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Occurrence of *Propionibacterium freudenreichii* Bacteriophages in Swiss Cheese

MICHEL GAUTIER,* ANNETTE ROUAULT, PHILIPPE SOMMER, AND ROMAIN BRIANDET

INRA Laboratoire de Recherche de Technologie Laitière, 35042 Rennes Cedex, France

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We isolated bacteriophages active against *Propionibacterium freudenreichii* from 16 of 32 Swiss cheese samples. Bacteriophage concentrations ranged from 14 to 7×10^5 PFU/g, depending on the sample and the sensitive strain used for detection. Only a few strains, 8 of the 44 strains of *P. freudenreichii* in our collection, were sensitive. We observed that multiplication of bacteriophages occurred in the cheese loaf during multiplication of propionibacteria in a warm curing room, but it seems that these bacteriophages have no adverse effect on the development of the propionic flora. We also found that sensitive cells, originating from either the starter or the cheese-making milk, were present at a high level (10^9 CFU/g) in the cheese.

Dairy propionibacteria, especially *Propionibacterium freudenreichii*, are widely used in Swiss cheese manufacture because of their ability to produce the characteristic flavor and holes (6, 7). They are also used in the fermentation industry for production of propionic acid and vitamin B_{12} (10, 14). Despite their use in cheese technology, no bacteriophage able to infect these bacteria had been described. Within the genus *Propionibacterium*, only bacteriophages infecting *P. acnes*, a cutaneous species, have been described (13, 15). However, the recent finding in our laboratory of a bacteriophage infecting *P. freudenreichii* indicated that, like many others, these bacteria are sensitive to bacteriophages (3). Because of the importance of dairy propionibacteria in cheese technology, it is worthwhile to evaluate the importance of bacteriophages in this group. This report describes the occurrence of propionibacterial bacteriophages in Swiss cheese.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used for phage detection came from the American Type Culture Collection; the Collection de l'Institut Pasteur; the Centre National de Recherches Zootechniques, Institut National de la Recherche Agronomique, Jouy en Josas, France; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; the Collection du Laboratoire de Recherche de Technologie Laitière, Institut National de la Recherche Agronomique, Rennes, France (TL); and private industrial collections. Strains were grown anaerobically at 30°C in Yel agar medium (4) in jars.

Cells were stored at −80°C in Yel broth containing 15% glycerol. Prior to being tested for phage sensitivity, they were subcultured twice in 2% Yel broth for 48 h each time at 30°C. Colonies were grown anaerobically (Anaerocult A; Merck, Darmstadt, Germany) on 1.5% Yel agar medium for 4 days.

Isolation of propionibacteria from cheese. Ten grams of cheese was dispersed with an Ultraturrax blender (Labo Moderne, Paris, France) at 20,000 rpm for 2 min in 90 ml of Yel broth. Propionibacteria were enumerated by plating aliquots of the suspension on selective lithium-glycerol-agar medium (8). On this medium, a yellow halo developed around propionibacterial colonies. Representative colonies were taken from the plates and purified by two successive isolations on Yel agar medium. Identification was performed by microscopic observation, propionic acid titration, and API 50 CH (API bio Mérieux) metabolic tests. Chromosome restriction patterns were then obtained to differentiate the strains.

Phage detection. For phage detection, 32 samples of Swiss cheese were used. Fifty grams of each sample was dispersed separately with an Ultraturrax blender at 20,000 rpm for 2 min in 60 ml of Yel medium. The suspension was then centrifuged for 20 min at 3,000 × *g*. After filtration on a 0.2-μm-pore-size filter (Millipore) each supernatant was tested on 44 strains of *P. freudenreichii* for bacteriophage detection.

* Corresponding author. Mailing address: INRA Laboratoire de Recherche de Technologie Laitière, 65 rue de Saint Brieuc, 35042 Rennes Cedex, France. Phone: (33) 99.28.75.82. Fax: (33) 99.28.75.78.

To detect phages, we used the soft agar layer method described by Adams (1). One milliliter of the supernatant was added to 0.2 ml of a mid-log-phase bacterial culture. After incubation for 20 min at 30°C, 3 ml of 0.8% agar was added. This mixture was spread on a Yel agar plate. Decimal dilutions of the supernatant were also used. After 2 days of anaerobic incubation at 30°C, the plaques were enumerated. Under these conditions, the lower limit of detection was one phage per milliliter of supernatant and, consequently, three phages per gram of cheese.

To enrich bacteriophage present at low concentrations in cheese (<1 PFU/ml of supernatant), the suspension was incubated at 30°C for 4 days, centrifuged, and treated as described above.

To search for bacteriophage in the starters, 2 g of the lyophilized starter was inoculated in 100 ml of Yel broth at 30°C. After 48 h of incubation, 6 ml of this culture was inoculated in 300 ml of Yel broth. The culture was incubated at 30°C for 4 days and centrifuged for 20 min at 3,000 × *g*. The supernatant was tested on indicator strains as previously described. However, to optimize bacteriophage detection we used for bacteriophage amplification the 8 sensitive strains identified among the 44 *P. freudenreichii* strains found during a search for bacteriophages in cheeses. Three hundred milliliters of Yel was inoculated with 0.5 ml of each of these eight sensitive strains and with 0.5 ml of a starter culture. This culture, containing both starter and sensitive strains, was incubated at 30°C for 4 days. After centrifugation, the supernatant was tested on the eight sensitive strains.

To search for bacteriophage in milk, we used 70 milk samples. These were pooled in seven lots, each containing 10 samples. Fifty milliliters of each lot was mixed separately with 300 ml of Yel broth, incubated at 30°C for 4 days, and centrifuged at 3,000 × *g* for 20 min; after filtration on a 0.2-μm-pore-size filter (Millipore), the supernatants were tested on the 44 strains of *P. freudenreichii*. If a lot gave rise to plaques on a sensitive strain, 3 ml of each sample composing this lot was incubated separately under the same conditions. After centrifugation and filtration, the supernatants were tested on the sensitive strain to detect the contaminated sample.

To increase the sensitivity of bacteriophage detection, another experiment was carried out. Each of the seven lots of milk mixture was inoculated with 0.5 ml of each of the eight sensitive strains. The mixtures obtained were incubated at 30°C for 4 days and centrifuged. The supernatants were tested on the eight indicator strains. If a lot gave rise to plaques on a sensitive strain, 3 ml of each sample composing this lot was inoculated with 0.2 ml of this strain. After 4 days of incubation at 30°C, the mixture was centrifuged and filtered. Finally, the supernatant was tested on the indicator strains.

Phage purification. A plaque was taken from the soft agar layer and incubated with 3 ml of an early-log-phase sample of the sensitive culture at 30°C until complete lysis, which generally occurred after 48 h. The lysate was filtered on a 0.2-μm-pore-size filter and tested again on the sensitive strain. After incubation, a plaque was taken and used to infect a culture of the same sensitive strain (called the propagating strain). After titration, a new plaque was taken and propagated again on the propagating strain. The filtrate obtained was titrated and served as a bacteriophage stock suspension.

High-titer lysates, from 10^{10} to 10^{12} PFU/ml, were needed for phage DNA or protein extraction or electron microscopy. These high-titer lysates were prepared by infecting an early-log-phase culture of sensitive cells with the phage at a multiplicity of infection of 0.1. The infected culture was then incubated at 30°C until complete lysis occurred.

DNase I (1-μg/ml final concentration; Boehringer, Mannheim, Germany) and RNase I (1-μg/ml final concentration; Boehringer) were added to 30 ml of a bacteriophage suspension, prepared as described above, and incubated at 37°C for 1 h. The lysate was centrifuged at 5,000 × *g* for 20 min to remove cell debris,

TABLE 1. Characteristics and contamination of cheeses tested

Cheese	Type of cheese	Milk heat treatment (temp [°C], time [s])	Ripening temp range (°C)	Presentation	Type of manufacturer[a]	Contamination level[b]
A2	Maasdam	72, 30	15–19		L	+
A3	Maasdam	72, 30	15–19		L	−
A4	Maasdam	72, 30	15–19		L	−
A1	Maasdam	72, 30	15–19		L	−
C1	Maasdam	72, 30	15–19		L	−
C2	Maasdam	72, 30	15–19		L	−
C3	Maasdam	72, 30	15–19		L	−
C4	Maasdam	72, 30	15–19		L	+++
B6	Emmental	63, 30	20–25	Grated	L	−
A5	Emmental	63, 30	20–25	Grated	L	−
C5	Emmental	63, 30	20–25	Grated	L	−
C6	Emmental	63, 30	20–25	Grated	L	−
A6	Emmental	63, 30	20–25	Grated	L	+
B7	Emmental	63, 30	20–25	Grated	L	+++
D6	Emmental	63, 30	20–25	Grated	L	+++
D7	Emmental	63, 30	20–25	Grated	L	+++
D2	Emmental	63, 30	20–25		L	−
E2	Emmental	63, 30	20–25		L	−
E5	Emmental	63, 30	20–25		L	−
E1	Emmental	63, 30	20–25		S	+++
E6	Emmental	63, 30	20–25		L	+++
E3	Emmental	None	20–25		S	+++
D4	Emmental	None	20–25		S	+++
B5	Emmental	None	20–25		ND	+++
E4	Emmental	None	20–25		S	+++
D1	Appenzell	None	15–19		S	−
B2	Comté	None	15–19		S	++
B4	Comté	None	15–19		S	++
B3	Comté	None	15–19		L	++
B1	Beaufort	None	10–12		S	−
D3	Fribourg	None	10–12		S	−
D5	Fribourg	None	10–12		S	++

[a] Type of manufacturer: L, large factory; S, small-scale production; ND, not determined.
[b] Contamination levels: −, no bacteriophage detected; +, $<10^2$ PFU/g; ++, $>10^2$ and $<10^4$ PFU/g; +++, $>10^4$ PFU/g.

and phages were pelleted by centrifugation at 30,000 × *g* for 3 h and then gently dissolved in 500 μl of TM buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.5). Phage particles were then purified by banding on CsCl gradients as described by Sambrook et al. (12).

Extraction of phage DNA. A 0.2-ml volume of STEP buffer (0.5% sodium dodecyl sulfate, 1 mg of proteinase K per ml [Boehringer], 50 mM Tris-HCl, 0.4 M EDTA, pH 7.5) (9) was added to 2 ml of a high-titer phage suspension. The suspension was then incubated at 56°C for 30 min and extracted twice with buffer-saturated phenol-chloroform. The DNA was precipitated from the aqueous phase by addition of 1/10 of a volume of 3 M sodium acetate (pH 7.0) and 2 volumes of ethanol. The precipitated DNA was recovered by centrifugation (10 min, 11,000 × *g*), washed in 70% ethanol, and resuspended in 500 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Restriction of phage DNA. Digestion of phage DNA with restriction endonucleases was performed in accordance with the manufacturer's (Boehringer) instructions. The DNA fragments were separated on a 0.8% agarose gel in TBE buffer (9).

Bacterial genomic DNA preparation, digestion, and pulsed-field gel electrophoresis (PFGE). Propionibacterial cells were embedded in agarose to prepare a DNA insert (plug) as described by McClelland et al. (11). Therefore, 10 ml of a culture was harvested at an optical density at 650 nm of 0.3 and treated as previously described (2).

Restriction enzymes were obtained from Boehringer. The agarose plugs were washed extensively with TE. Each plug was dialyzed at 4°C for 12 h in 500 μl of restriction enzyme buffer. For digestion, each plug was incubated overnight in 200 μl of restriction enzyme buffer with 20 U of restriction enzyme *Xba*I. PFGE was performed on a Bio-Rad CHEF DRII electrophoresis apparatus (5). The samples were electrophoresed through 1% agarose gels in 0.5× TBE buffer (9). The electrophoretic conditions used are described in the figure legends.

RESULTS

Occurrence of phages in cheese. Thirty-two cheese samples from different manufacturers were studied. They were collected and analyzed at the same time. Of these 32 cheeses, 16 were contaminated with bacteriophages (Table 1). Bacteriophage concentrations ranged from 14 to 7×10^5 PFU/g, depending on the cheese and the indicator strain used for detection. To determine whether this high frequency of bacteriophage contamination was usual, we searched for bacteriophages in different batches of the most heavily contaminated cheeses (cheeses B3, B5, C4, E1, E3, E6, and D6) at intervals of 1 to 2 months. We observed that the contamination remained constant, indicating the regularity of the contamination.

Indicator strains. Of the 44 strains of *P. freudenreichii* used, only 8 were effective in phage detection (Table 2). In addition, strain TL 303, which did not allow bacteriophage detection directly from the cheese samples, was found to be sensitive to one bacteriophage after phage enrichment of a mixture of cheeses for 4 days. Some of the eight indicator strains (TL 110 and TL 29 and TL 301 and TL 302) showed the same sensitivity pattern. To evaluate their relationship, we compared their DNA restriction patterns after treatment with infrequently cutting endonuclease *Xba*I (Fig. 1). The restriction patterns of TL 301 and TL 302 were identical, and the patterns of TL 110 and TL 29 were closely related, whereas the other strains showed various different patterns.

Bacteriophage detection is frequently hampered by a lack of indicator strains. To avoid this problem, it was tempting to isolate representative strains from the cheese and use them as indicator strains. To test this possibility, we isolated 10 colonies

TABLE 2. Bacteriophage numbers in Swiss cheese samples

Cheese sample	No. of bacteriophages (PFU/g) in indicator strain:							
	TL 110	TL 29	TL 19	TL 301	TL 302	TL 18	TL 105	TL 21
A2								50
C4	2×10^3	9.6×10^3						
A6	90	63						
B7	10^3	6.8×10^2		14		28		
D6	8.4×10^4	6.6×10^4		3.7×10^3				
D7	5.6×10^4	5.6×10^4		3.4×10^4	1.3×10^4			
E1	3×10^5	2.9×10^4	7×10^5	7.3×10^4	4.5×10^4	3.5×10^5		
E6	4.5×10^3	1.7×10^3		2.8×10^4	10^4	3.5×10^3	4.2×10^2	
E3				5.2×10^4	3.1×10^4	4.2×10^3		
D4	9×10^4	8.4×10^4						
B5	5.6×10^4	4.1×10^4	70	4×10^4	4×10^4	9.7×10^3		
E4	10^3	1.2×10^3	2.1×10^3	1.2×10^2	3.1×10^4	3.5×10^3		
B2	4.2×10^2		6×10^2	50				
B4			1.1×10^3					
B3	5.4×10^2	3.5×10^2	4.2×10^3	3.3×10^2	90	14		
D5					1.3×10^2			

from each of four infected cheeses and used them to search for phages in the corresponding filtrate. One to three isolates per cheese revealed bacteriophages, indicating that this method is well adapted for bacteriophage detection in Swiss cheese.

Origin of bacteriophages. Bacteriophages were detected in cheeses produced with either raw or heated milk. However, the proportion of contaminated cheeses was greater for cheeses made from raw milk (8 of 11) than for those made from milk heated at 72°C for 30 s (2 of 8) or at 63°C for 30 s (6 of 13). All of the cheeses made from raw milk and submitted to ripening conditions favoring the growth of propionibacteria, such as Emmental or Comté, contained bacteriophages. Grated cheeses were not more infected than sliced cheeses (4 of 8 versus 12 of 24, respectively). Cheeses produced in large factories were less frequently contaminated than those from small-scale production facilities. This might, however, reflect the fact that raw milk cheeses are more often produced by small manufacturers.

To study bacteriophage multiplication during the cheese-making processing, we monitored the production of cheese D6, which is contaminated with bacteriophages active against strains TL 110 and TL 301. These strains were used to detect bacteriophages during one cheese-making cycle. Bacteriophages were enumerated directly, without enrichment on a sensitive strain. Under these conditions, the detection limits were 1 PFU/ml in liquid samples and 3 PFU/g in solid samples. No bacteriophage was detected in raw milk during the production process (during clotting, after curd cooking, and after pressing and pickling) or after 2 weeks of ripening in a cold curing room (13 to 15°C). However, we enumerated 2×10^2 PFU/g of cheese after 4 weeks in a warm curing room. This observation is consistent with the view that bacteriophage multiplication occurs during multiplication of propionibacteria.

To better characterize the strains which had allowed bacteriophage multiplication in cheese, we isolated 20 colonies of *P. freudenreichii* from cheese D6 and tested their sensitivity to the bacteriophages in the corresponding filtrate. Nine of them were sensitive. Study of the restriction patterns of their DNAs revealed four different types (Fig. 2), which were all different from those of the eight indicator strains (Fig. 1). To determine whether or not these sensitive strains came from the starter which is widely used in Europe for Swiss cheese making, we isolated 20 colonies from the starter and characterized them by PFGE fingerprinting. We observed only two different but

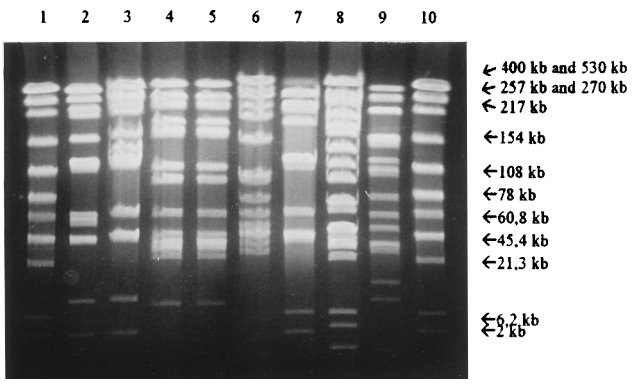

FIG. 1. PFGE separation of *Xba*I restriction fragments of genomic DNAs from *P. freudenreichii* strains sensitive to bacteriophages. Lanes: 1 and 10, DNA size standard TL; 2, TL 110; 3, TL 29; 4, TL 301; 5, TL 302; 6, TL 18; 7, TL 19; 8, TL 105; 9, TL 21. Electrophoresis was performed for 20 h at 200 V and 14°C in a 1% agarose gel. The pulse times were 2 to 20 s.

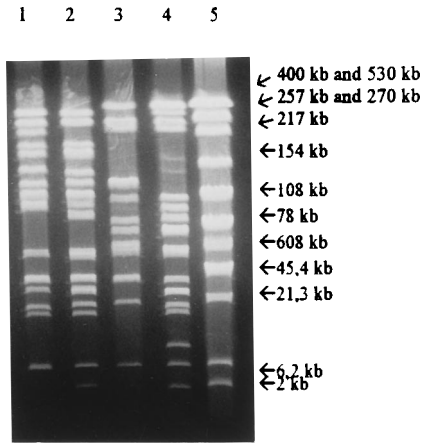

FIG. 2. PFGE separation of *Xba*I restriction fragments of genomic DNAs from four *P. freudenreichii* strains sensitive to bacteriophages isolated from infected cheeses. Lanes: 1 to 4, *P. freudenreichii* isolates; 5, DNA size standard TL. Electrophoresis was performed for 20 h at 200 V and 14°C in a 1% agarose gel. The pulse times were 2 to 20 s.

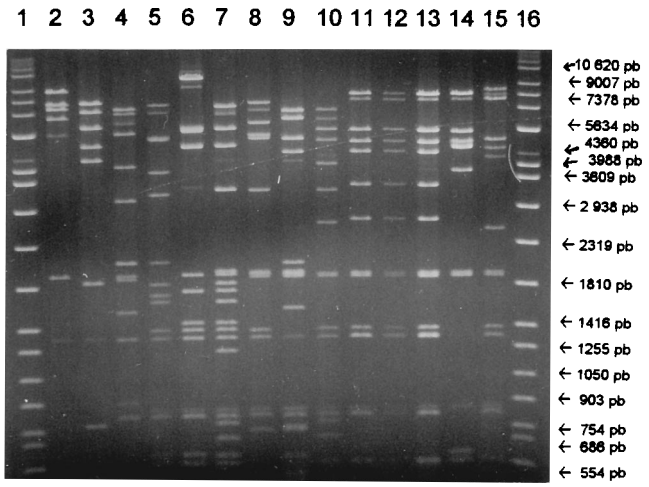

FIG. 3. Agarose gel electrophoresis of *Pst*I-digested DNAs of 14 bacteriophages. Lanes: 1 and 16, molecular weight marker Raoul (Appligène); 2, TL 110 B3; 3, TL 110 E1; 4, TL 110 B5; 5, TL 303 D7; 6, TL 19 E1; 7, TL 110 D6; 8, TL 19 B3; 9, TL 19 E4; 10, TL 110 E6; 11, TL 110 B7; 12, TL 110 L2; 13, TL 110 M; 14, B22; 15, TL 19 X.

closely related patterns (data not shown). One of these two strains was sensitive to the cheese D6 filtrate and had a pattern different from those of the sensitive strains isolated from the cheese. This indicates that bacteriophage multiplication can occur on either endogenous or starter-borne strains of propionibacteria.

To determine the primary origin of the bacteriophages, we searched for them in raw milk and in starters. We first tested 70 samples of raw milk from different regions of France. Each sample we received was a blend of milk from 15 producers. As raw milk is usually poor in propionibacteria, we supposed that it must be poor in bacteriophages as well. We therefore enriched the samples by inoculating them with a mixture of the eight indicator strains. Following incubation, only two samples produced plaques: in one sample, the bacteriophages were active against strains TL 110 and TL 29, and in the second one, the bacteriophages were active against strains TL 301 and TL 19. A similar experiment was used to detect bacteriophages in the starters. For this purpose, we used the two starters most frequently used in Europe. However, no bacteriophage active against any of the eight indicator strains was detected.

To estimate the importance of bacteriophages in Swiss cheese technology, it is essential to evaluate their diversity. To this end, we compared the restriction patterns of 19 bacteriophage DNAs cleaved with endonuclease *Pst*I (Fig. 3) or *Hin*dIII (data not shown). Most phages had identical or related restriction patterns. Five phages had restriction patterns identical to the one in lane 11. These bacteriophages were detected with indicator strain TL 110. Four came from different cheeses, and the fifth was isolated 2 years ago in our laboratory (lane 13). Moreover, one of the phages isolated from milk (lane 12) is similar to these bacteriophages. The pattern of phage B22, studied in a previous work (3), is shown in lane 14. However, only bacteriophages active on TL 110, TL 19, or TL 303 could be propagated and therefore included in this comparison. The bacteriophages active on the other strains may belong to different types, and we do not know the actual diversity within this group of bacteriophages.

DISCUSSION

We found bacteriophages active against propionibacteria in 50% of the samples of Swiss cheese examined. It is likely that some bacteriophages were not detected because of the lack of indicator strains. Only 8 different strains of the 44 tested were effective in revealing bacteriophages. We have shown that one solution to this problem is to use as indicators propionibacteria isolated from the cheese and to test their sensitivity to bacteriophages in the filtrate. However, it is still probable that our results represent an underestimate of the real contamination of Swiss cheese by bacteriophages.

Bacteriophages were detected only when the population of propionibacteria reached 10^8 to 10^9 CFU/g of cheese. All of the cheeses made from raw milk and ripened in a warm curing room for several weeks, which are known to contain high concentrations of propionibacteria, contained bacteriophages. By contrast, only one raw milk cheese ripened at a lower temperature, as are Fribourg and Beaufort, in which the propionibacterial population does not reach 10^8 CFU/g, contained bacteriophages. This, together with the observation that bacteriophages are detected in the cheese loaf only at the end of the ripening period, indicates that bacteriophages are produced during multiplication of propionibacteria in the cheese during ripening.

We have shown that multiplication of bacteriophages in cheese can occur on either endogenous or starter-borne strains of propionibacteria. This indicates that, in addition to starter-borne strains, spontaneous strains of propionibacteria might multiply and contribute significantly to the ripening flora. This involvement of spontaneous strains might render bacteriophage multiplication more difficult to control.

The isolation of bacteriophages in two samples of raw milk indicates that milk can constitute a significant source of bacteriophages. By contrast, the fact that no bacteriophage could be detected in the two samples of starter propionibacteria examined favors the view that commercial starters are not a major source of bacteriophage. This, however, does not exclude the possibility that propionibacteria harbor prophages, and study of the lysogenic status of these bacteria is of the utmost importance.

Although propionibacterial bacteriophages are common in Swiss cheese, their impact on cheese technology and quality is probably limited. We have shown that bacteriophages coexist in cheese with an abundant population of phage-sensitive cells, indicating that propionibacterial destruction is only partial. Because of the solid structure of Swiss cheese, bacteriophages cannot propagate throughout the cheese. Consequently, their multiplication occurs at different sites and only partially hampers propionibacterial development. The fact that only a relatively small population of bacteriophages was found in cheese samples suggests that they do not significantly disturb the cheese-making process.

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