Expanding the diversity of *Myoviridae* phages infecting *Lactobacillus plantarum* - a novel lineage of *Lactobacillus* phages comprising five new members

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Abstract: *Lactobacillus plantarum* is a bacterium with promising applications to the food industry and agriculture and probiotic properties. So far, bacteriophages of this bacterium have been moderately addressed. We examined the morphology and diversity of five new *L. plantarum* phages via transmission electron microscopy, whole genome shotgun sequencing and *in silico* protein predictions. Moreover, we looked into their phylogeny and their potential genomic similarities to other complete phage genome records through extensive nucleotide and protein comparisons. The five phages share a high degree of similarity among them and belong to the family *Myoviridae*. They have a long genome of 137.973-141.344 bp, a G/C content of 36,3-36,6% that is quite distinct from their host's, and, surprisingly, seven to 15 tRNAs. Only an average 35/174 of their predicted genes were assigned a function. Overall, the comparative analyses unraveled considerable genetic diversity for our five *L. plantarum* phages. Hence, the new genus "Semelevirus" was proposed, which comprises exclusively the five phages. This novel lineage of *Lactobacillus* phages provides further insight into the genetic heterogeneity of phages infecting *Lactobacillus* sp. Our results on *Lactobacillus* phages have a potential value for the development of anti-phage strategies as well as for the manipulation of *L. plantarum* fermentations.

Keywords: *Lactobacillus plantarum*; phage; new genus; annotation; comparative genomics; phylogenetics; isolation; diversity

1. Introduction

Lactobacillus plantarum is a gram-positive, non-sporeforming, lactic acid bacterium with probiotic qualities. It is commonly encountered in a variety of environments ranging from dairy products, meat, grape must and vegetable fermentations to the human mouth, gastrointestinal-tract and stool, as well as sewage and cow dung [1]. Lately, the species has received attention as a promising biocontrol agent in agriculture due to its favourable, stimulating effects on crop growth and yield and its activity against phytopathogenic microbes [2]. From a winemaking perspective, *L. plantarum* has been proposed and utilised as an alternative malolactic fermentation starter [3], [4], [5]. This is thanks to its wide variety of enzymes linked with aroma compounds, and its production of bacteriocins against spoilage bacteria. [3]. Lastly, like other lactic acid bacteria (LAB), *L. plantarum* can both be a traditional preservative of fermented food products [1] and a food spoiler [6].

While fermentation starter strains of *L. plantarum* and other LAB are of great industrial value, their use is not challenge-free. Bacteriophage (phage) attacks often hamper their activity and by extend cause economic losses because of fermentation failure or poor product quality [7]. Owing to the acute threat they pose to industrially-relevant starters, *Lactococcus* and *Streptococcus* phages have

been long and extensively studied [8], [9], [10]. On the other hand, the knowledge gaps on the biology of *Lactobacillus* phages and their genetic heterogeneity have negatively impacted taxonomic efforts [11], [12]. More than 200 published *Lactobacillus* phages have never been sequenced [12] and of the 56 complete genome sequences just eight are formally classified (NCBI search). Nevertheless, it is obvious that research on *Lactobacillus* phages is being revitalised. In their review in 2017, Murphy et al. [8] have documented 27 complete genomes of *Lactobacillus* phages; a year after, this number has been doubled.

As subset of *Lactobacillu*s phages, *L. plantarum* phages have also been affected by paucity of information and a moderate earlier interest on their host. The first reference on lytic phages infecting *L. plantarum* dates back to 1969 [13]. Since then, several lytic and lysogenic phages of *L. plantarum* have been discovered. A few of these phages were reported to have a high level of strain specificity [14]. Transmission electron microscopy (TEM) images of *L. plantarum* phages have principally placed them in the family *Siphoviridae*. The phages fri [15], phiPY1 and phiPY2 [16], phi22-D10 [17] and LP65 [18] are all lytic and currently the sole representatives of the family *Myoviridae*, and a single account of a *Podoviridae* phage [19] is probably inaccurate according to Villion and Moineau [12]. Confirmed sources of phages against *L. plantarum* are sewage, feces, silage, kimchi, sauerkraut, fermented coffee, chicha, whey, pear fruit, cucumber, plant materials, salami and meat [12], [20], [18]. Noteworthy is the latest publication on the lysogenic phage PM411, as this phage constitutes the first to be associated with an agriculturally-relevant strain of *L. plantarum* [20]. In this study, we describe new *L. plantarum* phages isolated from organic waste, and we provide further insight into the diversity of phages of *Lactobacillus*. On a global scale, our aim is to broaden current knowledge on the viral tree of life through a novel group of phages that infect *Lactobacillus*, a genus of probiotic bacteria with emerging roles in agriculture, as well as in the dairy and wine industry.

2. Materials and Methods

2.1. Bacterial Strains and Culture Media

Two bacterial strains of *L. plantarum*, strain L1 and MW-1, were used as indicator strains for phage isolation, propagation and characterisation. Both L1 and MW-1 were obtained from private collections and had initially been isolated from a wine fermentation sample and grapes, respectively. Bacterial cultures and phage propagations were performed in De Man, Rogosa and Sharpe (MRS) broth and agar (Difco Laboratories, Detroit, MI). All cultures and propagations were grown overnight at 37 °C without shaking to ensure minimum aeration, unless otherwise stated.

2.2. Environmental Sampling, Isolation, Purification and Enrichment of Phages

Organic waste samples were collected from two different organic waste treatment plants in Denmark in February and May 2017 and split into two subsamples. The first part of the samples was centrifuged (5000 × g, 15 min, 25 °C) and supernatants were passed through 0.45-µm pore size PVDF syringe filters (Merck Millipore, Darmstadt, Germany). The filtrates were stored in 4 °C until use. The other part was centrifuged (5000 × g, 15 min, 25 °C) and phages were precipitated with polyethylene glycol (PEG). Specifically, PEG 8000 (10% w/v) was dissolved in 250 mL of supernatant, stored for 1h at 4 °C and then centrifuged (10000 × g, 10 min, 4 °C). Formed pellets were dried for 15 min, carefully resuspended in 5 mL of SM buffer (0,05 M TRIS, 0,1 M NaCl, 0,008 M MgSO$_4$, pH 7,5) and stored overnight at 4 °C. Following that, they were treated as explained for the first part of samples, which resulted in concentrated filtrates. The presence of bacteriophages in both plain and concentrated filtrates was assessed using a double agar overlay assay [21]. Briefly, 0,1 mL of one indicator strain (10^6-10^7 colony-forming units (CFU)/mL) and 0,1 mL of one filtrate at a time were added in MRSΦ, i.e. MRS broth supplemented with 0,4% w/v agarose and 10 mM CaCl$_2$. Mixtures were poured on top of MRS agar and incubated overnight at 25 °C. To select for phages specific to *L. plantarum*, 16 mL of the plain filtrates were blended with 20 mL of 2x MRSΦ and supplemented with 4 mL of an overnight

culture of either strain L1 or strain MW-1. The mixtures were incubated overnight and their filtrates tested for phages using the aforementioned overlay assay.

For the purification of phages, all plates deriving from the overlay assay were examined and single plaques were picked based on their distinct morphology. Each plaque was then transferred into 0,7 mL of SM buffer, filtered through 0,45-µm pore size PVDF spin filters (Ciro, Florida, USA) and propagated on the same strain. Occasionally, 100 mM glycine were also added to promote plaque formation and visibility [22]. This procedure was repeated twice to ensure purity and phage stocks were stored at 4 °C. Generally, high titers were produced by infecting 0,1 mL of the indicator strains (10^6-10^7 CFU/mL) with 0,5 mL of each phage isolate (10^5-10^6 plaque-forming units (PFUs)/mL) in 10 mL of MRSΦ. In some cases though, phage lysates of high titer were produced as elaborated elsewhere [23] and kept at 4 °C until needed. The filtered phage lysates were further purified by a caesium chloride gradient according to the protocol of Sambrook [24].

2.3. Phage DNA Extraction, Library Preparation and Sequencing

For the DNA extraction, 0,3 mL of filtered phage lysates ranging from 10^8-10^{10} PFUs/mL were used and the extraction was performed following a standard phenol/chloroform protocol [25]. DNA pellets were resuspended in 2 µl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5). Libraries were built with the Nextera® XT DNA kit (Illumina Inc., San Diego, California, USA) and later sequenced on an Illumina MiSeq as a part of the flowcell using the MiSeq v2, 2x250 cycle chemistry. The library normalisation, pooling and sequencing were carried out as described elsewhere [26].

2.4. Assemblies and Annotations

Illumina reads from the DNA sequencing were cleaned using VecScreen (NCBI) and Cutadapt (v. 1.8.3) [27] and assembled with SPAdes (v. 3.5.0) [28] as detailed in [29]. SPAdes assemblies were cross-verified by Unicycler (v. 0.4.3) [30]. Furthermore, assemblies were compared against those generated by CLC Genomic Workbench (v. 9.5.3; CLC bio, Aarhus, Denmark), by first applying the "merge overlapping pairs" tool for overlapping reads and subsequently the "trim sequences" and the "de novo assembly" tools. Phage genomes were auto-annotated by the RAST annotation server v. 2.0 [31] using RASTtk annotation scheme and GeneMark [32] as a gene caller. Protein functions were ascribed only after manually corroborating RASTtk predictions with BLASTp [33] and HHpred [34], and occasionally with Pfam [35], TMHMM [36] and DELTA-BLAST [37]. The tool tRNAscan-SE (v. 2.0) [38] was used to search for existing tRNA genes. All phage genomes were scanned against ISFinder to identify insertion sequences using BLASTn and the default pipeline of ISFinder [39].

2.5. Trasmission Electron Microscopy

The caesium chloride-treated phage lysate was adsorbed to freshly prepared ultra-thin carbon film and treated with 1% (v/v) EM-grade glutaraldehyde (20 min) for fixation. Subsequently, negative staining was done with 1% (w/v) uranyl acetate. The specimen was picked up with 400-mesh copper grids (Plano, Wetzlar, D) and analysed using a Tecnai 10 transmission electron microscope (Thermo Fisher, Eindhoven, the Netherlands) at an acceleration voltage of 80 kV. Micrographs were taken with a MegaView G2 CCD-camera (EMSIS, Muenster, Germany).

2.6. Comparative Genomics and Phylogenetics to Distant Relatives

In accordance with ICTV [40], each phage sequence was initially compared to the viral nucleotide collection database (taxid:10239; nt/nr) using the BLASTn algorithm. Phage records that showed some degree of similarity to the new phage isolates of this study were selected and included in an all-against-all, quantitative DNA comparison by Gegenees (v. 2.1) [41]. The Gegenees analysis was done with a customised setting of 50/25, fragment size/sliding step size, and an "accurate" threshold of 0%. In addition, two phylogenetic trees, one for the major capsid protein and one for the large subunit terminase, were constructed using the default pipeline of "One Click mode" (http://phylogeny. Lirmm.fr/) [42]. The tree leaves comprised the phages of this study and selected

phage records from the Gegenees comparisons, which had earlier yielded average nucleotide similarity of at least 0.05 or higher by Gegenees. Homology between and within proteomes was assessed via a BLASTp comparison of the new phages and their closest phage relative using CMG-biotools system [43]. In this type of analysis, a pair of amino acid sequences is considered homologous (paralogous or orthologous) if the length of the alignment is at least 50% of the longest sequence and the identity of the alignment is at least 50%. Paralogue hits were determined using MAFFT [44] (v. 7.388) with the following arguments: algorithm FFT-NS-i x1000; scoring matrix 200PAM/k=2; gap open penalty 1.53; offset value 0.123, and then BLASTp. Finally, the genomes of the new phages and the two most related phage genomes were examined for common synteny with Easyfig by tandemly comparing phage pairs with BLASTn [45].

2.7. Phage Genomic Data Availability

Assembled and annotated genomes of Semele, Bacchae, Iacchus, Dionysus and Bromius have been uploaded to GenBank under accession numbers MG765279, MG765277, MH809529, MH809530 and MH809531, respectively.

3. Results and Discussion

3.1. Isolation of Phages and Basic Features

Bacteriophages Semele, Bacchae, Iacchus, Dionysus and Bromius were all isolated from the collected organic waste samples. All five isolates formed clear plaques with a diameter of approximately 0,5 mm on their respective host strains L1 and MW-1 (Figure 1).

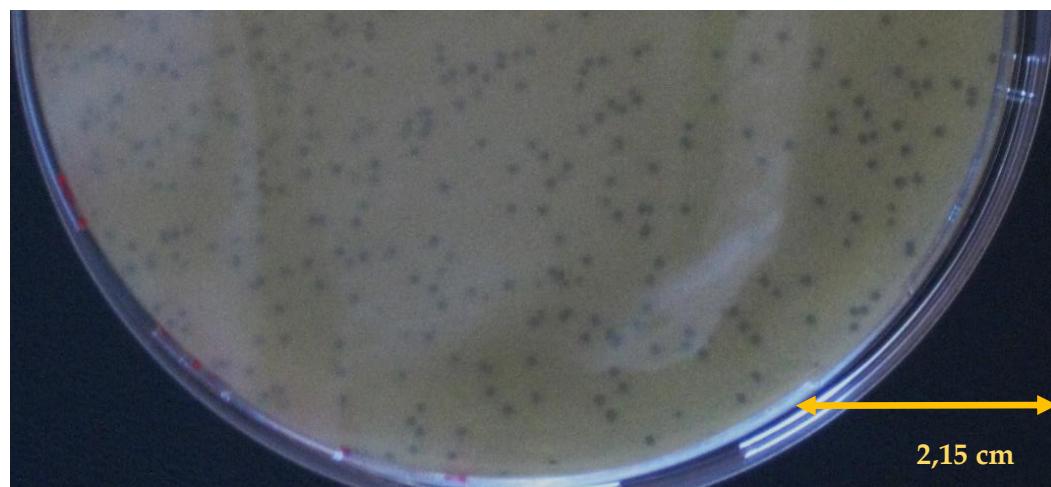



Figure 1: Plaques produced by phage Dionysus on *L. plantarum* MW-1.

Transmission electron microscopy imaging analysis was done with a representative phage, i.e. phage Dionysus (Figure 2, Table 1). The phage revealed a remarkably high stability, as only a low number of phage particles with contracted tail sheaths were detected (Figure 2e). Phage Dionysus has a large isometric capsid (diameter: 93 nm) with characteristic ("blackberry-like") surface decorations. At the distal end of the (contractible) tail (length 222 nm), a complex baseplate structure is visible, and unique flexible appendages (length ca. 22 nm) with terminal globular structures (ca. 9 nm in diameter) are attached under the baseplate. A flexible thin tail fiber (length: 30 nm) terminating with approx. 3 distinct knob-like structures is protruding under the baseplate. These morphological characteristics classify phage Dionysus into the order *Caudovirales* and the family *Myoviridae*. This is in agreement with existing records of phages infecting *L. plantarum* all of which fall into the order *Caudovirales*, while five of those belong to the family *Myoviridae* [12].

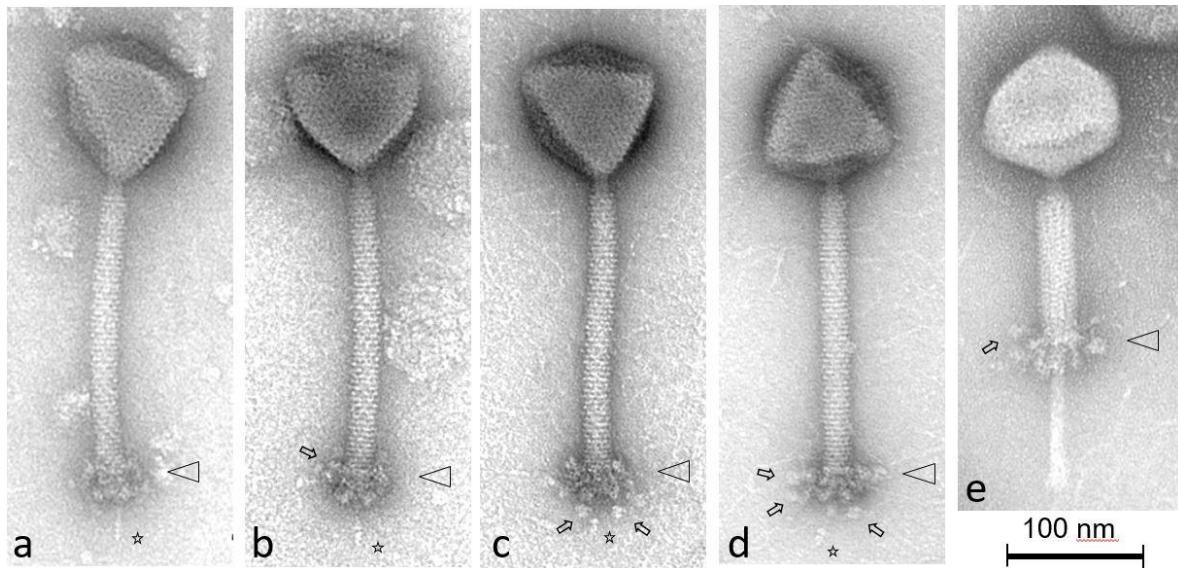



Figure 2: Transmission electron micrographs of *L. plantarum* phage Dionysus negatively stained with 2% (w/v) uranyl acetate. Triangles and arrows indicate the terminal baseplate structure and representative flexible appendages with terminal globular structures attached underneath them, respectively. The single distal tail fibers terminating with three distinct knob-like structures are indicated by the star symbols. Phage Dionysus is shown with extended tail sheath (a-d) and with contracted tail sheath (e), substantiating that it is a *Myoviridae* phage.

Table 1. Dimensions of phage Dionysus as measured with TEM.

	Dimensions (nm)
Head Diameter	92,7 ± 3,5
Tail Length (with Baseplate)	222,4 ± 9,0
Tail Width	20,9 ± 0,6
Baseplate Width	44,1 ± 3,9
Baseplate Length	25,0 ± 2,4
Baseplate Appendages Length	21,8 ± 1,8
Globular Structure Diameter	9,2 ± 1,3
Tail Fiber Length	30,4 ± 2,2

All five genomes of this study were assembled into a single major contig of size range 137.973-141.344 bp, G/C content range 36,3-36,6% and with average coverage range 77,1-600,6× (Table 2). The noted genome size is close to the longest observed genome for *L. plantarum* phages (145,162 bp, phage LpeD, accession number MF787246.1).

Table 2. Basic genomic characteristics of the five *Lactobacillus* phage isolates.

Phage Isolate	Ratio of Assigned Open Reading Frames	Genome Size (bp)	G/C Content (%)	tRNA Genes
Semele	32/177	139.450	36,3	11

Bacchae	34/180	141.124	36,3	15
Iacchus	37/170	137.973	36,5	7
Dionysus	37/172	141.344	36,6	7
Bromius	33/172	140.527	36,5	7

3.2. Analyses of DNA Sequences and Protein Predictions

Annotations of Semele, Bacchae, Iacchus, Dionysus and Bromius genomes assigned functions to 32 of the 177, 34 of the 180, 37 of the 170, 37 of the 172 and 33 of the 172 open reading frames (ORFs), respectively. Of the total number of ORFs, one third is harboured by the antisense strand. These genomes were also shown to encode clusters of 11, 15, seven, seven and seven tRNA genes, accordingly. Additional analyses that we are presenting in Section 3.3 suggest that these phage isolates are new species and could be members of a new *Lactobacillus* phage genus. Below, we discuss the different categories of proteins produced by these five phage genomes.

3.2.1. Transcription and Translation Takeover

A module of seven to 15 tRNA-encoding genes, often accompanied by one to three repeat regions, points to a potential mechanism of host transcription and translation takeover in all five *Lactobacillus* phage genomes. These tRNAs are predicted to carry seven to 11 different amino acids, with a common core of seven amino acids and the first two (Gly, Thr) and ultimate (Arg) maintaining their position across modules. Interestingly, similar observations were made for the genome of the distantly related phage LP65 of *L. plantarum*, wherein 14 tRNAs were identified. In particular, the equivalent LP65 module has the same core of tRNA genes and shares the same first two and last amino acids. Additionally, Semele, Bacchae, Iacchus and Bromius possess two additional transcription-related elements, an RNA ligase and an RNA polymerase sigma factor. The RNA ligase is homologous to the T4 RNA ligase by gene *mlA* (Pfam domain search). This enzyme has a double activity in T4. It participates in the repair of tRNAs to reverse the damage inflicted by host defence enzymes and it catalyses the attachment of tail fibers [46]. An RNA polymerase sigma factor has been described in phages before. In T4, the phage-encoded sigma factor serves the binding of the RNA polymerase to promoters different from the ones recognised by the host sigma factor [47]. In other words, the two sigma factors direct the transcription of the phage DNA independently and complementary to each other. A hypothetical connection between the T4 sigma factor and the phage's DNA packaging has been described, as well [48].

3.2.2. DNA Metabolism, Replication, Recombination and Repair

The five phage genomes seem to have a substantial DNA replication, recombination and repair system, which is fundamentally shared among them (Figures S1-S5). In confirmation to earlier observations, the genes of this system are arranged in modules [46] and are situated adjacent to DNA metabolism genes [46]. The replisome genes encode a DNA primase, a DNA polymerase, one or two DNA helicases, one or two DNA-binding proteins of unknown function and a recombinase A, not to mention putative recombination or repair-related proteins. Not least, a nucleotide precursor complex suggests that these phages may manipulate the nucleotide pool of their hosts and regulate DNA biosynthesis independently. Such a mechanism would be crucial given that their G/C content (Table 2) considerably differs from their host's (~44,5%) [46]. Indeed, the predicted phosphatase enzymes phosphatase/phosphodiesterase and deoxynucleoside kinase could allow for the dephosphorylation [16] and phosphorylation [17] of nucleotides, accordingly. Moreover, a gene associated with the nucleoside deoxyribosyltransferase of Lactobacillus phage ATCC 8014-B2 could catalyse the transfer between purines and/or pyrimidines, just as found for many lactobacilli, and thus contribute to nucleotide recycling [49]. The array of genes for a nicotinamide riboside transporter, a nicotinamide-nucleotide adenylyltransferase and ADP-ribose pyrophosphatase may be involved in a potential pyridine nucleotide (NAD+) salvage pathway. *In vitro* tests to support an analogous observation have been recently performed for a *Vibrio* bacteriophage [50]. A gene producing a nicotinamide

mononucleotide transporter, present in all our phages except for Semele, may be another unit of this pathway. At least 35 phages of different bacterial species have this transporter, including Lactobacillus phages LpeD and LP65, but its exact functionality has not been verified [51]. The only information available suggests that this transporter can pass nicotinamide mononucleotide molecules across membranes and that it is similar to its bacterial counterpart produced by *L. plantarum* [51]. The latter hints at a recent gene transfer between host and phages [51]. The fact that our phages and their host share a gene, and the striking difference in G/C content among our phages and *L. plantarum* may mean that they have a host range beyond *L. plantarum*. Hosts other than their primary host have been demonstrated for *L. plantarum* phages before [18,52,53].

3.2.3. Self-splicing/Selfish Genetic Elements

Self-splicing/selfish genetic elements are well-represented and interspersed throughout the genomes of the five phages (Figures S1-S5). One example is an unknown mobile genetic element protein, conserved in four of our phages and typically placed next to and upstream of the tRNA module. Another instance is that of transposases. Two transposases are observed in the genomes of Dionysus, Semele and Iacchus, and their neighbouring putative AAA ATPase probably controls their transposition to target DNAs [54]. None of these transposases returned any significant similarity to entries of inserted sequence elements in ISFinder. Since lysogeny is widespread in lactobacilli [55], transposable genetic elements from the chromosome could partially drive the evolution of lytic *Lactobacillus* phages from lysogenic ones, as reported for Lactobacillus phage φFSW [8]. However, our BLASTn search did not return any similarity of Dionysus, Semele or Iacchus to prophage records. In the genomes of Semele and Iacchus, a protein of unknown function that splits the DNA polymerase gene into two fragments may either be part of a self-splicing group I intron or a mini-intein. Group I introns are ribozymes capable of self-splicing from primary transcripts [56]. They interrupt tRNA, rRNA, and protein-coding genes and sometimes contain their own homing endonucleases (intron-homing) [57]. Mini-inteins are protein splicing elements that can ligate the polypeptide produced by the gene they disrupt post-translationally [58]. Their main difference from large inteins is the absence of a homing endonuclease domain, which enables large inteins to catalyse their self-excision [59]. HNH homing endonuclease genes are common in phage genomes [60]. In the genomes of Semele, Iacchus and Bacchae some of the HNH homing endonucleases reside next to hypothetical proteins, which may hint at the existence of spliced genes. Group I introns and inteins have already been discovered in phages with closest examples those of *Staphylococcus*, *Bacillus* and *Lactococcus* phages [29-32]. Regarding *Lactobacillus* phages, JCL1032 of *L. delbrueckii* is the only one proven to have group I introns so far [62]. On the other hand,many *Lactobacillus* phages have genes for HNH endonucleases with phage P2 predicted to have as many as eight in its genome (accession number KY381600.1) and phage LP65 of *L. plantarum* predicted to have two (accession number NC_006565.1).

3.2.4. Morphogenesis, DNA Packaging and Membrane Transport

Based on *in silico* analyses, the proteins that form the virion of the five *Lactobacillus* phages encompass a portal protein, a major capsid protein, a tail sheath and an unidentified tail protein, one or two baseplate proteins and one or two more structural proteins. The small and large subunit of a phage terminase are key enzymes of the DNA translocation and head filling [63]. A terminase DNA packaging subunit, often characterised as the large subunit terminase, has been used to define the start of the five genomes here.

3.2.5. Cell Wall and Membrane Degradation

A phage lytic cycle ends with the burst of the host cell and the release of progeny phages. The burst is accomplished due to the action of two groups of enzymes, holins and endolysins. Holins are responsible for the formation of transmembrane holes, which allows endolysins to reach and degrade the cell wall [64]. Out of the five different classes of endolysins, the *Lactobacillus* phages studied thus

far, utilise only two, muramidases and amidases [65]. Holins usually have two or three transmembrane domains, although a few cases of holins with just one transmembrane domain have also been found [66]. For *Lactobacillus* phages there are examples of two- or three-transmembrane domain holins [67], [68]. TMHMM search coupled with DELTA-BLAST attributed traits typical of a holin to a hypothetical protein in all five phages (accession numbers: AYH92097, AUV60238, AUV59966, AYH91927, AYH92270). This hypothetical protein is right upstream of the lysin gene and was predicted to have a pair of transmembrane domains and low similarity to a holin of *Pediococcus pentosaceus*. All five phage genomes of this study possess at least one putative endolysin. At the same time, absence of lysogeny-related genes (integrases, excisionases and repressors of phage reproduction and lysis genes) indicates that these phages are most likely purely virulent [69], However, since less than 22% of the genes in the five phages could be ascribed a function, it is possible that lysogeny-related genes remain undetected. New infections are initiated by the recognition and irreversible attachment of progeny phages to host bacterial receptors. The genomes of Bromius and Iacchus feature a glycerophosphodiester phosphodiesterase that has been described as a baseplate component of various bacteriophages against gram-positive bacteria, including *L. delbrueckii* phages [70]. Notably, this enzyme seems to facilitate the attachment of phage Ld17 of *L. delbrueckii* by degrading glycerophosphodiesters of the cell envelope [71]. Owing to glycerophosphodiester phosphodiesterases and other degrading proteins, receptor-binding proteins can access bacterial receptors masked by surface molecules and catalyse phage adsorption [72]. Peptidoglycan-degrading enzymes of the phage tail tip are indispensable for phage DNA ejection which occurs after adsorption [71]. We presume that a putative peptidoglycan hydrolase, which lies near a tail protein in the genomes of the five phages, is participating in this process [73].

3.2.6. Other predicted proteins

In the region close to the DNA metabolism, replication, recombination and repair genes, three coding sequences have been assigned a function but their exact purpose in the phage genome is unclear. One of them encodes a protein homologous with a DNA-binding ferritin-like protein from *Lactococcus lactis*, which provides protection against oxidative damage in bacteria and may help *L. plantarum* tolerate hydrogen peroxide [74]. The second of them encodes a protein that has homology with a low temperature requirement C protein of *Listeria monocytogenes*, which may be involved in lipid metabolism [75]. The protein of the third coding sequence is homologous with a YopX protein from *Bacillus subtilis*, an uncharacterised protein of bacteriophages and prophages of gram-positive bacteria [76]. This latter is solely produced by phages Dionysus and Iacchus.

3.3. Our Phage Diversity Supports the Introduction of a New Lactobacillus Phage Genus

According to BLASTn, the phages of this study show a low degree of similarity to Lactobacillus phage LpeD (70% identity over 51-55% query cover) and an even lower to Lactobacillus phage LP65 (83-88% identity over 20-23% query cover). Thereon, 24 phage records that returned some degree of similarity, as well as our five phages, were subjected to an all-against-all BLASTn comparison by Gegenees. In the resulting heatplot of phylogenomic data (Figure 3), the phages presented in this study fall into one group separate from the rest 24 phages. The calculated score distances within this group, which are equal to the average normalised similarities of fragments by BLAStn, are 44,92% or higher at a nucleotide level.

Figure 3: BLASTn heatplot generated using Gegenees. All-against-all comparisons were run with fragment length 50 bp, step size 25 bp, threshold 0%. Our phages (25-29) form a separate group.

2   The Gegenees distinction agrees with the outcome of the major capsid protein and large subunit
3   terminase trees (Figures 4 (**A**) and 4 (**B**), accordingly). In both phylogenetic trees phages Semele,
4   Bacchae, Iacchus, Dionysus and Bromius clearly cluster together and in the same clade with phage
5   LpeD. In order to clarify if LpeD pertains to the same genus as our phages, we have chosen to include
6   it in the remaining comparisons.

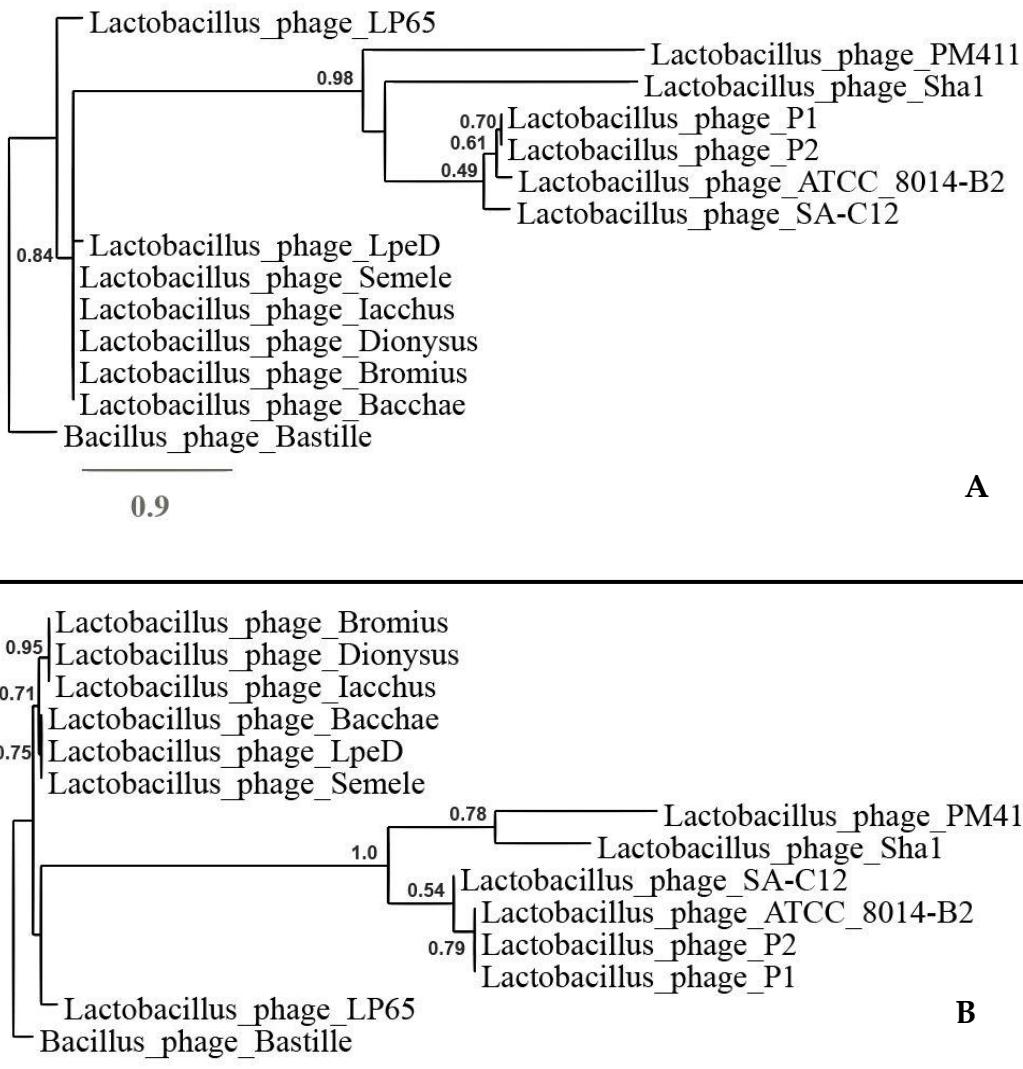
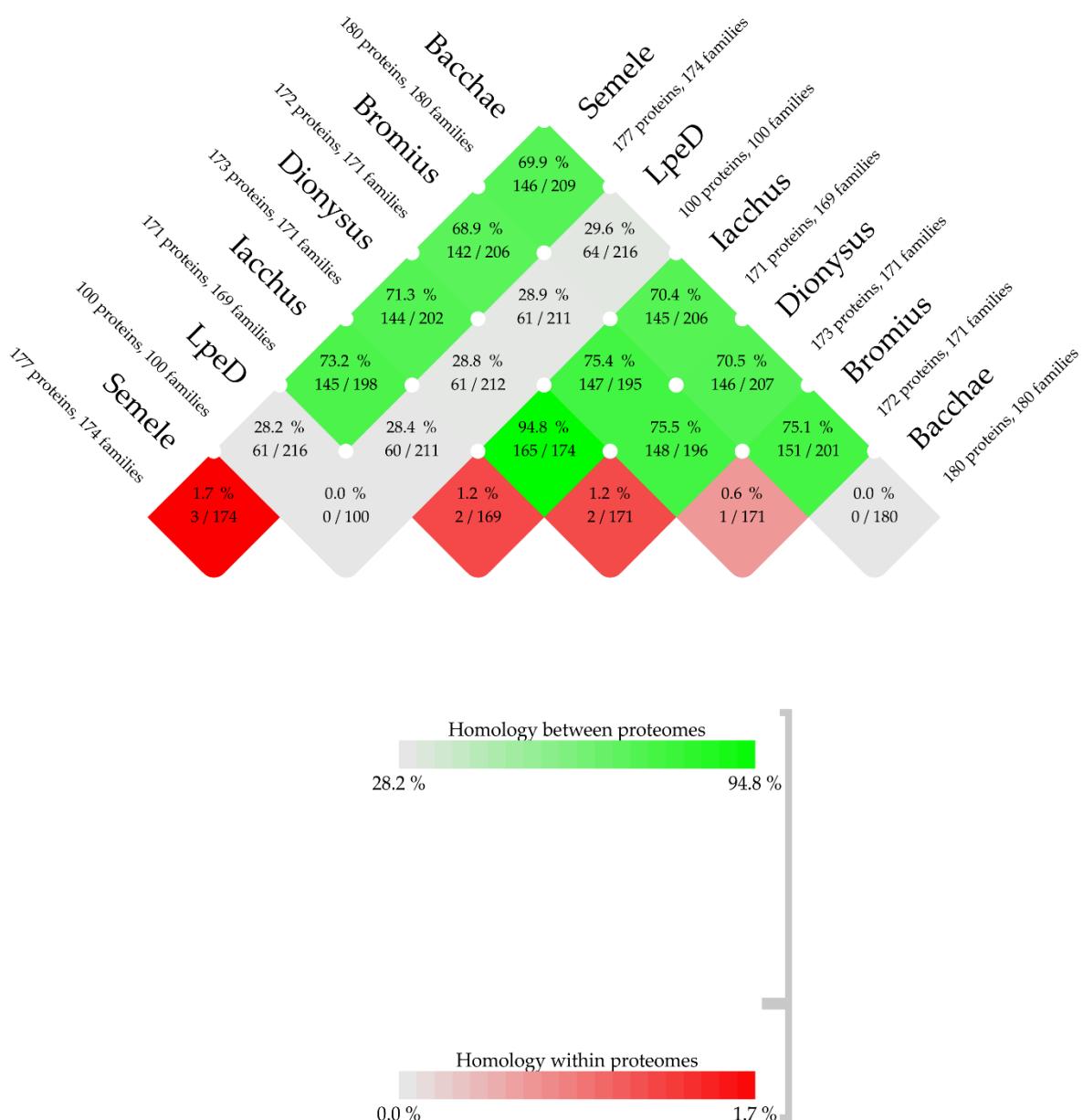



Figure 4: Phylogenetic trees for phages Semele, Bacchae, Dionysus, Iacchus and Bromius and other *Lactobacillus* phages yielding average similarity of at least 0,05 or higher by Gegenees. (**A**) Tree constructed using the major capsid proteins, (**B**) tree constructed using the large subunit terminases. The aminoacid sequences were compared with the "One Click mode" (http://phylogeny. Lirmm.fr/). *Bacillus* phage Bastille proteins were used as outliers.

7
8   Total protein checks done with CMG-biotools system revealed significant homology among the
9   proteomes of our five phages, while the homology between each of them and phage LpeD was
10   consistently less than 30% (Figure 5). Furthermore, homology within proteomes of Semele, Iacchus,
11   Dionysus and Bromius was traced to the family of transposases and one or two other hypothetical
12   protein families using MAFFT and BLASTp (Spreadsheets S1-S4). This is surprising, given that
13   duplicates (paralogues) in the same genome are rather rare for double-stranded DNA phages [77].
14   Transposases are motors of genome plasticity and adaptation [78]. Phages of *L. plantarum* are
15   parasites of a versatile bacterium that can grow and survive in a ream of environmental niches.
16   Hence, the abundance of transposases may assist phages Semele, Iacchus and Dionysus, which have

17 two paralogous transposases, to adapt to novel niches and hosts by facilitating horizontal gene
18 transfer with their hosts upon infection [79], [80]. Moreover, the over-representation of transposases
19 in the genomes of our phages could reflect an abundance of transposases in the genome of host
20 bacteria [81]. This scenario is coherent with the nature of the organic waste samples, where a plethora
21 of nutrients could translate to higher microbial densities, and thus higher rates of DNA exchange, as
22 reported for bacteria from the Baltic Sea [62]. We have investigated whether our phage transposases
23 have a bacterial version by revising our BLASTp results. It turned out that all examined transposases
24 showed significant homology to more than 20 different *Lactobacillus* sp., but unexpectedly not to their
25 immediate host, *L. plantarum*.
26

27
28 Figure 5: BLASTp comparison between and within proteomes of our phages and their closest phage relative
29 LpeD using CMG-biotools system.

30 Finally, yet importantly, Easyfig comparisons revealed a striking conservation of gene order and
31 nucleotide homology amongst our phages. When these were compared to phage LpeD, we could still
32 and to a large extent notice a gene order conservation but the nucleotide homology was generally
33 low (top genome; Figure 6). Concerning LP65, the genome of this phage displayed relatively low
34 synteny and nucleotide homology against our five phage genomes (bottom genome; Figure 6). Given

35   the distinctive characteristics of our five phage isolates, we propose a new *Lactobacillus* phage genus,

36   that we term "Semelevirus". The genus "Semelevirus" has as members the phages Semele (founder),

37   Bacchae, Iacchus, Dionysus and Bromius.

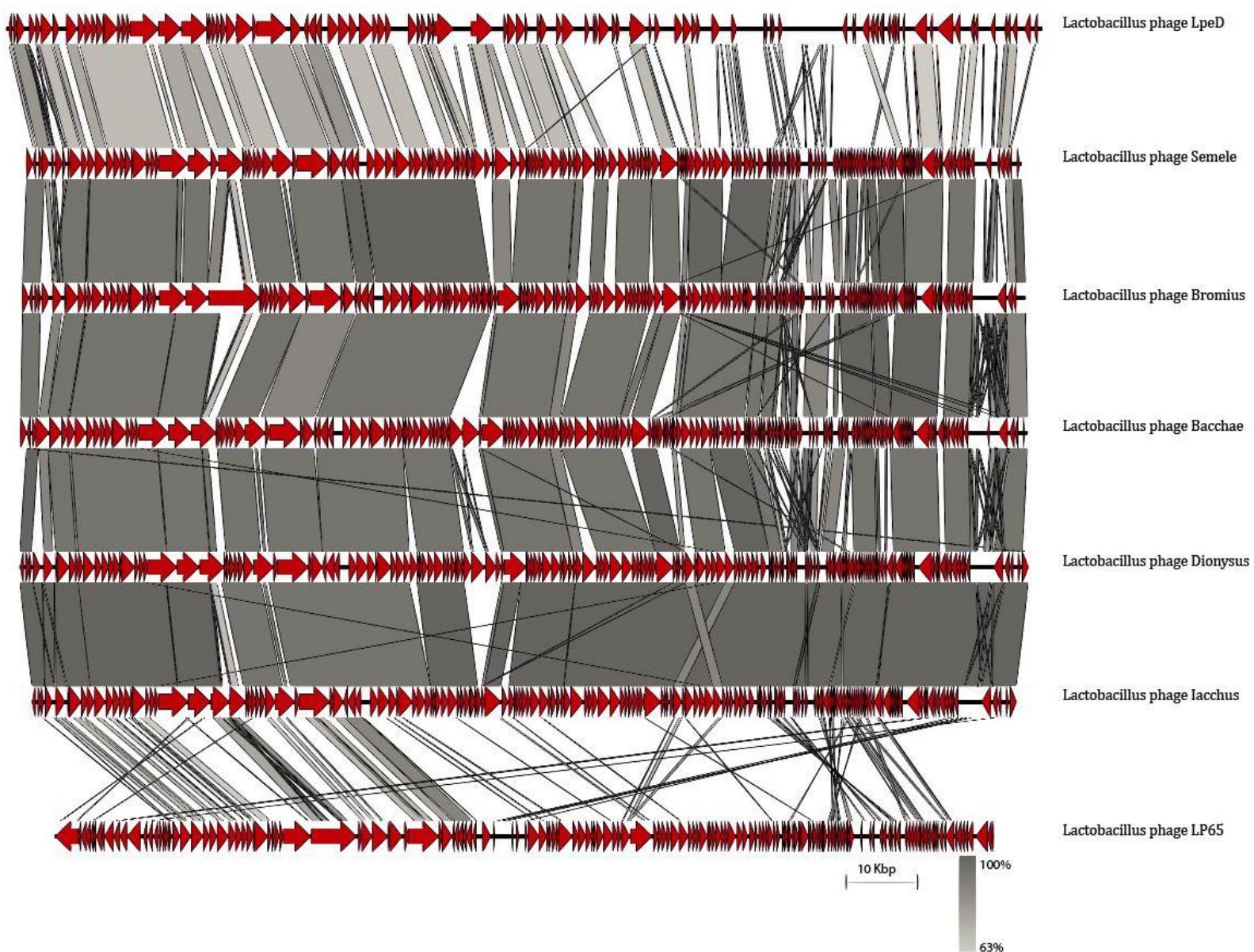



Figure 6: BLASTn comparisons of the genomes of our phages with distant relatives LpeD and LP65 using Easyfig. Arrows represent the locations of genes and lines the level of homology between each tandemly placed group of phages.

4. Outlook

Lactobacillus phages have been modestly addressed in comparison to other LAB phages. However, the doubling of publicly-available, complete-genome sequences from 2017-2018 could imply a boom in this field of research. The many displayed advantages of their hosts, and especially of *L. plantarum*, may justify the fueled interest in these phages. There are quite some arguments to support this trend in the coming years. *Lactobacillus* phages, just as other notorious LAB phages, can preclude from obtaining high quality fermentation products on a regular basis. Hence, a systematic analysis of existing and newly-discovered *Lactobacillus* phage genomes would supply researchers with a comprehensive dataset. This would guarantee a more thorough understanding of their origin, evolution, and relationships with other phages [7]. Eventually, better knowledge on the phages of *Lactobacillus* could help to choose starter strains, and adjunct or aroma cultures with different phage sensitivity for strain rotation, and develop more efficient phage cocktails for inhibition of unwanted bacterial growth. At the same time, we believe that improved classification schemes are crucial to design efficient control strategies against phages or aid phage therapy applications. Should we expect an increasing number of *Lactobacillus* phages in the future, this need is even more urgent.

Looking at *L. plantarum* phages, we can discern some cases where steady accumulation of knowledge is essential. As mentioned, *L. plantarum* is an emerging biocontrol agent, as well as biostimulation agent of crops. Even if phages can impede successful application of this bacterium in the field they can still be sufficiently anticipated and tackled when we know enough about their biological profile. The same is true for *L. plantarum* starters used in the food industry. On the other hand, *L. plantarum* phages could be valuable biocontrol tools for the food industry [82]. First, they could be used to prevent growth of their host in those biotechnological processes in which *L. plantarum* is undesirable, such as in meat, beer, wine or orange juice [6]. For example, phage cocktail preparations could minimise the addition of sulfur dioxide in those types of alcoholic beverages that should not undergo a malolactic fermentation. Studies on phages of beer spoiler strains of *L. brevis*, *L. paraplantarum* and *P. damnosus* have already introduced the concept of adding phages to inhibit these bacteria [83], [84], [85]. Secondly, phage therapy could offer the means to manipulate the malolactic fermentation in wine, cider and sour beer. Winemakers would have the chance to experiment by adding phages in aliquots of the same initial product but at different stages of the malolactic fermentation and later evaluating the effect in each one of the final wine products. Such an intervention may give rise to wines with novel characteristics, since through bacterial lysis phages can induce the release of enzymes for flavor development [86], [87].

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figures S1-S5, [88]: title "Genome maps of Phages Semele, Iacchus, Dionysus, Bromius and Bacchae and Visualisation of Encoded Proteins."; Spreadsheets S1-S4: title "MAFFT Results for Paralogous Proteins of Phages Semele, Dionysus, Iacchus and Bromius."

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