Influence of Milk Components on the Injury, Repair of Injury, and Death of *Salmonella anatum* Cells Subjected to Freezing and Thawing

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Fast freezing and slow thawing *Salmonella anatum* cells in various milk components inactivated from 20 to 98% of the cells and damaged 40 to 90% of the cells surviving the treatments. Injured cells failed to form colonies on a selective medium (xylose-lysine-peptone agar with 0.2% sodium deoxycholate) but did form colonies on a nonselective plating medium (xylose-lysine-peptone agar). The major milk components—lactose, milk salts, casein, and whey proteins—influenced the extent of injury, repair of injury, and death. The percentages of cells injured and inactivated were decreased by the presence of any milk components except whey proteins. Also, repair of injury was promoted by the presence of each milk component except whey proteins, which in contrast inhibited repair. Phosphate was the most influential milk salts component that protected the cells and promoted repair of injury. These individual milk components may have decreased the extent of freezing-induced death and cellular damage by stabilizing the *S. anatum* cell envelope.

Several environmental stresses are known to induce bacterial death and injury. Recent reports in the literature have indicated that exposure of bacteria to irradiation (8, 9), sanitizing compounds (23, 24), ethylenediaminetetraacetic acid (7), aerosolization (5), heating (15, 16), freezing (4, 10), and freeze-drying (20, 21) resulted in sublethal injury. Two types of injury were observed: (i) injury as measured by increased nutritional requirements for recovery, and (ii) injury as measured by increased sensitivity to selective agent in the recovery medium. In both types of injury, the damage was found to be repairable under specific conditions.

The extent of bacterial injury and death was influenced by the suspending medium used for freezing and freeze-drying. A number of compounds including sugar, milk proteins, peptone, gelatin, meat extract, glycerol, and phosphate protected bacteria from freezing or freeze-drying. These compounds apparently protected the cellular membranes from damage (4, 10).

The purpose of this investigation was to determine the influence of individual milk components and their interactions on the injury, repair of injury, and death of *Salmonella anatum* cells after freezing and thawing. A preliminary report of these findings has been made (D. W. Janssen and F. F. Busta, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 18, 1973).

**MATERIALS AND METHODS**

Freezing and thawing of cell suspensions. *S. anatum* MP3 cells were propagated and maintained in reconstituted nonfat dry milk (10% solids not fat) as described previously (20). A 1-ml portion of this culture was inoculated in 100 ml of tryptic soy broth containing 0.3% yeast extract (Difco) and grown for 16 to 20 h at 35 C. The cells were centrifuged, washed with sterile water, and suspended in the test solutions as described previously (19). Portions of 10 ml each were placed in test tubes (150 by 25 mm) and frozen rapidly in a dry ice-acetone bath for 10 min. The contents were thawed in a water bath at 4 C for about 45 min and tested for cell injury, death, and repair of injury.

**Determination of cell injury, death, and repair.**

The thawed test solutions were incubated in a water bath at 25 C. At indicated intervals, cells from the test solutions were counted on xylose-lysine-peptone agar (XLP) and XLP with 0.2% sodium deoxycholate.
added. A 0.1-ml quantity was surface plated in triplicate on each medium. Death, injury, and repair were determined as described previously (20).

Composition and preparation of the freezing and repair menstrua. To examine the effect of milk proteins in the suspending menstruum, 9-ml test solutions of several concentrations (wt/vol) of sodium caseinate (Fisher Scientific Co.), beta lactoglobulin (NBC Co.), alpha lactalbumin (NBC Co.), and bovine serum albumin (Sigma Chemical Co.) were prepared in sterile water. The pH was adjusted to 6.6 with sterile 0.1 N NaOH or HCl. Sodium caseinate was dissolved by a mild heat treatment and pH adjustment. Lactose (J. T. Baker Chemical Co.) was added in crystalline form to the test solutions to achieve the desired concentrations. A solution containing the salts present in milk was also prepared according to the following concentrations: KH₂PO₄, 0.158%; K₂SO₄, 0.018%; K₂CO₃, 0.03%; KCl, 0.10%; potassium citrate, 0.05%; ammonium citrate, 0.18%; magnesium citrate, 0.05%; CaCl₂, 0.13%. The milk salts were prepared in sterile water by the suggested method of Jenness and Koops (6). Concentrations of test media approximated those found in fluid milk.

Experimental design to determine the influence of milk components on injury, repair, and death. Full factorial designs, response surface designs, analysis of variance, and Yates analysis were used as described by Davies (3). All statistical computations were made utilizing programs provided by the Pillsbury Co., Minneapolis, Minn.

RESULTS

The influence of milk components on injury and death was examined. S. anatum cells were suspended and frozen in whole milk, skim milk, reconstituted nonfat dry milk (10% solids not fat), whey, sodium caseinate (2.5%), whey proteins (0.7%), beta lactoglobulin (0.4%), milk salts, and distilled water. The test solutions were thawed and incubated at 25°C for testing at 0, 1, and 2 h. The data are presented in Table 1. Whole milk, skim milk, and reconstituted nonfat dry milk (10% nonfat solids) afforded protection to the cells from injury and death and promoted repair. Whey or whey proteins also facilitated repair and protected the cells from freezing injury, but the number of damaged cells increased. The presence of casein (sodium caseinate) in the freezing and repair menstrua reduced the percentage of death to 26%, which was comparable to whole milk. Beta lactoglobulin in pure form was more protective than water alone, but did not promote repair to the extent that was observed with milk salts.

To investigate the importance of whey proteins on injury, repair, and death, S. anatum cells were frozen and thawed in 0.07% alpha lactalbumin, 0.03% bovine serum albumin (BSA), or 0.4% beta lactoglobulin. The data are presented in Table 2. Individual whey proteins showed similar effects on repair and death. The percentage of injury did not decrease with incubation at 25°C. Freeze-injured cells thus apparently could not repair in solutions of alpha lactalbumin, BSA, or beta lactoglobulin. The individual whey proteins also did not protect the cells from death by freezing and thawing. However, differences existed in the extent to which whey proteins protected the S. anatum cells from initial injury. All the proteins were more protective than water alone, but BSA was less effective than beta lactoglobulin or alpha lactalbumin. The combination of the three whey proteins reduced the percentage of death.

The data indicating the effects of whey proteins in combination with milk salts are presented in Table 3. In liquid medium containing milk salts alone and milk salts plus whey, the percentage of death was 24%. Table 3 also presents the percentage of death in milk salts solution plus BSA, and the combination of BSA and whey proteins. The combination of BSA and whey proteins reduced the percentage of death.

**Table 1. Effect of milk components on the injury, repair, and death of Salmonella anatum NF3 subjected to freezing and thawing**

| Freezing and repair menstrua | Percentage of injury | Percentage of death |
|-----------------------------|----------------------|---------------------|
|                            | 0 h      | 1 h      | 2 h      |          |
| Whole milk                  | 62*      | 16       | 10       | 26       |
| Skim milk                   | 62       | 22       | 11       | 22       |
| 10% Nonfat milk solids      | 61       | 20       | 10       | 20       |
| Whey                        | 67       | 22       | 15       | 42       |
| Sodium caseinate            | 73       | 42       | 20       | 26       |
| Whey proteins plus salts    | 76       | 63       | 14       | 55       |
| Beta lactoglobulin          | 76       | 73       | 69       | 90       |
| Milk salts                  | 85       | 59       | 48       | 81       |
| Water                       | 90       | 88       | 84       | 96       |

* Time at 25°C after thawing.
* Values are arithmetic means of four separate trials; range of data from individual trials did not vary greater than ±10% of the mean.

**Table 2. Effect of whey proteins on the injury, repair, and death of Salmonella anatum NF3 subjected to freezing and thawing**

| Freezing and repair menstrua | Percentage of injury | Percentage of death |
|-----------------------------|----------------------|---------------------|
|                            | 0 h      | 1 h      | 2 h      |          |
| Water                       | 90*      | 88       | 84       | 96       |
| Alpha lactalbumin (0.07%)   | 79       | 72       | 70       | 98       |
| Bovine serum albumin (BSA) (0.03%) | 88     | 80       | 79       | 96       |
| Beta lactoglobulin (0.4%)   | 76       | 75       | 71       | 93       |
| Alpha lactalbumin (0.07%)   | 87       | 77       | 69       | 98       |
| and BSA (0.03%)             |          |          |          |          |
| Alpha lactalbumin (0.07%), BSA (0.03%), and beta lactoglobulin (0.4%) | 77 | 72 | 68 | 84 |

* Time at 25°C after thawing.
* Values are arithmetic means of four separate trials; range of data from individual trials did not vary greater than ±10% of the mean.
sented in Table 3. All the test solutions were protective and promoted repair in comparison to the water control. Phosphate alone, at the concentration found in milk salts, appeared to be the important ingredient in the milk salts that was required for protection and repair. However, less death occurred in milk salts than in phosphate. The combination of milk salts and whey protein decreased death and promoted the repair of injury.

To determine the effect of milk components and their interaction on the injury, repair of injury, and death of *S. anatum* cells subjected to freezing and thawing, a two-level, full-factorial experimental design was used. The factors examined were milk salts, lactose, whey proteins, and casein. This design was used to determine the effects of these major components when they were either absent or present at normal milk concentrations (Table 4). A positive effect indicates greater death or injury when the factor was at the high level (i.e., present at the concentration levels in milk) in the test solution.

The data indicated that the single factors of lactose and whey proteins significantly influenced the injury of *S. anatum* cells. The significant interactions were: AD (milk salts and casein), BC (lactose and whey proteins), ABC (milk salts, lactose, and whey proteins), and ACD (milk salts, whey proteins, and casein). The presence of whey proteins increased the percentage of injury, whereas lactose decreased the percentage of injury. However, there was an interaction such that the effect of whey proteins was greater in the presence of lactose. Also, the effect of lactose was reduced in the presence of casein.

The effects of casein, lactose, whey proteins, and milk salts on the injury of frozen and thawed *S. anatum* cells after incubation for 2 h at 25°C (i.e., a measure of repair) also are evident in Table 4. The data indicated that all single factors except whey proteins significantly influenced the repair of injury. The negative effects of milk salts, lactose, and casein indicated that these compounds reduced the percentage of injury or facilitated repair. The significant interactions were: AB (milk salts and lactose), AD (milk salts and casein), BD (lactose and casein), and ABCD (all milk components). An analysis of two-factor interactions indicated that casein greatly enhanced the repair process and milk salts also aided repair, but the effect was not as great when in combination with casein.

The effects of the major milk components on the death of frozen and thawed *S. anatum* cells are also shown in Table 4. The data suggested that the single factors of milk salts, lactose, and

### Table 3. Effect of whey proteins and milk salts on the injury, repair, and death of *Salmonella anatum* NF3 subjected to freezing and thawing

| Freezing and repair menstrua | Percentage of injury | Percentage of death |
|-----------------------------|---------------------|---------------------|
|                             | 0 h*                | 1 h*                | 2 h*                |
| Water                       | 90                 | 88                 | 84                 | 96                 |
| K,HPO₄(0.158%)              | 75                 | 54                 | 37                 | 90                 |
| Milk salts                  | 87                 | 73                 | 48                 | 77                 |
| Beta lactoglobulin (0.4%) in milk salts | 75     | 59                 | 34                 | 65                 |
| Beta lactoglobulin (0.4%) in milk salts (without phosphate) | 91     | 74                 | 66                 | 72                 |
| Alpha lactalbumin (0.07%), and bovine serum albumin (BSA) (0.03%) in milk salts | 85     | 68                 | 32                 | 80                 |
| Alpha lactalbumin (0.07%), BSA (0.03%), and beta lactoglobulin (0.4%) in milk salts | 87     | 62                 | 36                 | 69                 |

*Time at 25°C after thawing.

*Values are arithmetic means of four separate trials; range of data from individual trials did not vary greater than ±10% of the mean.

### Table 4. Effect of casein, lactose, whey proteins, and milk salts on the injury, repair of injury, and death of *Salmonella anatum* cells after freezing and thawing

| Factor* | Effect on percentage of injury | Effect on percentage of injury after 2 h at 25°C |
|---------|-------------------------------|-----------------------------------------------|
| A (milk salts) | + | _*** | _*** |
| B (lactose) | _*** | _*** | _*** |
| C (whey proteins) | + | _*** | + |
| D (casein) | _*** | _*** | _*** |
| AB | + | _*** | + |
| AC | + | _*** | + |
| AD | + | _*** | + |
| BC | + | _*** | + |
| BD | + | _*** | + |
| CD | + | _*** | + |
| ABC | + | _*** | + |
| ABD | + | _*** | + |
| ACD | _** | + | _** |
| BCD | _** | + | _** |
| ABCD | _** | + | _** |

*Full-factorial design. Factor levels: A, 0% and normal milk concentration (i.e., Jenness and Koops' salt solution [6]); B, 0 and 5% (wt/vol); C, 0 and 0.5% (wt/vol) (i.e., beta lactoglobulin [0.4%], alpha lactalbumin [0.07%] and bovine serum albumin [0.03%]); D, 0 and 2.5% (wt/vol).

* Asterisks indicate the level of significance: *, 0.10 level; **, 0.05 level; ***, 0.01 level. Symbols: +, positive effect or increase in percent; −, negative effect or decrease in percent.
casein influenced the percentage of death. These factors had negative effects which indicated that their presence in the freezing menstrua decreased death. The significant interactions were: AB (milk salts and lactose), AD (milk salts and casein), and CD (whey proteins and casein). An analysis of the two-factor interactions suggested that casein greatly reduced death and milk salts also reduced death; however, the effect was greater when casein was absent. Also, the presence of lactose in combination with casein resulted in less death.

Figure 1 shows the results of a response surface experiment that demonstrated the influence of concentration of milk salts, lactose, whey proteins, casein and their interactions on the percent injury of S. anatum cells immediately after freezing and thawing. The experiments using five different concentrations of each variable were designed for the determination of quadratic equations which were then used to generate the response surfaces. Only sections of the response surfaces are shown. The axis for the dependent variable extends out from the page. Percentage of injury ranged from <40 to >65% under the test conditions. The differences in the surfaces indicated that high concentrations of lactose (8.54%) reduced the percentage of injury. Each section had curved response contours that suggested a casein-milk salts interaction. Lactose and whey protein concentrations influenced the percentage of injury. With high concentrations of lactose, injury was independent of casein and dependent on milk salts. High levels of whey proteins decreased the percentage of injury along with a high concentration of milk salts and a low concentration of casein. The different effects of increasing levels of lactose and whey proteins demonstrated a four-factor interaction among milk components.

Figure 2 shows the results of a response surface experiment to demonstrate the influence of the concentration of the major milk components and their interactions on the percentage of injury after 2 h at 25 C (i.e., repair).

**Fig. 1.** Effect of the freezing and thawing menstruum on the injury of Salmonella anatum NF3 cells. Responses are expressed as percentage of injury of the survivors immediately after thawing. Injured cells were capable of forming colonies on a nonselective plating medium, xylose-lysine-peptone agar (XLP), but were unable to form colonies on a selective plating medium, XLP with 0.2% sodium deoxycholate. The three surfaces in each horizontal row correspond to a fixed percentage of whey proteins (e.g., the three surfaces in the top row were obtained with 0.44% whey proteins). The three surfaces in each vertical column correspond to a fixed percentage of lactose (e.g., the three surfaces in the left vertical column were obtained at 1.46% lactose). F test of significance for model, significant at P = 0.10.
Fig. 2. Effect of the repair menstruum on the percentage of injury of repairing Salmonella anatum NF3 cells. Responses indicating repair are expressed as percentage of injury of the survivors after 2 h at 25°C. Injury was defined as the inability to form colonies on a plating medium containing deoxycholate as the selective agent; therefore, less injury indicates more repair. As in Fig. 1, the three surfaces in each horizontal row correspond to a fixed percentage of whey proteins (e.g., the three surfaces in the top row were obtained at 0.44% whey proteins). The three surfaces in each vertical column correspond to a fixed percentage of lactose (e.g., the three surfaces in the left column were obtained at 1.46% lactose). $F$ test of significance for model, significant at $P = 0.025$.

The percentage of injury after 2 h at 25°C ranged from 0 to 35%. A low percentage of injury after the incubation period indicated substantial repair of injury. The differences in surfaces suggested that the repair process was greatly influenced by lactose and whey proteins. Also, the curved response contours in each section indicated a milk salts-casein interaction with the greatest repair at low levels of lactose and whey proteins. With a high level of casein, the repair process was less dependent on milk salts.

The least repair occurred at low levels of casein (0.59 to 1.0%) and milk salts (0 to 20%), with whey proteins at a high concentration (2.56%) and lactose at lower levels (1.46 and 5.0%). Repair was inhibited at high levels of whey proteins as indicated by the steepness of the surfaces.

Figure 3 shows the results of a similar response surface experiment that demonstrated the effects of concentrations of milk salts, lactose, whey proteins, and casein on the percentage of death of frozen and thawed S. anatum cells. The percentage of death was from 33 to >51% under the test conditions. The curved response contours in each section indicated a milk salts-casein interaction. In all cases, low levels of casein and milk salts resulted in greater death at the various concentrations of whey proteins and lactose. The greater steepness of the surfaces with increased levels of whey proteins suggested that these proteins increased death. Lactose appeared to decrease death especially at higher levels of whey proteins.

**DISCUSSION**

Several investigators have reported the influence of the suspending medium on the recovery of bacteria after freezing or freeze-drying. Also, a great deal of information exists on the ability of specific nutrients to promote the repair of cells sublethally injured by freezing, irradiation, freeze-drying, and heating. However, the literature is lacking on the influence of constitut-
ments of the suspending medium on injury, repair of injury, and death of bacteria. Therefore, this paper presents the effects of milk components and their interactions on injury, subsequent repair of injury, and death of S. anatum. Janssen and Busta (manuscript submitted for publication) previously showed that milk solids had a protective influence on S. anatum cells subjected to freezing and thawing. In this study, the protective influence of milk systems was verified. Whole milk, skim milk, and reconstituted nonfat dry milk (10% nonfat solids) protected cells from injury and death and promoted repair. The presence of 2.5% sodium caseinate in the freezing and repair menstrua gave results comparable to whole milk. Whey was shown to protect the cells from freezing injury, but the extent of death in whey was greater than in whole or skim milk. The major whey proteins (beta lactoglobulin, alpha lactalbumin, and BSA) were more protective than water alone, and BSA was less protective than beta lactoglobulin or alpha lactalbumin. Whey proteins did not promote repair. Milk salts were the important ingredient of whey for protection and repair. In milk salts, phosphate was the most important ingredient for protection and repair. However, a combination of milk salts and whey proteins decreased death and enhanced the repair process.

The effects of milk salts, lactose, whey proteins, casein, and their interactions were evaluated by full-factorial and response surface designs. All of these factors affected the extent of S. anatum injury after freezing and thawing. The presence of lactose in the freezing menstrua decreased the percentage of injury, especially at a high level (8.54%). Several investigators have found that sugars were protective against immediate freezing injury (1, 10, 11, 17, 18). The mechanism of action remains obscure, but Mazur (10) suggested that sugars prevented injury to the cell membrane. The protective action of many compounds, including sugars, may be related to their ability to hydrogen bond (10). Mazur concluded that the ability of hydrogen bonding substances to protect cells from freezing injury must be related to their influence on the structure of water.

Milk salts alone or in combination with other milk components influenced injury. Phosphate was found to be the major protective component...
of milk salts. Moss and Speck (14) showed that *Escherichia coli* cells frozen in phosphate buffer were more resistant to freezing damage than those frozen in distilled water. They reported that protective peptides leaked from the cells suspended in phosphate buffer. Davies (2) suggested that the phosphate ion was capable of preventing the conformational destabilization of macromolecules.

The major milk protein, casein, also influenced injury of *S. anatum* cells. A significant casein-milk salts interaction was observed. Casein is a large protein with numerous exposed functional groups. The protective ability of this protein might be related to its association with phosphate, its hydrogen bonding properties, or its capacity to regulate cellular rehydration upon thawing. Several investigators (2, 4, 10, 12, 13) have reported the protective nature of milk proteins and other complex colloidal macromolecules, but the mechanism of protection remains obscure. Mazur (11) suggested that proteins might protect the cell from injury by interacting at specific membrane sites.

The effects of milk salts, lactose, whey proteins, and casein on the repair of freezing and thawing-induced injury of *S. anatum* were evaluated. At normal milk concentrations, all single factors except whey proteins promoted the repair process. However, as concentrations were increased, lactose and whey proteins slowed or reduced repair. The repair process was dependent on high levels of casein or milk salts. The least repair was observed at low levels of casein, milk salts, and lactose, with a high level of whey proteins. The repair inhibition by whey proteins might be related to an ability to bind the essential components of milk salts such as Mg$^{2+}$ and phosphate. It had been established previously that the repair of *S. anatum* or *E. coli* cells frozen and thawed in water required phosphate and MgSO$_4$ (19, 22).

The influence of milk components on the death of frozen and thawed *S. anatum* cells was evaluated. The results suggested that milk salts, lactose, and casein protected *S. anatum* cells from death induced by freezing and thawing. These same factors also protected the cells from injury. The mechanism of protection afforded by these compounds is only speculative. As mentioned previously, these components might regulate rehydration after thawing, stabilize the cell envelope by hydrogen bonding, or alter cell permeability.

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