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Title	Multiplex PCR to detect bacteriophages from natural whey cultures of buffalo milk and characterisation of two phages active against Lactococcus lactis, ϕApr-1 and ϕApr-2
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Publication date	2017-01-01
Original Citation	Aprea, G., Mullan, W. M., Murru, N., Fitzgerald, G., Buonanno, M., Cortesi, M. L., Prencipe, V. A. and Migliorati, G. (2017) 'Multiplex PCR to detect bacteriophages from natural whey cultures of buffalo milk and characterisation of two phages active against Lactococcus lactis, ϕApr-1 and ϕApr-2', Veterinaria Italiana, 53(3), pp. 207-214. doi: 10.12834/VetIt.315.1238.3
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.researchgate.net/profile/Giuseppe_Aprea2/publication/320551161_Multiplex_PCR_to_detect_bacteriophages_from_natu links/5a156b984585150052133d8e/Multiplex-PCR-to-detect-bacteriophages-from-natural-whey-cultures-of-buffalo-milk-and-characterisation-of-two-phages-active-against-Lactococcus-lactis-phApr-1-and-phApr-2.pdf - 10.12834/VetIt.315.1238.3
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Multiplex PCR to detect bacteriophages from natural whey cultures of buffalo milk and characterisation of two phages active against Lactococcus lactis, *ϕApr-1 and ϕApr-2*

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Veterinaria Italiana 2017, **53** (3), 207-214. doi: 10.12834/VetIt.315.1238.3
Accepted: 04.12.2015 | Available on line: 30.08.2017

Keywords
Bacteriophage,
Buffalo,
Lactic Acid Bacteria (LAB),
Lactococcus lactis,
Milk,
Natural starter culture,
PCR.

Summary
This work investigated bacteriophage induced starter failures in artisanal buffalo Mozzarella production plants in Southern Italy. Two hundred and ten samples of whey starter cultures were screened for bacteriophage infection. Multiplex polymerase chain reaction (PCR) revealed phage infection in 28.56% of samples, all showing acidification problems during cheese making. Based on DNA sequences, bacteriophages for *Lactococcus lactis* (*L. lactis*), *Lactobacillus delbruekii* (*L. delbruekii*) and *Streptococcus thermophilus* (*S. thermophilus*) were detected. Two phages active against *L. lactis*, ϕApr-1 and ϕApr-2, were isolated and characterised. The genomes, approximately 31.4 kb and 31 kb for ϕApr-1 and ϕApr-2 respectively, consisted of double-stranded linear DNA with pac-type system. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed one major structural protein of approximately 32.5 kDa and several minor proteins. This is the first report of phage isolation in buffalo milk and of the use of multiplex PCR to screen and study the diversity of phages against Lactic Acid Bacteria (LAB) strains in artisanal Water Buffalo Mozzarella starters.

PCR Multiplex per la rilevazione di batteriofagi in siero-innesto naturale di latte di bufala e caratterizzazione di due fagi attivi contro Lactococcus lactis, *ϕApr-1 e ϕApr-2*

Parole chiave
Batteriofago,
Batteri Acido Lattici,
Bufalo,
Colture starter naturali,
Lactococcus lactis,
Latte,
Reazione a catena della polimerasi.

Riassunto
Il presente studio ha esaminato il ruolo dei batteriofagi tra le cause principali di problemi di acidificazione riscontrati durante la produzione di mozzarella di bufala in alcuni stabilimenti lattiero-caseari del Sud Italia. Duecentodieci campioni di siero innesto naturali sono stati analizzati per la presenza di fagi mediante un test PCR multiplex. È stata rilevata una contaminazione fagica nel 28.56% dei campioni, tutti caratterizzati da problemi di acidificazione del latte durante la produzione del formaggio. DNA di batteriofagi contro *Lactococcus lactis (L. lactis), Lactobacillus delbruekii (L. delbruekii)* e *Streptococcus thermophilus (S. thermophilus)* sono stati rilevati e confermati con analisi di sequenza genomica. Due fagi attivi contro *L. lactis* e denominati ϕApr-1 e ϕApr-2 sono stati isolati e caratterizzati. Il genoma, di circa 31.4kb e 31kb rispettivamente per ϕApr-1 e ϕApr-2, è risultato essere costituito da DNA a doppio filamento lineare con sistema di tipo pac. L'elettroforesi su gel di poliacrilamide con sodio dodecil-solfato (SDS-PAGE) ha rilevato per entrambi i fagi una proteina strutturale maggiore di circa 32.5 kDa e numerose proteine minori. Questo è il primo studio d'isolamento di batteriofagi da latte di bufala e di valutazione dell'uso della PCR per la caratterizzazione della diversità della flora fagica attiva contro i Batteri Acido Lattici (LAB) utilizzati per la produzione di mozzarella di bufala.

Introduction

Buffalo mozzarella is an unripened 'pasta filata' cheese manufactured from whole and unpasteurised buffalo milk. It is produced traditionally and the unique organoleptic characteristics are guaranteed by the Protected Designation of Origin (PDO) from 1996, according to Reg. CE 510/2006. Natural starter cultures are used for milk acidification and derive from the whey of a previous and successful batch of cheese, stored at ambient temperature for 24 hours.

In this study we analysed the bacteriophage induced incidents of slow acid production encountered by artisanal mozzarella cheese makers. A preliminary work of characterisation of the whey starters revealed that they consisted of mixed strains of lactobacilli, streptococci, and lactococci, many of which contain inclusion bodies comprised of polyphosphate (Aprea *et al.* 2005).

The natural starters from buffalo milk have been extensively studied. They are a complex consortia of microorganisms playing an important role in cheese-making, especially due to acid production (Coppola *et al.* 1988, Mauriello *et al.* 2003). Milk contamination with phages of lactic acid bacteria (LAB) could result in inhibition of acid production, leading to curdling failures (Mullan 1986).

Spot and plaque assay methods are routinely used to isolate phages. However, modern biomolecular methods can support the classical microbiology techniques in order to obtain qualitative information on the presence of phages active against lactic acid and other bacteria. In particular, polymerase chain reaction (PCR) can be used to rapidly screen milk and to identify particular phage types.

The detection of phages against *Streptococcus thermophilus* in cheese whey and milk by PCR has already been reported in the relevant literature (Brussow *et al.* 1994, Labrie and Moineau 2000). Labrie and Moineau have developed a multiplex PCR method to detect, in a single reaction, the presence of the major lactococcal phage species in whey and phage lysate samples. More recently Del Rio and colleagues (Del Rio *et al.* 2007) have described a multiplex PCR technique for detecting and identifying, in a single reaction, the presence of phages for *Lactococcus lactis*, *Lactobacillus delbrueckii*, and *S. thermophilus*, in milk and dairy products.

To the best of our knowledge, no work on the role of phages in the manufacture of artisanal buffalo mozzarella has been finalised yet, and this is the first report of the diversity of the phage contamination in artisanal produced mozzarella cheese and of the isolation of phages against a *L. lactis* host, which is part of the LAB flora containing polyphosphate inclusion bodies (Aprea *et al.* 2005).

Materials and methods

Natural starter cultures

Two hundred and ten samples of buffalo whey starter cultures from 25 artisanal mozzarella cheese producers located in Southern Italy were analysed for bacteriophage contaminations. Ninety of them showed acidification delays during milk curdling.

Isolation and identification of lactic acid bacteria

Serial, decimal dilutions were made from samples using maximum recovery diluent (Straka and Stokes 1957).

Dilutions were plated onto De Man, Rogosa and Sharpe (MRS) agar (De Man *et al.* 1960) and Neutral Red Chalk Lactose Agar (NRCLA), and incubated at 30°C (MRS agar) and 37°C (NRCLA) for up to 3 days in microaerophilic conditions.

Colonies for characterisation were randomly selected using a Harrison disk (Harrigan and Cance 1998) and incubated in MRS broth (De Man *et al.* 1960) at 30°C for 24-72 hours and in Litmus Milk and purified by sequential colony purification on either MRS or Elliker agar (Harrigan and Cance 1998). Isolates were identified to genus level using API 50 CHL computer-assisted identification scheme (API Laboratory Products Ltd., Basinstoke, UK) and the scheme devised by Billie and colleagues (Billie *et al.* 1992).

One isolate identified as a phage host was analysed with a simplex PCR assay (Istituto Superiore di Sanità 2008) combined with 16S rRNA gene sequence analysis to confirm genus and species.

Spot and plaque assays for phage isolation and titre count

Whey samples were tested for the presence of phages by spotting 50 µl of filtered whey (0.45 µm filter) onto M17 agar plates containing soft agar overlays (Terzaghi and Sandine 1975) inoculated with the starter cultures (84 strains) isolated as potential hosts (Table I). Plaque assay was performed for phage titration using M17 agar (Terzaghi and Sandine 1975).

Plates were incubated at 30° and 37°C for 24 hours and host growth inhibition and plaque count were determined.

A 3-time sequential plaque purification was carried on 6 plaques randomly chosen after plaque assay performed from the whey sample that resulted contaminated with bacteriophages. Briefly, each of the 6 plaques was taken from the agar with a sterile loop, placed into 3 ml of BHI for 8 hours, filtered

with 0.45 µm filters, and the lysate was propagated and then assayed with Plaque Assay for 3 times consecutively.

Phage propagation

Phage stock suspensions were prepared using PLGYG broth as described by Mullan and colleagues (Mullan *et al.* 1981). Briefly the phage host was harvested overnight in PLGYG broth. The growth culture was then inoculated (0.1 ml) in 10 ml of fresh PLGYG broth with 0.1 ml of 1MCaCl2 and 0.1 ml of the phage lysate to propagate. The suspension was than incubated at 30 or 37°C for 6 hours. Then the new lysate was filtered (0.45 µm) and stored at +4°C.

Analysis of phage genome

The analysis of the viral genomes was carried out by digestion of the extracted DNA with EcoRV, EcoRI, AluI, HinfI, HindIII, Pst I, Sau3AI, BamHI, Sal I and Kpn restriction enzymes. DNA samples digested with each restriction enzyme were subjected to a 10 minutes heating treatment at 65°C in order to evidence the presence of cohesive ends and, consequently, differentiate between *pac*-type or *cos*-type phages (Forsman and Alatossava 1991).

DNA fragments were separated by agarose (1% w/v) gel electrophoresis in Tris-acetate-EDTA buffer. Fragments were visualized under ultraviolet light after ethidium bromide staining (Foschino *et al.* 2005).

DNA standard fragments of DNA molecular weight marker X (Roche, Welwyn Garden City, UK) were used to calculate the size of the fragments. The sum of molecular weights of fragments generated by digestion determined the total phage genome length.

Table I. *Lactic Acid Bacterium strains isolated from buffalo whey starter cultures and their sensitivity to phages Apr-1 and Apr-2.*

Lactic Acid Bacteria	Number (%)	Strains lysed by phages Apr-1 and Apr-2
Lactococcus lactis cremoris	29 (34.52)	0
Lactococcus lactis lactis	17 (20.23)	1
Lactobacillus paracasei	11 (13.11)	0
Lactobacillus delbrukii bulgaricus	7 (8.33)	0
Lactobacillus fermentum	6 (7.14)	0
Lactobacillus curvatus	3 (3.57)	0
Streptococcus thermophilus	10 (11.91)	0
Pediococcus spp.	1 (1.19)	0

Phage concentration and purification

High titre phage suspensions (about 10^9 pfu/ml) were obtained by using PLGYG broth. Sodium chloride was added to a final concentration of 0.5 M and centrifugation was used to remove cellular debris. Polyethylene glycol (PEG) 8,000 was then added to the lysate to form a solution of 50% final concentration. The PEG solution was then centrifuged at 800 g.

Pellets were reconstituted in TBT buffer [0.5 M-NaCl, 10 mM-Tris/HCl (pH 7-5), 10 mM-MgC12 and 10 mM-2-mercaptoethanol].

Ultracentrifugation tubes (Quick Seal, Beckmann, Munich, Germany) were filled up with lysate suspension, 3M CsCl, 5M CsCl and TBT buffer, heat-sealed and subjected to ultracentrifugation at 34,000 g for about 150 minutes.

Upper and lower phage bands were successively extracted through the plastic tube by syringe and the phage lysates were consequently purified overnight at 4°C by dialysis in cellulose tubes with buffer (Tris-HCL 10 mmol L^{-1}; pH 7.4; MgSO4 10 mmol L^{-1}; NaCl 0.5 mol L^{-1}).

Analysis of phage structural proteins

After purification, phage lysates were subjected to SDS-PAGE (Laemmli 1970). The bands were stained with Coomassie brilliant blue R250 and destained with 10% glacial acetic acid and 10% isopropanol. Prestained molecular weight marker (Broad Range, New England Biolabs, Hitchin, UK) was used to calculate phage protein sizes.

Transmission Electron Microscopy

For each phage a 200 mesh copper grid coated with carbon-stabilizer formvar was inserted into a tube for airfuge (Beckmann, Munich, Germany), filled with 120 µl of each lysate, centrifuged at 20 psi for 15 minutes and negative stained with 2‰ phosphotungstic acid. Each sample was observed with TEM EM 900 (Zeiss, Oberkochen, Germany) between 12,000x and 80,000x magnification.

Host range analysis

Purified ϕApr-1 and ϕApr-2 phage suspensions were spot-tested against the 84 lactic acid bacteria isolated and characterized as potential host in order to assess their host specificity.

Multiplex PCR analysis and sequencing

All 210 whey starters were analysed by multiplex PCR assay using the protocol described by Del

Rio and colleagues (Del Rio *et al.* 2007). DNA extraction was conducted by an automatic nucleic acid extractor (QIAcube, Qiagen, Hiden, Germany) using QIAmp DNA minikit protocol. The PCR reaction was prepared with Qiagen Multiplex Master Mix (Qiagen, Hiden, Germany). The primers used for the multiplex PCR were: 335A 5′ GAAGCTAGGCGAATCAGTAAACTTGCTAG 3′ and 335B 5′ CGGCTATCTCGTCAATTGTTCCGGTTGC 3′, 936A 5′ ATCAGTTGGCTCAATGGAAGACCAAGCGG 3′ and 936B 5′ GTTGCTTCTGCTGTTGGTGTCAAATGAGGA 3′, C2A 5′ CAATCGAAGCAGGTGTAAAAGTTCGAGAAC 3′ and C2B 5′ GCTTTATCCATTTGTAGGTATGCTTCTGCC 3′ for *L. lactis* phages, Lb1 5′ TCCCGGGCTAACCACCTCTACACTC 3′ and Lb2 5′ GGTGTAGTGACCATCCTTTGAGAGC 3′ for *L. delbrueckii* phages, Host 1 5′ GAATGATACTGCTGGCAGTATTTCGGTTGG 3′ and Host 5 5′CAGTCATGTAGCTATCGATGAAATTCCAACG 3′ for *S. thermophilus* phages (Del Rio *et al.* 2007). All the 5 primer couplets were used at the final concentration of 0.3 µM and 1 µL of the extracted DNA as template. Phage C2 was used as positive control.

The phages detected in this study were subjected to DNA sequencing using the following thermal protocol: Taq activation at 95°C for 15 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing 50°C for 1 minute and extension at 72°C for 1 minute, final extension at 72°C for 7 minutes.

The PCR products were analysed using the computerized system of capillary electrophoresis (QIAxcel; Qiagen, Hiden, Germany), purified with QIAquick PCR purification kit and used for the sequencing PCR (BigDye Terminator kit v.1.1 Cycle Sequencing, Applied Biosystem, Warrington, UK). The primers for the sequence analysis were identical to the one used for the conventional multiplex PCR. The sequencing thermal protocol was based on activation at 96°C for 1 minute and 25 cicles of 94°C for 10 seconds, 50°C for 5 seconds, 60°C per 4 minutes (Perkin Elmer gene amp PCR system 2,400 Thermo Cycler).

Amplicons resulted from the sequencing PCR were purified (kit DyeEx 2.0 Spin, Qiagen, Hiden, Germany), followed by formamide denaturation and sequencing phase with ABI PRISM 3130. The DNA sequences were assembled and analysed with the software SeqScape 2.5 version, Applied Biosystem.

Detection limit of the PCR method

To determine the sensitivity of the multiplex PCR assay, sterilised buffalo milk was contaminated with serial dilutions of ϕApr-1 and assayed by PCR (Forsman and Alatossava 1991).

Results

Phage isolation and plaque formation

The growth of 1 culture isolated from a whey starter was clearly inhibited when it was tested with 1 whey sample after filtration (0.45 µm filter). The host was identified as *L. lactis* by API 50 CHL computer-assisted identification scheme and by 16S rRNA gene sequence analysis. Plaques were visible after 24 hours incubation at 30° and 37°C, they were observed in the shape of very small circular areas of lysis of about 0.5 mm diameter.

Characterization of phage genomes

The digestions of phage DNA with the restriction enzymes Sau3AI, BamHI, Sal I and Kpn were negative. On the basis of the results of the genomic patterns related to the other 7 restriction tested enzymes (EcoRV, EcoRI, AluI, HinfI, HindIII, Pst I and BamHI), the 6 lysates were divided into 2 groups, each including 3 lysates. The lysates within the same group showed no genomic differences. The most important difference between the 2 groups was shown by EcoRV. From the analysis of these data, at least 2 phages were co-present in the same sample and they were named ϕApr-1 and ϕApr-2. Their genome sizes were estimated by the sum of molecular weights of fragments generated by digestion.

The genome lengths were approximately 31.4 kb and 31 kb for ϕApr-1 and ϕApr-2, respectively.

DNA did not show any heat-inducible fragment-dissociation with any of the 7 tested endonucleases for both bacteriophages, indicative of *pac*-type phage genomes (data not shown).

Protein composition of ϕApr-1 and ϕApr-2

The SDS-PAGE profiles of ϕApr-1 and ϕApr-2 contained 1 major protein with molecular weight of 32.5 kDa.

ϕApr-1 showed 23 other minor proteins (155 ,145, 103, 78.8, 66.2, 57.2, 54.7, 50, 45, 43.7, 42.5, 35, 37.5, 30.5, 30, 28, 27.5, 25, 22.8, 13.5, 11.5, 10.5, 6.5 kDa) for a total molecular weight of 1,152 KDa.

ϕApr-2 protein profile was constituted of other 14 minor proteins (103, 54.7, 52, 50, 43.7, 38, 30.5, 28, 24, 22.8, 13, 12, 10.5, 10 kDa) for a total molecular weight of 524.7 KDa (Figure 1).

Host range analysis

ϕApr-1 and ϕApr-2 showed activity only against their own *L. lactis* host revealing a very high host specificity (Table I).

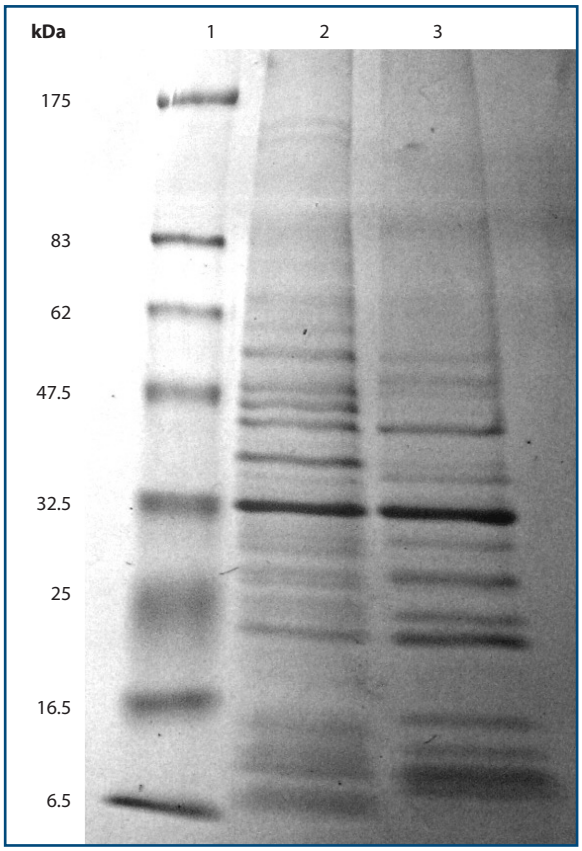


Figure 1. *Structural proteins of phage Apr-1 (lane 2) and phage Apr-2 (lane 3).* Lane 1 shows the molecular weight protein marker.

Phage morphological characterization

ϕApr-1 and ϕApr-2 were identical in shape. They presented an icosahedral-isometric head of about 50 nm diameter and a thin, long, non-contractile, flexible tail of about 110 nm lengths. The total phage length was about 160 nm. ϕApr-1 and ϕApr-2 belonged to the *Caudovirales* order of the *Siphoviridae* family (King *et al.* 2011) Group B, Morphotype B1 (Ackermann and Du Bow 1986). According to their morphological characterization, they are double stranded DNA viruses (Figure 2).

Multiplex PCR

Multiplex PCR assays detected DNA of phages active against lactococcocci (phage group P335), *S. thermophilus* and *L. delbrueckii* (Figure 3 and 4).

In particular, among 210 whey samples tested, 60 (28.56%) samples were positive to phage DNA. Phage DNA related to only group P335 was found in 10 (4.76%) samples, while *L. delbrueckii* and *S. thermophilus* phage DNA was found in 30 (14.28%) samples. In contrast, phage DNA belonging to all the three groups was detected in 20 (9.52%) samples (Table II).

Multiplex PCR performed on DNA derived from ϕApr-1

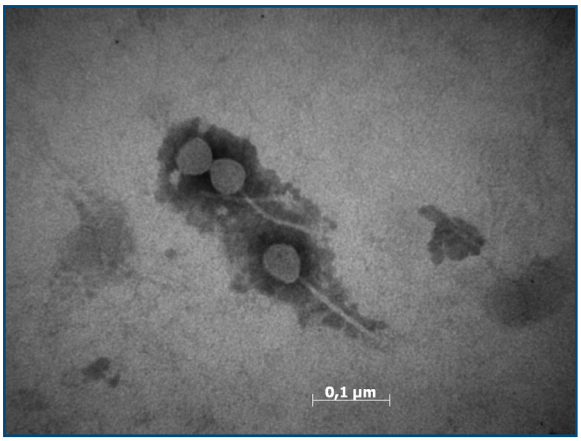


Figure 2. *Bacteriophage Apr-1 under Transmission Electron Microscope (50,000x).*

and the relative sequence analysis (data not shown) confirmed the relatedness of ϕApr-1 to phages active against *L. lactis* and belonging to group P335.

ϕApr-1 detection limit

The detection limit for ϕApr-1 was 102 pfu/ml in buffalo milk and whey.

Sequencing analysis.

The sequencing results confirmed that phages belonged to the groups determined by PCR, comprising the DNA related to Streptococcal phages (data not shown).

Discussion

This is the first report assessing the diversity of phage types against lactic acid bacteria in artisanal buffalo mozzarella starters although some preliminary findings were presented in the IV International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld) in Torremolinos, Spain, in 2011 (Aprea *et al.* 2011).

Multiplex PCR was used to screen 210 samples of natural whey starters from buffalo milk for bacteriophages. The results showed whey contamination by phages against *L. lactis*, *L. delbrueckii* and *S. thermophilus* in 60 (28.56%) samples, which were all related to starters from slow acid producing cheese batches. Interestingly, 9.52% of samples were positive to phages.

Lactococcal phages are currently classified into 10 species based on morphology and DNA homology (Deveau *et al.* 2006). Multiplex PCR identified the lactococcal phages in this study as belonging to the phage species P335. This was not surprising, since phages from this group belong to

1 of the 3 lactococcal phage species responsible for most cases of milk fermentation failure worldwide (Bisonnette *et al.* 2000, Brussow 2001, Casey *et al.* 1993, Moineau *et al.* 1996).

The bands identifying the streptococcal phage group revealed a high DNA concentration, up to 7.36 ng/ul and a length of 1,000 bp, larger than the 750 bp reported by Del Rio and colleagues (Del Rio *et al.* 2007). Nevertheless, the sequencing data confirmed the DNA relatedness to *Streptococcus thermophilus* phage OBJ.

Despite extensive efforts to identify host bacteria for the phages present in the starters, only 2 phages – ϕApr-1 and ϕApr-2 – active against a strain of *L. lactis* were isolated. Because of host specificity related to bacteriophages, isolation methods cannot be used

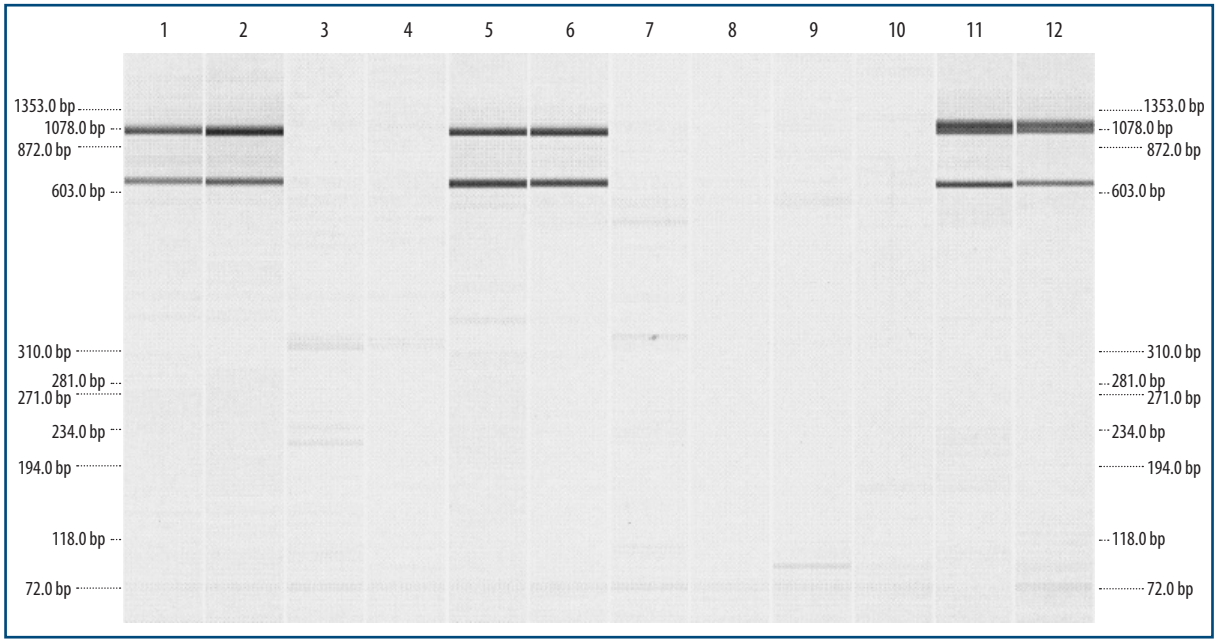


Figure 3. *Multiplex polymerase chain reaction results of a group of 6 whey samples positive to both* Lactobacillus delbrueckii *and* Streptococcus thermophilus *phages (lanes 1, 2, 5, 6, 11, 12)*. Lanes 3, 4, 7, 8, 9: negative samples. Lane 10: negative control (sterile water).

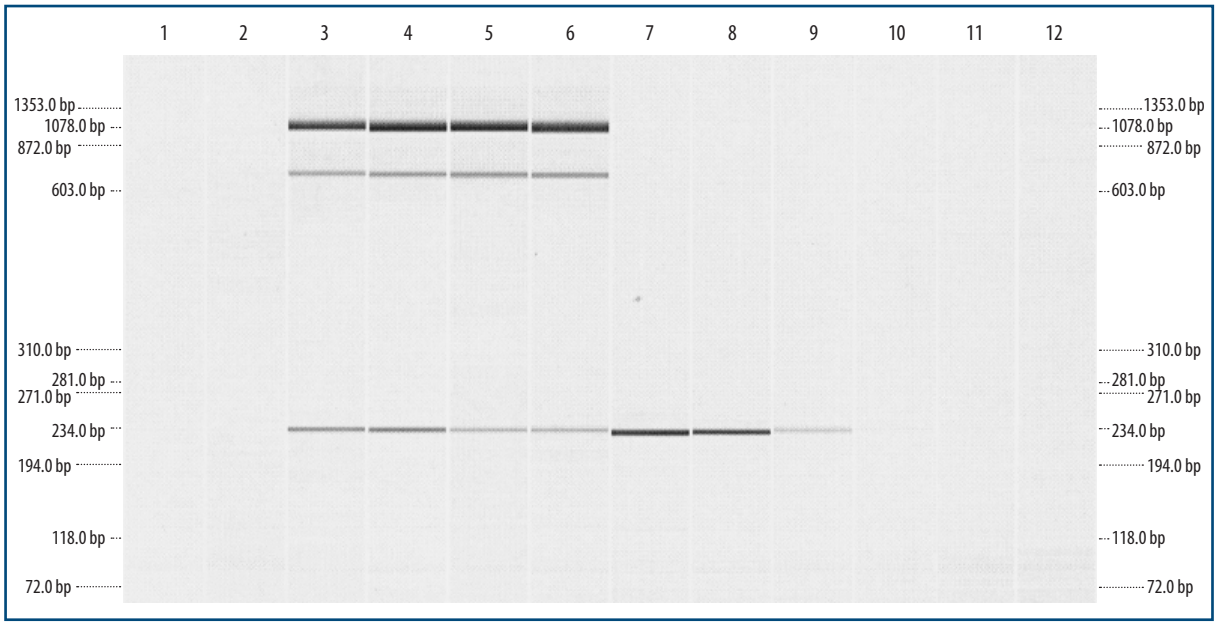


Figure 4. *Multiplex polymerase chain reaction results of 4 whey samples (lanes 3, 4, 5, 6) positive to phage group P335,* Lactobacillus delbrueckii *and* Streptococcus thermophilus *phages.* Lanes 7, 8, 9, 10 show the results of the polymerase chain reaction detection limit for phage ϕApr-1 (lane 7: 10^4 ufp/ml; lane 8: 10^3 ufp/ml; lane 9: 10^2 ufp/ml; lane 10: 10 ufp/m). Lanes 1 and 2: negative samples. Lanes 11 and 12: negative control (sterile water).

Table II. *Prevalence of P335,* Lactobacillus delbruekii *and* Streptococcus thermophilus *phage groups in whey samples with problems of slow acid production.*

Whey samples	P335 phage group (%)	*L. delbruekii* + *S. thermophilus* phage groups (%)	P335 + *L. delbruekii* + *S. thermophilus* phage groups (%)	Number (%)
Negative to phage detection				150 (71.44)
Positive to phage detection	10 (4.76)	30 (14.28)	20 (9.52)	60 (28.56)
Total (number)				210 (100)

as screening tools. In fact, false negative samples could occur if specific phage-hosts are not available during spot assays. However, isolation techniques still remain irreplaceable when novel phages need to be selected and characterized.

ϕApr-1 is included into P335 group by PCR. Its detection limit was 10^2 pfu/ml, demonstrating a significantly higher PCR sensitivity in buffalo milk than the one reported by Del Rio and colleagues for phages belonging to the same phage group (Del Rio *et al.* 2007).

The *pac*-type system for phages belonging to P335 group confirms their relatedness to lytic bacteriophages (Brussow *et al.* 1994).

In this study, we assessed the prevalence of phages in whey starters used in 25 buffalo mozzarella cheese factories experiencing slow acid production. Phage genomes have been detected in 28.56% of samples that showed milk acidification failures. Two phages active against *L. lactis* have been isolated in this research.

A previous paper reported that these starters contained strains of *S. thermophilus*, lactobacilli, and lactococci, both containing inclusion bodies comprised of polyphosphate (Aprea *et al.* 2005).

This work revealed 2 phages within the artisan starters studied and argues for the use of multiplex PCR to investigate phage diversity in complex microbial populations and to screen milk samples before their use in cheese making. The method may also be modified to obtain information on phage concentrations.

Raw milk is believed to be an important reservoir of phages. We are evaluating the potential of the described multiplex PCR method as a useful tool for screening farmers' milk in order to reduce the contamination in unpasteurized dairy products like the Italian buffalo mozzarella cheese.

Acknowledgments

The excellent technical assistance of Edmund Slaine (Loughry Campus, College of Agriculture, Food and Rural Enterprise, Cookstown, Co Tyrone, Northern Ireland), Paddy O'Reilly (University College Cork, Cork, Rep. of Ireland) and M. Gabriella Lucibelli (Molecular Biology-IZSM) is gratefully acknowledged. Particular thanks are due to 'Caseificio Vannulo' (Salerno, Italy) for supplying part of the whey starters.

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