# Use of Agglutination-Sensitive Bulk Starters in the Manufacture of Stirred Curd Cheese[1]

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## ABSTRACT

The effect of agglutinated bulk cultures on the manufacture of experimental stirred curd cheese was observed. To prepare three bulk starters, three aliquots of bulk medium were inoculated with cultures differing in agglutination sensitivity. Each bulk starter was divided into two portions, one of which was homogenized at 17.2 MPa (13.8 MPa, first stage; 3.4 MPa, second stage) pressure. Seventy grams (1%) of each starter were inoculated into 7000 g of milk, and stirred curd cheese was manufactured. The experiment was replicated three times. Cell chain length and clump size were markedly reduced in starters that were homogenized. No differences were observed in culture activity (acid production) during cheese manufacture between homogenized and unhomogenized cultures. Acid production was uniform throughout the vat of milk for both homogenized and unhomogenized cultures. The pH at the bottom of the vat was slightly lower when unhomogenized culture CH970 (a clump former) was used as the bulk starter. Large agglutinated complexes of bacteria and protein may have been physically buoyed up by the fat globules based upon photomicrographs of the cheese coagulum.
(**Key words**: lactic acid bacteria, bulk starters, agglutination, stirred curd cheese)

**Abbreviation key**: **ICM** = internal pH control medium.

## INTRODUCTION

Immunoglobulins, a type of natural inhibitor in milk, cause some cells of lactic acid bacteria to agglutinate (chain and clump together), resulting in irregular starter performance when cottage cheese is manufactured. Immunoglobulins involved in chaining and clumping problems are IgM (13, 30), IgG (23, 30), and IgA (30). Immunoglobulins occur naturally in milk and exhibit strain specificity for certain lactococci (30) that were formerly referred to as streptococci (18). Aggregated clumps of bacteria cause localized acid production and cause casein to coagulate around the clumps. As clumps amass, they fall to the bottom of the vat, forming a sediment or sludge layer and an uneven distribution of bacteria throughout the skim milk (11). Thus, the top portion of skim milk exhibits little acid development, but the bottom sediment or sludge layer has high acid development. The curd becomes grainy or mealy and shatters easily upon cooking (3, 4, 8, 19).

Stadhouders (26) reported that Ig lost their agglutinating activity during homogenization of skim milk. Russell-Campbell and Hicks (22) suggested that homogenization of skim milk affects the concentration of Ig that are available to bind to the culture surface because homogenization unfolds the skim milk membrane (5) or free milk fat globule membrane (13), which binds additional Ig. Culture agglutination may also be inhibited by homogenizing bulk starter because homogenization breaks apart chains and clumps of bacterial cells, retarding the rate of sedimentation (9, 19).

Culture agglutination problems are generally not observed during the manufacture of Cheddar cheese or other hard cheeses. However, several researchers (2, 7, 15, 31) have reported that inhibitory substances in milk associated with milk fat globules cause a decrease in starter activity during the manufacture of Cheddar cheese and other cultured dairy products. Stadhouders (26) proposed that uneven acid production occurred not only in skim milk, but also in whole milk. Some additional bacteria in whole milk were observed to be on the bottom of the vat, but most were carried into the cream layer, accelerating acid production in those areas. Wright and Tramer (32) reported that agglutinins cause fat globules to clump and accelerate cream rising. Other researchers (3, 27) suggested that euglobulins (Ig) appeared to be able to agglutinate bacterial cells and

Received June 3, 1996.
Accepted October 14, 1997.
[1]Published with the approval of the director of the Kentucky Agriculture Experiment Station as Journal Article Number 96-07-088.
[2]Reprint requests.

attach them to fat globules. Therefore, agglutination problems may also be evident in the manufacture of hard cheeses. The objective of this research was to determine the effect of agglutinated bulk starter cultures on the manufacture of stirred curd cheese.

## MATERIALS AND METHODS

### Experimental Design

A 3 × 2 split-plot design was used to determine the effect of agglutinated bulk cultures on the manufacture of stirred curd cheese. Three batches of bulk medium were prepared, and each was inoculated with a culture that was different in agglutination sensitivity (10). Resulting bulk starters were divided into two portions. One portion was not homogenized, and the other portion was homogenized at 17.2 MPa (13.8 MPa, first stage; 3.4 MPa, second stage). This procedure produced six different bulk starters. Seventy grams (1%) of each bulk starter were inoculated into 7000 g of pasteurized milk, and stirred curd cheese was manufactured. The experiment was replicated three times.

### Media Preparation

***Transfer culture medium.*** Nonfat dried milk (Difco Laboratories, Detroit, MI) was reconstituted to 10% solids with distilled water and autoclaved at 121°C for 10 min. After the milk was cooled to room temperature (approximately 25°C), the medium was stored at 2°C until needed to propagate starter cultures.

***Bulk starter medium.*** Thirty-four kilograms of commercial, internal pH control medium (**ICM**) (25) was prepared by mechanically mixing the medium with distilled water (75.7 g/L) in a 39-L stainless steel milk can. The medium was heated to 85°C and held for 45 min in a water bath. The heated medium was cooled to approximately 20°C, split into three portions of approximately 11 kg of each, and poured into sterilized stainless steel containers. All batches of medium were refrigerated at 4°C for several hours, then heated to 22°C, and inoculated with an appropriate culture.

### Culture Source and Preparation

A commercial single-strain culture, M37 (*Lactococcus lactis* ssp. *lactis*; Marschall Products, Rhône-Poulenc, Madison, WI), and two commercial mixed-strain cultures, M30 (containing one *L. lactis* ssp. *lactis* and one *Lactococcus lactis* ssp. *cremoris* strain; Marschall Products, Rhône-Pôulenc) and CH970 (containing three *L. lactis* ssp. *lactis* and one *L. lactis* ssp. *cremoris* strains; Chr. Hansen's, Inc., Milwaukee, WI), were maintained at –80°C.

Three vials containing 6 ml of sterilized reconstituted NDM (10% total solids) were each inoculated with 1 g of one of the three frozen stock cultures. Vials were incubated at 26°C for 2 to 3 h or until coagulated. Contents of these vials were transferred into 100-ml portions of the same medium and incubated at 26°C for 3 to 5 h or until coagulated. These active cultures were used to inoculate ICM bulk starter medium at a rate of 1%. Bulk starters (ICM) were incubated with continuous agitation at room temperature (24 to 26°C) for 12 h.

### Homogenized and Unhomogenized Bulk Starters

A 1-L portion was taken from each bulk starter and was designated as unhomogenized. The remaining portion (9.5 L) of each bulk starter was homogenized at 17.2 MPa (13.8 MPa, first stage; 3.4 MPa, second stage). A 1-L sample was collected midstream from each homogenized culture and designated as homogenized. In this experimental design, three bulk starters (M30, M37, and CH970) were divided into two portions (unhomogenized and homogenized) to produce six bulk starters.

### Milk Preparation

Grade A raw milk (mean fat content, 3.8%) was obtained from the University of Kentucky dairy research farm (Lexington) on three occasions and separated into skim milk and cream. Skim milk was used to standardize whole milk to 3.5% milk fat. This milk was pasteurized at 63°C for 30 min and cooled to 4°C overnight.

### Manufacture of Modified Stirred Curd Cheese

Milk (warmed to 32°C) was weighed (6996.5 to 7003.5 ± 0.1 g) into six prepared experimental cheese vats and placed in a thermostatically controlled water bath (28) to manufacture stirred curd cheese. Each vat of milk was randomly inoculated (1%) with one bulk starter. All vats were agitated for 2 min at the same time and speed (28). The milk was allowed to ripen for 1 h because of the slower acid-producing activity of agglutination-sensitive cultures. Single-strength calf rennet (Miles Laboratories, Inc., Elkhart, IN) was diluted (1:20, vol/vol) with distilled ice

water and added (0.5 ml/7000 g of milk) to the milks. Then, milks were stirred for 2 min to ensure even distribution of the rennet. Agitators were replaced with specially designed curd cutting knives (12). Samples from the top and bottom of the vat were collected (9) prior to coagulation to test for differences in pH, colony-forming units, and total solids. Coagulation occurred within 25 to 30 min. The curd was cut and allowed to heal for 15 min. The curd was agitated slowly, while the temperature was raised to 38°C in 30 min. The curd was held at this temperature until the pH reached 6.1, at which time the curd was drained, stirred intermittently until the pH reached 5.4, hooped, and pressed. The curd was not salted.

### Agglutination Determination

Differential measurements (9, 10) of pH, total solids, and colony-forming units between the top and bottom of each vat were determined to monitor the severity of culture agglutination. Determinations were made on the milk used for cheese manufacturing or the samples that were collected just prior to milk coagulation (coagulum sample). Samples were collected from the top (approximately 1 cm under the milk surface) and bottom of the vat via a glass tube as described (22).

***pH differentials.*** A pH probe (pencil-thin, gel-filled, polymer body, combination electrode; Fisher Scientific Inc., Pittsburgh, PA) attached to a pH meter (American™ pH II; Fisher Scientific Inc.) was used to measure pH. Plastic tips protecting the glass bulb of the electrode were trimmed even with the bottom of the bulb so that the pH of the sludge layer could be determined. pH measurements from the top and bottom of the vat were determined for ripened milks (1 h after inoculation, prior to the addition of rennet) and curd (prior to cutting). Differentials were calculated by subtracting pH values of the bottom from pH values of the top.

***Total solid differentials.*** Measurements of total solids in the curd were determined from duplicate samples collected from cheese coagulum. Samples (1 g) were placed in preweighed aluminum dishes and oven-dried at 98 to 100°C for 24 h (17) to compute total solids. Differentials of total solids were calculated by subtracting values for the total solids from the top of the vat from the values for total solids from the bottom (9, 10).

***Colony-forming unit differentials.*** One-gram samples were removed from coagulum samples and were diluted in phosphate buffer solution (17). Elliker agar was prepared to determine colony-forming units by the pour plate method (17). Differentials for colony-forming units were calculated by subtracting counts for the bottom portion of the milk from counts for the top.

### Microscopic Observation of Smears Prepared from Bulk Starters

Six bulk starters were observed for severity of agglutination from smears stained with crystal violet (1). Smears were prepared from samples taken from thoroughly agitated bulk starters. Photomicrographs were taken (Nikon Microphot-FXA microscope; Nikon Co., Instrument Group, Garden City, NJ) using the oil immersion objective (100× magnification) and Ektachrome™ film (5017, ISO 64; Kodak, Rochester, NY). The microscope lamp voltage was set at 9 V, and exposure time was approximately 11.6 s. A balance marker within the camera measured magnification.

### Interactions of Culture and Fat Globules

A second experiment was conducted to determine how fat globules interacted with agglutinated bulk cultures. Milk (pasteurized at 63°C for 30 min) containing 4% fat was tempered to 32°C and poured (990 ml per cylinder) into three 1000-ml graduated cylinders. Each cylinder was inoculated (10 ml) with one of the unhomogenized cultures (culture from replication 3) and incubated at 32°C. Milk samples were ripened for 1 h prior to renneting as previously described. Coagula was at the normal cutting point (32 min) when samples were collected. Samples (5 g) were taken from the top (1 cm below surface) of the coagula from the three 1000-ml graduated cylinders. Smears of cheese coagula were prepared from these fermentations to observe the structure and relationship among bacteria, fat globules, precipitated protein, and other milk components that form when agglutinated bulk cultures are used to manufacture renneted cheese.

Smears were prepared using a combination of two staining procedures (1, 6). Sudan III dye (Sigma Chemical Co., St. Louis, MO) was used to stain the fat globule membrane and protein precipitate. A solution of Sudan III dye was prepared by making a saturated solution in isopropanol. Dye was added to 100 ml of 99% isopropanol during agitation until excess powdered dye could be observed on the bottom of the flask. Six milliliters of saturated solution were diluted with 4 ml of distilled water and filtered (Whatman number 2 paper; W. & R. Balston, Ltd., London, England) after standing for 5 to 10 min (1).

Crystal violet was used to stain bacteria and help differentiate other milk components (6).

Smears were prepared from the three samples by streaking a loop of coagulated milk onto a slide. After 30 s, smears were stained with a drop of freshly prepared crystal violet dye for 30 s; then, excess crystal violet was carefully blotted with absorbent paper without touching the smears. Slides were immersed in a microscope slide container filled with tap water and very gently agitated for 15 to 30 s or until most of the crystal violet dye had diffused from the slide. Each slide was blotted without touching the smears. Before the smears dehydrated, two to three drops of Sudan III stain were applied to the smears by letting the drop lightly touch the surface and spread over each smear. A cover glass (1.0 mm thick) was placed over each smear (one drop of Sudan dye was added to each corner of the cover glass to prevent dehydration), and the cover glass was gently pressed onto smears to decrease the thickness of the liquid film under the glass. Slides were held for 2 to 3 min to allow the Sudan dye to stain the precipitated protein and fat globules. Slides were examined, and photomicrographs were taken as described.

### Statistical Analyses

Statistical analyses were performed using SAS software (24). Data were analyzed using the following general linear model procedures. The main statistical model was

$$\text{model } D_{PH}\, D_{TS}\, D_{CFU} = C \;\; H \;\; (C \times H) \;\; R/SS4$$

where

$D_{pH}$ = difference between pH from the top and from the bottom of the vat,
$D_{TS}$ = difference between total solids from the top and from the bottom of the vat,
$D_{CFU}$ = difference between colony-forming units from the top and from the bottom of the vat,
C = cultures used (M30, M37, and CH970),
H = homogenized and unhomogenized bulk starters,
$(C \times H)$ = interaction between cultures and homogenization, and
R = times the experiment was replicated (n = 3).

Least square means and predicted differences between means were computed for each dependent variable and interaction using type IV sums of squares.

## RESULTS AND DISCUSSION

### Experimental Model

Agglutination problems became more severe as bacterial chain length and clump size increased (10). The control or prevention of agglutination was achieved by breaking chains and clumps into smaller segments. Researchers (9, 10, 19) have reported that homogenization of bulk starter culture, at its recommended final pH, reduced bacterial chain length and clump size in the bulk starter and inhibited culture agglutination in cheese milk. A total homogenization pressure of 17.2 MPa (176 kg/cm$^2$) was optimum because colony-forming units increased 0.9 $\log_{10}$ at that pressure. Total pressures >17.2 MPa reduced cell numbers, probably because of increased cellular damage (19).

To determine the effect of adding agglutinated bulk starter to milk used for the manufacture of stirred curd cheese, three agglutinating cultures (M30, M37, and CH970) were selected (10) and propagated. Cultures M30 and CH970 were selected because they form large clumps of cells when agglutinated. Culture M37 was selected because it forms extended chains of cells when agglutinated. A portion of bulk starter was homogenized (control) at the optimal total pressure (17.2 MPa) proposed by Milton et al. (19) to break up chains and clumps and to inhibit agglutination of these cultures after their addition to milk. Culture performance of homogenized cultures should be similar to that exhibited by single and paired cells (nonagglutinated). Culture performance of agglutinated (unhomogenized) bulk starter should produce the worst-case agglutination response (9, 10).

### Bulk Starter Morphology

The undisturbed, unhomogenized bulk starters were observed to have a brown sediment layer that was distinct from the more homogeneous homogenized cultures. Microscopic examination showed that unhomogenized bulk starters were severely agglutinated for cultures M30, M37, and CH970 (Figure 1). Unhomogenized cultures contained long chains and clumps of chains that often exceeded 40 to 50 cells per chain (M30) and 250 to 300 cells per clump. Clumps and chains had protein precipitate around them that was similar to those reported by Russell-Campbell and Hicks (22) and Ustunol and Hicks (29) and those that settled to the bottom of cottage cheese vats within 15 min after bulk starter inoculation (19). Hicks and Ibrahim (10) stated that, when severely agglutinated bulk cultures were used to inoculate skim milk, a brown sediment (the color of bulk starter) could be observed

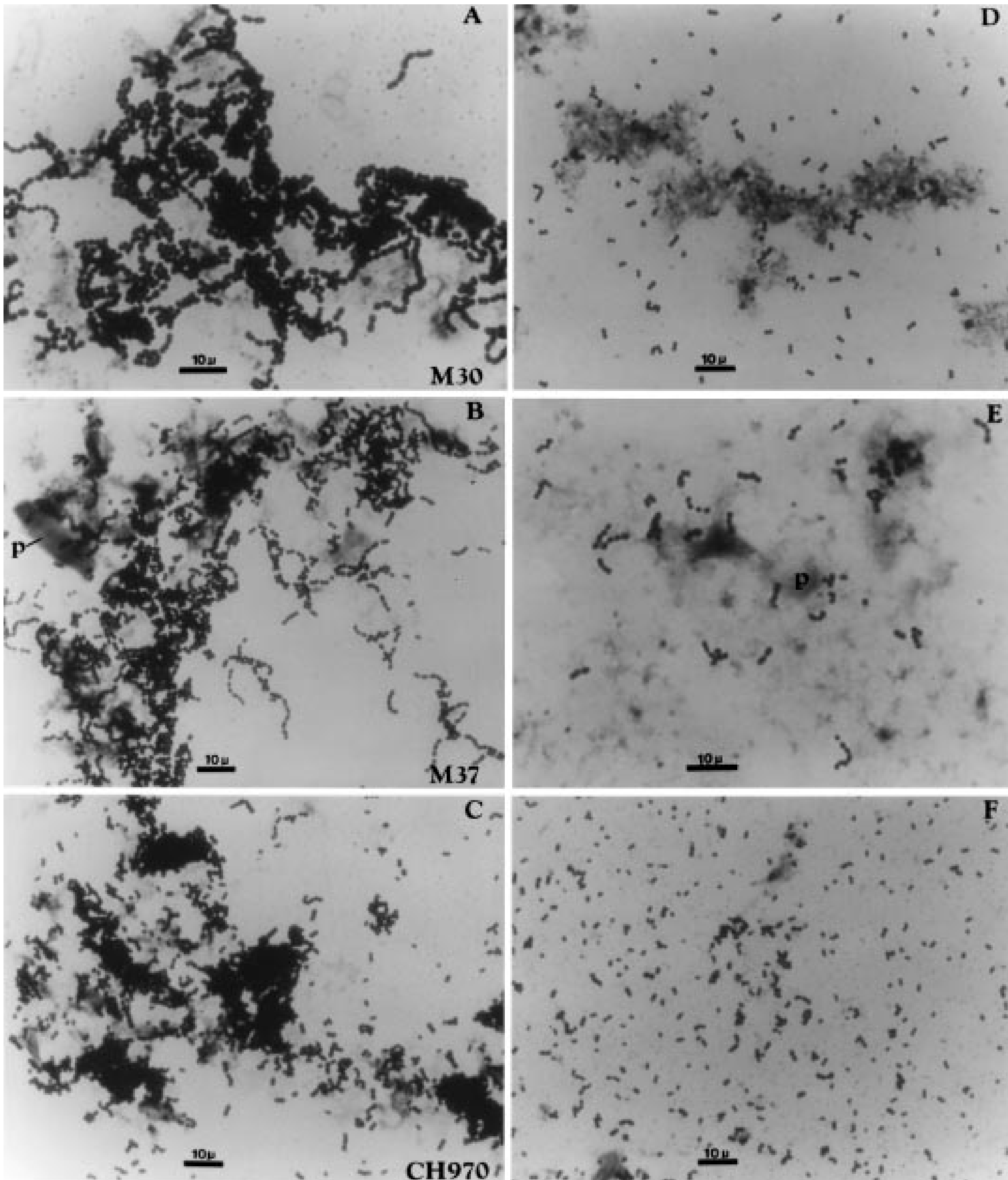



Figure 1. Photomicrographs A, B, and C show unhomogenized cultures M30 (A) (1500× magnification; 100× oil immersion lens, 1.5× intermediate magnification Bertrand lens, and a 10× eye lens), M37 (B) (1250× magnification; 100× oil immersion lens, 1.25× intermediate magnification Bertrand lens, and a 10× eye lens), and CH970 (C) (1250× magnification; 100× oil immersion lens, 1.25× intermediate magnification Bertrand lens, and a 10× eye lens) stained with crystal violet. Photomicrographs D, E, and F show the homogenized (17.2 MPa) cultures M30 (D) (1250× magnification; 100× oil immersion lens, 1.25× intermediate magnification Bertrand lens, and a 10× eye lens), M37 (E) (1500× magnification; 100× oil immersion lens, 1.5× intermediate magnification Bertrand lens, and a 10× eye lens), and CH970 (F) (1250× magnification; 100× oil immersion lens, 1.25× intermediate magnification Bertrand lens, and a 10× eye lens) stained with crystal violet. p = Protein deposits.

on the bottom of the vat because of the rapid settling. Thus, sedimentation probably occurred before casein could precipitate around clumps. Milton et al. (19) noted that agglutinated sediment contained >90% of the lactic bacteria present in the milk.

Homogenization effectively controlled agglutination of starters M30, M37, and CH970 by breaking apart the chains and clumps of cells (Figure 1). Only a few chains contained more than four cells per chain, and most chains contained only two cells per chain. Some clumps of chains contained two to four chains, but most chains were not in clumps. Cells from homogenized bulk starters were evenly scattered across microscopic smears, and no brown sediment was observed in the bottom of bulk starter containers. These photomicrographs verify that homogenized bulk starters were not agglutinated and were similar to photomicrographs of agglutination-resistant CH60 culture (commercial unhomogenized culture) (10). Unhomogenized bulk starters were severely agglutinated, and the photomicrographs were similar to those for culture M30 (9); for M30, M37, and CH1060 (10); and for UC310$^-$ (19).

### Determination of Culture Agglutination in Cheese Milk

***pH determination.*** Stirred curd cheese manufactured with homogenized bulk starters showed no significant differences between average pH measurements for the top and bottom of the vat for all three cultures. Cheese manufactured with unhomogenized bulk starter showed no differences between average pH measurements for both M30 and M37 cultures. However, bottom pH was lower ($P < 0.05$) than top pH (0.06 differential) when stirred curd cheese was manufactured with unhomogenized culture CH970 (Figure 2), suggesting that some agglutinated culture had settled to the bottom of the vat. Although a difference in pH for culture CH970 existed, no detectable decrease in starter activity was observed. Instead, this culture performed better than other cultures, reaching the desired whey drainage pH sooner. Figure 1C showed that culture CH970 bulk starter contained large masses of cells that were tightly clumped. When culture CH970 was added to the milk, some of the large clumps of cells apparently settled to the bottom of the vat. Because these bacteria were still active and producing acid, a pH differential occurred.

***Bacterial enumeration.*** No differences in bacterial number (colony-forming units) were observed between milks containing homogenized or unhomogenized cultures. Mean (homogenized and unhomogenized bulk starters) colony-forming units for top (7.12 $\log_{10}$) and bottom (6.93 $\log_{10}$) portions of milk were not significantly different for the three cultures. However, milks containing unhomogenized or homogenized M37 bulk starter (chaining culture) had higher ($P < 0.08$) counts in the top portion of the milk than in the bottom (Figure 3), indicating that bacteria were being carried to the top of the vat, probably by the buoyant action of milk fat. These data suggest that M37 (chaining culture) had a greater affinity for milk fat or that the net-like structure of M37 might have been lifted by milk fat to a greater extent than cultures M30 and CH970 (clumping cultures). Because culture CH970 produced a pH differential between the top and bottom of the vat of 0.06 units, a higher count might be expected for the bottom sample taken from this vat. However, if only the largest clumps of CH970 settled to the bottom of the vat and each clump of cells formed only one colony

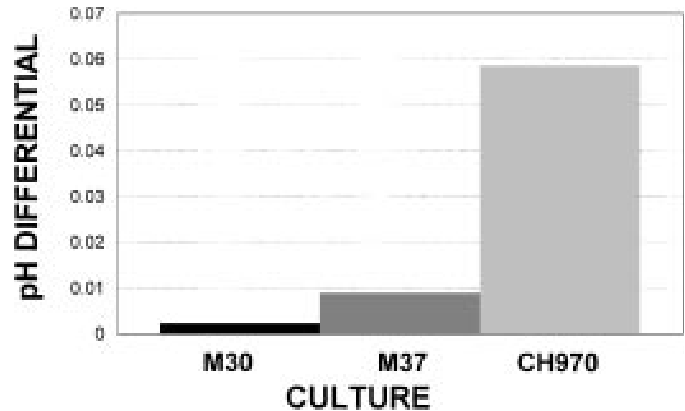



Figure 2. Severity of agglutination of unhomogenized culture as demonstrated by pH differentials between milk samples from the top and bottom portions of the vat.

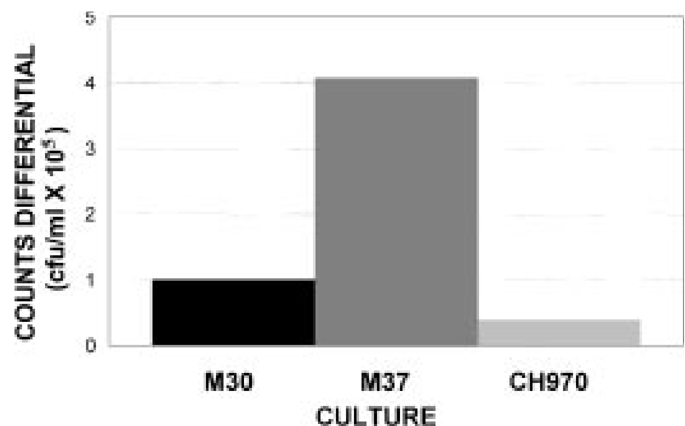



Figure 3. Severity of culture agglutination of unhomogenized culture as demonstrated by bacterial count differentials between the top and bottom portions of the vat.

when bottom samples were enumerated, it is not surprising that no differences in colony-forming units existed between the top and bottom samples.

***Total solids determination.*** For all cultures (both homogenized and unhomogenized), no differences ($P = 0.95$) were observed between mean values for the top and bottom total solids or between their differentials. Total solids from all milks (containing both homogenized and unhomogenized cultures) averaged 11.43% for top samples and 11.38% for bottom samples, indicating that the solids contents of all milks were distributed fairly evenly and that no detectable sedimentation occurred. Because the concentration of solids were slightly higher in the top portion of milk than in the bottom, slowly rising fat globules may have carried clumps of acid-producing cells that were surrounded by a precipitated protein mass to the milk surface. To prove that solids were being displaced, additional samples from the center of the vat and assays of fat would be needed. If only the largest spherical clumps of cells fell to the bottom of the vat when unhomogenized CH970 bulk starter was added to the stirred curd milk (as suggested by the pH differential), those clumps would not be expected to precipitate much additional protein around the clump because of their initial density and the speed at which they fell to the bottom of the vat. Therefore, very little, if any, distribution of total solids occurred in stirred curd vats when agglutinated bacteria were buoyed up by fat globules.

### Effect of Starter Culture Homogenization on the Cheese Manufacturing Process

No significant differences were observed between homogenized and unhomogenized bulk starters used to manufacture stirred curd cheese for any agglutination determinant. The probabilities of correlation between homogenization treatments and agglutination determinants (pH, colony-forming units, and total solids differentials) were $P = 0.78$, $P = 0.39$, and $P = 0.60$, respectively, suggesting that the homogenization of bulk starters had no influence on the mean distribution of bacteria in cheese milks over that of unhomogenized bulk starters. Although Figure 1 (D, E, and F) shows that homogenization decreased chaining and clumping of severely agglutinated cultures, no differences were observed on cheese manufacture schedules (time to drain and hoop) or pH of milk and curd during the manufacture of stirred curd cheese.

Several researchers (9, 16, 19) have demonstrated that homogenization of bulk starter reduces agglutination problems in skim milk when cottage cheese curd is manufactured. Hicks and Hamzah (9) showed the homogenization of agglutinated bulk starters was as effective as homogenization of the skim milk for inhibiting agglutination that occurred in the vat. Homogenization disrupts the bacterial clumps and tears the chains apart (9, 10, 19), and agglutination in the vat is inhibited because the cells do not form a critical mass and fall out of solution by the time (5 h) that the cottage cheese is manufactured (9, 10). Manufacture of many hard cheeses requires that the culture remain evenly distributed in the milk for 0.75 to 1 h (0.25 to 0.5 h during ripening and 0.25 h during coagulation) before the bacteria are immobilized by the coagulum. Previous research (9, 10, 16, 19, 22) on cottage cheese suggests that homogenized cultures would have optimal activity during manufacture of the stirred curd cheese. It was thus surprising that the severely agglutinated unhomogenized culture did not show any real agglutination problems, such as differences in manufacturing schedules or pH values. The unhomogenized cultures chosen and the experimental conditions used for this experiment typically form a sludge layer within 1 h in skim milk (9, 10). In skim milk, large differentials in pH, total solids, and colony-forming units would have been expected within 1 h. These differentials were not evident because of the shorter time before milk coagulation, and clumps and chains of bacterial cells may have been buoyed up by fat globules in milk used for stirred curd cheese; also, acid apparently diffused out from clumps of bacteria fast enough to prevent acid inhibition of the culture within clumps. However, no research has measured whether a sufficiently low pH exists within the clumps to inhibit the culture as has been demonstrated in a sludge layer. The present research suggests that acid diffused out of large spherical clumps, such as those observed for unhomogenized CH970 culture, was sufficient to maintain rate of acid development equal to the homogenized CH970 control. Therefore, homogenization of severely agglutinated bulk starter is not necessary in the production of hard cheeses, and agglutinated cultures do not appear to be a problem when hard cheeses are manufactured with them.

### Microscopic Examination of Cheese Curd

To determine whether agglutinated cells were attaching to fat globules or being physically displaced, smears were prepared from top areas of milk coagula inoculated with unhomogenized cultures. Photomicrographs of smears revealed that chains and clumps of cells were surrounded with coagulated protein, presumably casein, which formed a structure that was associated with several fat globules (Figure 4).

Two types of dyes were used to observe these relationships. Crystal violet stained bacterial cells a dark blue color, and Sudan III stained fat globules yellow. Sudan III also bound with casein, resulting in a reddish color. The phase contrast micrograph (Figure 4) shows that only a few, if any, single or paired cells of the culture may be attached to fat globules, which are red with green halos. Clumps of cells and chains found in coagula from the top portion of the milks in the cheese vat were as large as those viewed in bulk starter (Figure 1, A, B, and C). Most clumps of cells and clumps of chains in bulk starters were contained in heavy protein masses that enveloped the cells.

Complexes of agglutinated bacteria and protein continued to produce lactic acid during cheese manufacture. Protein from milk precipitated around the complexes, turning them into massive structures that appeared to be associated with the rising fat globules. Microscopic examination of these agglutinated cultures in cheese milk coagula revealed that these complexes appeared to be more developed than those in the bulk culture and were much larger than fat globules (Figure 4). Fat globules in the upper coagula were numerous and appeared to form a continuous cream layer. The photomicrographs show that the protein concentration in and around clumps of cells was much greater than that in the coagula matrices. These heavy protein deposits prevented attachment of the cells per se to fat globules. When fat globules aggregate, they rise (20, 27, 30) and enhance the rate that clumps and chains of bacterial cells are carried to the milk surfaces. However, under the conditions of this research, only a trace amount of cream could be observed on the surface after coagulation. Most fat globules were trapped within the coagulum before a cream layer could form.

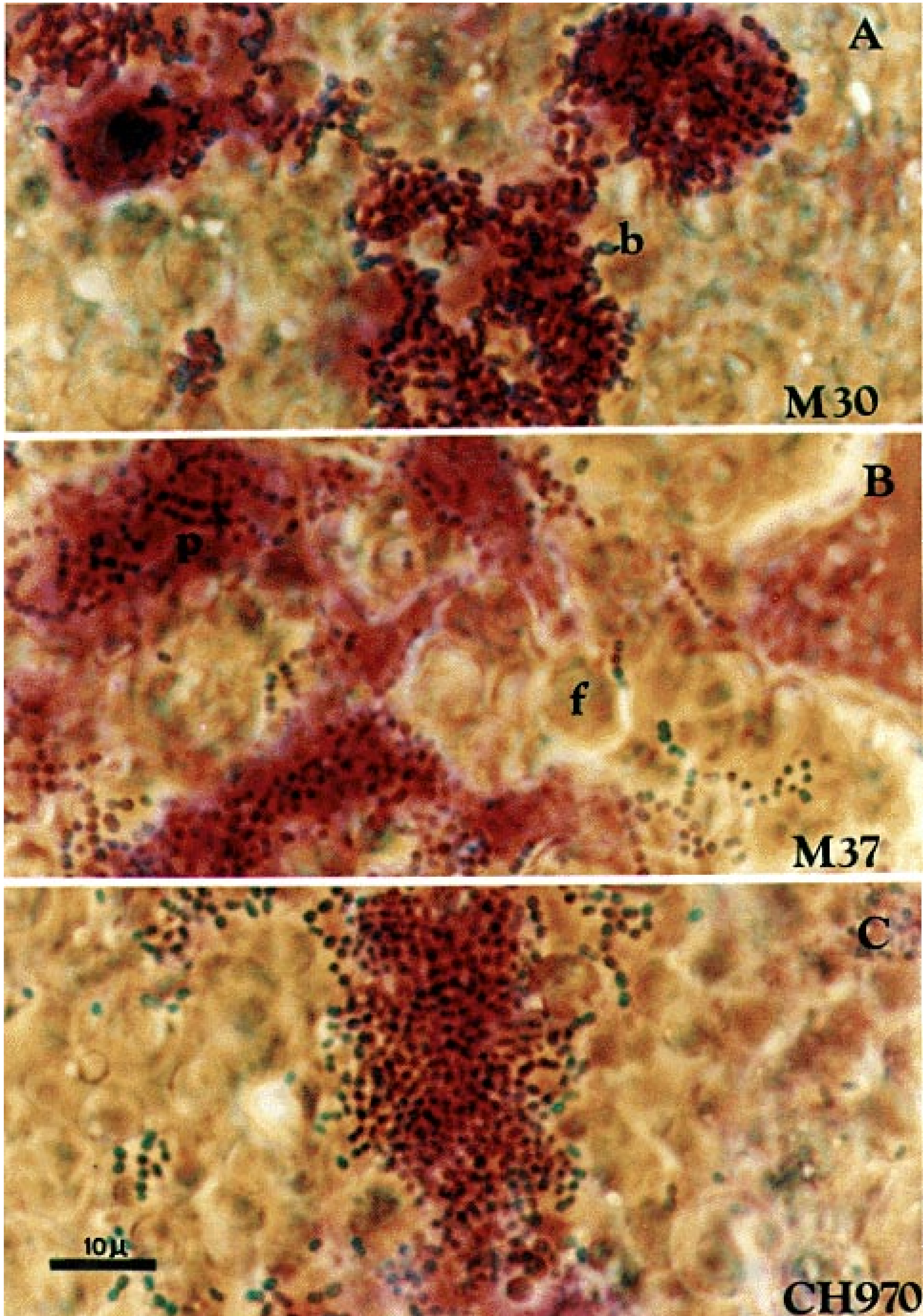
Wright and Tramer (32) and Walstra and Jenness (31) reported that inhibitory substances (Ig or agglutinins) in whole milk caused fat globules to agglomerate and to form an extended cream layer. Wright and Tramer (32) noted that agglutinated starter cultures (slow cultures) were carried with fat globules (6 h at 30°C) into a cream layer, decreasing acidity development. Keogh (14) proposed that the effect of creaming on bacteria appeared to be merely displacement; agglutinated bacteria were essentially removed from the milk body by rising cream. Culture performance decreased only when most of the bacteria were in the cream layer.

Payens et al. (20) found that increased fat globule clustering was accompanied by an enhanced adsorption of euglobulin onto the fat globule surface. They suggested that fat globules were aggregated by the formation of euglobulin bridges or bonds between the fat globules with the free ends of the peptide projecting into the milk plasma. Gillies (7) reported that bacterial cells were attracted to the surface of fat globules and that these cells rose with the fat globules to form the cream layer. Stadhouders and Hup (27) concluded that euglobulin could form a linkage between fat globules and culture bacteria. Reiter (21) confirmed the theory of Stadhouders and Hup (27) with a photomicrograph of an *Escherichia coli* bacterium attached to the milk fat globule membrane.

Although Ig can attach to fat globule membranes and to culture cell surfaces, the bond strength between Ig and these surfaces appears to be vastly different. Walstra and Jenness (31) reported that bonds between fat globules were easily disrupted at very low homogenization pressure (<0.01 MPa). However, Milton et al. (19) and Hicks and Hamzah (9) have reported that a pressure of 17.2 MPa (13.8 MPa, first stage; 3.4 MPa, second stage) was needed to tear apart agglutinated culture cells. Either the bond strength between bacteria cell surfaces and the Ig is much greater than that between fat globule membranes and Ig or the number of Ig associating with the cells is much greater.

In hard cheese manufacture, such as the stirred curd procedure, only a small amount of creaming occurs by the time the milk is coagulated; therefore, the effect of creaming would be minimal. Fat globules are rather evenly distributed throughout the cheese milk because stirring continues until rennet is added to the vat. When normal amounts of rennet are added, a soft coagula forms within 15 min, and fat globules are immobilized by the coagula. In the present study, milk was stirred for a total of 4 min between inoculation and cutting, and the total ripening time was 1 h to maximize settling and creaming problems. Even under these conditions, the use of severely agglutinated bulk cultures did not cause manufacturing problems. When these cultures were used in skim milk fermentations, large differentials in pH, total

Figure 4. Photomicrographs show cultures M30 (A), M37 (B), and CH970 (C) (1500× magnification; 100× oil immersion lens, 1.5× intermediate magnification Bertrand lens, and a 10× eye lens) in milk coagulant (4% milk fat) that had been inoculated with unhomogenized bulk starter. Smears were prepared from samples taken from the top portion of the coagula when pH was 6.1 to 6.2. b = Bacteria stained with crystal violet, f = fat globules stained with Sudan III, and p = casein stained with Sudan III (Sigma Chemical Co., St. Louis, MO).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10µ
CH970

solids, and colony-forming units occurred within the vat of milk (9, 10, 22, 29). Therefore, severely agglutinated culture masses may have associated with milk fat globules that prevented them from settling to the bottom of the vat during the ripening and rennet coagulation periods. Because the agglutinated cultures stayed rather evenly distributed within the vat, there should be no real problem when these types of cultures are used in the manufacture of hard cheeses.

## CONCLUSIONS

Homogenization of agglutinated bulk starters at 17.2 MPa (13.8 MPa, first stage; 3.4 MPa, second stage) dispersed agglutinated chains and clumps to the point that most cells were present as single cells or very short chains similar to commercial nonagglutinated cultures. Milk inoculated with unhomogenized CH970 (clump former) culture developed a lower pH at the bottom of the vat, suggesting that the larger clumps had settled to the bottom of the vat to form a thin sediment layer but continued to produce lactic acid, causing a pH differential. A slightly higher concentration of bacteria was found in top milk samples for culture M37 (chain former), which implied that culture M37 had a greater affinity for milk fat or developed a greater net-like structure that was carried up by rising fat globules. Photomicrographs taken from cheese curd suggested that the main reason that agglutinated cell masses did not settle to the bottom of the vat and that a higher bacterial cell concentration was sometimes observed at the milk surface was not due to direct attachment of bacterial cell chains and clumps to fat globules, but rather was due to the physical displacement of these chains and clumps by fat globules. Although unhomogenized cultures were severely agglutinated, the fat globules prevented the clumps of cells from settling and from causing defects from decreased starter activity and longer manufacturing schedules.

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