**Proteomic Characterization of Bacteriophage Peptides from the Mastitis Producer *Staphylococcus aureus* by LC-ESI-MS/MS and the Bacteriophage Phylogenomic Analysis**

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**Abstract:** The present work describes LC-ESI-MS/MS MS (liquid chromatography-electrospray ionization-tandem mass spectrometry) analyses of tryptic digestion peptides from phages that infect mastitis-causing *Staphylococcus aureus* isolated from dairy products. A total of 1933 nonredundant peptides belonging to 1282 proteins were identified and analyzed. Among them, 79 staphylococcal peptides from phages were confirmed. These peptides belong to proteins such as phage repressors, structural phage proteins, uncharacterized phage proteins and complement inhibitors. Moreover, eighteen of the phage origin peptides found were specific to *S. aureus* strains. These diagnostic peptides could be useful for the identification and characterization of *S. aureus* strains that cause mastitis. Furthermore, a study of bacteriophage phylogeny and the relationship among the identified phage peptides and the bacteria they infect was also performed. The results show the specific peptides that are present in closely related phages and the existing links between bacteriophage phylogeny and the respective *Staphylococcus* spp. infected.

**Keywords:** pathogen detection; LC-ESI-MS/MS; proteomics; mass spectrometry; phage peptide biomarker

1. **Introduction**

The vast majority of mastitis cases are due to an intramammary infection caused by a microorganism belonging to either the *Staphylococcus* or *Streptococcus* genus [1,2]. *Staphylococcus aureus* is considered one of the major foodborne pathogens that can cause serious food intoxication in humans due to the production of endotoxins; this pathogen remains a major issue in the dairy industry due to its persistence in cows, its pathogenicity, its contagiousness and its ease of colonization of the skin and mucosal epithelia [3–5].

It is well-known that *S. aureus* bacteriophages encode genes for staphylococcal virulence factors, such as Panton-Valentine leucocidin, staphylokinase, enterotoxins, chemotaxis-inhibitory proteins or exfoliative toxins [6]. These phages are usually integrated into bacterial chromosomes as prophages, wherein they encode new properties in the host, or vice versa, as transcriptions may hardly be affected by gene disruptions [7]. Phage-encoded recombinases, rather than the host recombinase, RecA, are involved in bacterial genome excisions and integrations [8,9]. These integrations may occur at specific bacterial genome sites that are identical to those present in the DNA of the phage, or, as in the case of phage...
Mu (as long as the given gene is not expressed), some phages can integrate randomly within the bacterial genome. In addition, bacteriophage and staphylococcal species interactions may substantially alter the variability of the bacterial population [10,11].

All known S. aureus phages are composed of an icosahedral capsid filled with double-stranded DNA and a thin, filamentous tail, and they belong to the order Caudovirales (tailed phages) [12,13]. Some Podoviridae family phages, such as the Staphylococcus viruses S13′ and S24-1, have been reported, characterized and used in phage therapy against S. aureus infections [14]. There are some well-known Siphoviridae phages of S. aureus, such as the prophage ϕSaBov, which is integrated into a bovine mastitis-causing S. aureus strain [15].

The interaction between bacteria and bacteriophages leads to an exchange of genetic information, which enables bacteria to rapidly adapt to challenging environmental conditions and to be highly dynamic [11,16]. As closely related phages normally occupy the same genome location in different bacteria, a specific site in different bacterial strains can be occupied by completely different phages or can be empty.

Conventional culture-based methods have been used for the detection of pathogenic bacteria [17,18] and their phages [19,20]; however, at this point, these procedures are time-consuming and laborious. For this reason, new, rapid molecular microbial diagnostic methods based on genomics and proteomics tools have been developed to achieve faster and more efficient bacterial and bacteriophage identification [1,21–24]. Specifically, phage typing is a classic technique for such purposes [25]. Moreover, biosensors based on phage nucleic acids, receptor-binding proteins (RBPs), antibodies and phage display peptides (PDPs) have been used for pathogen detection [26–30].

Mass spectrometry techniques, such as MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and LC-ESI-MS/MS (liquid chromatography-electrospray ionization-tandem mass spectrometry), have been used for the analysis and detection of specific diagnostic peptides in pathogenic bacterial strains [31,32]. In addition, LC-ESI-MS/MS methods have been employed for the identification and detection of bacteriophages [19]. In the case of bacteriophage detection and identification by a mass spectrometry analysis, the required production of viruses may be time-consuming. The detection of prophages based on protein biomarkers can be an alternative to genomic detection, and in this sense, proteomic techniques can be cheaper and faster and can ascertain different bacteriophage species by using a single analysis [33]. Based on the specificity of many bacteriophages with their hosts, bacteriophages are considered signal amplifiers; therefore, the detection of peptides from phages is suitable for pathogen identification. For example, Serafim et al. 2017 [33] identified bacteriophage lambda by a LC-ESI-MS/MS analysis. Moreover, the identification of peptides by means of LC-ESI-MS/MS from bacteriophage-infected Streptococcus has been performed, which revealed new information on phage phylogenomics and their interactions with the bacteria they infect [19]. However, no study has been published on S. aureus phage detection and identification by LC-ESI-MS/MS or on S. aureus phage characterization without a previous phage purification step. Viral genomic detection and phage display are time-consuming methods. Here, we describe an easy, fast and accurate method for the detection of bacteriophages without the need for the pretreatment of bacterial lysis for bacteriophage replication. This method led to the identification of putative temperate and virulent phages present in the analyzed strains.

A previously published work performed by our laboratory [3] studied the global proteome of several strains of S. aureus by shotgun proteomics. Important virulence protein factors and functional pathways were characterized by a protein network analysis. In this work, and for the first time, we aimed to use proteomics to characterize phage contents in different S. aureus strains to identify the relevant phage-specific peptides of several S. aureus strains and to identify both phages and bacterial strains by LC-ESI-MS/MS.
2. Materials and Methods

2.1. Bacteria

In this study, a total of 20 different *S. aureus* strains obtained from different sources were analyzed (Table S1 in Supplemental Data 2). These strains were previously characterized by MALDI-TOF mass spectrometry [1] after being obtained from the Institute of Science of Food Production of the National Research Council of Italy (Italy) and from the Spanish Type Culture Collection (Spain). The majority of the strains are from food origins, except for strain U17, which is a human clinical strain. Strains ATCC (American Type Culture Collection) 9144 and ATCC 29213 are classified as *S. aureus* subsp. *aureus*, while strain ATCC 35845 is categorized as *S. aureus* subsp. *an aerobius*. In previous works, the species identification of *S. aureus* and the presence of enterotoxins were evaluated by multiplex polymerase chain reactions (multiplex PCRs) [3,34,35]. The strains were reactivated in a brain–heart infusion medium (BHI, Oxoid Ltd., Hampshire, UK) and incubated at 31 °C for 24 h. Bacterial cultures were then grown on plate count agar (PCA, Oxoid) at 31 °C for 24 h [1,3,36]. Tubes of broth were inoculated under aerobic conditions.

2.2. Protein Extraction and Peptide Sample Preparation

Protein extraction was prepared as described previously [37]. All analyses were performed in triplicate. Protein extracts were subjected to in-solution tryptic digestion [38].

2.3. Shotgun LC-MS/MS Analysis

Peptide digests were acidified with formic acid (FA), cleaned on a C18 MicroSpin™ column (The Nest Group, South-borough, MA, USA) and analyzed by LC-ESI-MS/MS using a Proxeon EASY-nLC II Nanoflow system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) [3]. Peptide separation (2 µg) was performed on a reverse-phase (RP) column (EASY-Spray column, 50 cm × 75 µm ID, PepMap C18, 2-µm particles, 100-Å pore size, Thermo Fisher Scientific, San Jose, CA, USA) with a 10-mm precolumn (Accucore XL C18, Thermo Fisher Scientific, San Jose, CA, USA) using a linear 120-min gradient from 5% to 35% solvent B (solvent A: 98% water, 2% ACN (Acetonitrile) and 0.1% FA and solvent B: 98% ACN, 2% water and 0.1% FA) at a flow rate of 300 nL/min. For ionization, a spray voltage of 1.95 kV and a capillary temperature of 230 °C were used. Peptides were analyzed in the positive mode from 400 to 1600 amu (1 µscan), which was followed by 10 data-dependent collision-induced dissociation (CID) MS/MS scans (1 µscan) using an isolation width of 3 amu and a normalized collision energy of 35%. Fragmented masses were set in dynamic exclusion for 30 s after the second fragmentation event, and unassigned charged ions were excluded from the MS/MS analysis.

2.4. LC-MS/MS Mass Spectrometry Data Processing

LC-ESI-MS/MS spectra were searched using SEQUEST-HT (Proteome Discoverer 2.4, Thermo Fisher Scientific, San Jose, CA, USA) against the *S. aureus* UniProt/TrEMBL database (208,158 protein sequence entries in July 2020). The following parameters were used: semi-tryptic cleavage with up to two missed cleavage sites and tolerance windows set at 10 ppm for the precursor ions and 0.06 Da for the MS/MS fragment ions. These additional identified semi-tryptic peptides increased the sequence coverage and confidence in protein assignments. The variable modifications that were allowed were as follows: (M*) methionine oxidation (+15.99 Da), (C*) carbamidomethylation of Cys (+57.02 Da) and acetylation of the N-terminus of the protein (+42.0106 Da). To validate the peptide assignments, the results were subjected to a statistical analysis with the Percolator algorithm [39]. The false discovery rate (FDR) was kept below 1%. The mass spectrometric data were deposited into the public database PRIDE (Proteomics Identification Database), with the dataset identifier PXD023530.
2.5. Selection of Potential Peptide Biomarkers

For each peptide identified by LC-ESI-MS/MS, we used the BLASTp program to determine the homologies and exclusiveness with protein sequences registered in the NCBI (National Center for Biotechnology Information) database [40]. For the BLASTp search, the *Staphylococcus* taxon was included and excluded with the aim of finding the peptides that belonged to the *Staphylococcus* phages, *Staphylococcus* spp. and only to *S. aureus*.

2.6. Phage Genome Comparison and Relatedness

Genomes of all studied *Staphylococcus* spp. phages were downloaded from the GenBank database, analyzed and compared using the Web server VICTOR (Virus Classification and Tree Building Online Resource, http://ggdc.dsmz.de/victor.php, accessed on 27 November 2020) for the calculation of the intergenomic distances and the construction of the phylogenomic tree [41].

3. Results

3.1. *S. aureus* Proteome Repository

Protein mixtures from each of the 20 different *S. aureus* strains (Table S1 in Supplemental Data 2) were digested with trypsin and analyzed by LC-ESI-MS/MS.

A total of 1933 nonredundant peptides corresponding to 1282 nonredundant annotated proteins were identified for all *S. aureus* strains (see the Excel dataset in Supplemental Data 1). Among them, 79 phage peptides were identified. These peptides belong to proteins such as phage repressors, structural phage proteins, uncharacterized phage proteins and complement inhibitors. Figure 1 shows a comparative representation of the different types of phage proteins identified in this study. These phage peptides were selected and analyzed using the BLASTp algorithm. For the BLASTp search, *Staphylococcus* was included and excluded with the aim of finding peptides belonging to *Staphylococcus* bacteriophages.

![Figure 1](image-url)

**Figure 1.** Comparative representation of different types of phage proteins identified in this study for the different strains (represented by different colors). The number of each type of protein is shown in parentheses.
The obtained staphylococcal phage-specific peptides shared homology with the *Staphylococcus* phages and *Staphylococcus* spp. in the NCBI database. Among them, all shared homology with *S. aureus*; however, eighteen peptides were specific to *S. aureus* (IRLPYYDVK, LYVGVPFNPEATK, SIINGKLDWSQWTVPNEHK, M*NDSNQGLQANPQYTHYLSEQEIR, PCPALM*NKRNSIATHR, SQDNSNLPELSTKAPK, ESINANTYNQLK, VAVLSTPLVTSFESK, KDGELFRIAIDYLRNK, MPVYKDGNTGKYFSI, KTTSALKEVLSDT, EKPVP-DATGADDPLKPDDDRM*ITNFHANLVDKVSY, MSHNALTTGIGAGAG, VQHPGKLYNKVM*SGLNINFGGGGANATAK, QM*MGLSGVMDLAASVSGLASGTVDTGLTAGLKAKD, KSNVEAFSNAVK, GMVSMQMQVQVNVLTM*ELAQQNAMLTIQTELK and DIITVVC*PENGNTATDEY). Figure S1 shows the MS/MS spectra for these *S. aureus*-specific peptide biomarkers. Table 1 summarizes the list of 79 specific staphylococcal bacteriophage peptides, bacterial peptides with putative phage origins and bacteria and phages with 100% homology with respect to the NCBI protein database.

All staphylococcal phage peptides with 100% homology were found to belong to the *Siphoviridae* family: 52 staphylococcal phages belong to the *Phietavirus* genus, 37 belong to the *Biseptimavirus* genus, 30 are *Triavirus*, two are phieta-like viruses and one is a SPbeta-like virus, and the others are nonclassified *Siphoviridae* viruses (Table S2 in Supplemental Data 2). *Siphoviridae* genomes are usually organized into functional modules, such as lysogeny, DNA replication, packaging, morphogenesis and lysis modules [6,42].

Table 1. Phage origin peptides identified in *Staphylococcus aureus* strains. NCBI (National Center for Biotechnology Information).

| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|---------------------------------------------------------------|---------------------------------------------------------------|
| S4     | Uncharacterized phage protein | IRLPYYDVK | *Staphylococcus aureus* | *Staphylococcus* phage StauST398-2 |
| S4     | Uncharacterized phage protein | A VAELLKEINR | *Staphylococcus argenteus* *Staphylococcus simiae* *Staphylococcus aureus* | *Staphylococcus* virus 71 *Staphylococcus* virus 55 *Staphylococcus* virus 88 |
| S4     | Major capsid protein | LLHALPTGNDGSGKLLFK | *Staphylococcus aureus* *Staphylococcus xylosus* *Staphylococcus muscae* *Staphylococcus haemolyticus* *Staphylococcus argenteus* *Streptococcus pneumoniae* | *Staphylococcus* phage phiSa2wa_st72 *Staphylococcus* phage phiSa2wa_st121mssa *Staphylococcus* phage vB_Sau5_phi2 *Staphylococcus* phage StauST398-2 *Staphylococcus* phage LH1 *Staphylococcus* phage phiSa2wa_st30 *Staphylococcus* virus phi12 *Staphylococcus* virus 3a *Staphylococcus* virus phiSLT *Staphylococcus* phage ip310-2 *Staphylococcus* phage vB_Sau5_JSO2 *Staphylococcus* phage R4 *Staphylococcus* phage vB_Sau5_JPSau2 *Staphylococcus* phage SA137nuMSSA ST121PVL |
| S4     | Major capsid protein | RVSYLDDDDDFITDVETAKELK | *Staphylococcus aureus* 12S01399 *Staphylococcus aureus* *Staphylococcus aureus* A9299 *Staphylococcus aureus* A9765 *Staphylococcus aureus* A6300 *Staphylococcus* sp. *Terrabacteria* group *Escherichia coli* | *Staphylococcus* phage LH1 *Staphylococcus* phage StauST398-2 *Staphylococcus* phage vB_Sau5_phi2 *Staphylococcus* phage R4 |
| S7     | Major tail protein | LYVGVPFNPEATK | *Staphylococcus aureus* | *Staphylococcus* phage vB_Sau5_phi2 *Staphylococcus* virus phi12 *Staphylococcus* virus phiSLT *Staphylococcus* phage R4 *Staphylococcus* phage vB_Sau5_JSO2 *Staphylococcus* phage SH-S8 15644 *Staphylococcus* virus 3a *Staphylococcus* phage P240 |
| S8     | Uncharacterized phage protein | M*NDSNQGLQANPQYTHYLSEQEIR | *Staphylococcus aureus* | *Staphylococcus* phage phiN315 |
| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|-------------------------------------------------------------|---------------------------------------------------------------|
| S8     | Major tail protein | AVINITGLGFAK | *Staphylococcus aureus* *Staphylococcus argenteus Pararhedinema mesophilus* | *Staphylococcus phage phiNM3* *Staphylococcus phage P262* *Staphylococcus phage StaST398-4* *Staphylococcus phage phiNN315* *Staphylococcus phage phi7247PVL* *Staphylococcus phage phiSa2wa_st22* *Staphylococcus virus 77 Staphylococcus phage P954* |
| S9     | Major capsid protein | IYDRNSDTLDGLPVVINLK | *Staphylococcus aureus* *Staphylococcus argenteus* | *Staphylococcus virus 85* *Staphylococcus phage SF5* *Staphylococcus virus phiTA2* *Staphylococcus virus phiNM2* *Staphylococcus virus SAP26* *Staphylococcus phage SA12* *Staphylococcus virus Baq Saul* |
| S11 and S20 | Phage repressor, Cro/CI family | ELAEAIQVSQPTVSNWIIQTK | *Staphylococcus aureus* *Staphylococcus argenteus* *Staphylococcus sciuri* | *Staphylococcus virus IPLA35* *Staphylococcus phage SMSAP5* *Staphylococcus phage VB_Sau56_phl2* |
| S11 and S20 | Phage repressor, Cro/CI family | IQQLADYFNVPK | *Staphylococcus aureus* *Staphylococcus sciuri* *Staphylococcus pseudintermedius* | *Staphylococcus phage phiSA2wa_st72* *Staphylococcus phage phiNM2* *Staphylococcus virus IPLA35* |
| S12 S10 and S14 | Complement inhibitor | IYNEIDEALKSK | *Staphylococcus aureus, Enterobacter sp. IF2SW-B1 Klebsiella pneumoniae* | *Staphylococcus phage 13 Staphylococcus phage phiNM3 Staphylococcus phage StaST398-1* |
| S20 | Major capsid protein | VSYTLDDDDFDIDVTEAK | *Staphylococcus aureus* *Staphylococcus haemolyticus* *Staphylococcus saprophyticus* *Staphylococcus variornatus* *Staphylococcus pneumoniae* *Staphylococcus sciuri* | *Staphylococcus phage phiSa2wa_st72* *Staphylococcus phage phiSa2wa_st112mssa* *Staphylococcus phage VB_Sau56_phl2* *Staphylococcus virus 73 Staphylococcus phage phi2958PVL* *Staphylococcus virus PVL* *Staphylococcus phage SA137nuMSSAST121PVL* |
| S20 | Phage protein (DUF2479 domain) | SIINGKLDSQWTVIPNEHK | *Staphylococcus aureus* | *Staphylococcus phage DW2 Staphylococcus virus IPLA88* |
| S18 | N-acetylmuramoyl-L-alanine amidase | KEAGNYTVANVK | *Bacilli, Staphylococcus argenteus Staphylococcus aureus Staphylococcus hominis* | *Staphylococcus phage tp310-1 Staphylococcus phage tp310-2 Staphylococcus phage phi2958PVL Staphylococcus phage PVL Staphylococcus phage SA137nuMSSAST121PVL Staphylococcus virus IPLA35* |
| S4 | Phage protein NrdI | VETLENETNQNGLAM* SGGNRRNWGTNPFAAGDTISK | *Staphylococcus haemolyticus* *Staphylococcus hominis* *Staphylococcus aureus subsp. aureus Z172* | *Staphylococcus phage StaST398-1 Staphylococcus phage StaST398-1 Staphylococcus virus 13* |
| S12 | Complement inhibitor | IYNEIDEALK | *Staphylococcus. Aureus Klebsiella pneumoniae Enterobacter sp. IF2SW-B1* | *Staphylococcus phage StaST398-1 Staphylococcus virus 13* |
| S10 | Complement inhibitor | IYNEIDEALSKY | *Staphylococcus. aureus Klebsiella pneumoniae Enterobacter sp. IF2SW-B2* | *Staphylococcus phage StaST398-1 Staphylococcus virus 13* |
| S10 | DDE-type integrase/transposase/recombinase | PCPAMNKRNSIATHR | *Staphylococcus aureus* | *Staphylococcus aureus, Staphylococcus haemolyticus Staphylococcus capiti Staphylococcus epidermidis* |
| S9 | DNA primase phage-associated | LLHHFYPENTTALSFLNLDKFKPAALIQGKLYNIAD | *Staphylococcus aureus, Staphylococcus hominis Staphylococcus capiti, Staphylococcus epidermidis* | *Staphylococcus aureus* *Staphylococcus hominis* *Staphylococcus capiti* *Staphylococcus epidermidis* *Staphylococcus varani Staphylococcus sp. HMSC077D08 Cornubacterium propinquum* *Staphylococcus sp. U Staphylococcus lugdunensis Staphylococcus sp. HMSC077B09* | Uncultured Caudovirales Phage |
### Table 1. Cont.

| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|---------------------------------------------------------------|------------------------------------------------------------|
| S2     | Phage repressor, Cro/CI family | AAHLEGELTDEWQR | Staphylococcus haemolyticus Staphylococcus warneri Staphylococcus agnetis, Staphylococcus chromogenes Staphylococcus haemolyticus Staphylococcus sp. 58-22 Staphylococcus capitae Staphylococcus pasteuri Bacillales Staphylococcus chromogenes Staphylococcus agnetis Escherichia coli, Staphylococcus aureus | Staphylococcus virus 71 Staphylococcus phage phiSa2wa_s1 Staphylococcus phage phiSa2wa_s5 Staphylococcus phage Henu2 Staphylococcus phage ROSA Staphylococcus phage phi7401PVL |
| S2     | Phage repressor, Cro/CI family | VLDYADYIR | Staphylococcus aureus Staphylococcus epidermidis Staphylococcus warneri Staphylococcus agnetis Staphylococcus chromogenes, Staphylococcus spp. Staphylococcus schleiferi Staphylococcus simulans Staphylococcus haemolyticus, Staphylococcus viridans Escherichia coli | Staphylococcus virus 71 Staphylococcus phage phiSa2wa_s1 Staphylococcus phage phiSa2wa_s5 Staphylococcus phage Henu2 Staphylococcus phage ROSA Staphylococcus phage phi7401PVL |
| S9     | DNA-binding protein | SLDNM*SLK | Striga asiatica Staphylococcus aureus subsp. aureus 112608A Staphylococcus aureus A8819 Staphylococcus argenteus Staphylococcus spp. Pseudomonas aeruginosa Flectobacillus sp. BAB-3569 Escherichia coli | Staphylococcus phage vB_SauS_phi2 |
| S19    | DUF2479, Phage tail fiber, BppU family phage baseplate upper protein | HAGYVRC*KLF | Staphylococcus aureus, Staphylococcus sp. HMSC055H07 Staphylococcus argenteus, Staphylococcus sp. KY49P Staphylococcus sp. HMSC055F13 Pseudomonas aeruginosa Escherichia coli | Staphylococcus phage vB_SauS philippines Staphylococcus phage phiMR11 Staphylococcus phage SAP33 Staphylococcus phage 3MRA |
| S12    | Phage protein (DUF493 domain) | NSPIDLNSTEISLNNLER | Staphylococcus aureus Staphylococcus spp. Staphylococcus argenteus | Staphylococcus phage SuaST398-1 |
| S12    | Phage protein (DUF669 domain) | MNFNLNLQGAQELGN | Staphylococcus capitae Staphylococcus epidermidis Staphylococcus caprai Staphylococcus dermatis Staphylococcus warneri | Staphylococcus virus phiMR11 |
| S10    | GNAT family N-acetyltransferase | INVARQNNYESLITSIVSNNIGAK | Staphylococcus aureus Staphylococcus aureus subsp. anavorus Staphylococcus aureus subsp. aureus Mu50 Staphylococcus hominis Escherichia coli | Staphylococcus aureus Staphylococcus aureus subsp. anavorus Staphylococcus aureus subsp. aureus Mu50 Staphylococcus hominis Escherichia coli |
### Table 1. Cont.

| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|-------------------------------------------------------------|------------------------------------------------------------|
| S5     | Holin, phage phi LC3 family | SQDSNLTPLESTKAPK | Staphylococcus aureus | Staphylococcus phage HSA84, Staphylococcus phage SPF5 |
| S6     | ImmA/IrrE family metallo-endopeptidase | EKAKIFGDFMDNSGTVY DEENSTIIYNPPLSITR | Staphylococcus aureus subsp. aureus H19, Staphylococcus aureus | Staphylococcus aureus subsp. aureus 21204 |
| S16    | Involved in the expression of fibronogen-binding protein phage-associated | ESINANTYINQNLK | Staphylococcus aureus | |
| S16    | Involved in the expression of fibronogen-binding protein phage-associated | VAVLSTPLVTSFESK | Staphylococcus aureus | |
| S17    | N-6 DNA methylase; Nc_Mtase domain-containing protein | KDGEILFDAIDYLNK | Staphylococcus aureus | Staphylococcus phage phi-42 |
| S4     | Phage DNA-binding protein | GDPFVVITIMPMMQIK | Staphylococcus aureus, Staphylococcus warneri | |
| S9     | Phage terminase | KLYIIEEYKQCM | Staphylococcus aureus, Staphylococcus argenteus, Staphylococcus sp. HM58E11 Allobacillus sp. SKP4-8 | Staphylococcus virus Bag_Saul Staphylococcus virus phiETA2 Staphylococcus virus 69 Staphylococcus virus 11 Staphylococcus virus 80Alphap |
| S14    | Integrase | M*PVYKDNGTGTWYFSI | Staphylococcus aureus | Staphylococcus phage B166 Staphylococcus virus phiMR25 Staphylococcus virus 88 |
| S4     | Phage repressor | ISKVQQLADYFENVPK | Staphylococcus aureus, Staphylococcus chromogenes, Staphylococcus hyicus | Staphylococcus virus 80 |
| S13    | Toxin Phage protein; Pathogenicity island protein | NLGIVWLDLILIKRGLIDR | Staphylococcus aureus, Staphylococcus sp. HM58E11, Staphylococcus argenteus, Escherichia coli | Staphylococcus phage phiSa2wa_st58 Staphylococcus phage phiJF |
| S16    | Toxin Phage protein; Pathogenicity island protein | SDREKAGILFEELAHNK | Staphylococcus aureus, Escherichia coli | Staphylococcus phage phiSa2wa_st58 Staphylococcus phage phi7 |
| S6     | PBSX family phage terminase | QADNTYVHHYTLNRP FRKQPQAEASAKQR | Staphylococcus aureus, Staphylococcus sp. | Staphylococcus phage phiS2wa_st58 Staphylococcus phage phiB |
| S11    | PBSX family phage terminase | QGVSHLFKVTSP*M* | Staphylococcus aureus, Staphylococcus ventus, Staphylococcus sciuri | Staphylococcus phage phiS2wa_st58 Staphylococcus phage phiB |
| S20    | Phage-related cell wall hydrolase; Peptidase CS1; CHAP domain | EVPNEPDYVIVDVC^EDYSASK | Staphylococcus argenteus, Staphylococcus sp. HM58E11 | Staphylococcus viruss PLA88 Staphylococcus virus phiNM2 Staphylococcus phage SAP40 Staphylococcus phage phi53 Staphylococcus virus phiNM4 Staphylococcus phage SA12 Staphylococcus virus 69 Staphylococcus phage SA97 Staphylococcus phage TEM123 Staphylococcus virus 11 Staphylococcus virus phiMR25 Staphylococcus virus phiMR25 |
| S5     | Phage antirepressor Ant | QDNLAMPEVLPAIR | Staphylococcus aureus, Staphylococcus simulans Staphylococcus argenteus | Staphylococcus phage SA75 Staphylococcus phage SA13 |
| S11    | Phage capsid protein | M*PEITNSNVETEETVE | Staphylococcus aureus, Staphylococcus sp. | |
| S4     | Phage encoded lipoprotein | IHDEKELDPSSEESIKLTQEEENSI | Staphylococcus aureus, Staphylococcus capitis, Staphylococcus epidermidis, Staphylococcus cohnii, Staphylococcus haemolyticus | Staphylococcus phage SPFbeta-like |
| S2     | Phage head morphogenesis protein | KDQVRISVHT | Staphylococcus aureus, Staphylococcus argenteus | |
| S9     | Yrge/E/Pip, Phage infection protein | LNEYP^MPEIKLLN VASNDIPAGIKP | Staphylococcus aureus, Staphylococcus haemolyticus | Staphylococcus phage phiCMC02 |
| S14    | Minor structural protein | KKTEAIKELSST | Staphylococcus aureus | |
| S4     | Phage portal protein | EPKIPDATGADDLPLPDDRM^ITNFHANLVQDYSKSV | Staphylococcus aureus | |
| Strain | Protein Type | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|--------------|---------|---------|---------------------------------------------------------------|---------------------------------------------------------------|
| S5     | Phage        | VHISEFKPLYM*DFLGTKGELE | Staphylococcus aureus | Staphylococcus hominis, Staphylococcus epidermidis |
| S15    | Phage        | MSHNAIITGGGAGAG | Staphylococcus aureus | |
| S2     | Phage        | EITDGEISSVLTM*5 | Staphylococcus aureus, Staphylococcus hominis Staphylococcus epidermidis | |
| S20    | Phage recombination | KSSTTYEVNGETVK | Staphylococcus aureus, Staphylococcus sciuri | |
| S2     | Phage resistance | ESVDTGEITANTRTVK | Staphylococcus aureus, Staphylococcus pasteuri Staphylococcus epidermidis | |
| S13    | Tail tape measure | GM*PTGTVNYAVKGIADK | Staphylococcus aureus, Staphylococcus saprophyticus, Staphylococcus pseudogluco | Staphylococcus phage phi35a1Staphylococcus phage SH-15Staphylococcus virus 3n |
| S3     | Tail tape measure | QM*MEGLGVMIDLAWSGEDLG | Staphylococcus aureus | |
| S4     | Tail tape measure | QM*MEGLGVMIDLAWSGEDLG | Staphylococcus aureus | |
| S2     | Tail tape measure | AEEAGTVKQL | Staphylococcus aureus, Staphylococcus pasteuri, Staphylococcus epidermidis | Staphylococcus phage SFbeta-like |
| S10    | Phage repressor, Cro/CI family | QKNVNVYAEQILDEQNVK | Staphylococcus aureus Bacilli, Staphylococcus haemolyticus | Staphylococcus phage phiNM2Staphylococcus virus 53Staphylococcus virus 81Staphylococcus virus 89 |
| S13    | Phage protein | KSNVEAFPNAVK | Staphylococcus aureus | Staphylococcus phage phiNM1Staphylococcus phage phiNM2 |
| S11    | Phage protein | PYHDLSDERIM*EEELKK | Staphylococcus aureus Staphylococcus argenteus taphylococcus Schweiteri | Staphylococcus phage phiETA2Staphylococcus phage P630Staphylococcus virus SAP26Staphylococcus phage B26Staphylococcus virus 88Staphylococcus prophage phiLV83 |
| S4     | Minor structural | LNDNISINTIV | E. coli, Parathenothemera mesophilica Staphylococcus pseudointermedius Staphylococcus epidermidis, Staphylococcus aureus | |

**Table 1. Cont.**
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| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|----------------------------------------------------------------|------------------------------------------------------------|
| S9     | PhETA ORF58-like protein | GMVASMQMQQVVQVNLTM*ELAQQNAMLTQQTELK | **Staphylococcus aureus** | **Staphylococcus phage phi401PVL**<br>**Staphylococcus phage phiSa2wa_st121mssa**<br>**Staphylococcus virus 3a**<br>**Staphylococcus virus phi2LT**<br>**Staphylococcus phage phi310-2**<br>**Staphylococcus phage SA137ruM65AST121PVL**<br>**Staphylococcus phage phiSa2wa_st5**<br>**Staphylococcus phage SH-54 15644**<br>**Staphylococcus virus phi2958PVL**<br>**Staphylococcus virus IPLA35**<br>**Staphylococcus phage P240**<br>**Staphylococcus phage vB_SauS_JS02**<br>**Staphylococcus virus 42e**<br>**Staphylococcus virus phi12**<br>**Staphylococcus phage phiSa2wa_st72**<br>**Staphylococcus phage phiSa2wa_st30**<br>**Staphylococcus phage vB_SauS_53**<br>**Staphylococcus phage StauST398-2** |
| S4     | Phage portal protein | TEQLPRLLEML | Staphylococcus aureus, Staphylococcus sp. HMSC063A07, Staphylococcus lugdunensis, Staphylococcus sp. HMSC068D08, Staphylococcus sp. HMSC069E09 | Staphylococcus phage phiSa2wa_st1 |
| S4     | Prophage, terminase | KDRYSSVSY | Staphylococcus aureus, Staphylococcus delphini, Staphylococcus pseudintermedius, Staphylococcus agnetis, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus haemolyticus, Paenibacillus sophorae | Staphylococcus phage SPBeta-like |
| S4     | Prophage tail domain; Peptidase | VLEM*IFLGEDPK | Staphylococcus aureus | E. coli Bacilli |
| S15    | Site-specific integrase | VEELEDSEIHKK | Staphylococcus aureus, Staphylococcus epidermidis Staphylococcus haemolyticus Staphylococcus condimenti Staphylococcus sp. HMSC035D11 Staphylococcus warneri | uncultered Caudovirales phage Sequence ID: ASN72447.1 |
| S13    | Site-specific integrase | KEAGSIINHTINNAKSA*R | Staphylococcus aureus Staphylococcus sp. | |
| S6     | Site-specific integrase | YLRNRNFVFTNHK | Staphylococcus aureus, Staphylococcus argenteus Staphylococcus cohnii Staphylococcus haemolyticus Staphylococcus caeli Staphylococcus sp. 47.1 | |
| S9     | Terminase large subunit | KAMIKASPK | Staphylococcus aureus Escherichia coli Staphylococcus sp. HMSC74F04 Staphylococcus sp. HMSC055H07 Cutibacterium acnes Staphylococcus turneri Bacillus cluhemis Paenibacillus larvae | Staphylococcus phage vB_SauS_JS02 Staphylococcus phage phiSa2wa_st5 Staphylococcus phage phiSa2wa_st1 Staphylococcus phage phiSa2wa_st21mssa Staphylococcus virus IPLA35 Staphylococcus phage phi310-2 Staphylococcus virus phi2LT Staphylococcus phage StauST398-2 Staphylococcus phage vB_SauS_phi12 Staphylococcus phage phiSa2wa_st72 Staphylococcus phage phiSa2wa_st30 Staphylococcus phage vB_SauS_53 Staphylococcus phage SMA55 Staphylococcus phage phi2958PVL Staphylococcus virus 3a Staphylococcus phage YMC/09/04/R1988 |
| S20    | Phage repressor, Cmv/CI family | RIQQLADYFNVPK | Staphylococcus aureus Staphylococcus pettenkoferi Staphylococcus pettenkoferi Staphylococcus captitis Staphylococcus devriesi | Staphylococcus phage vB_SauS_phi12 Staphylococcus virus IPLA35 |
| Strain | Protein | Peptide | Bacteria with 100% Homology Based on the NCBI Protein Database | Phages with 100% Homology Based on the NCBI Protein Database |
|--------|---------|---------|------------------------------------------------------------|------------------------------------------------------------|
| S4     | Transposase B from transposon Tn554 O | WDRRN1LPDDK | Staphylococcus aureus, Staphylococcus epidermidis | Staphylococcus aureus, Staphylococcus epidermidis, Bacillus thermolatmus, Staphylococcus aureus, Staphylococcus epidermidis |
| S13    | Uncharacterized phage protein | C*VSGIAGGAVTGGTTLGLAGAG | Staphylococcus aureus | Staphylococcus aureus |
| S20    | Uncharacterized phage protein | QTDPWSWVPM*VLR | Staphylococcus aureus, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis | Staphylococcus aureus, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis |
| S12    | Uncharacterized phage protein | IIIHDEIDLL | Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis | Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis |
| S14    | Uncharacterized phage protein | TSIELITGFTK | Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus warneri | Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus warneri |
| S3     | Uncharacterized phage protein | EFRNKLNELGADK | Staphylococcus aureus, Streptococcus pneumoniae, Terrabacteria group | Staphylococcus aureus, Streptococcus pneumoniae, Terrabacteria group |
| S3     | Phage repressor, Cro/CI family | HLEEVDIR | Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa | Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa |
| S4     | YhgE/Pip Phage infection protein | APQSTSVKK | Staphylococcus aureus, Staphylococcus epidermidis | Staphylococcus aureus, Staphylococcus epidermidis |
| S4     | YhgE/Pip Phage infection protein | ALNFAADDVPAQFK | Staphylococcus aureus, Staphylococcus epidermidis | Staphylococcus aureus, Staphylococcus epidermidis |
3.2. Phage Peptides Determined from the Analyzed S. aureus Strains

For strains S2 and S3, six and three phage peptides were determined, respectively. For strain S4, seventeen phage peptides were determined, and three phage peptides were determined for strain S5. For strains S6 and S7, three and one phage peptide were determined, respectively. Moreover, for strains S8 and S9, two phage peptides and seven phage peptides were determined. For strains S10 and S11, five and three phage peptides were determined, respectively. For strains S12 and S13, five phage peptides and six phage peptides were determined, respectively. For strains S14 and S15, four and two phage peptides were determined, respectively. For strain S16, three phage peptides were determined, and one phage peptide was determined for strain S17. For strains S18 and S19, one phage peptide each was determined. Finally, for strain S20, seven phage peptides were determined.

A large number of phage peptides from structural proteins were identified (Table 1). Peptides from proteins such as the major capsid protein, major tail protein, minor structural protein, phage head morphogenesis protein, tail tape measure protein and phage tail fiber protein were determined. Moreover, different phage peptides from the major capsid protein and tail protein were determined (Table 1). Identifying these phage peptides is reasonable, as the major capsid protein and major tail protein are the most abundant proteins in mature virions [6].

There are a large number of uncharacterized protein sequences in databases, and more than 20% of all protein domains are annotated as “domains of unknown function” (DUFs). Several uncharacterized phage proteins and DUFs from Staphylococcus bacteriophages were identified for the analyzed strains (Table 1) [43,44].

Different peptides from repressor-type Cro/CI were determined. For strains S11 and S20 (both potential enterotoxin C producers), the same phage peptides of repressor-type Cro/CI were identified (Table 1). CI and Cro are encoded in the lysogeny module of lambdoid bacteriophages, particularly λ bacteriophages. Together, CII and CIII (that are formed through the anti-terminator role of protein N) act as an inducer that favors the first expression of the cl gene from the appropriate promoter; if the CI repressor predominates, the phage remains in the lysogenic state, but if the Cro predominates, the phage transitions into the lytic cycle, helped by the late Q regulator. The xenobiotic XRE regulator is extended in bacteria and has similarity to the Cro λ repressor, exhibiting a helix-turn-helix (HTH) conformation [45]. Peptides of the CI/Cro-repressor types are usually named XRE family proteins in the NCBI database for bacteria.

Three phage peptides of the complement inhibitor were identified (Table 1). Staphylococcal complement inhibitors are involved in the evasion of human phagocytosis by blocking C3 convertases, and a study reported that complement inhibitor genes were also found in Staphylococcal phages [46]. Another autolysin was determined in the present results, an N-acetylmuramoyl-L-alanine amidase that plays a role in bacterial adherence to eukaryotic cells [19]. The phage protein NrdI, which is a type of ribonucleotide reductase (RNR), was also identified. Several peptides of transposases, integrases and terminases were identified along with a DNA primase phage associated protein and a DNA phage binding protein. Moreover, peptides of other proteins, such as GNAT family N-acetyltransferase, holin, peptidase, methylase, anti-repressor protein (Ant), phage-resistant protein, phage-encoded lipoprotein, phage infection protein, phage portal protein, toxin phage proteins associated with pathogenicity islands and a protein involved in fibrinogen-binding proteins, were identified. A PBSX family phage terminase peptide was determined, and this protein is involved in double-stranded DNA binding, DNA packaging and endonuclease and ATPase activities [47].

As shown in Table 1, the vast majority of phage-specific peptides are not specific to S. aureus and can be found in other species of Staphylococcus. As an exception, the same peptides, such as peptide LLHALPTGNDSGGDKLLPK from a major capsid protein, were also found in Streptococcus pneumoniae, and peptide AYINITGLGFAK from a major tail protein was also found in Pararheinheimera mesophilae; whether these examples represent...
direct recombinations between bacteria belonging to different families or whether phage-mediated recombination occurs remains to be elucidated. Furthermore, as mentioned before, eighteen identified peptides were very specific for *S. aureus* based on the NCBI database (see Figure S1).

### 3.3. *Staphylococcus* spp. Phage Genome Comparisons and Their Relatedness

A phylogenomic tree of *Staphylococcus* spp. phages from the NCBI database (accession numbers in Table S2 in Supplemental Data 2) with 100% similarity to those found in this study was built (Figure 2). The phages identified in this study were classified in the order *Caudovirales* and the family *Siphoviridae*. Many of these bacteriophages were classified into the genera *Phietavirus*, *Bisetimavirus*, *Triavirus* phieta-like virus, SPbeta-like virus and unclassified genera. Genomes of well-known phages of the families *Siphoviridae*, *Myoviridae* and *Podoviridae*, such as phage Lambda, T4 and T7, respectively, were added for comparison purposes. The genome analysis showed three well-defined clusters that mainly divided the phylogenomic tree into different phage genera (*Phietavirus*, *Bisetimivirus* and *Triavirus*). Two principal branches separated Clusters A, B and C from D. Cluster A was formed by *Staphylococcus Phietavirus*, two phieta-like viruses and two unclassified *Staphylococcus* phages. Cluster B was formed by *Staphylococcus* phages classified as *Bisetimivirus* and by one unclassified *Staphylococcus* phage. Cluster C was formed by enterobacterial bacteriophages and one SPbeta-like virus. Finally, cluster D was formed by *Triavirus Staphylococcus* phages and two unclassified *Staphylococcus* phages. To the best of our knowledge, this is the first time that phages from mastitis-causing staphylococci were grouped in a phylogenomic tree.

Specific peptides were found in related *Staphylococcus* spp. phages (Table 2) located closely in the phylogenomic tree (Figure 2). Peptides HAGYRC*KLF and MPVYKDGNTGKWYFSI were found in phages of cluster A. Furthermore, peptides IYDRNSDTLDGLPVVNLK, QKNVLNYANEQLEDQNKV, EVPNEPDYVIDVC*EDYSASK, KSNVEAFSNAV and KLYIIEEYKQCM were found in *Staphylococcus* phages of the A.1 subbranch in cluster A. Additionally, peptide AWAELLEKINR was found in phages of the A.2 branch. The peptide AYINITGLGFAK was found in phages of cluster B.1, and TSIELITGFK was found in phages of cluster B.2. Peptides VSYTLDDDDFITDVETAK and LLHALPTGNDSGD-KLLPK, which belong to the phage major capsid protein, were found in the same 14 *Staphylococcus* phages of cluster D. Peptides ELAEAIGVSQPTVSNWIQQTK and IQQLAYFNVPK, which belong to the phage-repressor Cro/CI family of proteins, were found in the same bacteriophages of cluster D. Moreover, peptides LYVGVFNPEAT, RVSYTLD-DDDFTDVETAKELKL, LYVGVFNPEATK, VLEMIFLGEDPK, KAMIKASP, EFRNKL-NELGAD and GMPTGTNVAKGGIAKD were also found in phages of cluster D. Peptides IHDKELDDPSEEESKTQEEEINSI, IINHDEIDLL, KDRYSSSY and AEEAGVTVKQL are specific to *Staphylococcus* phage SPbeta-like.

**Table 2.** Phage biomarker peptides that belong to bacteriophages and phylogenomic tree clusters. Relationships between specific phage biomarker peptides and phylogenomic tree clusters.

| Protein          | Peptide                  | Phages                                                                 | Cluster Located |
|------------------|--------------------------|------------------------------------------------------------------------|-----------------|
| Major capsid protein | VSYTLDDDDFITDVETAK | *Staphylococcus* phage phiSa2wa_st72 |
|                  |                          | *Staphylococcus* phage tp310-2                             |
|                  |                          | *Staphylococcus* phage phiSa2wa_st121mssa |
|                  |                          | *Staphylococcus* phage vB_SauS_phi2 |
|                  |                          | *Staphylococcus* phage StauST398-2 |
|                  |                          | Staphylococcus virus 3a *Staphylococcus* phage LH1 *Staphylococcus* phage phiSa2wa_st30 |
|                  |                          | *Staphylococcus* virus phi12 *Staphylococcus* virus phiSLT |
|                  |                          | *Staphylococcus* phage vB_SauS_J602 |
|                  |                          | *Staphylococcus* phage R4 |
|                  |                          | *Staphylococcus* phage vB_SauS_FPSau02 |
|                  |                          | *Staphylococcus* phage SAI37rdsRSS21T21PV1 |
Table 2. Cont.

| Protein | Peptide | Phages | Cluster Located |
|---------|---------|--------|-----------------|
| Major capsid protein | LLHALPTGNSGGDKLLPK | *Staphylococcus* phage phiSa2wa_st72  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage VB_SauS_ph12  
*Staphylococcus* phage StauST398-2  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* virus phi112  
*Staphylococcus* virus phi 3  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage tp310-2  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage phiSa2wa_st121PVL | Cluster D |
| Major capsid protein | RSVYTLDDDDFTDVEATAELKL | *Staphylococcus* phage LH1  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* phage StauST398-2  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st30  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st30  | Cluster D |
| Major tail protein | LYGVFNPNEATK | *Staphylococcus* phage phiSa2wa_st72  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage StauST398-2  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage phiSa2wa_st121PVL  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  | Cluster D |
| Phage repressor, Cro/CI family | ELAEAGVSQPTVSNWIIIQTK | *Staphylococcus* virus IPLA35  
*Staphylococcus* phage SMSAP5  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage phiSa2wa_st121PVL  
*Staphylococcus* phage phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  | Cluster D |
| Phage repressor, Cro/CI family | IQQ指令YENF PK | *Staphylococcus* virus IPLA35  
*Staphylococcus* phage SMSAP5  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* phage phiSa2wa_st121PVL  
*Staphylococcus* phage phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  
*Staphylococcus* virus phi12  
*Staphylococcus* virus phiSLT  
*Staphylococcus* phage phiSa2wa_st121mssa  | Cluster D |
| Major tail protein | AYNINITGLGFAK | *Staphylococcus* phage phiNM3  
*Staphylococcus* phage StauST398-4  
*Staphylococcus* phage phiNM5  
*Staphylococcus* phage phiNM4  
*Staphylococcus* phage phiNM1  
*Staphylococcus* virus phiETA2  
*Staphylococcus* virus phiNM5  
*Staphylococcus* virus phiNM4  
*Staphylococcus* virus phiNM1  
*Staphylococcus* virus phiNM2  | Cluster A.1 |
| Major capsid protein | IYDRNSDTLDGLPVNLIK | *Staphylococcus* virus 85  
*Staphylococcus* virus phiETA2  
*Staphylococcus* virus phiNM1  
*Staphylococcus* virus phiNM2  
*Staphylococcus* virus SAP26  
*Staphylococcus* phage phi12  
*Staphylococcus* phage phi12  
*Staphylococcus* phage phi12  
*Staphylococcus* phage phi12  | Cluster A.1 |
| Phage terminase | KLYIIEEYVKQGM | *Staphylococcus* virus Baq Sau1  
*Staphylococcus* virus 85  
*Staphylococcus* virus phiETA2  
*Staphylococcus* virus phiNM1  
*Staphylococcus* virus phiNM2  | Cluster A.1 |
| Phage-related cell wall hydrolase; Peptidase C51, CHAP domain | EVPNEPDIVIDVC*EDYSASK | *Staphylococcus* virus phi12  
*Staphylococcus* phage phi12  
*Staphylococcus* phage phi12  
*Staphylococcus* phage phi12  | Cluster A.1 |
In addition, a correlation relating bacterial species for each cluster with all peptides found in the bacteriophages with 100% similarity was found. The results showed that clustered phages were related to specific species of *Staphylococcus*. All studied phages were found to be related to *S. aureus*; however, most of them were also found to be related to additional *Staphylococcus* species. *S. argenteus* was found to be related in all clusters of the phylogenomic tree. Cluster A phage peptides were found to be mainly related to *S. simiae*. However, different *Staphylococcus* species (*S. xylosus*, *S. muscae*, *S. haemolyticus*, *S. simiae*, *S. sciuri*, *S. pseudintermedius*, *S. deversi*, *S. warneri* and *S. capitis*) were found to be related to phages of cluster D.

### 3.4. Identification of Peptides of Virulence Factors

In this work, 405 peptides from *S. aureus* were determined to be related to virulence factors (Excel dataset Supplemental Data). Among these peptides, proteins such as staphopain, beta-lactamase, elastin-binding protein peptides and a multidrug ATP-binding cassette (ABC) transporter were identified.
Figure 2. Phylogenomic tree generated by the Virus Classification and Tree Building Online Resource (VICTOR) using the complete genomic sequences of the determined Staphylococcus spp. phages. The access numbers of the determined phage genomes are shown in Table S2 in Supplemental Data 2. Genomes of the lambda (NC_001416.1), T4 (NC_000866.4) and T7 (NC_001604.1) phages were added for comparison purposes. The VICTOR phylogenetic tree construction was based on an intergenic distance analysis with the GBDP tool (Genome BLAST Distance Phylogeny). The significance of each branch is indicated by a pseudo-bootstrap value calculated as a percentage for 1000 subsets. Bar, 20 nt (nucleotides) substitutions per 100 nt. Clusters are represented by different colors: light blue, cluster A, red, cluster A.1, purple, cluster A.2, light green, cluster B, yellow, cluster B.1, pink, cluster B.2, black, cluster C and orange, cluster D. Specific cluster peptides are represented by different color forms: , yellow-filled diamond IQQLADYFNVPK (cluster A-specific), , brown-filled diamond HAGYVRC*KLFL (cluster A-specific), , black-outlined diamond IYDRNSDTLDGLPVVNLK (cluster A.1-specific), , red-outlined diamond AWAELKINR (cluster A.2-specific), , pink-filled diamond KSNVEAFSNAVK (cluster A.1), , gray-filled diamond KVN-VLNYANEQLDENNQK (cluster A.1), , brown-outlined diamond MPVYKDGNTGWYFF (cluster A-specific), , dark gray-filled diamond KLYIIEEYVKQGM (cluster A.1-specific), , purple-filled diamond EVPNEPDYIVIDVC*EDYSASK (cluster A.1-specific), , orange-filled diamond AYINITGLGFAK (cluster B.1-specific), , yellow-filled diamond TSIELIT-GFTK (cluster B.2-specific), , red-filled diamond VSYTLDDDHTTDVETAK (cluster D-specific), , green-filled diamond LLHALPTGNDGDKLLPK (cluster D-specific), , black-filled diamond RVSYTLDDDHTDVE*AKELKL (cluster D-specific), , purple-filled diamond LYGVFNPEATK (cluster D-specific), , blue-filled diamond ELAEAI*QSTDSWNVQOQTK (cluster D-specific); , light green-filled diamond VLEMIFLGEDPK (cluster D-specific), , orange-filled diamond KAMIKASP (cluster D-specific) and , gray-outlined diamond GMPTGTVYVAKGGLADK (cluster D-specific).
4. Discussion

LC-MS/MS-based methods for bacteriophage identification offer several advantages compared with other approaches, since bacteriophages can be directly identified with this method without using genomic tools, which provides a new strategy for drawing the appropriate conclusions. In addition, the method proposed here may be applied for further analyses without the requirement of growing bacteria, since the samples can be collected directly from foodstuffs. The study of noninduced prophages provides a fast analysis and can detect specific temperate phage proteins produced by *S. aureus* while integrated in the bacterial genome or by phages that are infecting the bacteria. Both cases provide the identification of specific *S. aureus* species or strains—in this case, an *S. aureus* mastitis producer. In the proteomic repository of the 20 different *S. aureus* strains analyzed, 79 peptides from staphylococcal bacteriophages were identified. Among them, eighteen of these phage peptides were *S. aureus*-specific. As bacteriophages are host-specific, these putative diagnostic peptides could be good diagnostic biomarkers for the detection and characterization of *S. aureus* and *S. aureus* phages.

The results show that a given specific peptide is present in closely related phages (Table 2). These bacteriophage peptides can be used as specific markers to establish *S. aureus* bacteriophage relationships (Figure 2). Additionally, phages that show the same peptides and are specific to *Staphylococcus* spp. are located close to one another in the phylogenomic tree, suggesting that a link does exist between phage phylogeny and bacteriophages that can infect the same bacterial species.

The study shown here exemplifies how phylogenomic trees based on the genome analysis provide useful information, and the study corroborates previous investigations, which suggested that viral genomic or subgenomic region analyses provide the best tool for reconstructing viral evolutionary histories [48]. Nevertheless, the lack of knowledge of the phage genomic content [49] makes a phage analysis more difficult. The first priority must be the contribution of new large amounts of data for phages infecting bacteria [12].

In addition, there is an urgent need for novel therapies to treat and prevent mastitis [50]. Bacteriophage therapy is an alternative to the antibiotic treatment of bovine mastitis [51], with a high specificity and a low probability for bacterial resistance development [52]. Many studies have demonstrated the effectiveness of bacteriophages in a variety of animal models to fight several mastitis-causing pathogenic bacteria. Some studies have shown how virulent phages such as SPW and SA phages are active against bovine mastitis-associated *S. aureus*. Moreover, SAJK-IND and MSP phages have specific lytic activity against several strains of *S. aureus* isolated from mastitis milk samples [53]. Indeed, mouse-induced mastitis models decreased their bacterial counts after treatment with a vBSM-A1 and vBSP-A2 phage cocktail [54]. Finally, several temperate phage mixtures have been shown to be more effective than using a single temperate phage for inhibiting *S. aureus*. According to the data obtained for the different models of mastitis, phage therapy using bacteriophages in this study can be considered an innovative alternative to antibiotics for the treatment of mastitis caused by *S. aureus*.

Finally, the proteomic analysis by LC-ESI-MS/MS performed in this study provides relevant insights into the search for potential phage origin diagnostic peptide biomarkers for mastitis-causing *S. aureus*. In addition, this method may be useful for searching peptide biomarkers for the identification and characterization of mastitis-causing species and for finding new *S. aureus* phages useful as possible therapies for mastitis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10040799/s1: Figure S1: MS/MS spectrums for *S. aureus*-specific peptide biomarkers. The corresponding peptides were tested for specificity using the BLASTp algorithm. Excel Dataset Supplemental Data 1: Complete nonredundant peptide dataset. Supplemental Data 2: Table S1: *Staphylococcus aureus* (SA) strains used in this study. Table S2: Linage, authors and accession number of studied bacteriophages [55–88].
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