630.7
Or32ci
no.480
c.3

APR 15 1950

Bacteriophage and Flavor Problems in Cultured Milk Products

DOCUMENT
COLLECTION
OREGON

P. R. Elliker

DISCARD
Press or radio announcement of this
bulletin should not be made prior to
APR 23 '50

Agricultural Experiment Station
Oregon State College
Corvallis

Circular of Information 480 March 1950

630.7
Or32cL
no. 480
c. 3
DOCUMENT COLLECTION
OREGON COLLECTION

OREGON STATE LIBRARY

Bacteriophage and Flavor Problems
in Cultured Milk Products

By
P. R. Elliker
Department of Bacteriology

The flavor, body, and appearance of cultured-milk products, such as cultured buttermilk, cottage cheese, and cultured cream, are dependent on the fermentations of two important types of bacteria. One of these two types, the lactic-acid bacteria, is responsible chiefly for lactic-acid production from the milk sugar, lactose. The second type, the aroma bacteria, is responsible for production of the so-called aroma compounds, acetylmethylcarbinol and biacetyl. Biacetyl is the chemical that lends the culture a desirable butter aroma. Volatile acids, such as acetic and propionic acids, also contribute to the desired aroma. The chief source of the aroma compounds is citric acid present in the milk. The lactic-acid formers and aroma bacteria grow and produce their desired effects as a mixed culture. The quality and value of such a culture are largely dependent on maintaining the desired bacteria in a proper ratio and in an active state.

The lactic acid bacteria of the mixed culture may be either Streptococcus lactis or Streptococcus cremoris, two species that closely resemble each other in most respects. They produce chiefly lactic acid and relatively small amounts of acetic and propionic acids. The aroma bacteria may be either Leuconostoc citrovorum or Leuconostoc dextranicum. They produce most of the volatile acid and practically all of the acetylmethylcarbinol and biacetyl. In addition to aroma compounds L. dextranicum produces some lactic acid. L. citrovorum produces no lactic acid in milk.

Failure of a culture to actively produce either lactic acid or the aroma compounds usually results in serious loss of quality or in some cases complete loss of the cultured milk product. Insufficient acid production usually means a weak-bodied product with poor flavor and keeping quality. Insufficient production of aroma compounds usually results in a harsh acid or flat and sometimes metallic flavor. A study of the factors involved, together with careful handling of the culture, will usually reward the operator many times over in terms of a superior final product.

Combination of Cultures Employed

Care must be used in selecting the lactic acid and aroma bacteria that make up the mixed culture. The lactic-acid bacterium must be a vigorous acid producer and one that yields a clean acid flavor. The aroma bacteria should be active citric-acid fermenters and must be capable of producing the desired aroma

compounds in association with the active lactic-acid bacteria. Sometimes many combinations of lactic and aroma bacteria must be attempted before obtaining one that yields desired quantities of lactic acid and aroma compounds.

Contaminating organisms should be excluded from mother cultures. The spore-forming bacteria that survive pasteurization of bulk starter milk usually are restricted in growth due to the acid formed by the lactic acid bacteria.

Composition and Quality of Milk

The milk employed for preparation of starter cultures must provide those nutrients essential for active growth of both lactic acid and aroma bacteria. It has long been the practice to select milk from certain herds to provide a uniform, high quality milk for this purpose.

The milk employed for starter propagation may be fresh whole milk, fresh skim milk or reconstituted non-fat milk. High grade reconstituted non-fat dry milk solids for both mother and bulk starters may be favored for several reasons. First of all, a large lot of the milk may be obtained at one time. If tests prove the lot to be satisfactory, milk can be reconstituted daily from it for preparation of mother and bulk starter. Cultures prepared from such a powder will be more uniform from day to day because the composition of the starter milk is exactly the same from one day to the next. When a satisfactory culture is attained in such a milk, it may be transferred in the same manner month after month with no fear that the milk will upset the routine established. In a few instances certain batches of powder have proved unsatisfactory and in occasional instances the water employed for reconstituting the milk has inhibited the starter organisms. A few trials with a simple activity test (to be discussed later) will quickly indicate suitability of both dry milk solids and water. Some large plants that formerly employed selected whole milk for starters have been able to save more than $100 monthly by using non-fat milk solids instead.

Some strains of lactic acid bacteria growing in raw milk, on the farm or in the interval before it is processed, produce substances in the milk that inhibit growth of lactic-acid bacteria in starter cultures. The inhibitory substance is believed to be an antibiotic and is similar in effect to the antibiotics penicillin and streptomycin. The bacteria producing such inhibitory substances in raw milk may enter from poorly-cleaned farm equipment. Another source of antibiotics that has caused serious inhibition of starter cultures is penicillin carried over in the milk following treatment of cows for mastitis.

The aroma production by a starter culture may be increased by addition of citric acid or sodium citrate. The normal citric content of milk is usually about 0.2 per cent. Addition of 0.2 per cent more may greatly increase production of volatile acids, acetylmethylcarbinol, and biacetyl. It has been reported that addition of nicotinic acid or chlorides of manganese, iron, or copper also increases production of aroma compounds of starter cultures. A satisfactory culture, grown in a high grade milk, usually produces sufficient aroma compounds without addition

of such accessory substances. Aroma production also can be increased by aeration of a culture, but this is not practical under most circumstances. However, there should be sufficient access to air provided in the developing culture.

Temperature of Incubation

An incubation temperature of 70° to 72° F. is an important requirement. At temperatures below this range, the lactic-acid bacteria grow slowly and insufficient acid will be produced. At temperatures considerably above this range, the aroma bacteria may not develop readily, and the result of continued transfers above 72° F. may be a culture lacking in desirable aroma. The temperature of 70° to 72° F. represents the best workable compromise for maintaining both lactic acid and aroma bacteria in the mixture when using a convenient incubation time of about 14 to 16 hours.

Length of Incubation Period

No hard-and-fast rule can be employed for incubation time and final acidity of culture. The incubation period usually employed is in the range of 14 to 16 hours. If the cultures are in the proper ratio and are active, the titratable acidity will range from 0.75 to 1.00 per cent. This quantity of acid must be produced before appreciable amounts of the aroma compounds are formed. Presumably a high acidity (low pH) makes citric acid more readily available and favors conversion of some acetylmethylcarbinol to biacetyl. Extending the incubation period tends to increase the proportion of the aroma bacteria. Consequently, overripening may be employed to increase aroma production in a culture lacking in aroma. The aroma bacteria appear to be more acid tolerant than the lactic-acid producers. It also must be kept in mind, however, that the lactic-acid bacteria may be weakened by repeated overripening of the culture and an inactive culture may result. Further, the same bacteria that produce aroma compounds will destroy biacetyl by reducing it to acetylmethylcarbinol and 2,3 butylene glycol if the culture is incubated too long.

Bacteriophage and Starter Activity

The importance of bacteriophage as a cause of starter failure or inhibition in this country has been generally underestimated. Evidence indicates that a great many starter difficulties that have been attributed to various other causes actually have been due to bacteriophage. Isolations of bacteriophage continue to be made from dairy plants that never have suspected it as a cause of their starter difficulties.

Nature of bacteriophage

Bacteriophage, or "phage" as it is termed, actually is a virus that attacks and destroys bacterial cells. In the process of destroying the host cell, a great many new phage particles are formed—apparently from constituents of the host cell. Certain strains of phage are able to attack specific strains of lactic-acid starter bacteria. In so doing, they cause stoppage of acid development, frequently with serious damage to the product. Such phage attacks may involve any product dependent on S. lactis or S. cremoris for lactic-acid production. The worst, and most consistent, phage outbreaks usually have occurred in manufacture of cheeses like Cheddar made from pasteurized milk. Frequent and serious losses also have occurred in cottage cheese manufacture and somewhat less frequently in cultured buttermilk. A recent report also indicates that lack of aroma in starter cultures may be due to destruction of the aroma bacteria by phage strains specific for them.

Source of bacteriophage

Little is known regarding the ultimate source of bacteriophages specific for the lactic-acid bacteria. It is possible that they originally develop in the soil or on plants where lactic-acid bacteria grow in nature. One worker has reported phages for lactic-acid bacteria to be generated in the intestinal tracts of hogs. It is possible that phages enter dairy plants on dust particles. It is known that they enter the plant in the milk supply. Once they enter the plant, bacteriophage strains develop in the lactic-acid culture or cultured-milk product or on equipment if susceptible lactic-acid bacteria are present.

Heat resistance

Most phage strains demonstrate greater heat resistance than the host cell upon which they live. Consequently, the phages for lactic-acid bacteria are quite resistant to heat, and most of these phages will survive ordinary pasteurization of milk by the vat or holder or high-temperature short-time methods. As far as we know, they are destroyed by the so-called pasteurization of starter milk at about 190° F. for one hour.

Detection of bacteriophage

Presence of bacteriophage in a starter culture or cultured-milk product may be suspected whenever the lactic-acid bacteria suddenly slow up or completely fail to grow. Usually in such cases, a starter culture from a different source, containing other strains of lactic-acid bacteria, will provide temporary relief from the difficulty—providing no phage-strain specific for the new culture is present. In some cases, several strains of phage may be present in the plant. The phage sometimes may be one of multiple specificity and thus may be able to attack a number of different strains of starter organisms.

A few simple tests may be employed in the plant to provide presumptive evidence of the possibility of phage. A few drops of fresh starter may be added to about 10 ml. of sterile skim milk in a tube and if the milk fails to coagulate in 24 hours, the possibility of phage in the starter exists. If a second tube of milk is inoculated in the same manner and incubated at 86° to 98.6° F., microscopic examinations of the contents can be made at intervals over a period of about 8 hours. If the organisms begin to multiply and then lysis (disintegration of cells) is noted, the evidence is strong that phage has attacked and destroyed the bacteria. If phage is suspected in cottage cheese manufacture, duplicate tubes or small bottles or flasks containing sterile skim milk may be inoculated with about 0.5 per cent of fresh starter. One of the duplicate containers then may be inoculated with 2 or 3 drops of whey from a suspicious vat. The other container serves as a control. The cultures may then be incubated at 86° F. for 6 hours and titratable acidity determined. If acid developed is significantly greater in the control container, presence of phage in the whey is strongly suggested.

The most certain method of demonstrating presence of phage in a culture or product is to pass it through a bacteriological filter that will remove all micro-organisms. The phage particles, being smaller than bacteria, will pass through the filter with the filtrate. At the same time, the culture suspected of attack must be plated on agar and growth from a number of individual colonies transferred to sterile milk or broth to obtain single strains of the culture organisms. Duplicate sterile tubes of milk or broth then may be inoculated with the single strain cultures and a drop or two of filtrate added to one tube. If the control tube develops acid in significantly greater amounts or at a faster rate than the tube containing filtrate, there is a strong possibility of phage. The titer, or concentration of phage in the filtrate, may be determined by noting presence or absence of inhibition in tubes of milk or broth inoculated with the single strain and varying dilutions of phage. Another method involves plaque formation on agar plates.

The growth from the above broth or milk may be again passed through a sterile filter. If the inhibitory substance can be increased in concentration by successive filtrations and periods of growth on a susceptible culture, it is bacteriophage. If the inhibitory substance is diluted out and gradually becomes weaker by such successive passages through the filter, it may be an antibiotic.

Protective measures against bacteriophage

Bacteriophage develops upon susceptible bacteria. Thus it will be present not only in cultures or cultured milk products but also on growing organisms on equipment. It may lodge on floors, walls, ceilings, dust, and may even be carried on hands and clothes of workers. Apparently droplet infection from the contaminated product, especially whey, tends to spread it around the plant and even into the starter laboratory if it is located near the processing room of the plant. Whey separators are an especially difficult problem because they throw a fine atomized mist over the plant.

One measure found helpful in some plants has been thorough cleaning followed by hypochlorite treatment of floors and all equipment that comes in contact with the product. Brushing, soaking, or thoroughly spraying all equipment and tools

before use with 500-ppm hypochlorite solution is recommended where phage outbreaks occur. Another measure employed to reduce droplet infection is to spray the entire processing room with hypochlorite at the rate of at least 4 ml. of a 9 to 12 per cent solution per 1,000 cubic feet. The relative humidity of the room should be at least 80 per cent if possible for most effective penetration of the chlorine.

Some plants have reduced phage outbreaks and improved uniformity of starter cultures in general by obtaining mother culture daily from a central laboratory. This system greatly reduces the danger of phage contamination of mother culture and enables one laboratory to maintain closer control over the quality and selection of starter strains than would be true in scattered small plants. In some instances the mother culture is sent by air express from the central laboratory.

Another measure consists of removing the starter laboratory to some part of the plant away from the processing room to reduce chances of contamination of culture. In some cases the starter laboratory has been set up some distance away from the plant. Elaborate precautions, such as means for sterilization of the room, maintaining positive air pressure in the room to prevent air currents carrying phage in, and use of specially-constructed culture vessels with a small opening for inoculation and water seal of the lid, also have been employed.

If several different cultures can be carried in the laboratory, they can be rotated in such a way that one or two are used one day, another combination the next, and so on. In Cheddar cheese manufacture, as many as 8 or 10 strains may be carried. Two cultures are grown separately and mixed at the vat on the first day, two other strains the next, and so on. Then the original two are used again and the rotation is repeated. This tends to prevent a build-up of phage for one culture day after day in the plant.

Where facilities are available, tests may be run on whey or other products to determine whether or not phage is accumulating for a certain culture. As soon as evidence indicates such accumulation, another culture is introduced.

Strains of lactic-acid bacteria may be made resistant to bacteriophage by repeatedly exposing them to phage and growing the survivors. Such strains may be resistant to numerous phage types, but the possibility of attack by another phage specific for them always exists.

Tests for Activity of Lactic-Acid Starter Cultures

Various methods have been proposed for estimating activity of lactic-acid bacteria in starter cultures. The following is a relatively simple method used during the past several years: A series of tubes or flasks of sterile milk are inoculated with 3 per cent of the respective cultures to be tested. The milk used preferably should be prepared from high-grade non-fat milk solids and sterilized no longer than 10 to 12 minutes at 15 pounds pressure. Just before inoculation, it should be warmed to 100° F. After 3.5 hours of incubation at 100° F., the

titratable acidities of the inoculated tubes are determined. An active culture usually produces more than 0.35 per cent titratable acidity in 3.5 hours. An inactive culture usually produces less acid. When conditions are carefully standardized and controlled, the test provides an easy, rapid means of determining activity of the culture. Another variation of the method involves inoculation of 1 per cent of culture, incubation for 6 hours and titration with the same interpretation of results obtained.

Tests for Production of Aroma Compounds

One method employed for testing for content of aroma compounds produced by butter cultures is the modified creatine test of Hammer. In this method, 2.5 ml. of culture are placed in a test tube; a small quantity (about one-third the volume of a wheat kernel) of creatine and 2.5 ml. of 40 per cent sodium hydroxide added; and the mixture is shaken and allowed to stand for a few minutes. Development of a red ring about one-fourth inch deep indicates a considerable amount of aroma compounds. Less coloration occurs with lesser quantities of aroma compounds.

Another simple test is the modified Ritter method described by King. It may be employed for estimating production of aroma compounds (acetylmethylcarbinol plus biacetyl) in cultured milk starters. Two reagents are used: (A) Thirty per cent aqueous solution of potassium hydroxide; (B) A solution consisting of alphanaphthol 4.0 gm., amyl alcohol 10 ml., and ethyl alcohol 90 ml. In carrying out the test, 1 ml. of reagent A and 1 ml. of reagent B are added to 2 ml. of the untreated culture in a wide mouth test tube to permit sufficient contact with the air. The tube is placed in an 86° F. water bath and repeatedly shaken over a 30-minute period in order to incorporate air. The intensity of the red-lilac coloration that develops provides a measure of the relative quantity of aroma compounds. For more accurate estimation, the mixture can be filtered and compared with color standards. If the test is to be run on the serum, filtrate, or distillate of a culture, reagent B should include 0.1 gm. of dicyandiamide. Presence of the dicyandiamide also accelerates rate of color formation in testing untreated culture as outlined above.

Paper presented before Laboratory Section, 42nd Annual Convention, Milk Industry Foundation, Los Angeles, California, October 1949.