Journal of Dairy Research (2015) **82** 242–247. doi:10.1017/S0022029915000151

Biodiversity of *Lactobacillus helveticus* bacteriophages isolated from cheese whey starters

Miriam Zago*, Barbara Bonvini, Lia Rossetti, Aurora Meucci, Giorgio Giraffa and Domenico Carminati

Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca per le Produzioni Foraggere e Lattiero-Casearie (CRA-FLC), 26900 Lodi, Italy

Received 9 June 2014; accepted for publication 3 February 2015

Twenty-one *Lactobacillus helveticus* bacteriophages, 18 isolated from different cheese whey starters and three from CNRZ collection, were phenotypically and genetically characterised. A biodiversity between phages was evidenced both by host range and molecular (RAPD-PCR) typing. A more detailed characterisation of six phages showed similar structural protein profiles and a relevant genetic biodiversity, as shown by restriction enzyme analysis of total DNA. Latent period, burst time and burst size data evidenced that phages were active and virulent. Overall, data highlighted the biodiversity of *Lb. helveticus* phages isolated from cheese whey starters, which were confirmed to be one of the most common phage contamination source in dairy factories. More research is required to further unravel the ecological role of *Lb. helveticus* phages and to evaluate their impact on the dairy fermentation processes where whey starter cultures are used.

Keywords: *Lactobacillus helveticus*, bacteriophages, phage biodiversity, cheese whey.

Natural whey starters (NWS) are undefined cultures of lactic acid bacteria (LAB) obtained from the incubation of cheese whey collected at the end of the cheese-making process. NWS are characterised by the presence of thermophilic LAB and are used for the manufacture of Italian, long-ripened cheeses such as Grana Padano, Provolone and Parmigiano Reggiano (Beresford et al. 2001; Lazzi et al. 2004). Bacteriophage infections represent a serious problem in dairy fermentative processes, and their inhibitory effect against lactic acid starters has been recognised for more than 70 years (Moineau & Lévesque, 2005). Culture rotation programs and direct to vat-inoculation of starters are long-standing practices to control phage infections in industrial fermentation, whereas similar approaches cannot be applied to undefined cultures. Although it is believed that the richness and heterogeneity of the microbial composition may represent a natural barrier against phage infection problems, phage contamination of undefined starter cultures frequently occurs because they are propagated under non-aseptic conditions, without any control measures (Carminati et al. 2011). Thus, it is possible that a number of LAB strains that compose the NWS could be infected by phages, altering the metabolic activity of the culture. Zago et al. (2005) reported the presence of active lytic phages attacking *Lactobacillus helveticus* strains in NWS used for production of long-ripened cheeses. Similarly to other ecosystems, bacteriophages are suggested to play an ecological role within NWS, acting as a biological pressure, leading the spontaneous selection of phage-resistant strains, able to counteract the loss of the sensitive ones, thus preserving the overall technological performances of the cultures (Zago et al. 2008a).

Few studies on *Lb. helveticus* phages are available. Earlier investigations of Sozzi & Maret (1975) and Séchaud and colleagues (1992) described the characteristics of *Lb. helveticus* phages isolated, respectively, from Emmental starters and cheese whey in French factories, whereas Quiberoni et al. (1999) studied the inactivation of *Lb. helveticus* phages by thermal and chemical treatments. The aim of this work was to study the biodiversity of 21 *Lb. helveticus* phages isolated from different NWS by comparing host range, structural proteins and genome characteristics. This study contributes to increase the knowledge on the biodiversity of *Lb. helveticus* phages.

Materials and methods

Bacterial strains, bacteriophages and culture media

Twenty-one *Lb. helveticus* phages and their host strains used in this study are listed in Table 1. Sixteen bacteriophages

*For correspondence; e-mail: miriam.zago@entecra.it

Table 1. *Lactobacillus helveticus* bacteriophages and its host strains used in this study

Phage	Host strain	Origin	Cheese factory code
Lh55	CNRZ15807	CNRZ collection	
Lh56	CNRZ892	CNRZ collection	
Lh57	CNRZ493	CNRZ collection	
PA44	PA44	Provolone NWS	1
PS5	Lh144	Provolone NWS	2
G7	G7	Grana Padano NWS	3
G11	G11	Grana Padano NWS	3
H3	H3	Grana Padano NWS	4
W8	W8	Grana Padano NWS	5
AF3	AF3	Grana Padano NWS	6
U6	U6	Grana Padano NWS	7
T8	T8	Grana Padano NWS	8
AI7	Lh117	Grana Padano NWS	9
V15	V15	Grana Padano NWS	10
AA17	AA17	Grana Padano NWS	11
AA18	Lh1408	Grana Padano NWS	11
AC1	Lh99	Grana Padano NWS	12
AB9	Lh1407	Grana Padano NWS	13
AQ113	Lh1405	Grana Padano NWS	14
AQ114	Lh1406	Grana Padano NWS	14
733	Lh733	Grana Padano NWS	15

CNRZ, National Centre for Zootechnical Research; INRA, France; NWS, natural whey starter

were isolated from Grana Padano NWS, two from Provolone NWS after an enrichment procedure (as described by Zago et al. 2005) and three were from official culture collections. The real presence of *Lb. helveticus* phages was confirmed by specie-specific PCR as reported by Zago et al. (2008b). To determine phage host range, 100 genotypically diverse *Lb. helveticus* strains previously isolated from different NWS were used. The *Lb. helveticus* strains have been previously typed (data not shown) by RAPD-PCR fingerprinting according to Rossetti & Giraffa (2005). Strains and phages were maintained as frozen stocks at −80 °C in the presence of 15% (v/v) glycerol as cryoprotective agent. MRS-Ca broth and agar were routinely used to grow and plate bacteria or to propagate and count phages at 42 °C.

Host range determination

A microtitre plate assay (Zago et al. 2006) was used. Briefly, an overnight culture from different host bacteria with each phage were inoculated in microtitre plates at a m.o.i (multiplicity of infection) of 0·1, which had been filled with MRS-Ca broth containing bromocresol purple as growth indicator. All plates were incubated at 42 °C under anaerobic conditions and, after 8 h, scored for presumptive lysis of indicator strains (no colour change of the indicator). All presumptive lytic phage-host combinations were further confirmed by the turbidity test (Zago et al. 2005).

RAPD-PCR analysis

Phages were propagated on the appropriate indicator strains (Table 1) in 50 ml MRS-Ca broth. After lysis, the cultures were filtered, treated for 30 min with 1 µg/ml DNase I and 1 µg/ml RNase (Sigma-Aldrich, Milan, Italy), and concentrated by centrifugation (100 000 × ***g***, 1 h, 4 °C). Phage DNAs were extracted from concentrated phage particles and purified by the methods previously described (Zago et al. 2006). Phages were identified by species-specific PCR as described by Zago & colleagues (2008b) and typed by RAPD-PCR with primers M13 with sequence 5′-GAGGGTGGCGGTTCT-3′ (Huey & Hall, 1989) and OPL5 with sequence 5′-AACGCGCAAC-3′ (Gutiérrez et al. 2011). DNA bands were separated on 1·5% (w/v) agarose gel and visualised by GelRed™ solution (Biotium, Hayward, California, USA) staining. One-kb plus DNA Ladder and λ-*Hind*III DNA ladder (Invitrogen, Milan, Italy) were used as DNA molecular weight marker.

Restriction analysis of phage DNA

Phage DNAs were cleaved with *Eco*RI and *Eco*RV restriction enzymes (New England BioLabs, Hertfordshire, UK), according to manufacturer's instructions. Digested DNA fragments were heated for 10 min at 70 °C, held in ice, separated on 1·5% (w/v) agarose gel and visualised by GelRed™ solution (Biotium) staining. λ-*Hind*III DNA ladder (Invitrogen) was used as DNA molecular weight marker.

Bacteriophage structural proteins

Concentrated phage particles were used to extract structural phage proteins, which were analysed by SDS-PAGE electrophoresis according to De Antoni et al. (2010).

https://doi.org/10.1017/S0022029915000151 Published online by Cambridge University Press

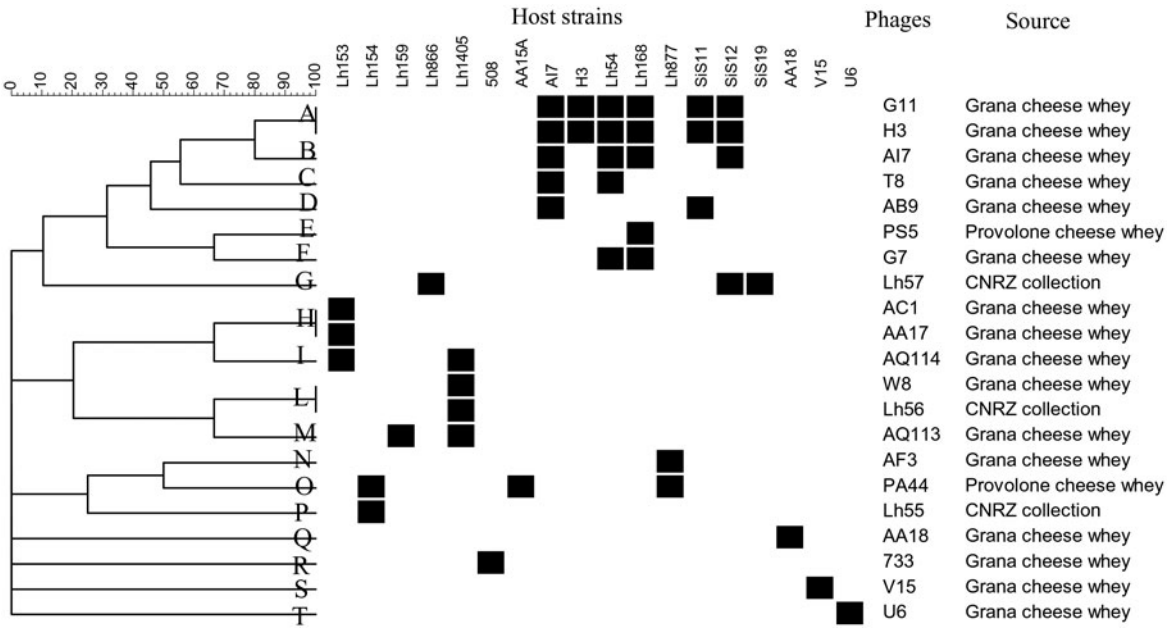

Fig. 1. Host range profiles of 21 *Lb. helveticus* phages against 100 *Lb. helveticus* strains. Positive lysis of the only susceptible strains is indicated by a black box. Cluster analyses were carried out by BioNumerics software using the Dice correlation coefficient, and the unweighted pair group method using arithmetic averages (UPGMA). On the top side of the branches the numbers indicated the % of similarity. Letters on the dendrogram indicated the different clusters.

One-step growth curve

One-step growth experiments were performed as described by Adams (1959). Briefly, each *Lb. helveticus* indicator strain was infected at a m.o.i. of 2 with its phage. After incubation at 42 °C for 30 min for adsorption, the infected culture was diluted to exclude secondary infection and subsequent decimal dilutions were made and incubated at 42 °C. The number of phages was determined every 60 min (up to 360 min) by the double-layer plate method. Assays were carried out in triplicate. The kinetic parameters of phage infection that could be determined by the one-step growth curve were the latent period, the burst time and the burst size. Latent period was defined as the time interval between the adsorption and the beginning of the first burst, as indicated by the initial rise in phage titre. Burst time was the time before the increase in the number of lysis plaques occurred. Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period.

Statistical analysis

Host spectra, RAPD and endonuclease restriction profiles of phage DNAs were analysed by BioNumerics software (version 6.6, Applied Maths, St. Martens-Latem, Belgium). Dendrograms were generated after cluster analysis using the Dice correlation coefficient (for host spectra) or the composite data set and the Pearson correlation coefficient (for RAPD and restriction endonuclease profiles), and the unweighted pair group method using arithmetic averages (UPGMA). For the determination of latent period, burst time and burst size on the one-step growth curves, DMFit web edition was used (http://modelling.combase.cc/DMFit.aspx); data were fitted using the complete model of Baranyi & Roberts (1994).

Results

Host range determination

The host range of the 21 phages enabled the grouping of the phages into 18 clusters (Fig. 1). Only three clusters (A, H, J) grouped two phages; notably, the cluster A included two phages (G11 and H3) capable of multiplying on six strains. The other 15 clusters were represented by only one phage. Overall, the 21 phages showed a narrow host range, with a lytic activity restricted to only one strain for 11 out of the 21 bacteriophages (Fig. 1).

Fingerprinting analysis

The cluster analysis of the RAPD profiles allowed a discrimination of all phages in seven clusters (Fig. 2). The clusters grouped from eight (cluster E) to two phages (cluster F) and three clusters contained only one phage (C, D, G). Phages grouped regardless of the NWS origin.

Characterisation of bacteriophages

Only six (ΦLh55, ΦLh56, ΦAQ113, ΦAQ114, ΦAA18, ΦAB9) out of the 21 bacteriophages were able to form

https://doi.org/10.1017/S0022029915000151 Published online by Cambridge University Press

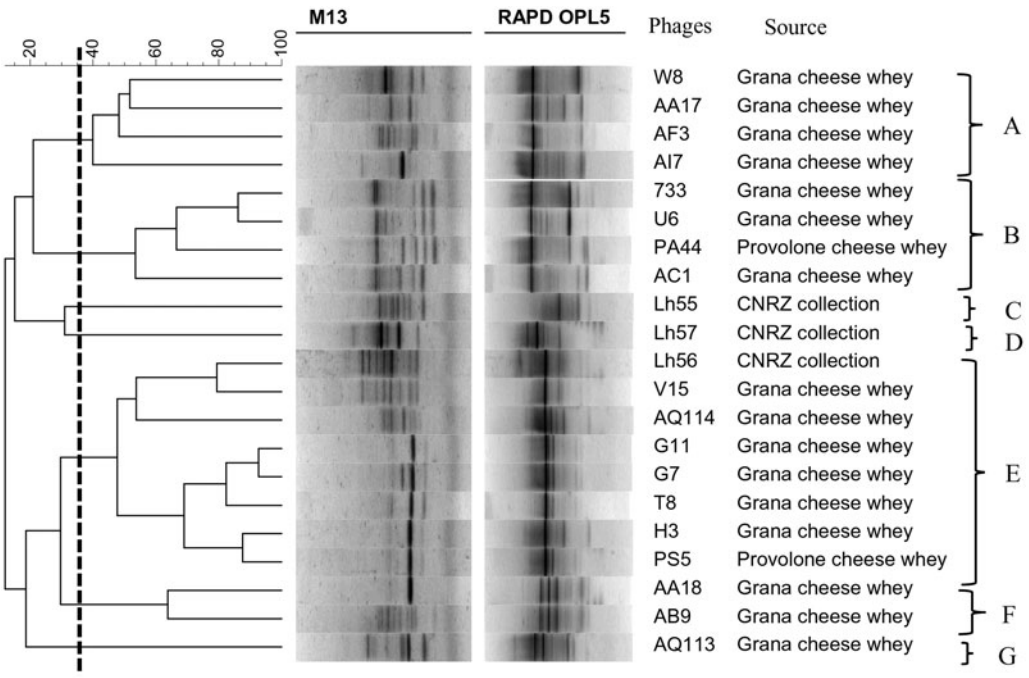

Fig. 2. Clusters analyses of the RAPD band patterns of 21 *Lb. helveticus* bacteriophages using the primers M13 and OPL5. Cluster analysis were carried out by BioNumerics software using the composite data set, the Pearson correlation coefficient, and the unweighted pair group method using arithmetic averages (UPGMA). On the top side of the branches the numbers indicated the % of similarity. The identity level for genotypes discrimination is represented by a dashed line at 35% of similarity as indicated by the software.

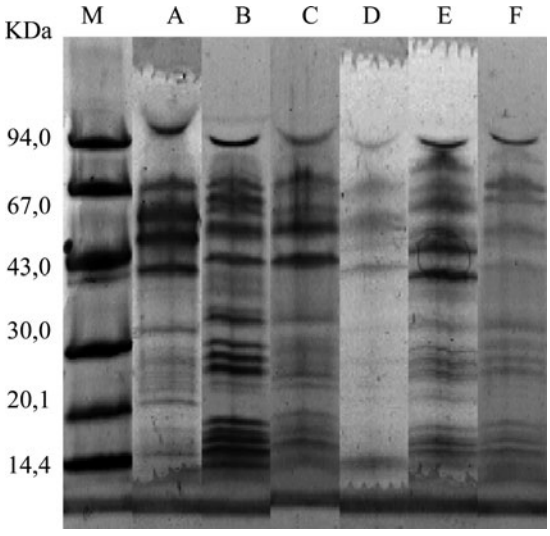

Fig. 3. Structural protein profiles of six *Lb. helveticus* bacteriophages, processed with BioNumerics software to normalise bands. After concentrations the purified phage proteins were separated by SDS–PAGE. Lanes: A, ΦLh55; B, ΦLh56; C, ΦAQ113; D, ΦAQ114; E, ΦAB9; M, Molecular Weight Marker (GE Healthcare, Italy).

lysis plaques by agar layer technique on MRS-Ca (data not shown). The six plaque-forming phages were then chosen for further analysis. These six phages belonged to six different host range clusters (D, I, J, K, N, O) and four RAPD clusters (C, E, F, G), but they showed a uniform structural protein profile, represented by three groups of similar bands sized around 14·4–20·1, 30 and 43–67 KDa (Fig. 3).

No evidence was found for the presence of cohesive, protruding ends (*cos*) in the six genomes, and ligation of its DNA did not alter the restriction patterns after the heat treatment of the digestion fragments (data not shown) as described by Kilic *et al* (1996). Therefore, the *Lb. helveticus* phages likely utilize the *pac* mediated DNA packaging. The composite dendrogram generated after the cleavage of the phage DNA with the enzymes *Eco*RI and *Eco*RV showed five different clusters, of which only the cluster A included two phages (Fig. 4).

As shown in Table 2, a latent period less than 150 min was observed for four out of the six phages, whereas phages ΦLh56 and ΦAA18 showed a latent period less than 100 min. ΦLh55, ΦLh56, ΦAA18 and ΦAB9 had a burst size greater than 100 PFU/infected cell, and in particular ΦLh55 and ΦLh56 had the highest burst size with a value of 135 PFU/infected cell. ΦAQ113 and ΦAQ114 showed a burst size around 90 PFU/infected cell. ΦLh56 appeared as the most virulent phage between the six studied, with the highest burst size occurring in less time (270·0 ± 15·5 min).

Discussion

The knowledge on the diversity and properties of *Lb. helveticus* phages is important for evaluating their characteristics

https://doi.org/10.1017/S0022029915000151 Published online by Cambridge University Press

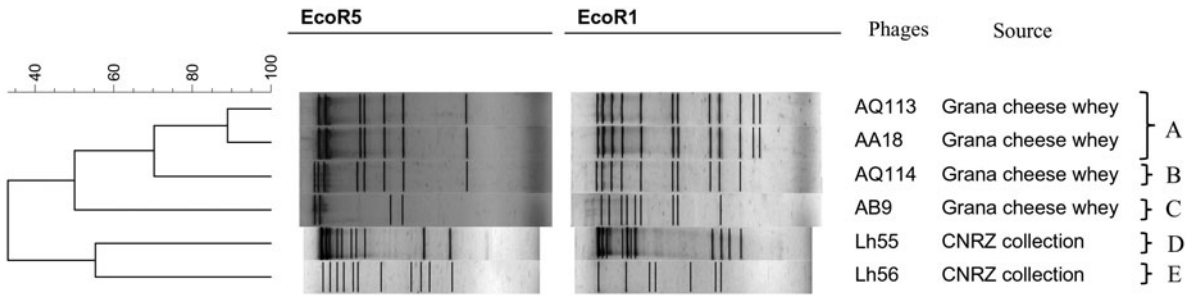

Fig. 4. Composite dendrogram of *EcoRI* and *EcoRV*, computer generated restriction profiles of 6 *Lb. helveticus* phages. Cluster analysis was carried out by BioNumerics software using the composite data set function, the Dice correlation coefficient, and the unweighted pair group method using arithmetic averages (UPGMA). On the top side of the branches the numbers indicated the % of similarity.

Table 2. Latent period, burst time and burst size of 6 *Lactobacillus helveticus* phages

Phages	Latent period†	Burst time†	Burst size‡
Lh55	214·6 (8·0)	360·0 (10·0)	135·0 (2·0)
Lh56	99·1 (20·0)	270·0 (15·5)	135·4 (5·5)
AQ113	181·0 (2·7)	258·0 (20·7)	93·2 (6·1)
AQ114	145·2 (34·3)	264·0 (10·4)	88·2 (4·5)
AA18	53·8 (6·0)	360·0 (10·0)	106·2 (8·7)
AB9	158·2 (24·1)	360·0 (10·0)	130·7 (2·9)

Data are the means of three determinations
†Minutes (±SD)
‡Indicated as PFU ml^{-1} infected cell (±SD)

and behaviour during the cheese-making process and the consequence of their presence on the final product quality. In particular, 18 of the 21 *Lb. helveticus* phages studied here were isolated throughout 10 years from NWS without acidification problems. This suggests the simultaneous maintenance of phages and sensitive strains within the cultures, as shown in previous studies (Zago et al. 2005, 2008a). Specifically, Φ AQ113 has already been characterised and sequenced in a previous work (Zago et al. 2013) and it belonged to *Myoviridae* family. Host range spectra of all the bacteriophages were narrow. These data differed from the results obtained by Séchaud et al. (1992) where most of the *Lb. helveticus* phages studied were able to lyse 60–90% of the strains. In their work, Séchaud and colleagues performed the sensitivity tests also on 4 strains isolated from CRA-FLC collection (formerly ISLC collection) and only one was sensitive to phages. These results could be explained by the different dairy sources from which phages were isolated in the two studies. Host range spectra evidenced a wide diversity within phages. This diversity was partly confirmed by genotyping of phage DNAs. Little correlation was observed between host range and RAPD grouping, which is in agreement with previous studies (Le Marrec et al. 1997). However, the above results are indicative of the great biodiversity between *Lb. helveticus* phages studied here. Only six phages, out of the 21 investigated, were able to form lysis plaque. This event is quite common in phages of thermophilic lactobacilli, especially *Lb. helveticus* (Carminati et al. 2011; Zago et al. 2012). Difficulties may range from the presence of small plaques difficult to distinguish, to the slow diffusion of the phage in the agar layer or simply to the failure of the plaque development (Abedon & Yin, 2009). Structural protein profiles of the six plaque-forming phages were very similar, showing three groups of similar bands. These data could suggest that the protein profile could serve as a marker of species for *Lb. helveticus* bacteriophages. In particular bands of 43 and 67 KDa were identified as putative tail sheath protein and major head protein, respectively as described for phage ΦAQ113 (Zago et al. 2013). The results obtained from the restriction analysis validated the genetic biodiversity between phages already underlined by RAPD data. At the same time latent period, burst time and burst size data evidenced that phages were active and virulent and confirmed once more the high level of phage biodiversity. In conclusion, this study underlined the biodiversity of *Lb. helveticus* phages isolated from different NWS. It also confirmed the widespread presence of phages in dairy factories and their virulence, that is counterbalanced by the presence in the NWS of an unknown number of strains, with different phage sensitivity, that represent a natural barrier against phage infection. The high level of resistance of starter strains to phage infection may hinder massive phage multiplication in NWS and explain the finding of phages in cultures without acidification problems. Occasionally, the concomitant presence of different phages active against the most part of strains within the same culture might however lead to the generation of a defective product (Madera et al. 2004). In this context, more research is warranted to further unravel the ecological role of phages and to evaluate their impact on the fermentation process. Concurrently, the abundance of the presence of phages in cheese whey starters underlines once again the importance of phage control strategies in the dairy industry.

This work was supported by partial grants from BIODATI Project (D.M. MIPAAF 15421/7303/11).

References

Abedon ST & Yin J 2009 Bacteriophage plaques: theory and analysis. In *Bacteriophages: Methods and Protocols, Vol. 1: Isolation, Characterization, and Interactions*, pp. 161–174 (Eds MRJ Clokie & AM Kropinski). New York: Humana Press, Springer Science+Business Media

Adams MH 1959 *Bacteriophages*. New York: Interscience Publishers, Inc

Baranyi J & Roberts TA 1994 A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* **23** 277–294

Beresford TP, Fitzsimons NA, Brennan NL & Cogan T 2001 Recent advances in cheese microbiology. *International Dairy Journal* **11** 259–274

Carminati D, Zago M & Giraffa G 2011 Ecological aspects of phage contamination in natural whey and milk starters. In *Bacteriophages in Dairy Processing*, pp. 79–97 (Eds AL Quiberoni & JA Reinheimer). New York: Nova Science Publishers, Inc

De Antoni G, Zago M, Vasek O, Giraffa G, Carminati D, Briggiler M, Reinheimer J & Suarez V 2010 *Lactobacillus plantarum* bacteriophages isolated from Kefir grains: phenotypic and molecular characterization. *Journal of Dairy Research* **77** 7–12

Gutiérrez D, Martìn-Platero AM, Rodrìguez A, Martinez-Bueno M, Garcia P & Martinez B 2011 Typing of bacteriophages by randomly amplified polymorphic DNA (RAPD)-PCR to asses genetic diversity. *FEMS Microbiology Letters* **322** 90–97

Huey B & Hall J 1989 Hypervariable DNA fingerprinting in *E. coli*. Minisatellite probe from bacteriophage M13. *Journal of Bacteriology* **171** 2528–2532

Kilic AO, Pavlova SI, Ma W-G & Tao L 1996 Analysis of *Lactobacillus* phages and bacteriocins in American dairy products and characterization of a phage isolated from yogurt. *Applied and Environmental Microbiology* **62** 2111–2116

Lazzi C, Rossetti L, Zago M, Neviani E & Giraffa G 2004 Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length-heterogeineity-PCR. *Journal of Applied Microbiology* **96** 481–490

Le Marrec C, Van Sinderen D, Walsh L, Stanley E, Vlegels E, Moineau S, Heinze P, Fitzgerald G & Fayard B 1997 Two groups of bacteriophages infecting *Streptococcus thermophilus* can be distinguished on the basis of mode of packaging and genetic determinants for major structural proteins. *Applied and Environmental Microbiology* **63** 3246–3253

Madera C, Monjardin C & Suarez JE 2004 Milk contamination and resistance to processing conditions determine the fate of *Lactococcus lactis* bacteriophages in dairies. *Applied and Environmental Microbiology* **70** 7365–7371

Moineau S & Lévesque C 2005 Control of bacteriophages in industrial fermentations. In *Bacteriophages: Biology and Applications*, pp. 285–296 (Eds E Kutter & A Sulakvelidze). Boca Raton, USA: CRC Press

Quiberoni A, Suarez VB & Reinheimer JA 1999 Inactivation of *Lactobacillus helveticus* bacteriophages by thermal and chemical treatments. *Journal of Food Protection* **62** 894–898

Rossetti L & Giraffa G 2005 Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *Journal of Microbiological Methods* **63** 135–144

Séchaud L, Rousseau M, Fayard B, Callegari ML, Quénee P & Accolas JP 1992 Comparative study of 35 *Lactobacillus helveticus*: morphology and host range. *Applied and Environmental Microbiology* **58** 1011–1018

Sozzi T & Maret R 1975 Isolation and characteristics of *Streptococcus thermophilus* and *Lactobacillus helveticus* phages from Emmental starters. *Lait* **55** 269–288

Zago M, Comaschi L, Neviani E & Carminati D 2005 Investigation on the presence of bacteriophages in natural whey starters used for the production of Italian long-ripened cheeses. *Milchwissenschaft* **60** 171–174

Zago M, De Lorentiis A, Carminati D, Comaschi L & Giraffa G 2006 Detection and identification of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophages by PCR. *Journal of Dairy Research* **73** 146–153

Zago M, Rossetti L, Bonvini B, Remagni MC, Perrone A, Fornasari ME, Carminati D & Giraffa G 2008a Il sieroinnesto di Grana Padano: una comunità di batteri e batteriofagi. *Scienza e Tecnica Lattiero-Casearia* **59** 277–286

Zago M, Rossetti L, Reinheimer J, Carminati D & Giraffa G 2008b Detection and identification of *Lactobacillus helveticus* bacteriophages by PCR. *Journal of Dairy Research* **75** 196–201

Zago M, Scaltriti E, Fornasari ME, Rivetti C, Grolli S, Giraffa G, Ramoni R & Carminati D 2012 Epifluorescence and atomic force microscopy: two innovative applications for studying phage-host interactions in *Lactobacillus helveticus*. *Journal of Microbiological Methods* **88** 41–46

Zago M, Scaltriti E, Rossetti L, Guffanti A, Armiento A, Fornasari ME, Grolli S, Carminati D, Brini E, Pavan P, Felsani A, D'Urzo A, Moles A, Claude JB, Grandori R, Ramoni R & Giraffa G 2013 Characterization of the genome of the dairy *Lactobacillus helveticus* bacteriophage Φ AQ113. *Applied and Environmental Microbiology* **79** 4712–4718