Influence of Milk Ultrafiltration on Bacteriophages of Lactic Acid Bacteria

ABSTRACT

Bacteriophages added to whole milk were partially concentrated during ultrafiltration. At 4:1 retentate, phage had concentrated 2.4:1. Thermal destruction at 54°C followed first order kinetics up to 6% protein, whereafter it deviated. When allowed to grow in retentate in the presence of an appropriate host, 3.5 generations of phage appeared after 12 h at 22°C compared with four generations in skim milk. In the presence of phage, lactic acid bacteria population increased to only 10⁷ cfu/ml compared with 3 x 10⁹ in their absence. Retentate starter prepared in the presence of phage was as active as skim milk starter prepared in the presence of phage.

INTRODUCTION

The influence of bacteriophage in cheese manufacture is well established (3, 6, 18). Bacteriophage attack specific host cells of a starter culture and render them inactive. When this phenomenon occurs during cheese making, it results in a dead cheese vat.

Several procedures have been developed to control bacteriophage attack. Among them, sanitation during starter preparation (2) and appropriate starter rotation are important. Other procedures have included the New Zealand system (8), where a known single strain is used to prepare starter cultures, and the Netherlands system (15), where phage insensitive strains are adapted to commercial conditions.

Phage inhibitory media (PIM) in starter culture preparation are used to control phage (4, 5, 13, 14). These media are based on the principle that bacteriophage require calcium ions to adsorb onto the bacterial cell surface (12). Phage inhibitory media are fortified with phosphates to chelate the calcium ions, thereby preventing phage attachment.

Retentates of ultrafiltration (UF) can be used to make bulk starter (10) and this starter in proper amounts is suitable for manufacturing Cheddar cheese (11).

Retentates, depending on the degree of concentration, are rich in calcium and phosphates. The purpose of the present study, therefore, was to investigate the growth of phages in retentates during starter culture manufacture. More specifically, the objectives were 1) to study the effect of ultrafiltration on phage population, 2) to study the growth of phage in 4:1 vol/vol UF retentate during starter preparation and its effect on activity of starter made from phage infected retentate, and 3) to study the activity of a clean retentate starter (i.e., one prepared in the absence of phage) in milk and 2:1 UF retentates that have been infected with phage.

MATERIALS AND METHODS

Propagation of Phage

Phage stocks and their corresponding hosts in the form of frozen concentrates were obtained from Miles laboratories (Elkhart, IN). Phage were propagated by incubating .5 ml of stock culture in the host culture grown in 50 ml M17 broth, a medium described by Terzaghi and Sandine (16), in the presence of 1 M CaCl₂ for 3 h at 30°C. After incubation, the mixture, which was originally turbid, cleared. The cleared mixture was filtered through a pre-sterilized .45-µ filter (Nalgene), and the filtrate was stored at 4°C in sterile dilution blank bottles until used.

Concentration of Phage

During Ultrafiltration of Milk

Approximately 115 kg raw whole milk obtained from the university dairy plant was
heated to 54°C in a jacketed vat and inoculated with the prepared phage to give approximately 10⁶ pfu/ml. This milk was ultrafiltered at 3.15 kg/cm² inlet pressure and 1.40 kg/cm² outlet pressure in a 5.6 m² spiral wound Acor UF unit of 20,000 daltons molecular weight cut-off. Samples were taken at two-, three-, and fourfold volumetric concentrations. Protein content of these samples was determined by the Kjeldahl method using 6.38 as the conversion factor, and the plaque assay of Terzaghi and Sandine (16) was applied to study the effect of UF on phage concentration in relation to protein concentration. Additionally, plaque assay was conducted on a portion of the 4:1 retentate that was heated to 85°C for 30 min and cooled to 25°C.

Growth of Phage in Whole Milk Ultrafiltration Retentate

A 4:1 retentate free of phage was heated to 85°C for 30 min and cooled to 25°C. After cooling, the retentate was inoculated with a known phage to give approximately 10⁶ pfu/ml. A retentate starter was thereafter prepared from this 4:1 retentate as described previously (10) using the corresponding host lactic culture. The retentate starter was assayed for phage population, lactic starter population (on All Purpose Tween agar), and activity in skim milk. As controls, starters were prepared from skim milk [referred to as nonfat dry milk (NDM)] starter and PIM and were also assayed for phage population, lactic acid bacteria population, and activity of starter.

Activity of Retentate Starter in Phage Infected Skim Milk and Skim Milk Retentate

Retentate starter was prepared in the absence of phage as previously described (10). Starter activity tests were conducted by inoculating 2% starter in skim milk that contained host specific phage at approximately 10⁴ pfu/ml. Titratable acidity and pH were determined after 4 h at 32°C. Titratable acidity was expressed as developed acid (difference between beginning titratable acidity and after 4 h at 32°C).

An activity test was conducted similarly in a skim milk retentate concentrated 2:1 by UF and the retentate starter was compared with skim milk starter. Activity tests were also conducted on skim milk or skim milk retentates not infected with phage.

RESULTS

Influence of Ultrafiltration on Phage

Whole milk was ultrafiltered to increase the protein concentration two-, three-, and fourfold (Table 1) while monitoring the concentration of added phages. At 2:1 protein concentration, the phage concentrated approximately 1.9:1. At 3:1 protein concentration, the phage concentrated to 2.6:1, whereas at 4:1 protein concentration phage concentration was 2.4:1 (Figure 1). When the 4:1 retentate was heated to 85°C for 30 min, its phage population, originally at 8.5 × 10⁵ pfu/ml, became 0. Permeates resulting from the UF of phage

<table>
<thead>
<tr>
<th>Total protein (%)</th>
<th>Protein concentration factor</th>
<th>Plaque-forming units per milliliter</th>
<th>Phage concentration factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.10⁴</td>
<td>1:1</td>
<td>3.5 × 10⁴ a</td>
<td>1.0:1</td>
</tr>
<tr>
<td>6.33</td>
<td>2:1</td>
<td>6.5 × 10⁴ b</td>
<td>1.9:1</td>
</tr>
<tr>
<td>9.61</td>
<td>3:1</td>
<td>9.2 × 10⁵ c</td>
<td>2.6:1</td>
</tr>
<tr>
<td>12.47</td>
<td>4:1</td>
<td>8.5 × 10⁵ c</td>
<td>2.4:1</td>
</tr>
</tbody>
</table>

a,b,cMeans with the same letter are not significantly different from each other (P<.05).

1 Control non ultrafiltered milk.
BACTERIOPHAGES IN ULTRAFILTERED RETENTATE

Figure 1. Concentration of phage of lactic acid bacteria during ultrafiltration of whole milk at 54°C.

containing milk contained no phage particles.

Phage population at each of the protein concentrations was corrected for the concentration effect of UF (plaque-forming units/protein concentration factor) and plotted against time to give the thermal death curve. Thermal death of phage at 54°C approximated first order kinetics over 22 min of UF (up to 6% protein) with D of 610 min. Thereafter it began to deviate from first order kinetics and D decreased to 62 min after 9% protein (Figure 2).

Growth of Phage in 4:1 Retentate

Phage present in retentate starters had greater ($P<.05$) plaque-forming units after incubation and decreased starter bacteria population as well as starter activity ($P<.05$). In retentate, phage population increased 3.54 generations compared with 4.05 generations in NDM and 1.25 in PIM (Table 2). Starter bacteria growth in PIM was unaffected by the moderate growth of phage. Phage inhibitory medium had $5.7 \times 10^9$ cfu/ml and pH 5.10. Starter bacteria growth in NDM and retentate were greatly retarded ($P<.05$) (Table 3). In retentate, the starter bacteria attained a population of $1.2 \times 10^7$ cfu/ml compared with $3.3 \times 10^9$ cfu/ml in retentate starter that was prepared in the absence of phage. Neither retentate starter nor NDM starter when prepared in the presence of phage formed any curd.

The influence of phage on starter activity is shown in Table 4. Within 4 h at 32°C the pH in skim milk by NDM starter and retentate starter had dropped by equal amounts. The retentate starter lowered pH by .18 units compared with .77 units by the control retentate starter. Likewise, there was a change of only .02% in titratable acidity by NDM and retentate starters compared with .2% by control retentate starter. The PIM starter changed pH by .29 units and acidity by .07%.

Figure 2. Destruction of phage during ultrfiltration at 54°C of phage containing whole milk.

TABLE 2. Growth of phage in 12 h at 22°C in starter media purposely infected with phage.

<table>
<thead>
<tr>
<th>Starter media</th>
<th>Initial count (^1) (pfu/ml)</th>
<th>Final count (^2) (pfu/ml)</th>
<th>Generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM(^3)</td>
<td>(1.6 \times 10^6) (^a)</td>
<td>(1.8 \times 10^6) (^A)</td>
<td>4.05</td>
</tr>
<tr>
<td>PIM(^4)</td>
<td>(1.4 \times 10^6) (^a)</td>
<td>(2.5 \times 10^7) (^B)</td>
<td>1.25</td>
</tr>
<tr>
<td>Retentate</td>
<td>(1.6 \times 10^6) (^a)</td>
<td>(5.5 \times 10^9) (^C)</td>
<td>3.54</td>
</tr>
</tbody>
</table>

\(^a\)Means with same letter are not significantly different from each other \((P<.05)\).

\(^1\)Phage count immediately after addition of phage.

\(^2\)Phage count after 12 h at 22°C.

\(^3\)Nonfat dry milk.

\(^4\)Phage inhibitory medium.

Activity of Retentate Starter in Milk Infected with Phage

Retentate starter prepared in the absence of phage had pH 5 after 12 h at 22°C. When inoculated into skim milk or skim milk retentate containing approximately \(3.9 \times 10^4\) pfu/ml, its ability to produce acid was retarded (Table 5). In skim milk infected with phage, the developed acidity was .1%, whereas with NDM starter it was .08%. Developed acidity in the presence of phage by retentate starter was greater \((P<.05)\) than that by NDM starter. Likewise, change in pH was also greater with retentate starter than with NDM starter. With PIM starter, pH and acidity change were not different from that with retentate starter \((P<.05)\). Results were similar when the activity test was conducted in skim milk retentate.

Although the presence of phage retarded acid production, the amount produced and the pH change were greater \((P<.05)\) with retentate starter than with NDM starter.

DISCUSSION

Ultrafiltration is a process wherein molecules of a certain molecular weight are retained. During UF of milk, all proteins are retained. Studies have shown bacteriophage proteins of lactic acid bacteria to have molecular weights of 23,000 to 77,000 (17). Therefore, phages present in milk would be retained during UF as no phage could be observed in the permeate.

TABLE 3. Growth of lactic bacteria in whole milk retentate, nonfat dry milk (NDM), and phage inhibitory medium (PIM) infected with phage.

<table>
<thead>
<tr>
<th>Starter media</th>
<th>pH (^1)</th>
<th>Colony-forming (^1) units per milliliter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM (^3)</td>
<td>6.35(^a)</td>
<td>(4.0 \times 10^7) (^A)</td>
</tr>
<tr>
<td>PIM (^4)</td>
<td>5.10(^b)</td>
<td>(5.7 \times 10^9) (^B)</td>
</tr>
<tr>
<td>Retentate</td>
<td>6.56(^a)</td>
<td>(1.2 \times 10^7) (^A)</td>
</tr>
<tr>
<td>Control (^2)</td>
<td>5.10(^b)</td>
<td>(3.3 \times 10^9) (^B)</td>
</tr>
</tbody>
</table>

\(^a\),\(^b\),\(^A\),\(^B\)Means with the same letter are not significantly different \((P<.05)\).

\(^1\)Measured after 12 h at 22°C.

\(^2\)Retentate starter prepared without added phage.

TABLE 4. Activity of phage containing and control cultures in 11% skim milk solids.

<table>
<thead>
<tr>
<th>Starter media</th>
<th>pH (^1)</th>
<th>Acidity (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM (^3)</td>
<td>.15(^a)</td>
<td>.015(^A)</td>
</tr>
<tr>
<td>PIM (^3)</td>
<td>.29(^b)</td>
<td>.070(^B)</td>
</tr>
<tr>
<td>Retentate</td>
<td>.18(^a)</td>
<td>.015(^A)</td>
</tr>
<tr>
<td>Control (^2)</td>
<td>.77(^c)</td>
<td>.200(^C)</td>
</tr>
</tbody>
</table>

\(^a\),\(^b\),\(^c\),\(^A\),\(^B\),\(^C\)Means with same letters are not significantly different from each other \((P<.05)\).

\(^1\)Represents change in pH and acidity in 4 h at 32°C.

\(^2\)Nonfat dry milk.

\(^3\)Phage inhibitory medium.
TABLE 5. Activity of retentate, nonfat dry milk (NDM) and phage inhibitory medium (PIM) starters in skim milk and 2:1 skim milk retentate infected with phage.

<table>
<thead>
<tr>
<th>Starter media</th>
<th>pH ¹</th>
<th>Acidity ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM ²</td>
<td>.40  a</td>
<td>.08 A</td>
</tr>
<tr>
<td>PIM ³</td>
<td>.49  b</td>
<td>.10 B</td>
</tr>
<tr>
<td>Retentate</td>
<td>.52  b</td>
<td>.10 B</td>
</tr>
<tr>
<td>Skim milk retentate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM</td>
<td>.45  a</td>
<td>.07 A</td>
</tr>
<tr>
<td>PIM</td>
<td>.50  b</td>
<td>.10 B</td>
</tr>
<tr>
<td>Retentate</td>
<td>.59  b</td>
<td>.12 B</td>
</tr>
</tbody>
</table>

¹,b,ABC Means with same letters are not significantly different from each other (P<.05).

¹ Represents change in pH and acidity in 4 h at 32°C.
² Nonfat dry milk.
³ Phage inhibitory medium.

This study confirmed that phage do not pass through the membrane. Phage added to raw whole milk became concentrated at approximately the same rate as milk protein up to about twice protein concentration. Thereafter, phage concentration was less than that of protein concentration. At 4:1 protein concentration, phage concentration was only 2.4:1. Concentrated phage in 4:1 retentate were completely destroyed at 85°C for 30 min, a treatment normally used for starter preparation. Thus, the concentration of phage during UF would not be a drawback during retentate starter manufacture.

Bacteriophage destruction at 55°C follows first order kinetics (7). This was observed during the first 22 min of UF at 54°C up to 6% protein. Thereafter destruction deviated from first order kinetics. Earlier Ledford et al. (9) with lactic streptococcal bacteriophages observed that phages underwent heat-induced clumping. The decrease in plaque-forming units observed at 9 and 12% protein could be an effect of clumping of phage particles or destruction by heat and by the increased salt concentration during UF. The latter is more probable, because growth of phage at 22°C in 4:1 retentate appeared to be suppressed compared with that in NDM. Effect of salt concentration is likely due to the presence of phosphates in retentates, but the phosphate concentration was probably not high enough to completely inhibit growth. A 4:1 retentate contains approximately .2% phosphorous. Ausavanodom et al. (1) reported that at least .5% phosphate was necessary to inhibit phage in a whey-based medium.

The slight reduction in phage growth was not sufficient to permit profuse starter growth. Retentate and NDM starters contained approximately equal numbers of lactic acid bacteria (10⁷), whereas an uninfected retentate starter had more than 3 × 10⁹ cfu/ml. Starter activity of retentate and NDM starters was also approximately equal (.015% acid in 4 h at 32°C) compared with .2% with an uninfected retentate starter. Complete protection against phage growth in retentates is, therefore, limited by the amount of phosphates present to bind calcium.

When a retentate starter is prepared in the absence of phage and used to ferment a phage infected skim milk or 2:1 skim milk retentate, its activity, although considerably less than that in a clean skim milk or skim milk retentate, is greater than that of a NDM starter. This is likely because retentate starter has greater number of lactic acid bacteria than NDM starter. The activity of a starter prepared in a PIM in these milks was also equal to that of the retentate starter.

In cheese making, presence of phage in cheese milk would retard the cheese making process regardless of the type of medium in which the starter is prepared. It would be essential, therefore, to practice starter rotation as well as proper sanitation in starter preparation.

ACKNOWLEDGMENTS

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REFERENCES

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