grow at low temperatures, it seems quite possible that their continued metabolism results in the production of additional CO_2 at low levels.

Formation of splits and checks in Swiss cheese is a cold-room phenomenon that may involve both physicochemical and biochemical changes in the cheese during finish curing. One important physical change that may have a direct bearing on the split defect is the loss in the elasticity of the cheese at low temperatures so that the continued production of CO_2 by low-temperature propionibacteria could create additional stress on the relatively inelastic body.

It is simple to accurately measure CO_2 production by pure bacterial cultures growing in broth. But currently available culture media cannot approximate the physical and chemical environment obtainable in the body of the cheese. For example, the complex buffering system in the cheese affords sustained microbial growth and enzyme action not possible in any broth system. Additionally, the cheese provides the proper degree of anaerobiosis for fermentation to continue. To study the actual production of CO_2 in cheese, it was necessary to develop a system that would collect all the CO_2 difusing from the cheese.

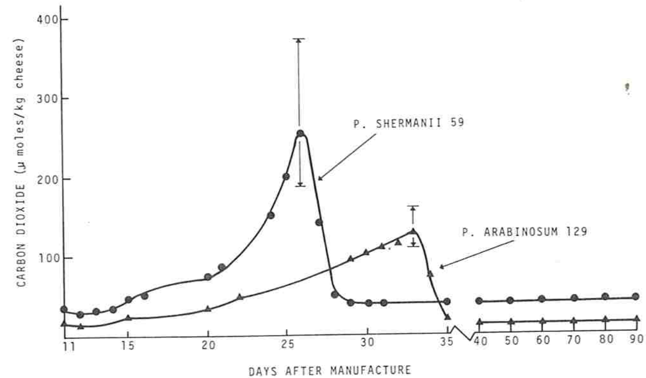

Figure 4. Average carbon dioxide production in Swiss cheese made with *P. shermanii* 59 and *P. arabinosum* 129 (eight lots each).

In developing this system, with the solubility of the gas in the cheese, the temperature, and the permeability remaining constant, it was assumed that the rate and amount of CO_2 diffusing from the cheese was directly proportional to the CO_2 tension within the cheese. This system was used to estimate CO_2 generated within several lots of cheese made with different *Propionibacterium* species and strains selected on the basis of their ability or inability to grow at low temperatures. Figure 3 illustrates the CO_2 production in two lots of Swiss cheese to which strain P-59 or P-129 had been added as starter. Figure 4 shows the average CO_2 production of eight lots of P-59 cheese and eight lots of P-129 Swiss cheese. Strain P-59 produced greater amounts of CO_2, developed eyes in the cheese more rapidly, and maintained a higher level of CO_2 production throughout the cold-room cure than did P-129. Figure 5 illus-

TABLE 5. COMPARISON OF FOUR DIFFERENT WRAPPING FILMS ON FOUR LOTS OF 3-MONTH-OLD COMMERCIAL SWISS CHEESE WITH EACH LOT MANUFACTURED WITH ONE OF FOUR STRAINS OF PROPIONIBACTERIA

Strain no.	Film	Oxygen permeability[a]	Comments on eye development[b]
P. arabinosum 129	Double-wound Saran	1	Heavyset, no splits or checks
	Saranex 29 duplex	1	Heavyset, no splits or checks
	Saranex 29 simplex	5	Heavyset, no splits or checks
	Polyethylene duplex	100	Heavyset, no splits or checks, moldy
P. shermanii 83	Double-wound Saran	1	Overset, many splits and checks
	Saranex 29 duplex	1	Overset, high incidence of splits
	Saranex 29 simplex	5	Overset, some splits and checks
	Polyethylene duplex	100	Overset, some checks, no splits, moldy
P. shermanii 59	Double-wound Saran	1	Overset, high incidence of splits
	Saranex 29 duplex	1	Overset, many splits and checks
	Saranex 29 simplex	5	Overset, splits and checks
	Polyethylene duplex	100	Overset, some checks, no splits
P. pentosaceum 5	Double-wound Saran	1	Overset, checks and splits
	Saranex 29 duplex	1	Overset, some checks, no splits
	Saranex 29 simplex	5	Overset, no checks, no splits
	Polyethylene duplex	100	Overset, no checks, no splits

[a]Approximate transmission rates of cc of oxygen per 100 sq in. in 24 h at 1 atm at 25 C.

[b]All four lots of cheese were graded "A" quality.

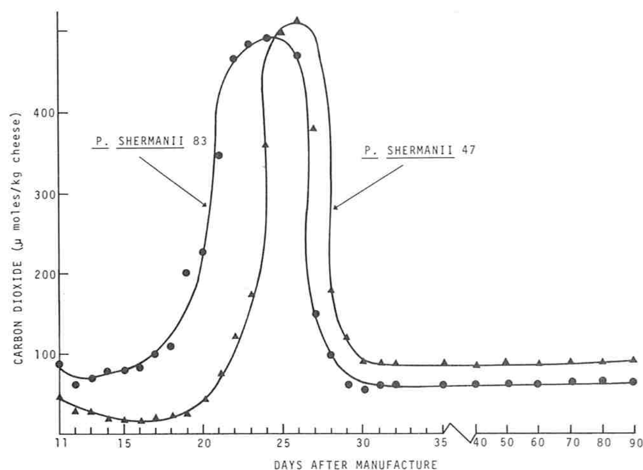

Fig. 5. Carbon dioxide production in single lots of Swiss cheese made with *P. shermanii* strains 47 and 83.

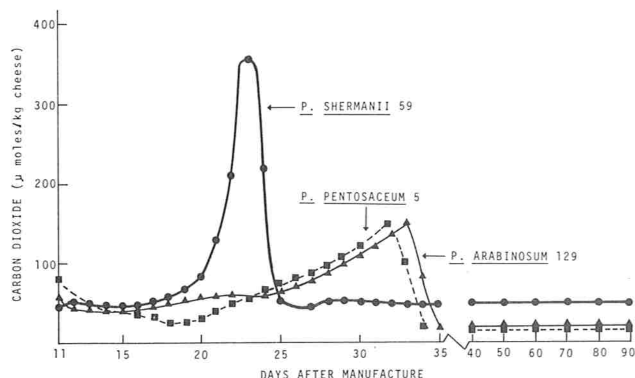

Figure 6. Carbon dioxide production in single lots of Swiss cheese made with *P. shermanii* 59, *P. arabinosum* 129, and *P. pentosaceum* 5.

trates the results for strains P-83 and P-47, which were selected for their ability to grow at low temperatures and for their capacity to produce large amounts of CO_2. Both demonstrated an ability to generate two or more times the amount of CO_2 that P-59 did, and the cheese made with both strains produced splits. This ability to produce greater amounts of CO_2 in cheese agreed with results obtained in pure culture. Although not noted elsewhere, all propionibacteria used in this study had been screened for gas production at different temperatures by growing in Sodium lactate broth in paraffin-petroleum jelly stoppered 50% cream-test Babcock bottles. The height of the gas bubbles collected in the bottle necks served as a rough indication of gas-producing ability.

Figure 6 presents the CO_2 production by P-5, P-59, and P-129 in Swiss cheese. Again, P-59 and P-129 exhibited their characteristic patterns for CO_2 production in cheese. Strain P-5, selected for lack of growth at low temperatures and poor ability to produce CO_2 in broth produced results similar to those obtained by use of P-129. Cheese manufactured with the P-5 strain did not show splits or checks, which supports the reliability of screening propionibacteria to be used as starters in Swiss cheese for inability to grow at low temperatures. Relatively greater CO_2 production by P-59 and other strains able to grow at low temperatures correlated well with a higher incidence of splitting and checking in cheeses made with these strains. This correlation again supports the hypothesis that continued augmentation of CO_2 pressure within the relatively nonresilient cheese in the finish cooler causes stresses leading to the formation of splits and checks.

In another experiment, the effect of four different wrapping films on the relative tendencies for splitting in commercial Swiss cheese was examined. Each of four lots of Swiss cheese were divided into four equal portions, and each portion was wrapped with a different film. Each lot of cheese was manufactured under identical conditions, except for the strain of *Propionibacterium* employed as starter. Wrapping films used were rated on a gas-transmissibility scale from impermeable to highly permeable. Results of the experiment are shown in Table 5. Cheeses wrapped in highly permeable film had less tendency to check or split as compared with cheeses wrapped in impermeable films. We had expected that, of the four strains used, P-59 and P-83 would produce cheese with the highest incidence of checks and splits and that cheese made with P-5 and P-129 would have little or no splitting tendencies. The results summarized in Table 5 were consistent with the hypothesis.

The more impermeable the wrapper, the less rind and mold formation that will occur. This is highly desirable, except that the more impermeable the wrapper (or the thicker and denser the rind in conventional wheel Swiss cheese), the greater will be the danger of splitting. Until a film is developed that is selectively permeable for CO_2, but limits the entrance of O_2; a compromise is necessary.

Several investigators *(8, 10, 16)* have demonstrated that strains of lactobacilli and micrococci may stimulate CO_2 production by propionibacteria. This could be another explanation why some cheeses split, whereas others made with the same cultures and under similar conditions do not.

It seems clear that there is no single factor or single strain of propionibacteria that may be responsible, but our work indicates that certain strains have a greater tendency to produce these defects. The presence of "wild" strains of propionibacteria in the natural flora of raw milk, and their ability to survive the usual heat-treatment processes, poses another problem. Bactofugation and (or) a hydrogen peroxide-catalase treatment of the milk do not seem logical preventative measures. Although not included in

this work, it is recognized that microorganisms other than propionibacteria may grow, metabolize, and produce CO_2 in Swiss cheese and could be of importance in the development of these defects.

In any event, because older cheeses generally split more readily than 2- or 3-month-old cheeses, temperature fluctuations must be avoided as the cheese ages. Small increases in temperature would induce a rapid increase in CO_2 pressure. If the cheese body is inelastic, splits are likely to occur because Swiss cheese is essentially supersaturated with CO_2 at temperatures below 5 C.

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STAPHYLOCOCCUS AUREUS IN COMMERCIALLY PROCESSED FLUID DAIRY AND NON-DAIRY PRODUCTS[1]

M. I. Sheikh[2] and L. O. Luedecke
Department of Food Science and Technology
Washington State University, Pullman, Washington 99163

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Abstract

Eight coagulase-positive staphylococci were isolated from 165 samples of commercially processed fluid dairy products and non-dairy whip. Seven of eight isolates tested for enterotoxigenicity by the microslide gel diffusion method did not produce enterotoxin A, B, or C, whereas one isolate produced enterotoxin C. None of the 8 isolates survived heating at 62.8 C for 30 min in raw low-count milk or in sterile milk.

Staphylococcal food poisoning attributed to the consumption of fluid milk is rare. Heat treatments applied to milk are undoubtedly partially responsible for this low incidence. However, occasional outbreaks do occur and some researchers have isolated *Staphylococcus aureus* from heat treated milk *(5, 9)*. Dabbah, et al. *(7)* using an inoculum of about 10^9/ml found that small numbers of S. *aureus* survived heating at 62.5 C for 30 to 120 min. Jackson and Woodbine *(11)* subjected 24-h cultures of enterotoxigenic strains of S. *aureus* to 60 C for 20 to 40 min and found survivors. Myhr and Olson *(13)* reported that 13 of 39 strains of micrococci isolated from milk survived pasteurization. In contrast, Heinemann *(10)* pasteurized 107 Grade A raw milk samples and found no surviving micrococci.

Most S. *aureus* found in raw milk probably come from mastitic infections. For example, Williams *(17)* reported that over 50% of the mastitic cows they examined shed staphylococci in their milk. Bell and Veliz *(1)* reported that 25 of 37 cultures of staphylococci isolated from raw milk produced enterotoxin. Casman *(3)* reported that eight of 190 staphylococci isolated frow raw milk produced enterotoxin A or B. Studies by Olson et al. *(14)* showed that 23 of 157 strains of staphylococci isolated from milk of mastitic udders produced enterotoxin, whereas 50 strains isolated from bovine mastitis samples were found to be nonenterotoxigenic by Casman *(3)*.

This study was done to determine the frequency of S. *aureus* in commercially processed fluid dairy products, to learn if these organisms produced recognized enterotoxins, and to find out if isolates from these products would withstand heating at 62.8 C for 30 min.

Experimental Methods

Isolation of Staphylococcus aureus

Five to 10 ml, from each of 162 commercially processed fluid dairy products and three non-dairy whips, were examined for staphylococci by spreading on prepared plates containing Staphylococcus 110 Medium (S-110) containing sodium azide that had been incubated at 32 C for 12 h. The amount of inoculum used for each plating varied from 0.3 to 0.5 ml per plate. Plates were incubated at 37 C for 48 h and then an additional 24 h at 22-25 C to promote pigmentation. Isolates found to be S. *aureus* were maintained on Trypticase Blood Agar Base (TBAB) slants at 5 C.

Testing of isolates for enterotoxin production

Each isolate was inoculated into Trypticase Soy Broth (TSB) after touching a loop to a stock slant. After 18 h of incubation at 37 C, a loopful of inoculum was transferred to a second tube of TSB and incubated 18 h at 37 C. A 1% (v/v) inoculum of the 18-h culture was inoculated to 100 ml of the medium described by Bergdoll et al. *(2)*. The inoculated medium was incubated 18 h at 37 C in a water bath shaker operated at approximately 55 revolutions per minute. Culture filtrates were prepared by the method of Bergdoll et al. *(2)* and tested for enterotoxin A. B, and C by the microslide gel diffusion method described by Casman and Bennett *(4)*. The antitoxin for enterotoxin D was not available.

Thermal resistance studies

Low count raw milk containing relatively low numbers of microorganisms and sterilized whole milk were used in the thermal resistance studies. Low count raw milk was obtained from individual cows by hand milking after carefully washing the udder and sanitizing the teat opening with 70% ethanol. The milk was held on ice until used which never exceeded 12 h. Sterilized whole milk was obtained by heating the low count raw milk at 121 C for 15 min.

A sterile 1,000-ml round-bottom, long-neck Pyrex flask containing 200 ml of low count raw milk or sterile milk was placed in a thermostatically controlled water bath at 62.8 C. When the flask contents were at 62.8 C, 1 ml of cell suspension was added to give a cell concentration of 10^3/ml. The contents of the flask were agitated continuously by a magnetic stirrer set at a speed that avoided foaming. After 30 min of heating, the flask was removed and contents cooled immediately in ice water to 5 C.

One milliliter of the cooled suspension in subdivided por-

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[2]Permanent address: Department of Food Technology, Agricultural University, Lyallpur, Pakistan.

TABLE 1. ISOLATION OF *Staphylococcus aureus* FROM DAIRY AND NON-DAIRY PRODUCTS IN CONSUMER MARKETING CHANNELS

Product	Number of samples examined	Number of *S. aureus* isolated
Low fat milk (2% fat)	20	0
Whole milk (3.5% fat)	66	3
Half and Half (11.5% fat)	46	2
Whipping cream (30% fat)	46	2
Non-dairy whip (30% fat)	3	1
Total	165	8

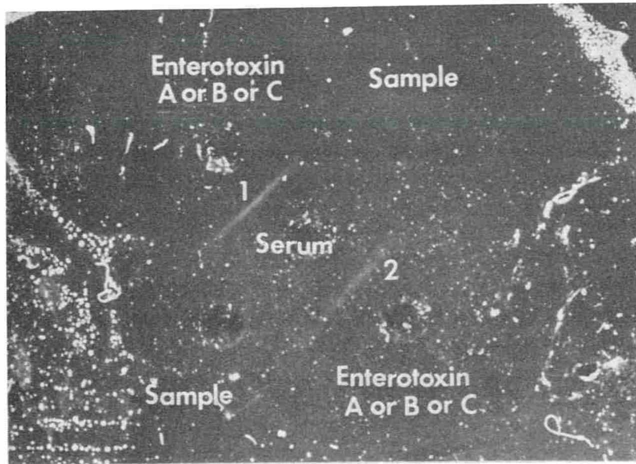

Figure 1. Reference lines of precipitate formed by the reaction of antigen and antiserum. Mixed antiserum, A, B, and C, was placed in the central well; enterotoxin A or B or C in peripheral wells as labelled. Sample from nonenterotoxigenic strains did not produce a line of precipitate.

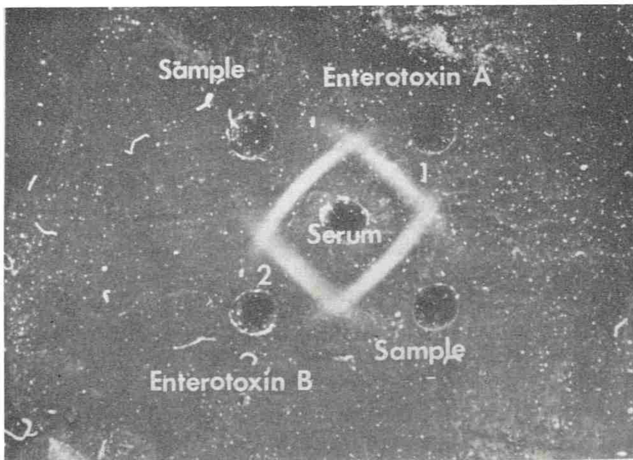

Figure 2. Samples tested for enterotoxin A and B. Enterotoxin A and B and sample were placed in the peripheral wells as labelled and the mixed antiserum was placed in the central well. The enterotoxin produced by one strain intersects reference lines 1 and 2, forming lines of "nonidentity" with the enterotoxin A and B reference lines.

tions was plated in duplicate on TBAB plates and incubated at 37 for 48 h. The remaining portion of the suspension was incubated at 37 C and at intervals of 8, 16, 24, and 48 h, 1 ml in subdivided portions were spread on TBAB pour plates and S-110 medium with sodium azide.

RESULTS

Isolation of Staphylococcus aureus from commercially processed fluid dairy and non-dairy products

Eight coagulase-positive staphylococci cultures were isolated from 165 commercially processed samples (Table 1). S. *aureus* was isolated from each type of product except the 2% milk. All three different commercial brands tested were found to contain S. *aureus*.

Using the binomial confidence limit tables, the data indicated that the level of staphylococci contamination was essentially the same for all types of dairy products examined and the level of contamination was essentially the same in the products processed under different brand names. Statistical analysis of the data for the non-dairy whip was not included because only a few samples were tested.

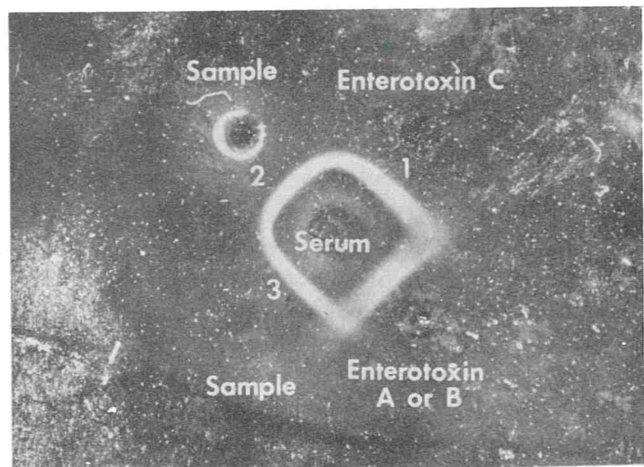

Figure 3. Serological specificity of enterotoxin. Reagents placed in wells as labelled. Sample containing unknown enterotoxin produced lines 2 and 3 which in coalescing with line 1 form a line of "identity" with reference enterotoxin C. In intersecting reference line of enterotoxin A or B, a line of "nonidentity" is formed.

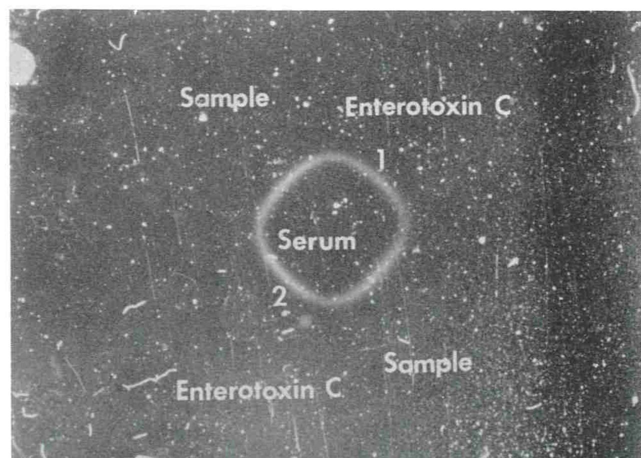

Figure 4. Identity of enterotoxin produced by one strain.

Enterotoxin production

Seven of the eight isolates did not produce enterotoxin A, B, or C, whereas one isolate produced enterotoxin C (Fig. 1, 2, 3, and 4).

Thermal resistance

Viable S. *aureus* cells were not found in low-count raw milk or in sterile milk that had been heated at 62.8 C for 30 min when S-110 medium containing sodium azide was used as the recovery medium. This was true for samples taken immediately after heating and after the heated cell suspensions were incubated 8, 16, 24, and 48 h at 37 C. When TBAB was used as the recovery medium, colonies of bacilli were obtained.

DISCUSSION

Isolation of Staphylococcus aureus

Coagulase-positive staphylococci were found in 4.9% of the samples examined. Comparable results were reported by Foltz et al. *(8)*, who found that 3.4% of the fluid dairy products examined contained coagulase-positive staphylococci. Presence of S. *aureus* in dairy products could result in pathological levels of enterotoxin production if the level of contamination was sufficiently high and the temperature and the period of holding were conducive to S. *aureus* growth. There is no indication that enterotoxin was present in the samples tested in this study; however, one of the 8 strains of S. *aureus* isolated from the products examined was shown to produce enterotoxin C.

Since all samples were taken at the consumer outlet, the source of contamination can only be postulated. Post-pasteurization contamination, inadequate pasteurization, or survival during pasteurization are possible causes. The most important reservoir of S. *aureus* is generally recognized as the nose and the upper respiratory tract of humans. These organisms may normally be discharged from the nose and transferred from person to person by contaminated droplets. Therefore, dairy plant workers could be a reservoir of contamination. Also, S. *aureus* is a frequent cause of acute and chronic bovine mastitis that results in contaminated raw milk and, therefore, must be considered as a source of staphylococci in dairy products.

As shown by data in Table 1, one isolate was obtained from one of the three non-dairy whip samples examined. The number of samples examined is small, and it is unknown whether this is a true pattern of staphylococcal incidence in non-dairy products or merely a matter of chance. Non-dairy whip is a synthetic product and the chances of contamination would be high if contaminated ingredients are used. Also, the human source of contamination should be considered in synthetic product preparations.

Enterotoxin production

Casman *(3)* reported that eight of 190 staphylococci isolated from raw milk produced enterotoxins A and B. Olson et al. *(14)* found that 23 of 157 isolates from mastitic cows were enterotoxigenic while in this study one of eight isolates from pasteurized products was found to be enterotoxigenic. No evidence was found in the literature giving the enterotoxigenicity of coagulase-positive staphylococci isolated from pasteurized fluid milk products.

Thermal resistance

It has been reported *(6, 15)* that bulk raw milk produced commercially contains staphylococci in the approximate range of $0 - 10^3$ cells/ml. Consequently, a concentration of 10^3 cells/ml in raw and sterilized milk was used for the heat resistant studies.

The results show that eight isolates tested did not survive pasteurization at 62.8 C for 30 min. These results are supported by those of other workers *(10, 16)*. Jackson and Woodbine *(11)* and Dabbah et al. *(7)* found survivors of S. *aureus* cultures heated to 60 C for 30 min and to 62.5 C for 30 to 120 min, respectively. The results obtained in this study using 10^3 cells/ml cannot be compared with those of Jackson and Woodbine *(11)* and Dabbah et al. *(7)*. Their inoculum contained about 10^9 cells/ml which may have resulted in a tendency to clump and increase the likelihood of finding survivors after 30 min at 60 or 62.5 C. Also, they used 24-h old cultures that probably were more heat resistant than the cells used in this study which were incubated for 18 h.

Moats *(12)* indicated that raw milk possesses a factor that offers thermal protection to bacteria but it is destroyed by autoclaving. When raw milk was used as the heating medium in this study, there were organisms that grew on TBAB plates but did not grow on S-110 medium containing sodium azide. These organisms were bacilli. The presence of bacilli could be attributed to contamination during sample collection and the germination of spores after the pasteurization treatment. Results of this study show that either the raw milk did not offer enough protection to S. *aupreus* during heating to survive pasteurization or if protection was offered, the bacilli did not allow the surviving S. *aureus* to grow.

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Appreciation is expressed to Dr. M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, Wis-

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REPORT OF THE COMMITTEE ON DAIRY FARM METHODS, 1972-1973

A. K. SAUNDERS, *Chairman*
A. E. PARKER, *Western Asst. Chairman*
J. B. SMATHERS, *Eastern Asst. Chairman*

In 1972-1973 we had 11 task committees working diligently to present members of the International Association of Milk, Food, and Environmental Sanitarians' membership with useful material.

ANTIBIOTICS, PESTICIDES, AND OTHER ADULTERANTS

M. W. JEFFERSON, *Chairman*

Antibiotics

Adulteration of milk supplies by antibiotics continues to concern the dairy industry and public health authorities. Control and use of antibiotics varies among areas of the country. Use of antibiotics for treatment of mastitis is under close supervision when used by a veterinarian, and is usually well supervised by the dairyman when used for such treatment. Cases have been reported where antibiotics were used to treat milking dairy animals for problems other than mastitis, and this has caused some adulteration. This has been particularly true when cattle were treated for breeding problems.

Education should continue to be emphasized and used to control and direct proper use of antibiotics. The Task Committee recommends that:

(a) Educational programs on use of antibiotics with dairy cattle should be continued and strengthened. Such programs should be directed to dairymen, practicing veterinarians, extension service workers, and other people dealing directly with milk producers.

(b) Antibiotics should be used as directed by the label, and all antibiotics should be properly labeled, giving clear and precise directions for use. Antibiotics, whether formulated by a veterinarian or a pharmacist, should carry a clear and precise label as to product and directions for use.

(c) Controls on a nationwide basis should be used to limit dosages of antibiotics, different types of antibiotics, and their use by dairy farmers.

(d) From time to time antibiotic residues have been detected in tissue of dairy animals slaughtered for meat. This has caused major concern to regulatory agencies responsible for meat inspection programs. Dairymen must be extremely careful with drugs and antibiotics, and use them in strict accordance with label recommendations and observe the withdrawal time before selling treated cows for slaughter. The most likely sources of antibiotics and drug residues in the cow's body are from injections and dry udder infusion products. Unless label directions for intramammary dry treatment specifically permit earlier release of treated animals, do not ship cows to market for 30 days following treatment. Treatments for diseases other than mastitis will cause tissue residues. Be sure you are informed of all treatments given to cattle. Cows given intramuscular injections of penicillin and dihydrostreptomycin must be held for at least 60 days following treatment to be safe.

Pesticides

Pesticides continue to be a problem in certain areas of the country; but indications are that controls placed on use and time of application have decreased the incidence of pesticide residues in milk supplies. Surveillance programs continue to play an important part in eliminating adulteration of milk supplies by pesticides. Compounds identified as polychlorinated biphenyls (PCBs) have been of concern to the dairy industry in many areas of the country. These substances are not classified as a pesticide, but are somewhat similar to DDT. Surveys have been conducted by the Food and Drug Administration and many state regulatory agencies to determine the incidence of PCBs in milk supplies. Indications are that these compounds have been in our environment for many years, and it will take considerable effort to eliminate PCBs from our environment.

Label directions continue to be very important in the use of pesticides. The Task Committee recommends that:

(a) Educational programs concerning the use of pesticides be continued and strengthened.

(b) Directions on labels of all pesticides be plain, simple, and distinct.

(c) Commercial applicators be licensed and controlled.

(d) Chlorinated hydrocarbon pesticides as well as PCBs be prevented from use where residues cannot be controlled:

Other adulterants

Use of automated equipment for cleaning and sanitizing milk contact equipment on dairy farms and in receiving and transfer stations as well as plants increases the possibility of adulteration of milk supplies by sanitizers and detergents. Continued vigilance is needed to establish and maintain control of the operation of such equipment to prevent adulteration of milk supplies. The Task Committee recommends that research be directed toward development of suitable test procedures for protection against chemicals in milk supplies.

Excess water in milk continues to exist in most areas of the country. Use of automated equipment to handle milk could contribute to adulteration of milk supplies with excess water. Use of plate and tubular coolers where the cooling medium is at a greater pressure than the milk could cause adulteration if leakage occurred. Indications are that tubular and plate coolers will be used much more in the future because of cooling requirements, and use of bulk holding and cooling tanks not designed for present requirements. Adulteration from excess water, chemicals, and microorganisms is possible unless proper controls are maintained.

It is recommended that:

(a) Serious attention be given to tubular and plate coolers that are used as a partial or complete cooling facility on dairy farms.

(b) That the dairy producer be educated as to use of external type cooling facilities in conjunction with farm bulk milk cooling tanks.

The Task Committee feels that clarification is needed among the federal agencies as to responsibility for pesticides, antibiotics, and other adulterants of milk. Changes have occurred on the federal level which have eliminated certain programs from one department and placed similar responsibility in other departments. The pesticide program, which formerly was a part of the U. S. Department of Agriculture and the Food and Drug Administration, is now supervised by the Environmental Protection Agency. The water supply and pollution program which existed in the Department of Interior is now a program in the Environmental Protection

Agency. Waste problems that were a concern of the U. S. Public Health Service are now dealt with by the Environmental Protection Agency. In view of these changes it is recommended that the Farm Methods Committee consider having someone from the Environmental Protection Agency as a representative on the Committee.

CLEANING AND SANITIZING OF FARM MILK EQUIPMENT

JAMES WELCH, *Chairman*

Pursuant to the 1972 Committee Report, the following recommendations are made:

1. Water usage requirements for pipelines
   A. Vacuum circulation systems

2-inch lines	3-inch lines	4-inch lines*
1/2 gal/10 ft	1 gal/10 ft	1-3/4 gal/10 ft

   In addition, allow 10 gal of water for wash vat. If weigh jars are used in conjunction with system, allow 1/2 gal of water for each jar.
   *Note: Allow 18 gal of water for wash vat when using 4-inch lines.
   B. Pressure circulation

2-inch lines	3-inch lines	4-inch lines
1-3/4 gal/10 ft	3-3/4 gal/10 ft	6-1/2 gal/10 ft

   Allow 10 gal of water for wash vat when 2-inch and 3-inch lines are to be cleaned and 18 gal of water for vat when cleaning 4-inch lines.
2. Air injection systems
   A. Mechanically controlled air injection system be installed providing a minimum of 10 slugs/min.
3. Water heating facilities
   A. A commercial type heater should be installed for CIP cleaning because of greater hot water requirements.
   B. Capacity of heater should be three times greater than one cycle requirement; i.e., if 25 gal are required for wash cycle, heater should be 75 gal in size.
   C. Separate facilities should be provided for heating of udder wash water and general milking parlor use.
4. Pre-coolers
   A. When tubular type coolers are in use, washing can be done in conjunction with pipeline cleaning.
   B. Plate type coolers should be washed as a separate circuit.
5. Temperature maintenance
   A. Temperature loss should not exceed that of recommendations made by the manufacturer of cleaner being used. This should be based on a 10-min wash cycle.
   B. If the temperature can not be maintained for the 10-min period, an auxiliary heater should be installed.
6. Temperature recording devices
   A. When enclosed tanks are to be cleaned in place (1,000 gal and over), a temperature recording device should be installed.
   B. If pipelines are 2 inches in size and over, consideration should be given to the installation of a temperature recorder.
   C. Consideration should be given to require fittings in the system to accommodate installation of recording devices.
7. Automatic cleaning equipment
   A. When pre-rinses are programmed into an automatic cleaning device, initial rinse temperature should be no higher than tepid.
   B. A complete educational program relative to operation of the unit should be conducted at time of installation.
   C. Establish a preventive maintenance program for all automatic equipment.
   D. Require water supplies to be of adequate volume and pressure.
8. Vacuum systems
   A. The vacuum pump or reservoir should be installed so that the operator has easy access for maintenance. This includes servicing of the unit, inspection, and cleaning. The area should have floor drain facilities.
   B. Consideration should be given to installation of glass or transparent vacuum lines for inspection of cleanliness.
   C. The vacuum system should be included as a part of the milking system and sanitation of the entire system be handled accordingly.

The Committee on Recommended Procedures for the Welding of Stainless Steel Milklines on Dairy Farms corroborated in these recommendations.

EDUCATION

VERNON D. NICKEL, *Chairman*

The Educational Task Committee of the Farm Methods Committee has the never ending task of making new educational material available to our membership through the Journal.

We have sent during the past year the name of much new material and where it can be obtained to Dr. Marth for your information.

We have added to the list a good selection of slide material and where it may be obtained. We have just recently obtained a list of new movie films which may be used by our membership.

The Educational Committee has continued to increase its efforts to gather this material and sincerely feels that by adding slides and movies the Committee has made an additional contribution to the Dairy Farm Methods Committee.

PLASTICS

BERNARD M. SAFFIAN, *Chairman*

Since 1971 this Committee has studied the potential effects that a phosphate ban on dairy cleaners would have on cleaning effectiveness and damage to plastics. If phosphates are banned in dairy cleaners, substitute systems may require higher alkalinity in the form of caustic content and higher cleaning temperatures. Both modifications would reduce the useful life of most plastics, especially flexible vinyl used as milk hoses.

One major cleaner manufacturer has been experimenting with a new product that affords satisfactory cleanability with no damage to plastics. However, tests are not extensive and no firm conclusion can be made at this time. Manufacturing cost is higher.

Another major manufacturer of cleaners and dairy farm equipment claims that banning of phosphates will have a negative effect on the ability to properly CIP dairy equipment.

A survey was made of legislation which bans phosphate in cleaners. No states ban phosphate in dairy cleaners; two counties have bans which do not appear to exempt industrial and institutional applications; eight cities or towns have bans. These county or city ordinances appear to concentrate on detergents purchased in those localities and probably are not

aimed at cleaners used on dairy farms, especially where effluent is not discharged into public sewer systems.

A table of phosphate legislation as of May 15, 1973, is attached.

TESTING FOR CLEANLINESS OF MILK PRODUCTION

MICHAEL H. ROMAN, *Chairman*

One of the important criteria for good milk quality is that the milk be produced clean and that it be produced by cows with healthy udders.

A maximum of 1.5 mg before-straining sediment by the mixed sample test method is recommended as indicative of clean milk production.

Too much emphasis is being placed by some dairymen on straining of milk instead of taking the necessary precautions to produce it clean. A mixed sample sediment test of a producer's milk as it is offered for sale is a good indicator of the degree of cleanliness of production since milk cannot be effectively cleaned by present straining methods so as to to cover up faulty milking practices.

The primary site of sediment contamination of milk is at cowside. Milk is free of sediment as it leaves the cow's teats. However, milk can become contaminated during the milking process. It is imperative that teats and adjacent udder portions be properly cleaned with a clean sanitizing solution followed by thorough drying. Single-service towels should be used. Improperly cleaned teats and wet udder surfaces allow for downward seepage of contaminants into the inflations and milk supply. Further, proper hygiene is most important in abnormal milk control.

In a study made to determine the degree of cleanliness of production, under sanitation compliance and noncompliance conditions, two groups of producers were selected. Those in group 1 were generally in compliance in that cows were clean and clipped, and preparation for milking consisted of proper washing and drying of teats and udders with single-service towels and with no reuse of the sanitizing solution. Those in group 2 were selected on the basis of mediocre sediment test records at the milk plant, and milking time investigations showed deficiencies in cow cleanliness and in teat and udder preparation for milking. In some instances no teat or udder cleaning whatever occurred before milking.

A sediment test was done on each bucket of milk before straining. Milk samples were collected at milking time but tests were not made until all of the milking had been completed. Group 1 consisted of seven producers and group 2 of eight.

The following is a tabulation of results obtained from the two groups:

TABLE 2. RESULTS OF SEDIMENT TESTS ON MILK FROM TWO GROUPS OF PRODUCERS

Sediment Grade/gal. basis	Group 1	Group 2
Total samples tested	258	285
Less than 1.5 mg	84.3%*	51.9%
1.5 - 2.9 mg	14.5	25.6
3.0 mg	0.6	8.8
More than 3.0 mg	0.6	13.7
% of samples resisting filtration-high abnormal milk reaction	6.5	21.3

*Approximately 85% of these tests were at less than 0.5 mg level.

Conclusions:

(a) Production of milk which is practically sediment-free before straining is attainable if proper preparation of teats and udder is practiced and other precautions observed.

(b) A milking time investigation should include some before-straining tests. A combination sample of any four cows should be $<$ 1.5 mg/gal. Otherwise, the test indicates that improvement in practices is needed.

(c) Reasonably clean milk from any individual cow which fouls the test disc bears further investigation as such milk is likely to be abnormal.

(d) Producers with sediment test problems should be encouraged to monitor their own milk cleanliness. Standard sediment test discs should be used for this testing. A mixed sample test as small as 4 oz of milk passed through a 0.2-inch diameter test disc can be used for such monitoring.

RECOMMENDED PROCEDURES FOR WELDING STAINLESS STEEL MILK LINES ON DAIRY FARMS

KENNETH HARRINGTON, *Chairman*

I. Welder-installer recommendations

A. Farm pipeline welder-installers should be approved by official regulatory agencies. In addition, farm pipeline welders should be able to demonstrate proper techniques to regulatory agencies and submit sample welds for approval.

B. Farm pipeline welder-installer's name and address should be submitted as part of the CIP installation application. The installation should not be started until tentative approval of the application has been received.

C. The welder-installer should notify the enforcement agency when the installation is to be started.

D. The farm pipeline installer should certify to the producer upon completion of the installation, in writing, that the installation meets 3-A Standards.

II. Regulatory agency inspection

A sanitarian need not be present when the installation is made, but the installer should make a sample weld at the start of each day's operation to be sure the welding device is operating properly. These welds should be labeled as to the approximate location of the welds for that particular day's operation. These samples should be left at site of installation for the supervising sanitarian's inspection.

III. Machine or hand welding are satisfactory methods of installation. The entire milk line is to be welded; however, an optional standard milk line union could be installed in the milk house to provide quick disassembly for inspection. There should be no restrictions in the pipeline which will impede the velocity. The receiving receptacle should not be welded into the pipeline and the milk pump should be so mounted that it can be easily inspected.

IV. Unsatisfactory welds from the standpoint of appearance as well as noncompliance with 3-A Standards should be replaced by the installer. The installation should be inspected as soon as possible after completion by the supervising sanitarian.

V. The following are considered unacceptable:

A. Grinding of welds
B. Welds sagging
C. Pin holes
D. Scorching or discoloration
E. Welds showing ridges which would impede or restrict solution flow.

VI. Borescope examination

The committee believes most sanitarians think that bore-

TABLE 1. DETERGENT LEGISLATION SUMMARY (FOR COMPLETE INFORMATION SEE INDICATED SDA LEGISLATION REPORT

	Intermediate phosphorus Limit				Total phosphate Ban			Labeling		Comments	Rpt.
Jurisdiction	% P	Eff. Date	Dish washer Exemp.	I&I Exemp.	Eff. Date	Dish washer Exemp.	I&I Exemp. ****	Phos-phorus	All Ingred.		WR/ ER
Arkansas *HCR 49	–	–	–	–	–	–	–	–	–	*Resolution-Encourages Congress to pass anti-phosphate legislation	
*HR 48	–	–	–	–	–	–	–	–	–		37**
Connecticut H.6699	8.7	2/1/72	yes	yes	6/30/73	yes	yes	2/1/72	no		47**
Florida H.3020 (amends Comm. Sub. H.191 & H.449)	8.7	12/31/72	yes	yes	–	–	–	–	–	Water Pollution Control Dept. authorized to adopt regulations to insure that no harmful detergents are sold after Dec. 31, 1972. Would preempt certain local ordinances.	33***
Indiana H.1032	–	–	–	–	4/12/73	yes	yes	yes	yes		
Iowa S.85	–	–	–	–	–	–	–	–	–	Provides that the Dept. of Env. Qlty. to issue phosphorus labeling requirements.	43***
Kentucky HR 60	–	–	–	–	–	–	–	–	–	Study Resolution	31***
Maine S.564	8.7	8/1/72	no	yes	–	–	–	no	no	–	47**
Mass. H.5596	–	–	–	–	–	–	–	–	–	Study bill.	92***
Michigan S.20	8.7	7/1/72	–	–	–	–	–	–	–	Pre-empts enactment & enforcement of future or existing ordinances	7***
Minnesota *H.1088	–	–	–	–	–	–	–	By posting	–	*Effective 6/7/71 Pollution Control Agency may prescribe maximum nutrient content in any cleaning agent or water softener. Letter out from Agency req. info.	53**
Montana HJR 33	–	–	–	–	–	–	–	–	–	Study Resolution	35**
New York A. 6963	8.7	1/1/72	no	no (1)	6/1/73	no	no (1)	yes	*Possible	*Not required at this time. (1) Some exemptions authorized (Dairy Cleaners)	72**

Oklahoma SR 61	–	–	–	–	–	–	–	–	Study Resolution	60**
Oregon HB 1336	–	–	–	–	–	–	–	yes	no Effective 9/9/71. Dept. of Environmental Quality to adopt rules & regulations re labeling which will require % P & recommend use level labels on all cleaning agents. Also all cleaning agents must be biodegradable.	60**
Tennessee SJR 83	–	–	–	–	–	–	–	–	Study Resolution - Encourages Fed. Gov't. to avoid restricting use of phosphates.	46**
COUNTIES										
Dade, Fla.	8.7	4/30/71	yes	yes	1/1/72	no	no	4/30/71	8/20/72 Dade County Pollution Control Hearing Board on 4/13/72 granted a one year extension (through 7/1/73) exempting dish washing compounds and I&I products from a Phosphate content limit or ban.	36** 64** 83***
Lake, Fla.	8.7	3/31/71	yes	yes	12/31/72	no	no	3/31/71	no No ban without leg. action.	67**
Orange, Fla.	8.7	5/31/71	yes	yes	12/31/72*	no	no	5/31/71	no *Repealed total ban 12/12/72.	41**
Lake, Ill.	8.7	6/1/71	yes	yes	6/30/72	no	no	6/1/71	no –	35**
Prince Georges, Maryland	8.7	1/1/72	yes	yes	1/1/74*	no	no	1/1/72	no *Amended ordinance signed extending effective date.	50** 100***
Erie, NY	8.7	4/30/71	yes	yes	1/1/72	yes	yes	no	no –	29**
Rockland, New York	–	–	–	–	–	–	–	–	– Resolution - Ask Congress to prohibit phosphate.	23**
Suffolk, New York	–	–	–	–	–	–	–	–	– Prohibit after 3/1/71 ABS, AS, MBAS, Non-Ionics.	4**
Temple City Calif.	–	–		–	–	–	–	–	– Retailer post listing phosphate content.	7**
Cocoa Bch Fla.	8.7	9/30/71	no	no	–	–	–	9/30/71	9/30/71 –	70**
Kissimmee, Fla.	8.7	1/1/72	yes	yes	12/31/72*	no	no	1/1/72	no Enforcement of O P ban suspended to 12/31/73 by City Commission 1/9/73.	8***
Macon, Ga.	–	–	–	–	–	–	–	–	– Resolution	50**

Aurora, Ill.	8.7	7/1/71	yes	yes	6/30/72	no	no	7/1/71	no	—	116***
Pinellas Co., Fla.	8.7	9/1/71	yes	yes	*	—	—	—	—	*repealed 12/14/71	
Chicago, Ill.	8.7	2/1/71	yes	yes	6/30/72	*no	*no	2/1/71	no	12 month extension granted for certain dishwasher & I&I products on 12/29/72 effective 1/1/73 to 12/31/73	62**
Chic. Ill. Metro San. District	—	—	—		—	—	—	—	—	Resolution	20*
Elgin, Ill.	8.7	7/1/71	yes	yes	12/30/72	no	no	7/1/71	no	—	21**
Elmwood Pk., Ill.	—	—	—		10/1/72	no	no	no	no	—	80**
Franklin Pk.,Ill.	8.7	7/1/71	yes	yes	*	no	no	7/1/71	no	*City Council repealed on 6/5/72.	50**
Hillside, Ill.	8.7	2/1/71	yes	yes	6/30/72*	no	no	2/1/71	no	*Board on 7/3/72 postponed enforcement pending further clarification.	81*
Harwood Hts., Ill.	8.7	7/1/71	yes	yes	6/30/72	no	no	7/1/71	no	—	80**
Joliet, Ill.	8.7	5/1/71	yes	yes	*	no	no	6/1/71	no	*City Council repealed on 6/6/72.	39**
Kankakee, Ill.	8.7	10/1/71	yes	yes	1/1/73	no	no	10/1/71	no	—	38**
Lombard, Ill.	8.7	7/1/71	yes	yes	*	no	no	7/1/71	no	*Village Board repealed on 9/18/72.	6***
Northlake, Ill.	8.7	9/1/71	yes	yes	9/30/72*	no	no	9/1/71	no	*Enforcement of O P ban suspended to 12/31/73 by City Council on 1/4/73.	115***
Highland Pk., Ill.	8.7	6/1/71	yes	yes	*	no	no	6/1/71	no	Total phosphate & NTA ban repealed 10/24/72.	29** 113*** 134***
Morton Grove, Ill.	8.7	1/1/73	yes	yes	*	no	no	7/1/71	no	*Total ban repealed 12/19/72.	103***
Niles, Ill.	8.7	7/1/71	yes	yes	12/30/72	no	no	7/1/71	no	Board of Trustees unanimously voted to REPEAL ordinance, 1/9/73.	35**
Park Forest, Ill.	8.7	5/1/71	yes	yes	6/30/72*	no	no	5/1/71	no	*Trustees (6/26/72 granted 6 mo. ext. from 6/30/72 to 1/1/73 for I&I products.	29**

Skokie, Ill.	8.7	6/30/71	yes	yes	6/30/72	no	no	6/30/71	no	Ord. repealed 5/22/72 to be considered by Board in 6 mo.	39**
Bridgton, Maine	–	–	–	–	6/1/71	yes*	yes*	–	–	*Ban laundry detergents only.	29**
Kennebunk-port, Me.	14 P04	6/2/71	no	no	–	–	–	no	no	*Ban: Nitrate & NTA.	24**
Naples, Maine	–	–	–	–	7/1/71	no	no	no	no	–	29**
Duluth, Minn.	–	–	–	–	–	–	–	–	–	Posting phosphorus content.	69*
Robbins-dale, Minn.	–	–	–	–	–	–	–	–	–	Posting phosphate at point of sale.	69*
Orene, Minn.	–	–	–	–	–	–	–	–	–	Posting phosphate at point of sale.	
Center Harbor, NH	–	–	–	–	6/1/71*	no	no	–	–	*Ban covers laundry detergents only, provided that other towns bordering on Lakes Winnipesaukee, Squam and Waukegan restrict in like manner.	87**
Gilford, NH	–	–	–	–	6/1/71	yes	no	–	6/1/71	Prohibits soaps & detergents containing phosphate in any substantial quantity.	35**
Laconia, NH	8.7	6/1/71	*	*	6/1/72	*	*	6/1/72	–	*Exemptions for essential uses may be prescribed.	45**
Moultonboro, NH	–	–	–	–	6/1/71*	no	no	–	–	*Ban covers laundry detergents only.	87**
Alton, NH	–	–	–	–	6/1/71*	no	no	–	–	*Ban covers laundry detergents only.	
Meredith, NH	–	–	–	–	6/1/71*	no	no	–	–	*Ban covers laundry detergents only, provided that the majority of the cities & towns in the lakes region take similar action.	87**
Sanbornton NH	–	–	–	–	6/1/71*	no	no	–	–	*Ban covers laundry detergents only.	29**
Baltimore, Maryland	–	–	–	–	–	–	–	–	–	Directs City Purchasing Agent to phase out high phosphorus detergents.	40**
Bayville, New York	–	–	–	–	–	–	–	–	–	ABS, AS, MBAS & nonionics prohibited after 3/1/71.	29**
Syracuse, New York	8.7	7/1/71	yes	yes	7/1/72	no	no	7/1/71	no	Ban presumed preempted.	37**

Akron, Ohio No. 923	8.7	2/1/71	yes	yes	1/15/73*	no*	no*	2/1/71	no	*Council (1/9/73) extended effective date for dishwasher & I&I products to 7/15/73.	81*
Akron, Ohio Res. #94-1971	–	–	–	–	–	–	–	–	–	Resolution - urges FTC Act in favor - labeling.	22**
Berea, Ohio	8.7	6/1/71	yes	yes	7/1/72*	no	no	6/1/71	no	*Will not impose until notice is given to the trade.	41**
Euclid, Ohio	8.7	8/1/71	yes	yes	12/31/73*	no	no	8/1/71	no	*Amended (12/4/72) to extend effective date for O Ban from 12/31/72 to 12/31/73.	43**
Fairlawn, Ohio	–	–	–	–	2/1/73	yes	yes	2/1/73	no		ER-54
Independence, Ohio	8.7	6/1/71	yes	yes	7/1/73	no	no	6/1/71	no		43**
Painesville, Ohio	8.7	7/1/71	no	no	–	–	–	5/1/71	no		39**
Stow, Ohio	8.7	1/16/73	yes	yes	1/16/73	no	no	1/16/73	no		ER-55
Willowick, Ohio	8.7	6/1/72	yes	yes	–	–	–	6/1/72	no		116***
City of Erie, Pa.	–	–	–	–	–	–	–	–	–	Point of sale posting of phosphate content.	54*
New Shoreham, RI	–	–	–	–	–	–	–	–	–	ABS, AS, MBAS & nonionics prohibited (4/1/71)	36**
Madison, Wis.	8.7*	7/1/71*	yes*	yes*	7/1/71*	no*	no*	no*	no*	*Only applies to city purchases.	33**
Milwaukee, Wis.	8.7	8/1/73	–	–	–	–	–	3/1/73	no	Prohibit pH of greater than 10.9 - based on 1% solution 3/1/73.	142***

NOTES:
*1970 Series
**1971 Series
***1972 Series
****Industrial and institutional uses (includes dairy)

scope examinations are too costly and should not be necessary if the above procedures are enforced. The borescope should be optional as a tool for regulatory officials.

CLEANING AND SANITIZING OF FARM PICKUP TANKERS

BOYD M. COOK, *Chairman*

The farm bulk pickup tanker, backbone of the milk collection system in the United States, is the vital link in the quality chain from producer to processor. This tank must be cleaned, sanitized, and maintained in a condition where it cannot cause any product deterioration during pumping or transporting. Responsibility for these actions must be clearly defined to reach this objective.

The Task Committee attempted to determine the preferred procedures and methods for cleaning and sanitizing the pickup tanker. The driver-receiver has the direct responsibility to be certain the pump, tank, and other milk contact surfaces are cleaned and sanitized before pumping milk into the truck. The Task Committee is aware that numerous opportunities for failure are present due to use of relief drivers, deliveries at various times of day and night, and various cleaning facilities provided at different dairies. Sanitarians have found that the following procedures have worked:

I. Driver responsibility before loading

A. The driver must be certain that the tank is completely clean and sanitized before departure for milk pickup.

B. If the unit is found to be deficient in cleaning, remedial action should be taken to clean, sanitize, and properly drain the unit before loading.

C. At times units are not used for several days. If a tank has been out of use for >2 days, it should be rewashed and sanitized before use.

D. The driver should keep the outside of the unit as clean as practical. Driver appearance should be clean and neat.

II. Responsibility of the driver and/or plant personnel following receipt of the load

A. The Task Committee finds that most successful programs clearly define responsibility for cleanliness of the tank interior, manhole area, pump, hose, and pump compartments. Plant personnel normally are assigned responsibility for cleanliness of the tank.

1. Plant personnel should clean the interior of the tank by hand washing or CIP cleaning. Care must be given to proper sizing of pumps, solution volumes, and piping to do an adequate cleaning job on tanks of various sizes and dimensions. Built-in nozzles or spray heads should be installed and maintained to assure proper distribution of cleaning solution within the tank.

2. Plant personnel should be required to hand wash CIP ports, manhole covers, gaskets, and other manhole accessories and the tank.

3. The health authority should establish a tagging procedure calling for the plant employee to sign that he has cleaned and sanitized his area of the truck.

B. The driver operator should be required to be certain the pump compartment, hoses, and pump are thoroughly cleaned and sanitized following each delivery.

1. The proper wrenches, brushes, mat, or soft container necessary to dissassemble and wash the pump, valves, hoses, and other accessories should be provided by the trucker so that he can be assured that they are available at each delivery location. The mat or soft container is to hold the parts of the pump to prevent damage.

2. The plant should provide a method of cleaning the pump hose by recirculation within a tank containing a hot cleaning solution of proper strength and temperature. This method of cleaning assures internal and external cleanliness of the hose and tends to increase the life of the hose. Where pump hoses are hand washed, the length should be restricted to a length that can be properly cleaned with a brush and stiff handle. A recording device should be installed to assure proper time and solution temperature. Stainless steel baskets may be provided to suspend pump parts in the solution tank to allow cleaning by circulation.

3. Following cleaning, pump parts should be assembled and sanitized by spray or circulation of a sanitizing solution. Responsibility for seeing that this is accomplished rests with the driver even though the requirement to do the work in some markets may be assigned to plant personnel.

4. The driver should be required to sign the truck cleaning tag indicating that the truck and equipment have been cleaned.

5. The driver must be certain that the sanitizing solution is completely drained.

III. Responsibility of the dairy plant reload station, transfer station, or receiving station

A. The station should furnish the necessary material and facilities to allow proper cleaning and sanitizing of all tankers.

B. The station should establish a routine quality control check to assure that the cleaning solutions are in compliance with the manufacturer's recommendations and that personnel are completing the required work. Recording thermometers on CIP lines may be used to compare cleaning cycles to the load receiving reports as a means of determining compliance with the plant cleaning procedures. Routine laboratory checks of cleaners and sanitizer strength should be a requirement.

IV. Responsibilities of the regulatory authority

A. Regulatory authorities should meet with plant management and haulers to establish equipment criteria and procedures that will minimize the problems in the market such as:

1. Standardize CIP systems in the area to be compatible with all truck units and adequate to handle the largest unit in operation.

2. Establish location requirements for truck CIP ports so that equipment at various plants will properly wash all truck units.

3. Establish a spot check procedure to determine compliance by all personnel involved.

4. Meet with plant management and truck operators to amend procedures to meet new problems with minimum inconvenience and expense to industry.

5. Work with all segments of industry to establish solution temperatures, solution concentrations, and facility requirements to make the best use of today's technology.

6. Require that detailed cleaning-sanitizing regimen be posted adjacent to clean-up pump or make-up tank at each cleaning location.

7. Require that pump compartment door gaskets and dust cover gaskets be maintained in good repair.

B. The Task Committee recommends that CIP cleaning systems include the following:

1. Valve sizes should be standardized in each marketing area.

2. Proper size CIP pumps for adequate coverage of tanker walls with cleaning solution. Manufacturer's recommendations should be followed to insure proper volumes and pressures in the cleaning system.

3. Initial rinse water should be discarded down the drain.

4. Proper positioning of the spray ball or tear drop spray head within the tank is critical for complete coverage. Open-

ings in spray balls or tear drop spray heads must be kept clean to prevent deflection of spray jets.

5. Burst wash and rinse cycles are recommended to allow the return pump to keep flooding of long tankers to a minimum and to increase turbulence of the cleaning and sanitizing solutions.

V. Pump cleaning procedure

The following cleaning procedure for the tanker pump and pump parts is recommended as a guide.

A. Disassemble and rinse the pump and parts.

B. Clean all parts using suitable brushes and cleaning solution or by recirculation.

C. Rinse with potable water.

D. Brush pump housing with an acceptable sanitizing solution.

E. Hand dip pump parts in an acceptable sanitizing solution and assemble the pump.

F. It is recommended that the entire pump compartment be cleaned and sanitized.

SAMPLING OF BULK TANK MILK

HELENE UHLMAN, *Chairman*

The following recommendations are made by the Task Committee on Sampling of Bulk Tank Milk.

(a) There should be uniformity throughout the country on sampling methodology and techniques.

(b) Study is needed on the length of agitation time before sampling for proper distribution in all parts of tanks—farm tanks, farm pickup tanks, transport tanks, and plant tanks.

(c) Since tank sizes, agitators, and speeds are such variables, each tank should carry information on the identification plate and/or the calibration chart showing the recommended agitation time for a homogeneous sample.

(d) Installation of an interval timer to control agitation will help solve problems of the quiescent time and milk level in the tank.

(e) A study should be made on the feasibility of a sampling valve on the discharge line. Consideration should be given to problems of accuracy in butterfat sampling and sanitation in bacteriological sampling from farm tanks.

(f) A study should be made to adequately and technically take samples from all types of tanks conveying milk. Extreme care should be exercised in construction to avoid cleaning and cooling problems.

(g) Development of universal sampling procedures should be encouraged. Agreement is needed between regulatory agencies and industry on type and size of sample containers.

(h) A study is needed on adequate temperature and handling techniques for control of samples in transit.

WATER TREATMENT AND PROTECTION

DALE TERMUNDE, *Chairman*

Water treatment and protection are becoming increasingly important to the Dairy Industry. The continued trend toward consolidation of dairying is closely correlated with installation of highly complex automated CIP dairy farm equipment having additional demands for consistently high quality water.

A review of water treatment and protection procedures, as prepared by this Task Committee, is furnished as suggested guidelines to help improve total water quality standards for dairy farm usage.

Bacteriological testing of dairy farm water supplies is established in the U. S. Public Health Service publication #229, *Grade "A" Pasteurized Milk Ordinance, 1965 Recommendations,* requiring bacteriological testing for new permits or upon any repair of a water supply. Additional bacteriological testing of water supplies at a specific designated time should be considered.

Knowledge of pH, degree of hardness, total mineral content, and similar factors is necessary for the determination of proper germicidal treatment and is essential for specialized conditioning of water to provide efficient and economical cleaning, compatibility with cleaners and sanitizers, to reduce corrosion, and to improve taste and odor.

Metering devices should supply water without having a submerged inlet. An air break between the inlet and the level of the tank overflow is most satisfactory. If an air space is not practical, an approved anti-siphon valve should be installed to insure against cross contamination of water supplies.

Removal of hardness minerals through the ion exchange process (water conditioning) can improve the effectiveness of cleaners and bactericides by eliminating mineral precipitation. Water conditioning equipment should be thoroughly disinfected before use to insure against contamination of the water supply.

Iron filtration may be feasible in water supplies with high iron content (over 0.3 ppm) to eliminate oxidation of iron caused by hypochlorite sanitizing solutions. Iron filtration units should be disinfected before use to insure against contamination of the water supply.

Neutralization of acid water supplies (below pH 6.5) with chemical feed pumps or neutralizing filters has proven beneficial in reducing cleaner consumption and increasing the life expectancy of water piping and plumbing fixtures.

Sand filtration is important to eliminate clogging of water inlet screens, for effective cleaning ability of equipment, and to prevent abrasive action on milk contact surfaces. Coarse sand removal may normally be accomplished through the centrifugal process. However, colloidal sand particles require cartridge or media filtration.

The most common method to control bacterial contamination of farm water supplies is chlorination. Many factors must be considered if this system is to operate successfully.

(a) Chlorine demand in raw water supplies may vary greatly, resulting in increased levels of chlorine residual which may be objectionable to taste and odor.

(b) Sufficient contact time is necessary for maximum killing efficiency. The capacity of retention tank is important.

(c) Chlorine residual must be checked and equipment inspected and serviced on a regular basis.

(d) Highly alkaline and extremely cold water supplies may require increased levels of chlorine residual.

(e) Hard water supplies may require additional maintenance to prevent the clogging of injection fittings and check valves.

Super chlorination followed by dechlorination (on drinking water) is the most widely recommended on farm use treatment.

Additional research is suggested to determine if iodine residuals may prove practical for germicidal teatment of water supplies.

Ultra-violet sterilization of farm water supplies is gaining in consumer acceptance. However, there are many limiting factors determining its total effectiveness. The USPHS has written criteria for specific installation and operation. Turbidity, iron content, and water color are important factors in determining effectiveness of this unit. Periodic disinfection of the water distribution system may be required, since ultraviolet treated systems have no residual action.

Present pasteurization systems for bacterially contaminated water supplies have proven uneconomical for farm application.

High quality water, available in quantity, has become a requirement for installation of highly automated CIP dairy farm equipment. Water treatment and protection of farm water supplies, therefore, greatly increases in importance.

Water sources for milkhouse use should be properly constructed and protected. However, where such action fails to yield a potable supply, properly engineered water treatment equipment should be installed and used. Since there is growing evidence that contamination of drinking water is a factor in the spread of livestock disease, adequate treatment of such waters should be provided to destroy organisms or chemicals that might affect the health or production of farm animals as well as that of the milk supply.

The National Academy of Sciences is presently preparing a report, *Quality Criteria For Water To Be Used For Agricultural Purposes*. This report is to be presented to the Environmental Protection Agency during 1973. Additional research to better define the relationship between quality water supplies and milk supplies is required so that this new information may be brought to the attention of the dairy industry.

The Task Committee on Water Treatment and Protection recommends that the following reference material is for review.

(a) Water Quality Criteria. 1968. Report of the National Technical Advisory Committee to the Secretary of the Interior —Federal Water Pollution Control Administration, Section 4, "Agricultural Uses."

(b) Manual of Individual Water Supply Systems—U.S. Public Health Service publication #24.

(c) Water Supply and Plumbing Cross Connection, U. S. Department of Health, Education and Welfare—Public Health Service.

(d) Rural Water Supplies, circular #145, University of Vermont.

(e) Evaluating Water Treatment Methods, H. V. Atherton, University of Vermont.

ANIMAL WASTE DISPOSAL

RUSSEL E. LOCK, *Chairman*

Over the past 2 years, the Animal Waste Disposal Task Committee has been involved in accumulating information and in establishing its role in the development of environmental guidelines directed at disposal of dairy farm animal wastes.

In 1972, through assistance of the National Milk Producers Federation, information was distributed to the Task Committee on the following: *(a)* model State Feedlot Environmental Control Act, *(b)* questionnaires from Environmental Protection Agency, *(c)* uniform terminology for rural waste management, and *(d)* copies of all available state regulations.

It was obvious in reviewing this information that governmental agencies responsible for regulating animal waste disposal are not the same regulatory officials who have traditionally administered the pasteurized milk ordinances.

In October 1972, a new Federal Water Pollution Act was enacted into law. The committee subsequently offered a resolution to members of the International Association of Milk, Food, and Environmental Sanitarians that outlined the main provisions of this act as it related to agriculture.

This resolution was published in the *Journal of Milk and Food Technology* and a letter was sent by our president, Mr. Walter Wilson, to William D. Ruckelshaus, Administrator of EPA, Washington, D. C. offering consultation service of the IAMFES. In addition, we resolved as members of the International Milk, Food, and Environmental Sanitarians that we offer our assistance and cooperation to area EPA officials.

In recent months, a concerted effort has been made to accumulate information concerning area recommendations and regulations on animal waste disposal. Several outside experts were contacted to secure this information and we wish to acknowledge and thank the following for their assistance: George M. Turner, University of Kentucky; Darrell O. Turner, Washington State University; E. O. Olson, University of Nebraska; Roger A. Nordstedt, University of Florida; Parker Pratt, College of Biological and Agricultural Sciences; Robert M. George, Cooperative Extension Service; Donald W. Bates, University of Minnesota; James S. Boyd, Michigan State University; James W. Crowley, University of Wisconsin; W. R. Jenkins, USDA-ARS Federal Extension Service; R. O. Martin, Agway, Inc.; C. M. Christy, University of Missouri; and Charles E. Fogg, USDA—Soil Conservation Service.

Currently, a contract has been let by the Federal EPA Effluent Guidelines Division with Hamilton Standards to develop national guidelines for animal waste management and on May 3, 1973 a proposed order establishing minimum effluent guidelines was published in the *Federal Register*. These guidelines establish that all dairy herds of $>$ 700 head of cattle will be required to obtain a permit. States will also be required to seek out cases of significant discharge of polluted water from agricultural sources. Each case, when established, will be required to obtain a permit, regardless of size.

The preliminary set of guidelines has been distributed for review and comment to all members of the Animal Waste Management Committee. Since the EPA will utilize such guidelines to enforce certain conditions of the 1972 Federal Water Pollution Act (specifically concerning pollution discharge permits which will be issued to livestock operators including dairy farmers), the opinions and comments of this committee should have a considerable bearing on enforcement of this act.

Finally, we reiterate our previous recommendation that all members of the International Milk, Food, and Environmental Sanitarians contact local agencies now working on livestock waste management and pollution abatement programs, and that they offer their advice and assistance in establishing programs and policies.

LIAISON COMMITTEE ON UDDER INFECTION PREVENTION

L. A. SKEATE, *Chairman*

Duties of the Liaison Committee on Udder Infection as designated at the 1972 Farm Methods Committee meeting were as follows. It was our charge to solicit and collect all the teat dip labels from products offered for sale throughout the United States.

Response was good and the members of the committee contributed a great deal of time and effort to this assignment. Labels were collected, listed, and directed to the National Mastitis Council before February 1, 1973. There were in excess of 100 different types of teat dip labels collected.

Most labels were collected from 27 different chemical companies. We are certain, however, that we do not have the listing of all the teat dips available today.

A. K. Saunders, *Chairman,* The De Laval Separator Company, 5724 North Pulaski Road, Chicago, Illinois 60646

A. E. Parker, *Western Assistant Chairman,* 10818 N. E. Thompson Street, Portland, Oregon 97220

James B. Smathers, *Eastern Assistant Chairman,* Maryland & Virginia Milk Prod. Assn., Inc., 1530 Wilson Boulevard, Arlington, Virginia 22209

Dr. J. C. Flake, *Farm Methods Report Editor,* Evaporated Milk Association, 910 Seventeenth Street N. W., Washington, D. C. 20006

John Adams, National Milk Producers Federation, 30 F Street Northwest, Washington, D. C. 20001

R. L. Appleby, The De Laval Separator Co., 350 Dutchess Turnpike, Poughkeepsie, New York 12602.

William L. Arledge, Suite No. 506, Portland Federal Building, Louisville, Kentucky 40202

Henry V. Atherton, Department of Animal Sciences, University of Vermont, Burlington, Vermont 05401

Richard L. Ayres, Los Angeles Co. Health Dept., Branch Office, Tulare, California 93274

Sidney E. Barnard, Pennsylvania State University, 213 Borland Laboratory, University Park, Pennsylvania 16802

R. A. Belknap, Food and Drug Administration, Dept. of Health, Education and Welfare, 1090 Tusculum Avenue, Cincinnati, Ohio 45226

Phillip Bergner, Alameda County Health Department, 15001 Foothill Boulevard, San Leandro, California 94377

James A. Black, Oregon State Dept. of Agriculture, 635 Capital Street, Salem, Oregon 97310

A. Richard Brazis, Department of Health, Education and Welfare, Food Microbiology Branch, 1090 Tusculum Avenue, Cincinnati, Ohio 45226

Glenn R. Briody, Multnomah County Milk Sanitation Section, 104 S. W. Fifth Avenue, Portland, Oregon 97204

James Burkett, Northwest Iowa Milk Sanitation Unit, 3340 Stone Park Boulevard, Sioux City, Iowa 51104

Glenn Cavin, Cedar Valley Cooperative Milk Association, 1936 Hawthorne, Waterloo, Iowa 50704

Boyd M. Cook, Maryland Cooperative Milk Prod. Assn., Inc., 1717 Gwynn Avenue, Baltimore, Maryland 21207,

M. R. Cooper, Virginia Dept. of Agriculture & Commerce, Box 7, Broadway, Virginia 22815

Floyd M. Copenhaver, Kansas City Health Dept., 10th Floor, City Hall, Kansas City, Missouri 64106

Robert Dawson, Babson Brothers, 2100 South York Road, Oak Brook, Illinois 60521

Pat J. Dolan, Bureau of Dairy Service, California Department of Agriculture, 1220 North Street, Sacramento, California 95814

C. W. Dromgold, Milk Control Service, 1220 Carr Lane Avenue, St. Louis, Missouri 63104

William J. Ernst, Jr., Technical Service Department, Chemical Specialities Division, BASF Wyandotte Corporation, 1532 Biddle Avenue, Wyandotte, Michigan 48192

Harold L. Faig, Cincinnati Training Facility, Food and Drug Administration, 1090 Tusculum Avenue, Cincinnati, Ohio 45226

E. J. Fincher, Bureau of Food Inspection, State of Wisconsin Department of Agriculture, 801 West Badger Road, Madison, Wisconsin 53713

Clarence C. Gehrman, Dairy and Food Division, Washington State Department of Agriculture, P. O. Box 128, Olympia, Washington 98501

Charles R. Gilman, Associated Milk Producers, Inc., Southern Division, P. O. Box 7617, Houston, Texas 77007

Harry D. Gleason, State of Washington Department of Agriculture, Court House Annex, Box 708, Chehalis, Washington 98532

F. P. Godfredson, Kendall - Fiber Products Division, Walpole, Massachusetts 02081,

Buck Greene, Louisiana Cooperative Extension Service, Knapp Hall, Louisiana State University, Baton Rouge, Louisiana 70803

W. J. Harper, Department of Food Science and Nutrition, Ohio State University, 2121 Fyffe Road, Columbus, Ohio 43200

Kenneth Harrington, Babson Brothers Company, 2100 South York Road, Oak Brook, Illinois 60521

Keith A. Harvey, Environmental Improvement Division, Idaho Department of Health, Statehouse, Boise, Idaho 83707

M. W. Jefferson, Commonwealth of Virginia, Division of Animal Health and Dairies, 1444 East Main Street, Richmond, Virginia 23219

R. E. Lock, De Laval Separator Company, 350 Dutchess Turnpike, Poughkeepsie, New York 12602

Ben Luce, Dairy & Food Division, Department of Agriculture, P. O. Box 128, Olympia, Washington 98501

Clarence Luchterhand, Division of Health, Department of Health and Social Services, State of Wisconsin, P. O. Box 309, Madison, Wisconsin 53701

Omer Majerus, Universal Milking Machine Division, 408 First Avenue South, Albert Lea, Minnesota 56007

C. M. Mecham, Maryland and Virginia Milk Prod. Assn., Inc., 1530 Wilson Boulevard, Arlington, Virginia 22209

David E. Monk, Environmental Health Services, Department of Community Health, 1900 E. 9th Street, Wichita, Kansas 67214

Melvin Neff, Upper Florida Milk Producers Assn., P. O. Box 6962, 4851 Nolan Street, Jacksonville, Florida 32205

Vernon D. Nickel, St. Louis Department of Public Health, 416 Tenth Street, Crystal City, Missouri 63019

Vernal S. Packard, Jr., Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55101

Lester E. Peik, Alpenrose Dairy, 6149 S. W. Shattuck Road, Portland, Oregon 97200

Loren E. Pine, 1020 West Sixth Street, Ontario, California 91761

Mark L. Prescott, Safeway Stores, Inc., Milk Department, P. O. Box 275, Clackamas, Oregon 97015

R. G. Raup, The De Laval Separator Company, 5724 N. Pulaski Road, Chicago, Illinois 60646

Jim Reeder, Maryland & Virginia Milk Prod. Assn., Inc., 1530 Wilson Boulevard, Arlington, Virginia 22209

Richard Rintelmann, Klenzade Products, Division of Economics Laboratory, Inc., Osborne Building, St. Paul, Minnesota 55102

M. H. Roman, State of New York Department of Agriculture, 18 Eugene Street, Lowville, New York 13367

Robert J. Ryan, Bureau of Milk and Food Sanitation, State of New York Department of Health, 845 Central Avenue, Albany, New York 12206

Bernard Saffian, Norton Company, P. O. Box 1624, Stow, Ohio 44224

Bernard J. Scheib, West Agro Chemical, Inc., 267 Warren Avenue, Kenmore, New York 14217

Paul W. Scherschel, Associated Milk Producers, Inc., Tri-State Region, 8550 W. Bryn Mawr Avenue, Chicago, Illinois 60631

L. A. Skeate, The De Laval Separator Company, 5724 N. Pulaski Road, Chicago, Illinois 60646

Stephen B. Spencer, 213 Borland Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802

Richard Stucky, Sep-Ko Chemicals, Division of H. B. Fuller Company, 3900 Jackson Street N. E., Minneapolis, Minnesota 55421

Donald K. Summers, Department of Health, Education and Welfare, Food and Drug Administration, 513 W. S. Custom House, Denver, Colorado 80202

Dale Termunde, Babson Brothers, 2100 South York Road, Oak Brook, Illinois 60521

Alvin E. Tesdal, Dairy & Consumer Division, State Department of Agriculture, Agriculture Building, Salem, Oregon 97310

Harold E. Thompson, Jr., Milk Sanitation Branch, Dept. of

Health, Education, and Welfare, Food and Drug Administration, Washington, D. C. 20204

Leon Townsend, Kentucky State Department of Health, 275 East Main Street, Frankfort, Kentucky 40601

Helene Uhlman, Gary Health Department, 1429 Virginia Street, Gary, Indiana 46407

J. A. Vaughan, The De Laval Separator Company, 1815 Rollins Road, Burlingame, California 94010

R. J. Weaver, Associated Suburban Boards of Health, 75 East Lancaster Avenue, Ardmore, Pennsylvania 19003

James Welch, Klenzade Products, Division of Economics Laboratory, Inc., Osborne Building, St. Paul, Minnesota 55102

Robert L. West, Bureau of Dairy Service, Department of Agriculture, 2550 Mariposa Street - Room 3080

Harvey J. Wilhelm, Mountain Empire Dairymen's Association, Inc., 945 - 11th Street, Denver, Colorado 80204

G. J. Zivtins, Diversey Chemicals, 1855 South Mt. Prospect Road, Des Plaines, Illinois 60018

TASK COMMITTEES 1971-72 AND 1972-73

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AMERICAN CULTURED DAIRY PRODUCTS INSTITUTE 1974 CONFERENCE SET

The Annual Conference of the American Cultured Dairy Products Institute will be held September 9-10, 1974, at the Hilton Inn, St. Louis, Missouri.

This year's program will include general conference sessions as well as specific panel presentations relative to technical subjects and manufacturing procedures for cottage cheese, sour cream, yogurt and other cultured dairy products.

The ACDPI annual conference, the only one of its kind, will cover yogurt marketing, increasing shelf-life of cottage cheese, directly acidified products, nutritional labeling and a panel of consumers who will give their opinions of cultured dairy products.

In announcing the annual conference, Secretary C. Bronson Lane invited not only members of the Cultured Dairy Products Institute, but representatives from all cultured dairy products companies to attend the sessions and participate in the discussions.

The final program will be announced shortly and nonmembers of ACDPI should write the Institute office for further meeting information.

FLAVOR AND SHELF LIFE OF FLUID MILK[1, 2]

SIDNEY E. BARNARD
Dairy Science Extension
The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

Today's consumer determines the acceptance of milk by flavor and shelf life. Rancid, oxidized, and strong feed tastes of raw milk need to be eliminated. To prevent rancid and psychrotrophic flavor development, milk should be processed within 24 h of collection. Hot water sterilization of processing and filling equipment is essential. Product temperatures below 40 F at the filler extend shelf life. Proper handling during delivery and in stores is necessary to maintain good taste and keeping quality. Temperatures below 40 F, minimal fluorescent light and daylight exposure, and proper rotation are the key factors. Containers vary in their ability to protect the flavor and change in milk composition. Holding quality programs of 14 days at 45 F should be adopted by industry. Ten-day coding or open dating should be practiced by all processors. Regulatory agencies need to permit tests which will indicate shelf life. More emphasis must be placed on factors which determine consumer acceptability. These are flavor and shelf life.

Our present marketing system has made quality the key to fluid milk sales. We can no longer produce, process, and deliver milk to consumers within 24 h. For this reason we need milk which will have good flavor and shelf life for 10 days or more after processing.

Milk is usually collected from farms every other day and processed on a 5-day per week schedule. Some plants are closed on weekends. This means that some raw milk is 4 days old when processed.

More than 80% of all milk is sold wholesale. Delivery to supermarkets, neighborhood, and convenience stores may be two or three times a week. Store sales have brought other people into the handling process, that is, store personnel. This means of distribution has also lengthened the age of milk between the time of processing and consumption.

EDUCATIONAL PROGRAMS

We have had a Pennsylvania Milk Flavor Program for more than 9 years. Printed materials and slide sets have been prepared and used with producers, processors, distributors, and consumers. All of this has been a cooperative effort in the Northeast, primarily with David Bandler at Cornell. Copies of the materials are available and the flavor and quality handbooks and slide sets may be purchased.

Primary emphasis in the last 5 years has been with processing personnel. Recently this has shifted to store employees as we secure the contacts to reach them. Mr. Bandler is concentrating on handling of milk in schools in New York state.

QUALITY SURVEYS

Much information has been gained from purchasing milk samples at stores. In addition to observing handling practices; temperature, flavor, bacterial, and recently compositional determinations have been made. Our confidential judgments have been sent to the individual processors of most of the nearly 2,700 samples examined.

These samples were purchased at more than 500 retail markets and represented over 340 brands and processing plants. Samples have been iced, and evaluated within 24 h. Our three-member trained panel classifies the flavor as good to excellent, fair, or poor. The results for 1972 and 1973 are shown in the following tables.

TABLE 1. FLAVOR OF 532 SAMPLES DURING 1972

Criticism	Number	Percent
Good to excellent	211	39.7
Fair	204	38.3
Poor	117	22.0

TABLE 2. FLAVOR OF 337 SAMPLES, JAN.-JUNE, 1973

Criticism	Number	Percent
Good to excellent	140	41.5
Fair	98	29.1
Poor	99	29.4

A breakdown of the criticisms into off-flavor categories helps pinpoint the problems. Results for 1972 were similar to those of 1973.

TABLE 3. FLAVOR CRITICISMS OF 337 SAMPLES, 1973

Criticism	Number	Percent
Good to excellent	140	41.5
Strong feed	59	17.5
Strong cooked	24	7.1
Unclean	46	13.7
Oxidized	55	16.3
Rancid	13	3.9

This points out the continuing farm problem of feed tastes. This can be reduced by keeping silage and green chop away from cows for 2 h before milking. Of greatest concern should be the unclean and oxidized flavors. These are caused primarily by im-

[1]Presented at the 60th Annual Meeting of the International Association of Milk, Food and Environmental Sanitarians, Rochester, New York, August 13-16, 1973.
[2]Authorized for publication on October 19, 1973 as Paper No. 4556 in the journal series of the Pennsylvania Agricultural Experiment Station.

proper handling in stores. These account for most of the samples in the poor category. Oxidized and unclean are primarily responsible for the increasing incidence of poor tasting samples.

Temperatures of store purchased samples have shown a slightly increasing percentage of samples over 45 F. This is a primary factor in determining shelf life. Results of 250 samples during 1973 are shown in Table 4.

TABLE 4. TEMPERATURE OF 250 SAMPLES DURING 1973

Range	Number	Percent
40 F or less	84	33.6
41 to 45 F	105	42.0
Over 45 F	61	24.4

Nearly one-fourth of the samples were over 45 F. Yet, one-third were at the ideal temperature of less than 40 F. This shows it can be done.

OXIDIZED FLAVOR

The tallowy taste caused by light exposure accounts for the greatest percentage of samples with an objectionable taste. It is also a serious problem with school milk.

Oxidized flavor becomes more common as less opaque containers are used and exposure times to fluorescent lights in stores increase. Some stores now stock shelves at night further lengthening exposure time. The trend to lighter colored paper containers and relatively clear plastic bottles is an important factor.

The incidence of oxidized flavors in various containers shows a much higher percentage in blown molded plastic.

TABLE 5. INCIDENCE OF OXIDIZED FLAVORS

Container	Number	Oxidized	Percent
Plastic coated paper	254	28	11.0
Blown molded plastic	19	16	84.2
Glass	49	9	18.4
Plastic bag	15	2	13.3

It should be noted that milk in glass and plastic bags was purchased at farm retail stores. Most of these samples had been processed for <24 h. Results should not be compared with samples purchased from supermarkets in the other two container types.

Recent research at North Carolina and Pennsylvania State Universities has shown changes in milk composition of milk with an oxidized flavor. Significant changes in vitamin C and riboflavin content were noted. Milk in blown molded plastic and glass containers developed an objectionable tallowy taste in as little as 12 h. This compared with about 48 h for plastic coated paper.

Consumer preference studies show that the majority of people can differentiate between milk with a good taste and one which is oxidized. They prefer that which we call good over the tallowy taste.

Two consumer studies have recently been completed at Pennsylvania State University. In one, over 300 persons preferred the control sample over others exposed to fluorescent light for 12 or 24 h. Many were unable to differentiate between the two exposed samples. However, strong preference was shown for the unexposed sample.

In a second study over 300 persons were asked to rank a single sample. No preference was noted. However, since we conventionally judge products by comparison, this is not surprising.

PROCESSING PRACTICES

The trend of plants toward shutting down two successive days each week is detrimental to milk quality and flavor. Raw milk held from time of receipt on Friday, for example, until processed on Monday occasionally develops unclean and rancid flavors. There is no simple solution. When milk is held on farms for two days, it should be pasteurized on the third day. Both bacterial and chemical changes take place causing objectionable tastes.

There is increasing evidence that heat stable enzymes are produced from some organisms in raw milk. These may cause bitter, unclean tastes even though bacterial counts are satisfactory.

Most large plants use silo tanks which do not provide the flexibility of many smaller raw storage tanks. The problem seems to be where to unload fresh raw milk when no tanks are empty. The occasional practice of putting fresh milk on top of old milk and not emptying raw tanks each processing day causes quality problems. These are difficult to trace, but in a few cases have been very costly practices to processors.

Hot water sterilization of processing equipment is essential for 10 day shelf life. This should include all processing, pasteurized storage, and filling equipment. Temperatures of at least 175 F for 5 min at the discharge end are essential. In most plants this means starting with water of at least 190 F. Circulation and reheating with steam are usually essential to maintain proper temperature.

Chemical sanitizers are satisfactory for farm use and raw milk storage tanks. Unfortunately, they don't always kill all bacteria. Even one organism per container in pasteurized milk hastens spoilage. This is the reason for hot water, to destroy most bacteria which might later grow at refrigeration tem-

peratures. Hot water also heats assemblies which are not contracted by chemical sanitizers.

Milk temperatures should be below 40 F at the time of filling. This is in the container, thus requiring 37 F or less in the filler bowl. More glycol refrigeration sections are being added. This permits temperatures in containers of below 35 F for all except pints and half pints. These should still be below 40 F.

QUALITY ASSURANCE

Quality programs need to stress shelf life tests. Initially you may be holding samples collected from the filler line at 45 F for 7 days. Flavor and odor checks are usually sufficient. If you have a laboratory, you may wish to check coliform and Standard Plate Counts. Set your goals for a shelf life of 14 days at 45 F. Many plants now achieve this on a regular basis. They follow the suggestions mentioned earlier or an even more rigid program of plant sanitation and equipment sterilization. Don't conduct shelf life studies at 40 F. Higher temperatures are common during distribution, so results are not valid.

Many plants now use open dating. Usually this indicates a sell-by-date which is 10 days following the date of processing. The average age of store purchased samples in our survey varies between 4 and 5 days. This leaves the consumer an average of another 5 days to use it. Some plants indicate their fluid milk will keep for 5 or 7 days after the day of expiration.

All processors should use a code or date on containers to permit proper rotation. This is critical for store distribution, but is important to the consumer for home handling. Make delivery-sales personnel and store personnel aware of your method of coding or dating. We urge the industry to adopt a universal dating method, so consumers will understand it, and use the oldest milk first. Presently there is much confusion on the part of consumers. Many aren't aware dairy products are dated or coded.

REGULATORY

Some state and federal agencies need to make changes in their quality standards and methods of determining quality. The objective of protecting public health through rigid standards should not be altered. However, the emphasis on quality checks should be shifted from *as processed* to *as purchased*. Processors cannot be held responsible for quality as purchased at stores. However, checking will reveal the source of the problem. Then corrections can be made.

Consumers should be assured of good quality as they purchase milk. Open dating will not assure this, although some agencies apparently have been convinced this is the solution. Mandatory open dating was not adopted in Pennsylvania last fall. No evidence was presented at the public hearing to indicate it would improve quality. Proper handling was stressed as the key to assuring consumers of good quality and flavor.

The regulations covering retail handling of dairy products in Pennsylvania are effective as of August 14, 1973. They provide that all dairy products, except frozen desserts, shall be held at 45 F or below at all times after delivery to stores. They also provide that dairy products be protected from unreasonable exposure to contamination.

This brief regulation would appear to prevent delivery of dairy products to stores unless personnel were there to place them under refrigeration. Unreasonable exposure may include animals or sunlight. It would seem to require that dairy products be under refrigeration and not displayed in the aisle for sale. The essentials are there. However, it will take much time and manpower to inspect thousands of stores.

We have made copies of regulations available to retail associations. Our recommendations for store handling practices to comply with the regulations were included. They are as follows: *(a)* Place milk in a cooler immediately after delivery and maintain at 40 F or below. *(b)* Fill the dairy case in the refrigeration zone only. In most dairy cases this is one layer of quart or larger containers. *(c)* Don't block air circulation ducts and keep air temperature below 30 F. *(d)* Place no more than a 2-day supply in the dary case. Rotate the stock to sell the oldest first. *(e)* Maintain product temperature below 40 F for maximum shelf life. Every 5 F rise cuts shelf life in half. *(f)* Observe open dates and codes. Aim to sell milk within 3 to 5 days of processing. This leaves the customer 5 days to use it. *(g)* Never use the fluorescent lights in the dairy case as a "night light" for the store. This will cause a tallowy (oxidized) taste. Paper containers provide about 48 h protection, but blown molded plastic less than 12 h. *(h)* Refrigerate any returned product and notify the processor. They may want to check flavor and quality to determine the reason for the consumer complaint.

State regulatory agencies should permit use of other tests to determine potential shelf life. These would be in addition to the four bacterial tests every 6 months required by the Public Health Service. For example, these might be psychrotrophic or holding quality tests to satisfy a once monthly bacterial determination. Such changes could greatly lengthen

the shelf life of milk from dairies not now performing holding quality tests.

SUMMARY

We must aim for fluid dairy products which will have good taste and keeping quality at least 10 days after processing. It seems that the weak link has been store handling. In Pennsylvania, new regulations and educational efforts should strengthen this weak link. We must convince store personnel of the need for proper rotation, maintaining temperatures below 40 F, and minimizing light exposure.

I have stressed other steps which the dairy industry should take to assure shelf life and flavor. It does involve everyone—producers, processors, store personnel, and consumers. One weak link in handling dairy products causes reduction in keeping quality, loss of sales and lower consumption.

The important factors are flavor and shelf life when consumers use milk. We need to stress in all our efforts these factors which determine consumer acceptability—taste and keeping quality.

ACKNOWLEDGMENTS

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ADVANCE REGISTRATION OPENS FOR FOOD AND DAIRY EXPO '74

Advance registration for Food & Dairy Expo '74, to be held Oct. 20-24, 1974, at Dallas Convention Hall, is now open.

Forms are available upon request from Dairy and Food Industries Supply Association (DFISA) and will also be mailed to thousands of food and dairy processors worldwide in a folder describing the exposition.

The advance registration folder is available in two formats—English, for use in the U.S., and multilanguage, for overseas visitors. The multilanguage version is printed in French, German, Spanish and English.

Complete hotel reservation and additional Expo information will be sent to advance registrants.

Registration is free to food and dairy processors, public health officials, sanitarians, educators and students, laboratory and testing personnel and visitors from outside the U.S.

More than 235 exhibiting companies have reserved space for the 29th biennial exposition. Exhibits will consist of processing and handling equipment and components, container and packaging machinery and materials, ingredients, merchandising and refrigeration equipment and promotion, transport and delivery, services and supplies, and cleaning and sanitizing systems and materials.

For registration information write or call DFISA, 5530 Wisconsin Avenue, Suite 1050, Washington, D. C. 20015, telephone 301/652-4420.

3A UPDATES TANK, THERMOMETER STANDARDS

The first complete revision since 1960 of the 3A Sanitary Standards for farm cooling and holding tanks and the first total rewriting of the 1950 standards for thermometer fittings were given final approval by the 3A Sanitary Standards Committees at their spring meeting at Omaha May 7-9.

Both documents were updated to reflect technological developments for criteria, such as materials definitions and new fabricating techniques, and to conform with the current 3A standards format, according to Don Williams, secretary to the 3A committees.

The significance of the new thermometer fittings standards is reflected in two major areas. The new standard now encompasses all types of sanitary instrumentation, including pressure sensors and level indicators, and it has consolidated separate supplements issued since the first publication of the standard 24 years ago.

The new standards will be prepared for official signing and publication at the earliest opportunity. They will become effective one year later.

In other action, the 3A committees reviewed an agenda of tentative sanitary standards for dry product bins, conveyors, colloid mills, continuous blenders, ice cream freezers, and sanitary tubing. Further work will be conducted by processors and by the technical committee of Dairy and Food Industries Supply Association. All will be scheduled for action at the next 3A meeting.

PROBLEMS IN THE RECOVERY AND IDENTIFICATION OF ENTEROPATHOGENIC *ESCHERICHIA COLI* FROM FOODS[1]

Ira J. Mehlman, Nicholas T. Simon, Arvey C. Sanders,
and Joseph C. Olson, Jr.
Division of Microbiology, Bureau of Foods, Food and Drug Administration, Washington, D. C. 20204

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Abstract

During a recent outbreak of gastroenteritis associated with serogroup 0124:B17 of *Escherichia coli,* various problems complicated recovery and identification of the pathogen. Standard methods for *E. coli* were of limited value because of atypical behavior of isolates. Two modified recovery procedures have been presented. For rapid lactose fermenters, pre-enrichment in MacConkey broth with subsequent transfer to lauryl sulfate tryptose broth and incubation at 44 C is recommended. For slow lactose fermenters, pre-enrichment in nutrient broth with subsequent transfer to Mossel's enteric enrichment broth and incubation at 41.5 C is tentatively proposed. Isolation agars include Levine's eosin methylene blue for lactose fermenters and MacConkey agar for non-lactose fermenters. The merits of a direct streak are considered. To facilitate rapid differentiation of *E. coli* from closely related *Enterobacteriaceae* within 3 days a modified Lundbeck procedure is offered. Isolates are first screened for H_2S formation, indole production, arabinose fermentation, urease and ONPG-ase. Secondary characterization based on results of the indole and TSI reactions includes Voges-Proskauer test (22 C), lysine decarboxylase activity, KCN tolerance, and fermentation of adonitol, cellobiose, sorbitol, or glucose. Confirmation by gram-reaction nitrate reduction, and cytochrome oxidase activity is required to differentiate from members of other families. Critical factors of serological analysis are stressed. Prospects for future research are discussed.

When an outbreak of gastroenteritis associated with the consumption of soft-ripened cheeses occurred in November 1971 *(13, 21),* a program to develop methods for enrichment of *Shigella* had been in progress at the Washington headquarters of the Food and Drug Administration (FDA). After preliminary reports had suggested that serotype 3 of *Shigella dysenteriae* was suspect, our laboratory became involved in the project. None of the isolates from samples of the food or clinical specimens were confirmed biochemically and serologically as species of *Shigella* or *Salmonella,* although most of the other genera of *Enterobacteriaceae* were present. In the meantime other laboratories had re-evaluated their data on the basis of additional physiological reactions and had concluded that the bacterium was a member of serogroup 0124:B17 of *Escherichia coli.* Serological examination of cultures isolated in our laboratory confirmed this diagnosis. In addition, however, there was evidence that the potentially pathogenic serogroups 055:B5, 0112:B11, and 0125:B15 were also present in the product.

To confront this crisis Fishbein developed a tentative procedure to be used in FDA district laboratories for surveillance of enteropathogenic *E. coli* (EEC). Personnel were familiar with the rather specialized procedures required for serological identification because of an earlier project on *E. coli.* During collaborative studies involving various FDA and public health laboratories, questions were posed of such a probing nature as to necessitate a re-assessment of *E. coli* methodology. The questions concerned the following aspects: *(a)* adequacy of currently recommended temperatures for enrichment, viz., 44 C *(20),* 44.5 C *(19)* and 45.5 C *(8)*; *(b)* relevance of presently used media, viz., MacConkey *(20),* lauryl tryptose (LST) and elevated coliform (EC) broths *(8)*; *(c)* the ability of available procedures *(8, 17)* to recognize biochemically typical and atypical *E. coli* amid a mixture of closely related enteric bacteria; and *(d)* modification of the rather complex serological procedures to facilitate implementation into standard laboratory operations *(1, 4, 11).*

While facets of these problems had been examined before onset of the outbreak, it rapidly became apparent that a comprehensive approach to rapid enrichment and identification of these bacteria had not been developed. The "state of the art" was probably similar to that available for *Salmonella* in 1950.

The intention of this manuscript is to present the problems as they arose, to indicate our approach to their solution, and to stimulate enough interest in the subject to promote further effort.

Enrichment

During the outbreak some laboratories reported that direct streak of a 1:10 dilution of the food was at least as effective in the recovery of the pathogen as enrichment (for reasons discussed below). The standard method for *E. coli (8)* involves pre-enrich-

[1]Presented at the 73rd Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, May 6-11, 1973.

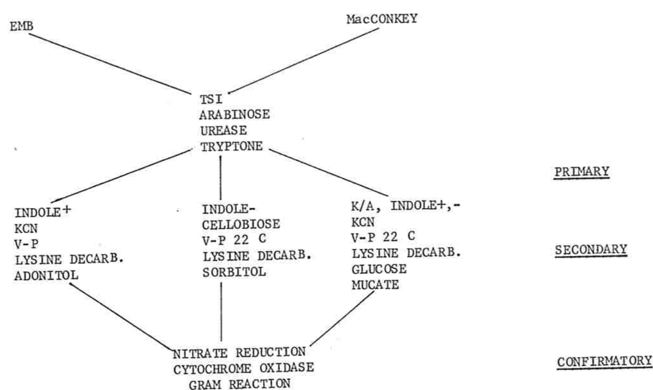

Figure 1. Biochemical-morphological characterization (modified from Lundberg's proposal)

TABLE 1. RECOVERY OF PRESUMPTIVE PATHOGENIC HUMAN SEROTYPES OF *E. coli*

Temperature	% Cultures recovered
44 C	87 (33/38)
45.5 C	76 (29/38)

ment in LST at 35 C with subsequent transfer to EC and incubation at 45.5 C for a maximum of 48 h. Material from the tubes showing gassing is streaked to Levine's eosin methylene blue agar (EMB). Colonies with the typical metallic sheen are characterized by using reactions discussed below.

Failure of recovery could be explained as follows: *(a)* Many of the isolates of 0124:B17 fermented lactose slowly, the lag lasting from several days to a month. These cultures generally did not produce gas from lactose. Thus, they could be overgrown during enrichment or could be completely overlooked unless a lactose fermenter was also present. *(b)* Some isolates failed to grow at 45.5 C. *(c)* Even if present in the enrichment the culture could be lost at the time of picking from EMB because of atypical appearance of the colony or insignificance with respect to the total microflora.

In a preliminary study, controlled temperature tests were made on strains isolated from feces of ill children under 3 years old to determine the capability of growth at the recommended values, 44 and 45.5 C, and the extent of recovery starting with small inocula. The approach involved recovery in LST and EC broths using the most probable number technique *(9)*. A positive result was represented by visible growth after 18 h of incubation. The control was recovery in trypticase soy broth at 35 C.

The parameter of inhibition was a 10-fold or greater difference between values obtained at 35 C and 44 or 45.5 C; lesser values were not considered significant. In several instances there was a 3-log (base 10) difference. The strains used in the preliminary study were isolated from feces of ill children under 3 years of age. Data, presented in Tables 1 and 2, show that at 45.5 C only about 3/4 of the cultures were quantitatively recovered whereas nearly 7/8 were recovered at 44 C. Also, with the possible exception of 0112:B11, temperature sensitivity did not appear to be confined to any serogroup but was encountered among members of 0124:B17, 0125:B15, 0126:B16, and 0128:B12.

No significant difference was observed between recoveries in LST and EC. However, at 44 C LST tended to support three times as much growth as EC. This finding favors serological analysis of the enrichment before streaking, which diminishes the labor entailed in subsequent examination. Based on these data, maintenance of 45.5 C for enrichment could be justified if the loss of about one-fourth of the strains could be accepted. Fishbein *(3)* found that some biotypes of *E. coli* (serological identity undetermined) were temperature-sensitive but was willing to accept this compromise because of the pronounced sensitivity of other *Enterobacteriaceae* and the absence of other reliable selective factors.

The second phase of the study utilized *E. coli* strains of both human and animal origin for which other workers had demonstrated pathogenic potential. Recoveries at 44, 44.5, 45, and 45.5 C were determined. The data, in Tables 3 and 4, indicate that at 45.5 C only about half of the cultures were quantitatively recovered, while at 44 C nearly 3/4 were recovered; 45 C was more inhibitory than 44.5 C. In contrast to the earlier results, pathogenic strains may be more temperature-sensitive than strains of unproven potential, although additional

TABLE 2. PRESUMPTIVE PATHOGENIC HUMAN SEROTYPES OF *Escherichia coli* RECOVERED

Recovered at 45.5 C				Recovered at 44 C but not at 45.5 C		Not recovered at 44 C	
Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains
018:B21	3	0111:B4	2	0112:B11	2	0127:B8	1
020:B7	1	0124:B17	4	0124:B17	1	0125:B15	2
026:B6	3	0125:B15	4	0128:B12	1	0126:B16	1
044:K74	1	0126:B16	4			0144	1
055:B5	2	0128:B12	3				
086:B7	1	0119:B14	1				

study would be required for corroboration. The significant finding was that even 44 C might be excessive for a third of the strains. Limited studies indicate that 42 C would appear to be the maximum value for these cultures, although additional selective factors would probably have to be considered to increase selectivity.

COMPOSITION OF THE MEDIUM

Since several strains of EEC ferment lactose slowly, the value of conventional lactose-containing media, such as MacConkey, LST and EC broths, might be questioned. This hypothesis was examined in a cultural interaction between a rapid lactose fermenter (an isolate of 0125:B15) and a slow lactose fermenter (an isolate of 144:K). Both were added to various media to give a final level of approximately 100 cells/ml. The mixed cultures were then incubated at 35 C for 18 h and streaked to EMB. Colonies were randomly selected and serologically characterized. The data are in Table 5. As hypothesized, the slow lactose fermenter was not recovered in LST and EC broths. Although slight differences were observed between nutrient, trypticase soy, G-N, and indole-nitrite broths, these media tended to preserve the initial proportion of both cultures. Thus other media would be required for enrichment of slow lactose fermenters.

Therefore, standard methods appeared to be unsuitable for recovery of some EEC biotypes. Because of the peculiarities of the cultures a multiple approach might be necessary, at least temporarily. This would include: *(a)* Direct streaking to both EMB agar (for lactose fermenters) and MacConkey agar (for non-lactose fermenters). This method will be successful only if the concentration of *E. coli* in the specimen is at least 3000/g and this population represents about 20% of the microflora. *(b)* Recovery of rapid lactose fermenters by pre-enrichment in MacConkey broth at 35 C for 18 h, enrichment in LST at 44 C for 18 h, and streaking to EMB agar. These media were chosen because of their tendency to minimize roughness and increase growth yield with the possibility for serological examination of the enrichment. *(c)* Recovery of slow lactose fermenters or temperature-sensitive biotypes by pre-enrichment in nutrient broth at 35 C for 6 h, enrichment in Mossel's EE (enteric enrichment) broth *(15)* at 41.5 C for 18 h, and streaking to EMB and MacConkey agars. Media were selected for their relative toxicity. Nutrient, trypticase soy, and indole- nitrite broths were unsatisfactory because of their non-selectivity; LST and EC were of little value on the basis of earlier data. G-N *(6)* was suggested by

TABLE 3. RECOVERY OF ENTEROPATHOGENIC *E. coli*

Temperature	% Recovery
44 C	70 (16/23)
44.5 C	70 (16/23)
45 C	63 (14/23)
45.5 C	52 (12/23)

TABLE 4. ENTEROPATHOGENIC STRAINS[a] OF *Escherichia coli* RECOVERED

Not recovered at 44 C
- 078:K:H11
- 07:K:H
- 0144:K:H (1624)
- 0115:K:H11
- 08:K87:K88ab:H19 (P307)
- P155
- H10407

Recovered at 44.5 C, not at 45 C
- 06:K:H16 (B2C)
- 339

Recovered at 45 C, not at 45.5 C
- 4042
- 334

Recovered at 45.5 C
- 08:K87, K88:H19 (263)
- 0148:K:H28 (B7A)
- 09:K:H (P16)
- 08:K87, K88ab:H (P18)
- 0147:K88ab, K89:H (P54)
- 0141:K85ab, K88ab:H (P99)
- 0A1:KA1, K88ac:H (P115)
- 085:K:H7
- 08:K87, K88ab:H
- 0143:K:NM (4608)
- E57
- PS274

[a]Designation in parentheses refers to identification number used in private collections.

TABLE 5. RECOVERY OF DELAYED LACTOSE FERMENTERS

Enrichment[a]	Final ratio[b] 0144/0125
Nutrient broth	3:2
Indole - nitrite	1:2
Trypticase soy	1:3
G-N broth	1:1
Lauryl tryptose	<1:100
EC broth	<1:100

[a]Cultures of slow fermenter 0144 and rapid fermenter 0125: B15 added to give initial counts of 80 and 120 cells per ml, respectively. Enrichments incubated 18 h at 35 C.

[b]Broths streaked to EMB agar; colonies selected at random and serotyped.

Hajna for enrichment of *Shigella;* it contains both glucose and mannitol as energy sources and citrate and deoxycholate as inhibitors for gram-positive organisms. Therefore it was discarded because of its toxicity at elevated temperature (possibly because of the large concentration of citrate). EE is similar to brilliant green lactose bile broth except that glucose is substituted for lactose.

This multiple enrichment approach is obviously untenable in a surveillance program for EEC. The search for additional selective factors, chemical and/or physical, deserves priority in future studies.

BIOCHEMICAL-PHYSIOLOGICAL CHARACTERIZATION

Various schemes are used to identify *E. coli.* In clinical laboratories enrichment is rarely used; specimens are streaked to EMB, and, after incubation, colonies with a metallic sheen are selected. Diagnostic tests include indole production, citrate assimilation (usually Simmons' agar), reactivity in EEC sera, and other tests, depending on the resources available. Methods of the Association of Official Analytical Chemists *(8)* and the American Public Health Association *(17)* used in food and water control laboratories specify enrichment followed by streaking to EMB or endo agar. Representative colonies are characterized by the IMViC set of reactions (indole, methyl red, Voges-Proskauer, citrate) and production of gas from lactose within 48 h at 35 C. Because of the potentially large number of serotypes in a specimen, at least 10 colonies are selected from each plate with suspected *E. coli.*

The problem in biochemical identification may be considered from three points of view: *(a)* philosophy underlying the taxonomic system, *(b)* framework of the diagnostic scheme and *(c)* cultures present in the material. The IMViC reactions and lactose fermentation were valuable where the main concern was with *E. coli* and *Enterobacter aerogenes.* Although difficulties were encountered, modifications had been accepted by several investigators. The modifications include the following: *(a)* The recognition of indole-negative biotypes of *E. coli* (Type II). *(b)* Difficulties in the citrate test. Freshly isolated cultures of interfering bacteria frequently give a negative result. After subculture they may give a position reaction. *(c)* The acceptance of anaerogenic and/or slow lactose-fermenting biotypes. *(d)* The tolerance of a 4- to 5-day period for the citrate and methyl red reactions.

Probably the most critical factor compromising the usefulness of the standard tests was the recent reorganization of the family *Enterobacteriaceae (1),* entailing recognition of additional genera and species, the flexibility of the taxa, and a revised concept of interrelationships. During this transition several problems became apparent: Additional differential tests were required; the relative importance of some standard reactions, e.g., methyl red, was minimized; distinctive positive behavior for some taxa, including *Escherichia* and *Shigella,* was lacking; and some keys placed undue reliance on exclusive reactions.

As an example of the situation that the worker may encounter, the following members of the *Enterobacteriaceae* were recovered from soft-ripened cheeses analyzed during the outbreak: *(a)* The pathogen, a member of serogroup 0124:B17, an atypical *E. coli* with respect to lactose fermentation. Some isolates were non-motile; others possessed flagellar antigens 30 or 32. *(b)* A potentially pathogenic culture, serotype 0125:B15:H11, a biochemically typical *E. coli.* *(c)* Biochemically typical *E. coli* not belonging to recognized enteropathogenic serotypes. *(d)* Several biotypes of *Enterobacter hafniae* with respect to IMViC reactions, lactose-fermented with production of gas. One biotype was − + − − and therefore was reported as *E. coli* Type II. Many isolates also gave agglutination reactions in 0112:B11 and 0124:B17 *E. coli* sera. *(e) Citrobacter diversus.* *(f) Citrobacter freundii.* *(g) Proteus vulgaris.*

E. coli could not be differentiated from the other enteric bacteria by the five standard tests. Undoubtedly, other workers had been confronted with a similar situation since 14-16 reactions have been recommended *(1, 4, 11, 18),* an obvious difficulty for the routine food microbiology laboratory.

A scheme used by H. Lundbeck at the Statens Bakteriologiska Laboratorium in Stockholm (personal communication) that appeared promising incorporated the indole, V-P, H_2S, urease, and ONPG-ase tests. However, it was unable to differentiate *C. diversus* and *E. hafniae* from *E. coli.* A modified version, outlined in Fig. 1, is proposed by the authors. It entails the following.

At the primary stage of screening, H_2S+, urease+, ONPG-ase−, non-arabinose fermenters are discarded after 18 h at 35 C. Secondary screening is dependent on results of the indole and triple sugar iron (TSI) agar reactions. Indole-positive cultures are tested for adonitol fermentation, indole-negative cultures for sorbitol and cellobiose fermentation. *C. diversus* ferments sorbitol and adonitol and produces indole *(2)* *E. hafniae* does not ferment sorbitol and does not produce indole *(1).* *E. coli* generally ferments sorbitol but not adonitol or cellobiose and usually produces indole *(1).* Additional secondary screening reactions include KCN-sensitivity, V-P (at 22 C), and lysine decarboxylation to provide a safety factor for atypical cultures. Isolates that would ap-

pear to be *Shigella* on the basis of the TSI reactions (alkaline slant, acid butt, no gas or H_2S) are tested in glucose and mucate broth. *E. coli* produces an acid reaction in mucate and usually ferments glucose with production of gas. Reactions of *Shigella (1)* in the above tests are identical to those obtained with *E. coli* except that arabinose fermentation is variable, gas production from glucose is negative, and mucate is usually negative.

Secondary screening tests are incubated for 2 days. It is suggested that the primary screening reactions be incubated for an additional 2 days at 22 C (concomitant with the secondary tests) to detect delayed reactions, and that the indole reaction be repeated at 72 h if negative at 18 h.

For cultures conforming to the description of *E. coli* on the basis of the above tests and giving satisfactory reactions in EEC sera the following confirmatory reactions are recommended: *(a)* gram stain: gram-negative, non-spore-forming rod; *(b)* nitrate reduction: positive; and *(c)* cytochrome oxidase: negative in contrast to *Aeromonas* and *Vibrio.*

Serological Identification

Most aspects have been already discussed in treatises by Kauffmann *(11)* and by Edwards and Ewing *(1)*. Adaptation of the standard procedures is contained in the method developed by FDA for use in its district laboratories *(14)*. Some aspects, however, deserve emphasis.

(a) Because of extensive relationships within the family *Enterobacteriaceae (1, 10, 22)* and with other gram-negative bacteria including members of the genera *Pseudomonas (23), Vibrio (12, 24), Neisseria (5)*, and *Hemophilus (16)*, and some gram-positive bacteria such as *Pneumococcus (7)*, it is advisable first to ascertain the identity of cultures by standard biochemical-physiological tests. This, however, may be impractical because of the time factor.

(b) Colonies from selective agar should be subcultured to blood agar base with incubation at 35 C for 18 h and subsequently at 22 C (or room temperature) for 24 h to enhance formation of capsular antigen (personal communication from F. Orskov).

(c) To determine the presence of capsular factor, slide agglutinations must be done both with O (contains factor to somatic antigen) and OB (contains factors to both somatic and capsular antigens) sera. Many cultures upon isolation lack detectable capsular antigen.

(d) Results of slide agglutination reactions must be confirmed by tube agglutination tests. Four titrations involving somatic and capsular antigens in O and OB sera are required.

(e) Motility must usually be enhanced by multiple passage in semi-solid medium because a third or more of the isolates may be non-motile. Motility gelatin infusion medium has been found to be satisfactory. If an isolate is motile, it is necessary to identify the flagellar antigen since within a given serogroup only a limited number of H groups are associated with illness. Quantitative titrations are generally unnecessary. Because of forensic implication it is necessary to identify the somatic, capsular, and flagellar (if motile) antigens. Because of the relative newness of the problem it is necessary to differentiate EEC from other serotypes of *E. coli* that may be closely related.

Conclusions

Some of the problems involved in the present effort to evaluate the incidence of EEC in food have been discussed. Because of the "atypical" behavior of some isolates, modifications of the enrichment medium and temperature have been necessary. A simplified biochemical scheme to differentiate *E. coli* from closely related members of the *Enterobacteriaceae* has been offered. Some of the difficulties involved in serological identification have been alleviated. The procedure has been offered to regional laboratories for evaluation.

Many problems that hinder implementation of a surveillance program require additional research. The following should be given immediate attention: *(a)* A search for additional selective factors to be used in conjunction with elevated temperature to increase specificity and sensitivity. *(b)* Minimization of roughness. A conservative estimate is that a quarter of the isolates are untypable. It is not unlikely that selective factors currently used, e.g., 45.5 C and bile salts, promote roughness. *(c)* Quality of commercial antisera. Although standard EEC cultures and sera generally available are of excellent quality, some modification of the system, involving preparation of B rather than OB sera, may be necessary to reduce the amount of labor. Some absorptions may be necessary because of the rather complicated interrelationships. For example, some isolates of 0144 gave reactions in 018:B21, 0119:B14, and 0124:B17 sera. For positive identification, cultures considered to be members of recognized EEC groups must be examined in the more complete schema of Kauffmann *(11)*, Edwards and Ewing *(1)*, and Glantz *(4)*. *(d)* Serotyping of *E. coli* cultures recovered from foods and especially injury samples should be done either at the laboratory of origin or at reference centers. *(e)* Detection of capsular factor. Precise conditions for production of capsular antigen should be elucidated.

The association of a particular capsular antigen with a given somatic factor has been demonstrated in cultures associated with illness. However, it is beginning to appear that two or more capsular factors may be present in a given 0 serogroup, e.g., B factors 11 and 13 in serogroup 0112. Because of this observation and because the analyst is dealing with cultures from food that may not have been previously associated with illness, it is essential that the presence and identity of the capsular factor be established. *(f)* Pathogenicity. It is becoming apparent that only a fraction of EEC isolates may be pathogenic. At present, no legally defensible routine test is available to ascertain the potential to induce one of the two recognized syndromes, viz., cholera-like and dysentery-like illness. *(g)* Collaborative effort. Because of the difficulties discussed above, cooperative studies between interested parties are essential. It is of interest that a referee has recently been appointed by the Association of Official Analytical Chemists for development of standard methods for the recovery and identification of enteropathogenic *E. coli.*

ACKNOWLEDGMENTS

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We also thank the following for contribution of cultures: Dr. S. B. Formal, Walter Reed Army Institute of Research, Washington, D. C. 20012; Dr. H. Dupont, University of Texas Medical School at Houston, Houston, Texas 77025; Dr. C. L. Gyles, University of Guelph, Ontario, Canada; Dr. R. B. Sack, Baltimore City Hospital, Baltimore, Maryland 21224; Dr. W. H. Ewing, Center for Disease Control, Atlanta, Georgia 30333; Dr. H. W. Smith, Houghton Poultry Research Station, Huntington, United Kingdom; Dr. C. E. Park, Food and Drug Directorate, Ottawa, Canada; Mr. G. Contronì and the staff of the bacteriology laboratory at Children's Hospital, Washington, D. C. 20009.

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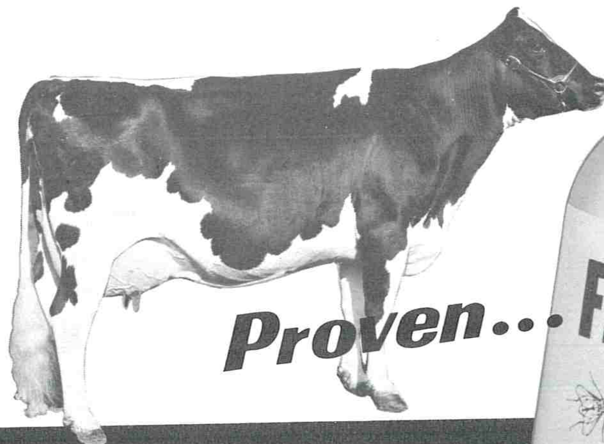

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3-A HONORS PIONEER LEADER

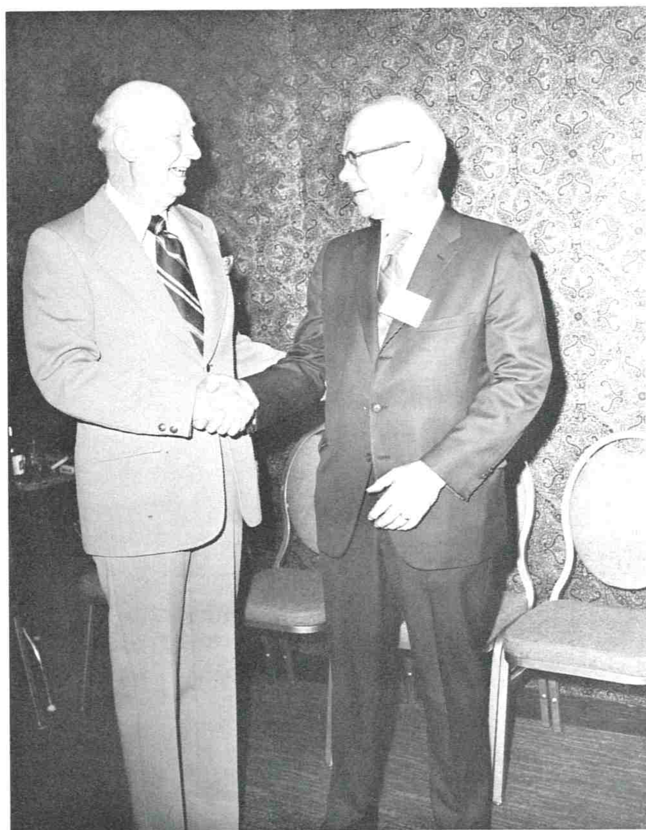
H. L. (Red) Thomasson, who served as executive secretary of the International Association of Milk, Food and Environmental Sanitarians from 1951 to his retirement this year, was honored by the 3-A Sanitary Standards Committees at a testimonial dinner May 7 during the 3-A spring meeting at Omaha.

Red was presented with the bronze 3-A Honor Plaque for outstanding leadership and years of service to the 3-A program. He was one of the pioneers in developing 3-A and was instrumental in securing the patent rights for the 3-A Symbol which serves as the trade mark on equipment used in the processing of dairy products.

In his capacity as chief executive officer of IAMFES, he was a leading force in initiating new developments and programs to aid the dairy and food industries. He will continue to serve during retirement as a consultant to the society's executive board.

Red lives in Shelbyville, Ind., with his wife Peggy. They have four children and eleven grandchildren.

H. L. (Red) Thomasson, left, displays the 3-A Honor Plaque he received in recognition of outstanding leadership and years of service by the 3-A Sanitary Standards Committees at the group's spring meeting May 7, 1974, at Omaha. Making the presentation is Earl Wright, president of the International Association of Milk, Food and Environmental Sanitarians.



H. L. (Red) Thomasson, receives congratulations from Robert L. Nissen, chairman of the technical committee of Dairy and Food Industries Supply Association.

H. L. (Red) Thomasson, receives congratulations from Gordon A. Houran, president of Dairy and Food Industries Supply Association.

LEON TOWNSEND RECEIVES AWARD

Plaque is presented to Mr. Townsend (L) by KAMFES President, Dr. James Hartley, University of Kentucky Department of Dairy Science, Lexington, Kentucky.

The Kentucky Association of Milk, Food and Environmental Sanitarians, Inc. recently presented their Secretary-Treasurer for the past 10 years, Leon Townsend, 110 Tecumseh Trail, Frankfort, Kentucky with a plaque in recognition of his service to the association.

KAMFES is composed of over 250 members, representing local and state regulatory officials, milk & food industry managers & fieldmen, related service companies, and university officials.

Mr. Townsend is presently Director of Manufacturing Milk, Environmental Branch, Bureau of Health Services, Department for Human Resources.

FARRIS BIGGART RECEIVES MERLE P. BAKER AWARD

The Iowa Association of Milk, Food and Environmental Sanitarians awarded the Outstanding Sanitarians Award, known as the M. P. Baker Award to Farris Biggart.

Farris Biggart is being presented the Dr. Merle P. Baker Sanitarian's Award by Earl Wright, advisor of the Iowa Association of Milk, Food and Environmental Sanitarians and Executive Secretary of the International Association at the 33rd annual meeting of the Iowa Affiliate on March 20, 1974 at the Holiday Inn, Ames, Iowa.

Mr. Biggart, recently retired, has been active in the Iowa Dairy Industry in field quality control since 1944. He was a leader in introducing stainless steel cans, then farm bulk tanks to the milk producers in Iowa.

Mr. Biggart was local chairman for the 1970 International Association of Milk, Food and Environmental Sanitarians convention held in Cedar Rapids, Iowa and is a past president of the Iowa Association.

20 Year Continuous Service Awards were presented to Walter Suntken, Mississippi Valley Milk Producers Association; Art French, Mid-America Dairymen, Inc.; Edwin Wegermann, Sanitation Laboratories; Glenn Cavin, Associated Milk Producers, Inc.; and R. L. Sanders, Food and Drug Administration.

NOTICE TO IAMFES MEMBERS

There will be open discussion of Articles II of the By-Laws at the annual meeting at the Hilton Hotel, St. Petersburg, Florida on Wednesday, August 14, 1974 at 10:20 a.m.

Earl O Wright, President

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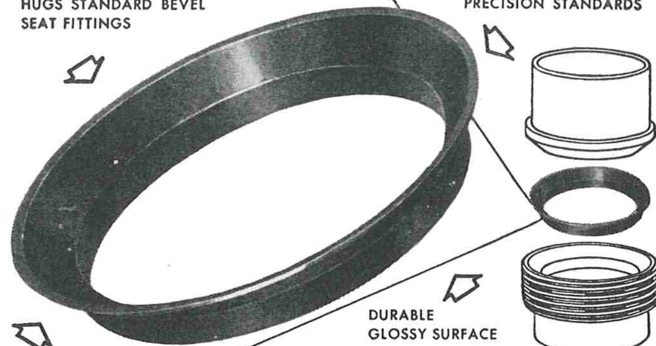

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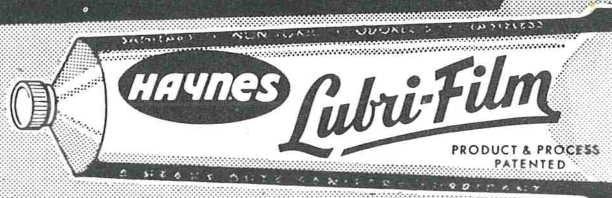

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Dairy authorities speak out on better cow milking

Dr. Elmer H. Marth
Department of Food Science,
University of Wisconsin/Madison

Rapid cooling can help maintain high quality of raw milk

The two most important things a dairyman can do to insure milk quality at his dairy are:

1. Put the cleanest possible milk into the bulk tank.
2. Cool it as quickly and efficiently as possible.

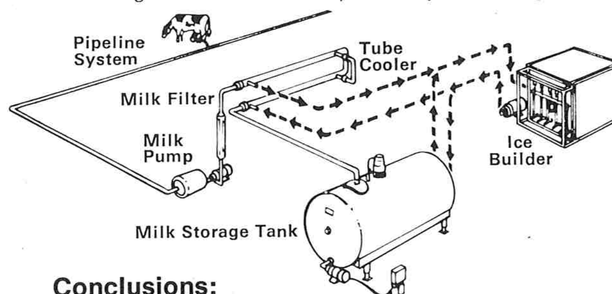
Much has been said about the necessity of sanitary milk handling through the entire milking operation. However, even under the most sanitary conditions, milk from a healthy cow will contain several hundred to several thousand bacteria per milliliter. Certain strains can cause undesirable conditions such as rancidity or other off-flavors unless their growth is retarded.

Follow the rules

The best way to retard bacterial growth is by cooling milk as rapidly as possible, without freezing it.

1. *Milk must be cooled promptly.* Delays result in bacterial growth. Some of the bacteria in milk can multiply in as little as 20 to 30 minutes if the milk is warm.
2. *Cooling should be rapid,* so further appreciable bacterial growth does not occur during the cooling process. Care must always be exercised so that milk does not freeze.
3. *Milk must be cooled to and maintained at a safe temperature.* Cooled milk must be held at a 40° F. and preferably 36-38° F. This temperature must be maintained throughout the storage period. When freshly drawn milk is added to milk already in the bulk tank, the rise in temperature of the initial milk must be minimal and the temperature of all milk in the tank must be rapidly reduced to 36-38° F. (Again, milk must not be frozen in the process.)
4. *Raw milk should not be stored for excessive periods* and should be moved from the bulk tank to the tank truck under conditions which preclude additional microbial contamination.

The refrigerated bulk cooling tank is the most widely used device to cool milk on the farm today. However, it is limited in its ability to meet some of the demands outlined above. New equipment available makes it possible to "pre-cool" milk on the way to the tank. Instant coolers using chilled water from an ice-builder help make rapid cooling practical on the farm. This type of cooling also eliminates the possibility of freezing milk.



Conclusions:

Even though all conditions needed for effective rapid cooling are met, some bacteria can still grow in refrigerated milk. Two points already discussed bear repeating. Be certain that good sanitary practices are followed during production of milk to insure that few bacteria of the kind able to grow at refrigeration temperatures are present. Do not hold raw milk refrigerated for excessive periods.

The successful marketing of milk depends on everyone doing his part at each step along the way. Even though you, as an individual dairyman, may not benefit *directly* from each of your efforts to improve sanitation and milk handling, you most certainly will benefit *indirectly* through greater total consumer acceptance of milk and dairy products thanks to fewer flavor problems and the absence of other negative factors caused by improper handling.

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This is one of a series of topics developed by noted Dairy authorities. For a complete set write for a free booklet.