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Evaluation of an Inoculation System for a Closed Bulk Starter Vessel¹

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ABSTRACT

Stainless steel vessels (open, closed and control, i.e. not exposed to aerosol) containing 10% reconstituted nonfat dry milk were inoculated with *Streptococcus cremoris* at 10⁶ CFU/ml. Milk in the closed vessel was specifically inoculated through a rubber seal in a port using a sterile needle and syringe in the presence of an aerosol containing bacteriophage, *Escherichia coli* and milk or whey solids. The lid of the open vessel was removed for 2 min to expose milk to the aerosol. Milk was incubated at 22°C for 16 h. Samples were tested for pH, titratable acidity, and *E. coli*, host and bacteriophage numbers. *E. coli* and bacteriophage were recovered from the open vessel, and not the control and closed vessels. This inoculation system minimized exposure of bulk starter medium to an aerosol and reduced the risk of producing bulk starter contaminated with bacteriophage.

A recognized source of bacteriophage (phage) contamination originates in cheesemaking with the inoculation of bulk starter medium. In many cheese factories, bulk starter medium may be exposed to contaminated air when starter culture is poured through an opened port of the bulk starter vessel. Potential contamination also exists from the handling of the starter culture. When contamination includes phage, growth of the bulk starter could be inhibited, thus producing a "dead vat" of cheese. In 1950, Elliker (4) stated the problem of phage appeared to be universal in the dairy industry. Over 30 years have elapsed since this statement was made, and phage contamination is still a concern. Various methods have been designed to inhibit or eliminate phage contamination during bulk starter preparation and cheesemaking (10). Targets for change have included bulk starter media, cheesemaking procedures, equipment, and lactic acid bacteria used as starter cultures.

Bulk starter media may be based on skim milk or whey (3). Key to phage attack of host cells is the availability of calcium, and modified bulk starter media (2) inhibit phage by making calcium unavailable. An area of ongoing research involves the development of phage-resistant lactic acid bacteria. According to Klaenhammer (5), systems encoded by plasmid DNA in lactic acid bacteria, primarily streptococci, interfere with phage adsorption, direct restriction and modification of phage DNA, and disrupt the lytic cycle. Natural selection of strains that are suitable for dairy fermentations and are phage-resistant occurs slowly. In contrast, genetic engineering may be a method to more quickly construct strains with desired characteristics. Earlier work in New Zealand focused on procedures in the cheese factory which would reduce phage propagation (6). The spread of phage was discovered from whey aerosols and from inadequately cleaned and sanitized cheese vats and other pieces of equipment. Proper sanitization of the vat with 200 ppm of chlorine reduced phage contamination. Additional changes in cheesemaking procedures were elimination of the ripening period (i.e., time the inoculated milk sat in the vat before adding rennet) and the use of paired starters in rotation.

In 1974 the annual report of the New Zealand Dairy Research Institute (9) mentioned several improvements of bulk starter vessels: cleaning-in-place, design alterations which reduced corrosion and fatigue cracks, and use of a lower stirrer shaft speed to reduce phage entering the machine through the water seal. Previously, Robertson (8) described a bulk starter vessel that eliminated exposure of medium to air. Phage-free bulk starter is essential to industrial cheesemaking. The design of a bulk starter vessel which allows aseptic inoculation of the starter culture and eliminates contamination from aerosols may reduce the build-up of phage in the cheesemaking environment. For our research, a closed bulk starter vessel was designed in which starter culture was aseptically inoculated through a rubber seal in an inoculation port using a sterile needle and syringe. The objective of this study was to evaluate the inoculation system in the presence of an aerosol carrying potential bulk starter contaminants. A similar method of aseptic

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inoculation of starter medium was reported by Robertson et al. (9).

MATERIALS AND METHODS

Detection of bacteriophage in milk

Nonfat dry milk (NDM) was reconstituted to 10% and placed in rolling steam for 30 min (final temperature 99°C). Steamed milk was divided among seven sanitized stainless steel vessels (3L) and cooled to 22°C for use as bulk starter medium. Milk was inoculated with *S. cremoris* 804 (Nordica) at 10^7 CFU/ml. Six vessels were inoculated with serial dilutions of homologous phage 804 to give phage concentrations ranging from 10^2 PFU/ml to 10^{-3} PFU/ml (ca. 1 PFU/L). The seventh vessel served as the phage-uninoculated control. Milk was incubated for 16 h at room temperature (24°C). Titratable acidity (TA) was measured by titrating with 0.1 N sodium hydroxide to the phenolphthalein endpoint, and pH was determined using an Orion Research microprocessor ionalyzer (model 901, Orion Research Incorporated, Cambridge, MA) (7). Host CFU were enumerated by spreading serial dilutions of milk on M17 agar, and incubating at 30°C for 2 d (11). Phage were enumerated by the method of Adams (1) using M17 top and bottom agars containing 0.01 M CaCl_2 , incubated 12 to 24 h at 30°C. Coagulation of milk was noted.

Generation of aerosol

A portion of the same steamed milk used for bulk starter medium was diluted in sterile phosphate buffer (7) to reduce the solids to 1% v/v. Cottage cheese whey, obtained from the University of Minnesota food science pilot plant and stored at 4°C, was placed in rolling steam for 30 min, decanted from the protein mass that precipitated during steaming, and used without dilution. The aerosol solution was composed of phage (10^4 - 10^5 PFU/ml), *E. coli* ATCC 12955 (10^6 CFU/ml), and whey or milk solids. These solutions were propelled by pressurized freon into a laboratory hood to generate an aerosol. M17 agar, seeded with host homologous to the phage in the aerosol, and Trypticase Soy agar (TSA) plates were opened at intervals through 60 min after aerosolization. TSA was overlaid with Violet Red Bile agar (VRBA), incubated at 37°C for 24 h, and colonies typical of *E. coli* were counted (7). M17 agar plates were incubated at 30°C for 12 to 24 h and plaques were counted.

Evaluation of the inoculation system

Two vessels containing 3 L of reconstituted NDM were exposed to the aerosol in the laboratory hood. One vessel was preinoculated with host (*S. cremoris* 023 homologous to phage 316 and/or *S. cremoris* 4-12 homologous to phage 322, obtained from T. Cogan, Moorepark Research Centre, Fermoy, County Cork, Ireland), at a concentration of 1% (10^6 CFU/ml), and the inoculated milk was exposed to the aerosol by removing the vessel lid for 2 min. The second vessel remained closed, and milk was inoculated to a concentration of 1% (10^6 CFU/ml) by injecting a broth culture through a rubber seal in the inoculation port with a needle and syringe. A sample of inoculated milk was obtained through the port using a second sterile needle and syringe. A control vessel containing inoculated milk (10^6 CFU/ml) remained outside the hood to avoid exposure to the aerosol. Each experiment consisted of a control (outside hood), open (in hood), and closed (in hood) vessel; three trials were done. The vessels were incubated at 22°C for 16 h. TA and pH were measured after inoculation and incubation. *E. coli*, phage, and host CFU were

enumerated before and after inoculation and after incubation using the same media and incubation conditions as described previously.

RESULTS AND DISCUSSION

Detection of bacteriophage in milk

The primary purpose of this part of the study was to determine whether one phage particle could be detected after contaminating 3 L of bulk starter medium and after 16 h of potential propagation. Phage were detected in our system by plaque assay. Milk was inoculated at a host concentration of 10^7 CFU/ml, and the seven initial phage concentrations ranged from the lowest concentration of ca. 1 PFU/L to the highest concentration of 10^2 PFU/ml; control milk was not inoculated with phage. Figure 1 illustrates the data obtained after 16 h. Excluding the control milk, phage were present at a concentration of $>10^7$ PFU/ml. At lower initial phage concentrations (10^{-3} to 10^{-1} PFU/ml) measurements of pH and TA were similar to those for the control milk and did not indicate the presence of phage.

Final host concentrations decreased (Fig. 1) from 10^9 CFU/ml to $<10^3$ CFU/ml and milk remained fluid, when initial phage concentrations were $>10^{-1}$ PFU/ml. Milk in three vessels (initial phage concentrations of 10^{-3} to 10^{-1} PFU/ml) and control milk were coagulated after 16 h. In instances where milk was fluid, phage contamination would be suspected, while milk that showed coagulation and suitable pH and TA readings would not be suspect for phage contamination. Despite all indications to the contrary, phage were, in this study, present at high concentrations.

The conclusion is that at low initial phage concentrations, TA and pH did not indicate the presence of phage after 16 h of host growth. In these instances phage can be detected by performing a plaque assay, as was done here. In our system where 3 L of milk was used as bulk starter medium, the presence of phage contamination was detected after 16 h from the lowest possible initial concentration of 10^{-3} PFU/ml (ca. 1 PFU/L).

Generation of aerosol

When phosphate buffer, without milk solids, was used as the aerosol solution, all phage fell from the aerosol in 1 min, and none were detected through the remaining 90 min. In contrast, when 1% milk solids comprised the solution, phage fell from the aerosol and were detected after 30 min (Table 1). Because of the observation that solids were necessary to suspend the phage in air, milk or whey solids were included in the aerosol solution. *E. coli* was added as a second potential contaminant and its fate was followed along with that of phage. For each experiment the presence of phage and *E. coli* was monitored through 60 min after aerosolization; in all instances both were detected through 15 min and occasionally >45 min (data not shown). These data are similar to that shown in Table 1 for 1% reconstituted NDM. During evaluation of the inoculation system, inoculation and sampling of milk were completed before 15 min.

Evaluation of the inoculation system

Before inoculation and aerosolization, steamed milk did not contain *E. coli*, phage homologous to host strains, or other bacteria capable of growing on M17 agar at 30°C. Table 2 shows the data obtained after inoculation and aerosolization. Phage and *E. coli* were only detected from milk exposed to the aerosol (open vessel), immediately after aerosolization (Table 2) and after 16 h at 22°C (Table 3).

In contrast to the first part of the study, TA and pH did indicate the presence of phage in milk from the open vessel (Table 3), and this was due to the relatively high phage concentration of 1-8 PFU/ml (Fig. 1, Table 2) after aerosolization. In the second trial of the experiment (Table 3, middle rows), TA was low and pH was high from all three milk samples. Phage were not detected from the

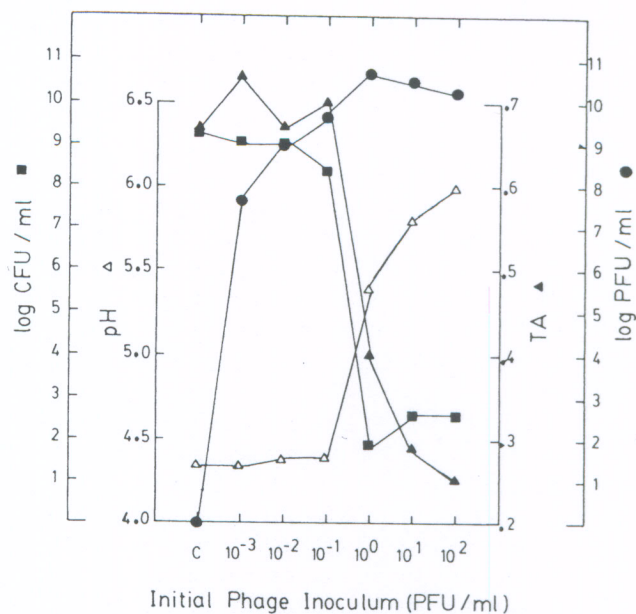


Figure 1. Numbers of phage (●) and host (■), pH (Δ) and titratable acidity (TA) (▲) for reconstituted nonfat dry milk (10% solids) inoculated with 1% host and with serial dilutions of phage and incubated at 24°C for 16 h. Control (C) was not inoculated with phage.

TABLE 1. Number of phage particles recovered after aerosolization of phosphate buffer and 1% reconstituted nonfat dry milk (expressed as PFU/cm²).

Phosphate buffer		1% reconstituted nonfat dry milk	
Time (min)	PFU/cm ²	Time (min)	PFU/cm ²
Control	0	Control	0
0-1	8.4	0-1	8.5
2-15	0	2-30	2.3
15-30	0	30-45	0.1
30-45	0		
45-60	0		
60-75	0		
75-90	0		

TABLE 2. TA, pH, and host, phage and *Escherichia coli* numbers from samples of milk obtained after inoculation and aerosolization (data from three trials).

Vessel	TA	pH	Host CFU/ml	Phage PFU/ml	<i>E. coli</i> CFU/ml
Control	0.16-0.19	6.4	10 ⁶ -10 ⁷	0	0
Open	0.16-0.19	6.4	10 ⁶ -10 ⁷	1-8	38-65
Closed	0.16-0.19	6.4	10 ⁶ -10 ⁷	0	0

TABLE 3. TA, pH, and *Escherichia coli*, phage and host numbers from samples of milk after 16 h at 22°C. Data are from three trials.

Vessel	TA	pH	<i>E. coli</i> log CFU/ml	Ø316 log CFU/ml	Ø322 log CFU/ml	host log CFU/ml
Control	0.61	4.6	-	-	-	9.1
Open	0.19	6.3	2.8	9.9	10.2	1.0
Closed	0.69	4.5	-	-	-	9.1
Control	0.33	5.5	-	-	nd*	8.6
Open	0.27	5.9	4.0	10.6	nd	5.0
Closed	0.38	5.4	-	-	nd	8.8
Control	0.68	4.4	-	nd	-	9.2
Open	0.22	6.2	4.0	nd	10.3	4.0
Closed	0.66	4.9	-	nd	-	9.1

*Not done.

control or closed milk, which suggests the host homologous to phage 316 was producing acid slowly. Comparing the microbiological data of the second trial to the first and third trials, decreased acid production did not change the results, since the final phage concentration in milk from the open vessel was 10^{10} PFU/ml and host numbers in milk from control in closed vessels were 10^8 CFU/ml. In this experiment, acid production was not important for indicating the presence of phage, since phage were enumerated by plaque assay. However, in bulk starter preparation, acid development as measured by TA/pH would be an important factor to evaluate the use of the starter in cheesemaking.

Contamination by *E. coli* followed phage contamination since both were detected only from open vessel samples. Numbers of *E. coli* increased from 10^1 CFU/ml after aerosolization to $\leq 10^4$ CFU/ml in 16 h. The small population increase reflected the decreased rate of growth of *E. coli* at 22°C compared to its optimum growth temperature near 37°C. A 3-log cycle increase of *E. coli* in milk indicated that contamination by one cell/L could be detected at a final concentration of 1-10 CFU/ml, although this was not determined separately. It is safe to conclude that milk from control and closed vessels was not contaminated with *E. coli*.

CONCLUSION

A bulk starter vessel was designed with a lid and an inoculation port that excluded contaminants from an aerosol created in a laboratory hood. The aerosol carried phage and *E. coli*. Starter culture was aseptically injected through the inoculation port using a sterile needle and syringe in the presence of the aerosol. After 16 h of growth, the bulk starter was not contaminated with phage or *E. coli*. While phage contamination may originate from sources other than

aerosols in cheese factories, this inoculation system in which the starter medium was not exposed to aerosols reduced the risk of growing a contaminated bulk starter. The design of the inoculation port described here is an improvement on that described by Robertson (8) and Robertson et al. (9). The improved design eliminates inoculation through a chlorine solution which, if of low concentration, could serve as a source of contamination. Use of this inoculation system, together with packaging of frozen concentrated starter cultures in sterile syringes or other injection devices, could further reduce contamination of starter media by eliminating the exposure of cultures to air, handling, and other potential sources of contamination.

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