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Multiplex PCR for the detection and identification of dairy bacteriophages in milk

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Abstract

Bacteriophage infections of starter lactic acid bacteria are a serious risk in the dairy industry. Phage infection can lead to slow lactic acid production or even the total failure of fermentation. The associated economic losses can be substantial. Rapid and sensitive methods are therefore required to detect and identify phages at all stages of the manufacture of fermented dairy products.

This study describes a simple and rapid multiplex PCR method that, in a single reaction, detects the presence of bacteriophages infecting *Streptococcus thermophilus* and *Lactobacillus delbrueckii*, plus three genetically distinct 'species' of *Lactococcus lactis* phages commonly found in dairy plants (P335, 936 and c2). Available bacteriophage genome sequences were examined and the conserved regions used to design five pairs of primers, one for each of the above bacteriophage species. These primers were designed to generate specific fragments of different size depending on the species. Since this method can detect the above phages in untreated milk and can be easily incorporated into dairy industry routines, it might be readily used to earmark contaminated milk for use in processes that do not involve susceptible starter organisms or for use in those that involve phage-deactivating conditions.

Keywords: Dairy bacteriophages; Multiplex-PCR; Detection; Identification; *Streptococcus thermophilus*; *Lactobacillus delbrueckii*; *Lactococcus lactis*

1. Introduction

Bacteriophage infection of lactic acid bacteria (LAB) is a serious risk in the industrial production of fermented dairy products. Dairy fermentations are susceptible to phage infection since the starting material is not sterile. In addition, the continued use of the same starter cultures provides a constant host for phage proliferation (Coveney et al., 1994; Neve et al., 1995). Phage infection can lead to slow lactic acid production or even the total failure of fermentation, and the ensuing economic losses can be substantial (Josephsen and Neve, 1998). Rapid and sensitive methods are therefore required to detect and identify phages at all stages of milk product manufacture.

Lactococcus lactis, Streptococcus thermophilus and *Lactobacillus delbrueckii* are three of the most important dairy LAB species. *L. lactis* is used as starter for the production of many different types of cheese, buttermilk and sour cream (Gasson and de Vos, 1994), but it is attacked by specific bacteriophages. The lactococcal phages have been classified into 11 genetically unrelated species or groups (Labrie and Moineau, 2002). However, of these, only three lytic phage species (P335, 936 and c2) have been mainly detected in dairy plants (Moineau et al., 1992). *S. thermophilus* and *L. delbrueckii* are mainly used as starters in the production of yoghurt and fermented milk. In recent years, however, *S. thermophilus* strains have also been extensively used as starters in the production of different cheeses, so the number of fermentation failures caused by *S. thermophilus* phages has increased (Moineau, 1999). *L. delbrueckii* is also sensitive to phage attack, although few studies have focused on its phages since the economic losses they cause are less important (Suárez et al., 2002). There is therefore great interest in the development of techniques that can detect dairy phages.

Usually, milk is examined for phages using standard microbiological methods (plaque assays, activity tests, etc.)

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(Everson, 1991), but these assays are time-consuming. The polymerase chain reaction (PCR) is a much faster candidate for this kind of task. Indeed, PCR techniques have already been successfully used to detect and identify viruses and bacteria in a number of food environments (Starbuck et al., 1992; Brüssow et al., 1994; Allmann et al., 1995). The technique has been adapted to detect *S. thermophilus* phages in cheese whey (Brüssow et al., 1994) and milk samples (Binetti et al., 2005; Dupont et al., 2005).

Besides, Labrie and Moineau (2000) have developed a multiplex PCR method to detect, in a single reaction, the presence of the main lactococcal bacteriophage species in phage lysates and whey samples. The present work describes a multiplex PCR technique for detecting and identifying, in a single reaction, the presence of *L. lactis*, *L. delbrueckii* and *S. thermophilus* phages in milk and dairy products.

2. Materials and methods

2.1. Bacterial strains and bacteriophages

Table 1 shows the bacterial strains and bacteriophages used in this study. *L. lactis* was grown at 30 °C in M17 broth (Oxoid, Basingtoke, Hampshire, England) containing 0.5% glucose (w/v). *L. delbrueckii* was grown at 37 °C in MRS broth (Oxoid). *S. thermophilus* was grown at 42 °C in M17 broth (Oxoid) supplemented with 0.5% lactose. Phages were enumerated by the double layer plaque titration method (Fayard et al., 1993) in the appropriate medium, in the presence of 10 mM $CaCl_2$.

2.2. Phage multiplication and concentration

Phages were propagated in their hosts as previously described (Binetti et al., 2005). The lysate was centrifuged (10,000*g* for 30 min at 4 °C) and the supernatant precipitated by adding 4% polyethylene glycol 6000 in 0.2 M NaCl (48 h at 4 °C). It was then centrifuged at 17,000*g* for 60 min at 4 °C. The pellet was resuspended in SM buffer (170 mM NaCl, 50 mM Tris pH 7.5, 9 mM $MgSO_4 \cdot 7H_2O$) and stored at 4 °C until use (Sambrook and Russell, 2001).

2.3. Multiplex PCR

PCRs were performed with the primer pairs designed from the conserved regions shown in Table 2. The primers Host 1 and Host 5, designed by Binetti et al. (2005), were used for *S. thermophilus* phage detection. All primers were supplied by Sigma-Genosys (Cambridge, England).

For the simultaneous detection of all the bacteriophage species mentioned, a one-step multiplex PCR technique was developed using all the above primers. PuReTaq Ready-To-Go™ PCR Beads (Amersham-Biosciences, Buckinghamshire, England) were used (with some modifications of the manufacturer's recommendations) in a final reaction volume of 25 µl (0.4 µM concentrations of

Table 1
Bacterial strains and bacteriophages used

Bacteriophages	Reference or source
L. lactis subsp. *lactis*	
IL1403	Chopin et al. (1984)
MG1614	Gasson (1983)
F4-2	Moineau (1999)
L. delbrueckii subsp. *bulgaricus*	
Ab_1	Quiberoni et al. (2003)
YSD V	Quiberoni et al. (2003)
L. delbrueckii subsp. *lactis*	
LKT	Alatossava and Pythilá (1980)
S. thermophilus	
LMD-9	NCCB[a]
5-C	Suárez et al. (2002)
M1-C	Suárez et al. (2002)
M10-C	Suárez et al. (2002)
M11-C	Suárez et al. (2002)
Sth10	Suárez et al. (2002)
799	Suárez et al. (2002)
Abc2	Suárez et al. (2002)
cLy1	Suárez et al. (2002)
cLy7	Suárez et al. (2002)
10.3	Binetti et al. (2005)
13.2	Binetti et al. (2005)
3.1	Binetti et al. (2005)
cLy4	Binetti et al. (2005)
MiC7	Suárez et al. (2002)

Bacteriophages	Host strain	Reference or source
L. lactis subsp. *lactis*		
P335 phage specie		
P335	F4-2	Braun et al. (1989)
104/37	F4-2	Madera et al. (2004)
27CYL	IL1403	Madera and Suarez[b]
114/4	IL1403	Madera and Suarez[b]
936 phage specie		
bIL170	IL1403	Bidnenko et al. (1995)
070/6	IL1403	Madera and Suarez[b]
125/0	IL1403	Madera and Suarez[b]
074/5b	IL1403	Madera and Suarez[b]
123/0	IL1403	Madera and Suarez[b]
268/9	IL1403	Madera and Suarez[b]
174/37	IL1403	Madera and Suarez[b]
224/40	IL1403	Madera and Suarez[b]
184/26	IL1403	Madera and Suarez[b]
114/37	F4-2	Madera and Suarez[b]
234/14	F4-2	Madera and Suarez[b]
c2 phage specie		
c2	MG1614	Pillidge and Jarvis (1988)
056/5	MG1614	Madera and Suarez[b]
262/4	MG1614	Madera and Suarez[b]
100/8	MG1614	Madera and Suarez[b]
116/8	MG1614	Madera and Suarez[b]
92/47	MG1614	Madera and Suarez[b]
154/26	MG1614	Madera and Suarez[b]
154/18	MG1614	Madera and Suarez[b]
110/2	MG1614	Madera and Suarez[b]
L. delbrueckii subsp. *bulgaricus*		
YAB	Ab1	Quiberoni et al. (2003)
BYM	YSD V	Quiberoni et al. (2003)

Table 1 (*continued*)

Bacteriophages	Host strain	Reference or source
L. delbrueckii subsp. *lactis*		
LL-H	LKT	Alatossava and Pythilá (1980)
S. thermophilus		
OBJ	LMD9	Suárez et al. (2002)
021-5	5-C	Suárez et al. (2002)
CP	M1-C	Suárez et al. (2002)
CQ210	M10-C	Suárez et al. (2002)
CQ211	M11-C	Suárez et al. (2002)
FcSth10	Sth10	Suárez et al. (2002)
799-M1	799	Suárez et al. (2002)
Abc2	Abc2	Suárez et al. (2002)
Ly1	cLy1	Suárez et al. (2002)
Ly7	cLy7	Suárez et al. (2002)
P10.3	10.3	Binetti et al. (2005)
P13.2	13.2	Binetti et al. (2005)
P3.1	3.1	Binetti et al. (2005)
Ly4	cLy4	Binetti et al. (2005)
Mi1	MiC7	Suárez et al. (2002)

[a]NCCB: The Netherlands Culture Collection of Bacteria.
[b]Unpublished results.

each of the five primers and 0.5 U of pyrophosphatase (Biotools, Spain)). One microlitre of the template was then added. This consisted of either artificially infected sterile skimmed milk, industrial pasteurized milk used to make yoghurt, or commercial yoghurt. A negative control (no phages) was included in all reactions. All reactions were performed in an iCycler™ thermocycler (Bio-Rad, Hercules, California, USA) under the following conditions: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and a final step of 7 min at 72 °C. The PCR products were separated on 1.5% agarose gels in TAE buffer (40 mM Tris–Acetate, 1 mM EDTA), stained with ethidium bromide, and visualized under UV light (Sambrook and Russell, 2001). To determine the detection limit of the PCR method, sterile skimmed milk (Oxoid) was inoculated with serial dilutions of each phage suspension and 1 µl sample used directly in the multiplex PCR reaction. The lowest concentration visible in the agarose gels was taken as the detection limit.

2.4. Nucleotide sequence analysis

PCR fragments were purified using the GelElute PCR Clean-up Kit (Sigma-Genosys). The nucleotide sequence was obtained using an ABI Prism 373 A Strech automated sequencer (performed by the *Servicio de Secuenciación Automática de DNA*—the Automatic DNA Sequencing Service (CIB, CSIC, Spain)).

Sequence data were assembled and analysed using the sequence analysis software package available from the EMBL Spanish node (CNB, CSIC, Spain). Sequence alignments were performed using the CLUSTAL W algorithm (Thompson et al., 1994).

3. Results and discussion

3.1. Primer design

Primer pair selection is crucial in this multiplex PCR technique. Different regions of sequenced bacteriophage genomes were compared to look for conserved regions. Previously, Labrie and Moineau (2000), analysed conserved regions of P335, 936 and c2 lactococcal phages species genomes and selected three conserved genes as target to design three pairs of primers. In order to obtain PCR products of different size to be able to identify the detected phages, another set of primers were designed in the same conserved genes. Thus, for lactococcal phages belonging to the P335 species group, the *orf21* gene of Tuc2009 (accession no. NC002703) and the homologous gene of bIL285, ul36A, Q30 and r1t (accession no. AF323668, AF152415, AF152414 and U38906, respectively) were compared. For lactococcal phages belonging to the 936 species group, the *msp* gene of p2 and Q7, the *orf11* gene of sk1, the *orf13* gene of bIL170, and the *mcp* gene of F4-1 (accession no. AF152407, AF152409, AF152409, AF009630 and M37979 respectively) were compared. For the lactococcal phages belonging to the c2 species group, the *mcp* gene of eb1, Q38 and Q44 (accession no. AF152410, AF152411 and AF152412, respectively), the *orf15* gene of c2 and *orf26* gene of bIL67 (accession no. L48605, L33769, respectively) were compared. For *L. delbrueckii* phages, the *mur* gene encoding the muramidase of LL-H (accession no. M96254) and the *lysA* gene of mv4 and mv1 (accession no. Z26590 and Z26590, respectively) were chosen because were the most conserved genes of the available sequences. The primers Host1 and Host 5 used in this work, were previously designed by Binetti et al. (2005), to amplify the antireceptor gene of the two groups of *S. thermophilus* phages (Le Marrec et al., 1997).

Based on these regions, a pair of primers for each phage species was designed. The primers have no mismatches with most of the phage sequences compared. In some cases (Q7, F4-1, Q38, Q44, mv1 and mv4) there is one mismatch but it does not affect the amplification. The 936 specie primers have three mismatches when compared with phage bIL170 sequence. Since it is the most unfavorable case, bIL170 was the phage used for 936 detection limit experiments. Primers were also designed to obtain PCR products of different sizes in order to check, in a single reaction, for the presence of all these phage species. For these reason it was necessary to design this new set of primers, although three sets of primers had been previously described for the detection of *L. lactis* P335, 936 and c2 phages species (Labrie and Moineau, 2000).

PCR products of the expected sizes were obtained using phage suspensions as templates. The 335 (A, B), 936 (A, B) and c2 (A, B) primers used to amplify the *L. lactis* P335, 936 and c2 phages gave PCR products of 196, 318 and 444 bp, respectively. Lb1 and Lb2 amplified a 621 bp region of *L. delbrueckii* phage YAB, and Host 1 and Host 5 amplified a 750 bp region of *S. thermophilus* phage OBJ. Primers pairs

Table 2
List of primers used in multiplex PCR

Primer	Sequence	Phage	Amplicon (bp)	Reference
335A	5′ GAAGCTAGGCGAATCAGTAAACTTGCTAG 3′	P335	196	This work
335B	5′ CGGCTATCTCGTCAATTGTTCCGGTTGC 3′	P335		
936A	5′ ATCAGTTGGCTCAATGGAAGACCAAGCGG 3′	sk1	318	This work
936B	5′ GTTGCTTCTGCTGTTGGTGTCAAATGAGGA 3′	sk1		
c2A	5′ CAATCGAAGCAGGTGTAAAAGTTCGAGAAC 3′	c2	444	This work
c2B	5′ GCTTTATCCATTTGTAGGTATGCTTCTGCC 3′	c2		
Lb1	5′ TCCCGGGCTAACCACCTCTACACTC 3′	LL-H	621	This work
Lb2	5′ GGTGTAGTGACCATCCTTTGAGAGC 3′	LL-H		
Host 1	5′ GAATGATACTGCTGGCAGTATTTCGGTTGG 3′	DT1	750	Binetti et al. (2005)
Host 5	5′ CAGTCATGTAGCTATCGATGAAATTCCAACG 3′	DT1		

were tested against all the different phage types and found to be specific for the target species. Therefore, the size of the amplicons allows the phage species to be identified.

3.2. Multiplex PCR detection of dairy bacteriophages in milk

The five pairs of primers were then used together in multiplex PCR to analyse the milk artificially infected with each phage (P335, bIL170, c2, YAB and OBJ). DNA products of the expected size were only obtained in the presence of the respective phage (Fig. 1). The multiplex PCR method was successfully tested with milk contaminated with 10^7 PFU ml^{-1} titters for four distinct P335 phages, 11 different 936 phages, nine different c2 phages, three different *Lactobacillus* phages and 15 different *S. thermophilus* phages (Table 1). Since Host 1 and Host 5 amplify a variable region of the antireceptor-encoding gene, the amplicon size ranged between 700 and 800 bp. Although milk contaminated with different phages would not be expected in practice (Bissonnette et al., 2000), the capacity of the method to detect different phage species in the same sample was examined via a PCR experiment involving different virus combinations (Fig. 2). Milk samples artificially contaminated with P355, bIL170 and c2 at 10^6 PFU ml^{-1} (Fig. 2, lane 3), with YAB and OBJ at 10^7 PFU ml^{-1} (Fig. 2, lane 4), and with all phages together (Fig. 2, lane 5), were used as template sources.

No interference was observed in these multiple PCR assays. The five phages together in the same reaction were easily detected. It is important to note that when milk is used as a template source for the PCR reaction, the sample volume is critical. In fact, if a 2 μl sample is used instead of 1 μl sample (always in a 25 μl final volume reaction) the method sensitivity decreases by 100 fold (data not shown). This effect is attributable to the inhibition of Taq polymerase by proteinases, calcium and plasmin in the milk (Powell et al., 1994).

3.3. Detection limit of the proposed method

To determine the sensitivity of the multiplex PCR assay for each of the species selected, serial dilutions of purified

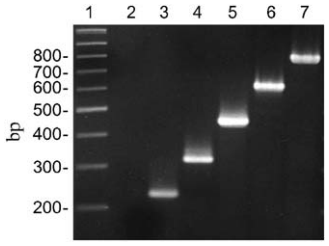

Fig. 1. Multiplex PCR of milk contaminated with 10^7 PFU ml^{-1} of each phage. *L. lactis* phages: P335 (P335 phage species), bIL170 (936 phage species), and c2 (c2 phage species). YAB represents the *L. delbrueckii* subsp. *bulgaricus* phage species and OBJ represents the *S. thermophilus* phage species. Lane 1: 100 bp molecular marker (BioRad); Lane 2: negative control (non-contaminated milk); Lane 3: P335; Lane 4: bIL170; Lane 5: c2; Lane 6: YAB; Lane 7: OBJ.

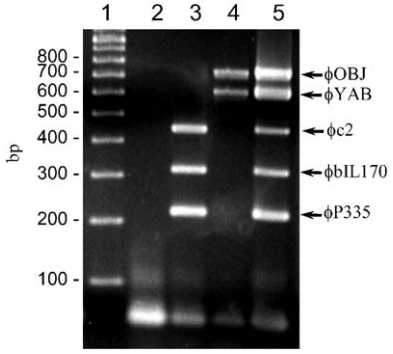

Fig. 2. Multiplex PCR assay with different combinations of phage species in the same sample. Reactions were performed with phage titres of 10^6 PFU ml^{-1} (*L. lactis* phage species) and 10^7 PFU ml^{-1} (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* phage species). Lane 1: 100 bp molecular marker; Lane 2: negative control (without phages); Lane 3: P335 plus bIL170 and c2; Lane 4: YAB plus OBJ; Lane 5: All five phages.

phages in milk were assayed. Although all phages were successfully detected, the detection limit was different for each phage type: 10^4 PFU ml^{-1} for P335 and c2, 10^3 PFU ml^{-1} for 936 (bIL170), 10^4 PFU ml^{-1} for YAB and 10^3 PFU ml^{-1} for OBJ (Fig. 3). The detection limit of the 936 amplification product was 1 log order better than that indicated in previous reports (Labrie and Moineau,

2000), even though whey was used instead of milk in this earlier work. It could be due to the use of pyrophosphatase in the reaction. With respect to the *S. thermophilus* phage, the detection limit was 2 log orders lower than that reported by Binetti et al. (2005), although the bacteriophages studied were not the same.

Since phage concentrations below 10^5 PFU ml^{-1} in whey or milk are not considered a threat to fermentation (McIntyre et al., 1991; Suárez et al., 2002), the proposed multiplex PCR method (with its detection limit of 10^3–10^4 PFU ml^{-1}) can be used to guarantee minimum quality requirements.

3.4. Detection of bacteriophages in industrial samples of milk and yoghurt

The multiplex PCR method was then evaluated for use with industrial samples of milk and yoghurt (kindly provided by the company *Corporación Alimentaria Peñasanta S.A.*, Spain). Samples were assayed to detect and identify streptococcal, lactococcal and lactobacilli bacteriophages. Since milk is the most likely source of phage contamination (Moineau et al., 1996), 1 μl samples of the milk used in yoghurt production were analysed. Twenty of the 54 samples (37%) of milk tested were positive. According to their size, the amplicons corresponded to *S. thermophilus* phages (37%) (Fig. 4A, lane 2; Fig. 4C, lane 2), and the lactococcal phages 936 (27%) and P335 (36%) (Fig. 4C, lane 2). This putative identification based on amplicon size was confirmed by sequencing the purified PCR products. These results for phage presence in milk (37% of positive samples) agree with those obtained by other authors (Madera et al., 2004). Previous studies on lactococcal phages interfering with milk fermentation have shown a prevalence of P335 and 936 type phages, their incidence depending on the source (Labrie and Moineau, 2000; Madera et al., 2004). In the present case, both species were present in similar percentages. No data is available on the frequency of *S. thermophilus* phages in milk. However, an incidence of up to 79% has been reported in cheese samples (Suárez et al., 2002).

In the yoghurt, 13% of the 30 samples were positive. The size of the amplicons obtained corresponds to the same phage types found in the milk, although in different proportions. Half of the positive samples were contaminated with *S. thermophilus* phages, according to the size of the PCR product (Fig. 4B, lane 2; Fig. 4C, lane 3). The other half were contaminated with lactococcal phages 936 (17% of samples) and P335 (33% of samples) (Fig. 4C, lane 3). These results were confirmed by sequencing the purified PCR products. The detection of lactococcal phages in yoghurt samples is not surprising. Many phages (especially the 936-like phages) can survive pasteurization and even spray-drying (Chopin, 1980; Madera et al., 2004).

L. delbrueckii phages were not detected, indicating that the numbers of these phages in dairy samples to be low, as described by other authors (Suárez et al., 2002; Quiberoni et al., 2003). Although these phages seem to be very resistant to pasteurization, their absence could be a consequence of the current trend in the composition of commercial yoghurt starters. Nowadays, these tend to be composed of *S. thermophilus* and a very low proportion of

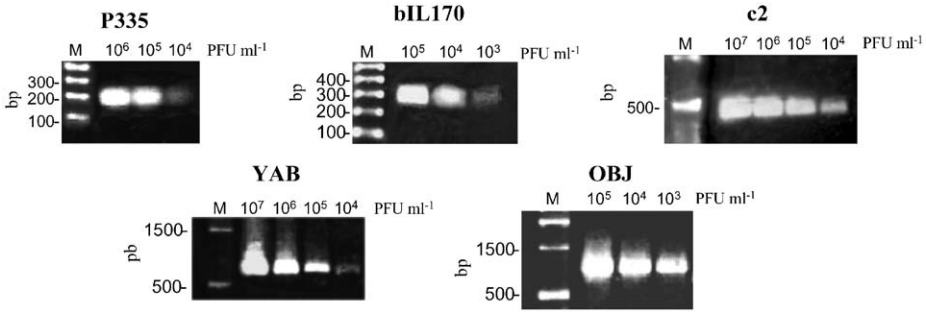

Fig. 3. Detection limits of the multiplex PCR method. Serial dilutions were made of each bacteriophage suspension: P335, bIL170, c2, YAB and OBJ. M: 100 bp molecular marker.

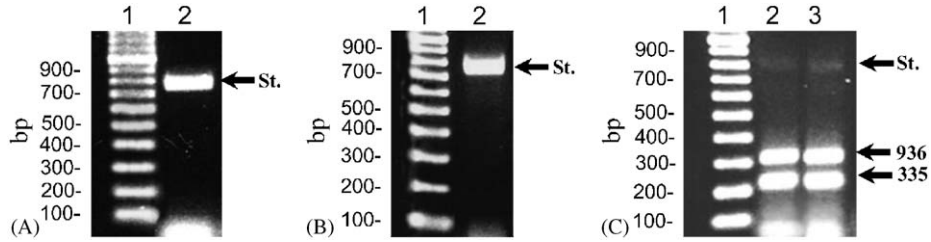

Fig. 4. Multiplex PCR used with milk and yoghurt samples. A: lane 1: 100 bp molecular marker; lane 2: 1 μl of milk. B: lane 1: 100 bp molecular marker, lane 2: 1 μl of yoghurt. C: lane 1: 100 bp molecular marker; lane 2: 1 μl of milk; lane 3: 1 μl of yoghurt. St. represents *S. thermophilus* phages, P335 and 936 represent P335 and 936 lactococcal phages.

L. delbrueckii subsp. *bulgaricus* strains (Quiberoni et al., 2003). Furthermore, these phages have a remarkably high sensitivity to pH (Quiberoni et al., 2004), which could be another reason for their absence in yoghurt samples. The high proportion of *S. thermophilus* bacteriophages detected in the present study reflects the high incidence of these phages reported in dairy processes over the last decade (Brüssow et al., 1994; Suárez et al., 2002).

Phage monitoring is particularly important in the dairy industry. However, these assays are time-consuming and mostly rely on the availability of single indicator strains. In contrast, the multiplex PCR assay described in this paper can detect bacteriophages in starting milk samples in just 4 h. An additional benefit is that neither phage particle concentration (purification by precipitation, CsCl gradient, filtration, etc.) nor any other procedure is required to enrich the samples—this is a great advantage. The correct and rapid identification of bacteriophages potentially able to attack starter cultures allows for speedy decisions with regard to the destination of contaminated milk. Such milk might be earmarked for use in processes in which phages are deactivated, processes that do not require starters, or processes that employ starter bacteria insensitive to the detected phage. For example, if a sample of milk is contaminated with *L. lactis* phages, it could undergo UHT treatment, be used as pasteurized milk for drinking, or be employed in yoghurt production.

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