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Characterization of lactic and acetic acid bacteria and their phages from a California winery

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Abstract

Spoilage of wine from volatile acidity or off flavors caused by uncontrolled growth of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) results in significant economic losses. We aim to survey the persistence of LAB and AAB throughout the wine processing stages, and to evaluate the potential of phages as targeted microbial control. Cabernet Sauvignon samples were collected from a winery in Livermore, California during 2025 harvest season, covering grape rinse, juice/must, the end of primary fermentation and malolactic fermentation steps. Bacterial 16S amplicon sequencing of these samples identified key species that persist through the wine processing steps, including *Lactiplantibacillus plantarum* and *Levilactobacillus lactis* among LAB, and *Acetobacter oryzoeni* and *Acetobacter pasteurianus* among AAB. Phages targeting *L. brevis*, *A. pasteurianus* and *A. fabarum* were isolated. *L. brevis* phages Indaco_L1 and Indaco_L2 (genome size of 135,623 bp and 141,184 bp, respectively) are related to phages in the *Tybeckvirus* genus of the *Herelleviridae* family, but distinct enough to form two novel species. The two novel *Acetobacter* phages, a first reported *Acetobacter* jumbo phage Vinphasol_A1 (genome size 397,952 bp) and Vinphasol_A2 (genome size 56,636 bp), share little DNA sequence similarity to phages in the current NCBI database. These isolated phages were able to inhibit the growth of *L. brevis, A. pasteurianus* and *A. fabarum* for up to 7 days in conditions simulating wine fermentation, suggests their potential application in controlling microbial spoilage during wine making processes.

Introduction

Despite good management practices, uncontrolled growth of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during winemaking leads to high volatile acidity (VA), stuck fermentations, or the development of other “off flavors”, which results in wine spoilage and significant economic losses across the industry (1–3). Controlling LAB in winemaking is uniquely complex in that while many species cause spoilage and quality degradation, *Oenococcus oeni* (formerly *Leuconostoc oenos*) is explicitly favored to convert malic acid to lactic acid during secondary fermentation, or malolactic fermentation (MLF), a critical process for the sensory and chemical profile of the majority of red wines and select white wine varietals (4–6). Consequently, targeted control capable of differentiating between LAB and AAB at the species or strain level is essential for effective microbial management in these complex fermenting communities. Current industry practice relies primarily on addition of sulfites and enzymes (such as lysozyme) to suppress unwanted bacteria (7). While these methods do reduce the populations of spoilage species, they also can inhibit beneficial MLF species and microbial fermentation. These treatments are also highly pH sensitive, have narrow windows for application, have poor consumer perception, and can require downstream processing for wine clarification (7–10).

Bacteriophages (or phage), viruses that specifically infect bacteria, have potent antimicrobial activity and are already widely recognized for their potential in food safety, animal health, and human clinical settings (11–13). One untapped application is the use of phage for the reduction of bacteria frequently responsible for various wine faults. The narrow host range of phage allows them to target their specific

host in complex communities, i.e. selectively knockdown undesirable LAB and AAB strains while leaving desirable malolactic fermentation bacteria safely intact. The robust and self-replicating nature of phage allows them to retain antimicrobial activity overtime and across a wide range of temperatures, pH, and ethanol concentrations (14–17). These properties suggest they could be a desirable alternative or supplement to standard antimicrobials used throughout the winemaking process.

To date there have been several microbiome studies analyzing the bacterial dynamics during wine fermentation wine microbiome (18, 19). The studies suggest a progression of bacteria, where a high level of bacterial diversity at the outset at the grapes/must stage gives way to select species or genera capable of surviving the harsh pH and high alcohol conditions associated with a developing wine. Microbiome studies such as these, as well as individual isolates from spoiled wines, show LAB genera such as *Lactobacillus*, and *Leuconostoc*, AAB species *Acetobacter pasteurianus* and *Acetobacter aceti*, are frequently the cause of high VA or stuck fermentations, and thus the critical targets for phage. Phages capable of controlling the wine-associated AAB and LAB species have not been well studied. LAB phage research has been heavily focused on the strains of dairy origin due to their industrial significance as starters and probiotics (20–25). In contrast, reports on phages targeting LAB related to wine/ethanol fermentation remain minimal. Limited studies have isolated phages for *Lactobacillus plantarum* (now *Lactiplantibacillus plantarum*) and *Oenococcus oeni* of wine origin (26, 27), and *Lactobacillus fermentum* from industrial ethanol fermentation (14). One study demonstrated the inhibition of *Lactobacillus* and *Oenococcus oeni* growth in laboratory must and wine cultures using their specific phages (16). Compared to LAB phages, reports on AAB phages are limited, among which are reports on isolated phages for *Gluconobacter oxydans* (28, 29), *Acetobacter methanolicus* (30), and the examination of widely distributed temperate phages characterized in *Acetobacter* spp. used for industrial vinegar fermentation (31, 32). There is no report on lytic phages for AAB associated with wine-making according to our knowledge.

This study aims to characterize the LAB and AAB populations present across wine fermentation processes longitudinally, and to isolate and characterize phages specifically targeting LAB and AAB strains from species which persist through these processes. The ultimate goal is to evaluate the potential of phage as precision microbial control during wine fermentation.

Materials and Methods

Wine sample collection, LAB/AAB enrichment and isolation

Fresh samples throughout different wine making stages (grape rinse/mash, must/juice, end of primary fermentation, end of malolactic fermentation) were collected and were either plated directly or enriched and then plated on selective media. Selective media used to isolate or enrich LAB and AAB strains, respectively, are de Man, Rogosa, and Sharpe (MRS, Hardy Diagnostics, Santa Maria, CA) and glucose-yeast extract-calcium carbonate (GYC) media. GYC was further supplemented with glacial acetic acid (0.1% v/v), ethanol (5% v/v), and penicillin (5 µg/ml) to further select for AAB capable of surviving in the

wine environment in a similar manner described previously (33). MRS and GYC media are supplemented with 10 mg/ml cycloheximide to inhibit yeast and mold growth. For enrichment, each wine sample was inoculated in liquid MRS or GYC broth at 1:20 inoculum and incubated at 30 ºC overnight for up to seven days until culture turbidity indicated bacterial growth was evident. LAB cultures were incubated in a low-oxygen candle jar (a container into which a lit candle is introduced before sealing the airtight lid) either in static liquid culture or on agar plates at 30 ºC. AAB enrichments were cultured aerobically with shaking. To obtain predominant representative LAB/AAB strains, representative bacterial colonies from the predominant colony morphological types on the highest dilution plates were subject to several rounds of sequential colony purification. Following isolation and subculturing, strains were routinely cultured and maintained on standard MRS or GYC media without supplement, unless necessitated by the bacterial host.

LAB and AAB microbiome composition

Cells in the AAB or LAB enrichment cultures (1 ml each) were pelleted by centrifugation (8,000 × g, 3 min). After removing the supernatant, the cell pellet was resuspended in 500 µl of Zymo DNA/RNA Shield before being submitted for DNA extraction and Microbiome 16S Amplification & Sequencing at Plasmidsaurus (https://plasmidsaurus.com/, Oxford Nanopore, R10.4.1). The obtained raw sequencing reads were quality controlled (using the Super-Accurate basecalling model and run the raw reads through a basic quality filter) and classified against rrnDB (v5.6) and NCBI Targeted Loci database of bacterial, archaeal, and eukaryotic species using emu (v3.5.1) to determine their taxonomic classifications. Taxonomic data is compiled from all raw reads to generate metrics on the relative abundance of all species that comprise at least 0.1% of the microbial community. Samples from each wine making step were enriched independently and the enrichment cultures from each sample type were used to calculate average population abundance.

Bacterial 16S rRNA identification

Genomic DNA from the individual AAB and LAB isolates were extracted using a Promega Wizard™ Genomic DNA Purification Kits following the manufacturer's protocol. PCR amplicon of the 16S rRNA (V1-V9) was generated using primers Bact8F (5'-AGAGTTTGATCCTGGCTCAG-3') and Bact1492R (5'-GGTTACCTTGTTACGACTT-3'), and were sequenced at Plasmidsaurus for PCR amplicon sequencing (Oxford Nanopore, R10.4.1). The obtained 16S DNA sequences were analyzed against NCBI's 16S ribosomal RNA sequences database via BLASTn to determine their identities.

Phage isolation and purification

Phages were isolated by enriching wastewater and fermented food samples, using a modified version of the previously described enrichment method (34). Briefly, enrichments were set up with 10 ml of filter-sterilized sample liquid and 40 ml MRS or GYC broth to which 1 ml of an overnight LAB or AAB host

bacteria culture was added. Following 24 h incubation at 30°C, bacterial cells were removed by centrifugation (10,000 × g, 5 min) and the supernatant was passed through a 0.22 µm filter. The obtained filtrate was subjected to plaque purification and propagation using the double agar overlay method (35), using MRS or GYC agar (1.5% agar) as bottom plates coupled with MRS or GYC soft top agar (0.5% agar with 5 mM $CaCl_2$ and 5 mM $MgSO_4$).

Phage genome sequencing and annotation

Phage genomic DNA was prepared using a modified Promega Wizard DNA cleanup kit protocol as described previously (36) and was sequenced via Illumina miSeq with pair-end 2 × 150-bp reads at SeqCenter (https://www.seqcenter.com/). The reads obtained were analyzed by FastQC v0.11.19 (www.bioinformatics.babraham.ac.uk/projects/fastqc) for quality control and then assembled using SPAdes v3.5.0 (37). The genome was closed either bioinformatically or by PCR and Sanger sequencing off the ends of the contig (36), which was the case for phage Indaco_L1 and Indaco_L2 (PCR forward primer: 5'-AGAAACAGTATATTCGGACGTTGA-3', reverse primer: 5'-CCTAAGACAGTAGATTATGAAGATGCTA-3'). For phage Vinphasol_A1, the extracted genomic DNA was also sequenced at Plasmidsaurus (Oxford Nanopore, R10.4.1) due to its large genome size, and the result was analyzed together with the Illumina assemblies for genome closure. Putative protein-coding genes were annotated using GLIMMER v3 (38) and MetaGeneAnnotator v1.0 (39), and tRNAs were detected with ARAGORN v2.36 (40). Sequence homology analysis from BLASTp (41) against the NCBI nonredundant (nr), conserved folding analysis using HHpred v3.2.0 (42), and conserved domain analyses using InterProScan v5.33 (43) and TMHMM v2.0 (44) were used to assign the putative gene functions. Minus HHpred, all tools were accessed in the Phage Galaxy-Apollo interface (https://phage.usegalaxy.eu/), or at the Center for Phage Technology at Texas A&M university (https://galaxy.cpt.tamu.edu/) (45–47). Genome-wide DNA sequence identity to other organisms in the NCBI nucleotide database was determined via BLASTn, and the comparison of the predicted proteomes to other phages was determined using the Center for Phage Technology (CPT) Galaxy's comparative genomics workflow. Intergenomic similarities/distances of selective phage genomes were analyzed using VIRIDIC (48). Phage genome maps were generated using a visualization tool (49) and comparison genome maps were plotted using clinker & clustermap.js (50).

Phage inhibition of LAB and AAB

The *L. brevis*, *A. pasteurianus* and *A. fabarum* strains were inoculated to MRS, commercial white wine or white grape juice supplemented with MRS (at a ratio of 8:2, wine/juice to MRS, v/v) at a starting bacterial levels of approximately 10^5 cfu/ml and aliquoted into a Costar 96-well microtiter plate. Thirty minutes following inoculation with their respective hosts, *L. brevis* phage Indaco_L1, *A. pasteurianus* phage Vinphasol_A1, and *A. fabarum* phage Vinphasol_A2 were added to bacterial cultures at a target MOI (multiplicity of infection, or bacteria to phage ratio) of 100. The cultures were incubated statically at ambient temperature of 21.1°C for 90 h, and the bacterial growth (OD550nm) was monitored using

BioTek Synergy LX multimode plate reader. In a separate experiment, *A. pasteurianus* and *A. fabarum* were inoculated at 10^2 -10^3 cfu/ml to pure white grape juice (pH4.2) without MRS supplementation, and phages Vinphasol_A1 and Vinphasol_A2 and were added to a target MOI of 1000 to their respective host cultures. The cultures were incubated at 21.1°C, and the bacterial growth (OD600nm) was monitored daily for 7 days. For all the phage inhibition assays, bacterial cultures without phage additions were included as controls. All experiments were performed in biological triplicate (n = 3), error bars displayed represent 1 SD from the mean.

Results

Characterization of LAB and AAB populations from wine fermentation process

Figure 1. Sample collection, identification and isolation of LAB and AAB from different wine processing stages. (A) Processing steps of isolating and characterizing LAB and AAB. **(B)** Microbiome compositions of the LAB and AAB enrichment samples determined by 16s amplicon sequencing. Each pie chart represents the average population percentage of 2–3 independently processed samples for each sample type. Arrows indicate the species present from early to end of the processing stages, and other species present at < 1% of total population are grouped together as “other”.

Cabernet Sauvignon fermentation processing samples were collected from a winery in Livermore, California during 2025 harvest season, covering different processing stages of wine fermentation, which includes grape rinse/mash (2 samples), juice/must (3 samples), the end of primary fermentation (3 samples) and the end of malolactic fermentation (3 samples). Grapes used in the process were of local origin grown in Livermore, California. Each wine sample was enriched for LAB and AAB populations separately. Total LAB or AAB populations in each enriched sample were determined via 16S amplicon metagenomic sequencing (**Fig. 1A**). For LAB or AAB, results of the two enriched rinse/mash samples and the three enriched samples from each of the rest processing stages (must/juice, end of primary fermentation, end of malolactic fermentation) were pooled together and the average values were illustrated (**Fig. 1B**). In samples enriched for LAB, *Lactococcus lactis* dominated the LAB in the grape rinse/mash, juice/mast samples and *Lactiplantibacillus plantarum* at the end of primary fermentation (both > 34% in each sample). LAB species that were present throughout from the early stages (rinse/wash or must/juice) to the end of malolactic fermentation were *L. plantarum* and *Levilactobacillus lactis*. In samples enriched for AAB, *Acetobacter oryzoeni* and *Acetobacter pasteurianus* are the two most dominant AAB during the early wine process steps (grape rinse/mash and juice/must) comprising 18–60% of the population in each enrichment. They are also the two AAB species that persisted through all processing steps, though *A. oryzoeni was* not detected at the end of primary fermentation, but re-appeared at the end of malolactic fermentation. Due to the deliberate inoculation of *O. oeni* by the wine makers at the later fermentation stage, and despite their growth being limited under AAB enrichment conditions, *O. oeni* dominated at the end of the malolactic fermentation by taking over 97% and 72% of

the total population in the LAB and AAB enrichments, respectively (**Fig. 1B**). Detailed breakdown of bacterial population in each sample type can be seen in **Supplementary Table S1**. The wine samples (either before enrichment or after enrichment) were also selectively plated for purified LAB and AAB isolates. LAB and AAB strains isolated from these samples were identified for their taxonomy placement based on the 16S rRNA sequencing. Strains representing the species that persisted throughout the fermentation processes (*L. brevis* and *A. pasteurianus*) and an additional *A. fabarum* strain, were selected as the hosts for phage isolation.

Characterization of two lytic Levilactobacillus brevis phages, Indaco_L1 and Indaco_L2, and one temperate phage Brevisa

Using a *L. brevis* strain isolated from juice/must stage as host, two phages named Indaco_L1 (L1) and Indaco_L2 (L2) were isolated from fermented kimchi and wastewater sample, respectively. L1 and L2 have a genome size of 135,623 bp and 141,184 bp, respectively. The genome characteristics of these two phages are summarized in **Table 1** and illustrated in **Fig. 2**. Pairwise intergenomic similarity analysis using VIRIDIC (48) revealed an overall 84.4% DNA similarity between these two phages (Fig. 3B). Most genes with predicted functions are shared between these two phages, including virion morphogenesis modules (terminase, head and tail proteins) and DNA regulatory genes (**Fig. 2A**). Nucleotide level comparison indicated the closest relative in the current NCBI database to these two phages are three other *Lactobacillus* phages under the ICTV Genus *Tybeckvirus*, SAC12B (NC_048754.1), 521B (NC_048752.1) and ENFP1 (OM293948) (**Fig. 2B**). L1 and L2 sharing 73.3%-81.3%, which is less than 95% overall DNA identity with these three phages, suggesting L1 and L2 each comprises a new ICTV species under the Genus *Tybeckvirus*. On protein level, L1 shares 162–168 (out of 196 total proteins), and L2 shares 168–173 (out of 200 total proteins) conserved proteins with these three phages. L1 and L2 are predicted to have a strict virulent lifestyle, as no lysogenic associated genes (such as integrase and repressors) were identified in their genomes.

Table 1. Genome characteristics of the phages reported in this study.

The *L. brevis* strain used in this study also produced a temperate phage, named phage Brevisa, which was spontaneously induced from a prophage on its genome. Brevisa has a genome length of 45,654 bp with 71 predicted gene-coding sequences (**Table 1**). Structural (head and tail) genes were identified, the presence of genes coding for a tail tube, tail sheath and the several baseplate components indicate Brevisa is a myophage (Fig. 3). Brevisa is not related to any phages currently in the database on the nucleotide level. It shares only 23 proteins with *Lactobacillus* phage Lb (MG020111.1), and 17 proteins with *Lactobacillus* phage LBR48 (GU967410.1). Conservation between Brevisa and the prophage elements located in the genome entries (> 50% overall DNA identity) was evident in three strains, *L. brevis* strains CHEE98 (CP168078.1), PL102 (CP103391.1), and CGMCC NO3414 (CP195892.1), where Brevisa shared > 95% identity over 72–81% genome length. Similar to the phage lambda repressor regions, it has two Cro/CI like repressor proteins encoded on divergent transcripts indicating the presence of a lysis/lysogeny switch. Another interesting feature of this phage is that it encodes two

adjacently located Asp23-like alkaline shock proteins (InterPro IPR005531), which was characterized as a membrane-associated protein in *Staphylococcus aureus* controlling the response to environmental stress such as alkaline shock (51, 52). The Asp23 protein in *Lactobacillus casei* was shown to be important in regulating the antibiotic-induced stress (53, 54). The possession of this protein in Brevisa as a prophage form may aid in the host bacteria's resistance to environmental stress including the low pH wine-making environment.

Figure 2. Genomic comparison of LAB phages Indaco_L1, Indaco_L2, 512B, SAC12B, and ENFP1. (A) comparative genome maps of phage Indaco_L1 and L2. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend. Shared protein between two genomes are linked and the percent identifies are illustrated. **(B) Pairwise intergenomic similarity analysis of the LAB phages with DNA sequence similarity calculated by VIRIDIC.**

Figure 3. Genome map of phage Brevisa. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend.

Characterization of two novel Acetobacter phages, Vinphasol_A1 and Vinphasol_L2

Two AAB strains isolated from the early processing stages (grape rinse and grape mash), identified as *A. pasteurianus* and *A. fabarum*, respectively, were used for phage isolation. Two phages named Vinohasol_A1 (targeting *A. pasteurianus*) and Vinphasol_A2 (targeting *A. fabarum*) were isolated and characterized (**Table 1**). Both genomes appeared to be highly novel and unclassifiable by the International Committee on Taxonomy of Viruses (ICTV) as they share no DNA relationships to any phages in the current *Caudoviricetes* class.

Vinphasol_A1 is a "jumbo" phage with an extremely large genome of 397,952 bp. Its genome has a GC content of 26%, significantly lower than the ~ 52% GC content of its *A. pasteurianus* host (unpublished host genome data). It forms clear plaques surrounded by halos on its *Acetobacter* host lawns. There is little detectable DNA sequence similarity between phage A1 and any other organisms in the NCBI nucleotide database (< 2% alignable DNA sequence identity by BLASTn). On the protein level, it shares 113 proteins (out of 579 total proteins) with another jumbo phage *Agrobacterium* phage Atu_ph07 with a 490 kb genome (accession number MF403008). The A1 genome lacks clear modular organization, with identifiable head and tail genes separated apart throughout the genome. Out of the lysis genes, only an endolysin gene and an outer membrane protein sharing homology with o-spanin genes were identified. One notable feature of this jumbo phage is that it has multiple genes that appear interrupted, with separate genes encoding N-terminal and the C-terminal fragments of the same protein, respectively. These types of interrupted genes include genes for recA protein, terminase large subunit, DNA topoisomerase II, ribonucleotide-diphosphate reductase subunit beta, tRNA synthetase, and TnpB-like nuclease. Phage A1 also has genes that have duplicated copies, such as three copies of terminase large

subunits, five copies of DNA polymerase, and 14 copies of the RNA-guided DNA endonuclease. Due to its large size, the detailed genome map of phage A1 is provided as the **Supplementary Fig. 1**.

Figure 4. Genome map of phage Vinphasol_A2. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend.

Vinphasol_A2 has a genome size of 56,636 bp and a GC content of 47%. Its detailed genome map is illustrated in **Fig. 4**. Structural (head and tail) genes and lysis genes (endolysin and holin) were identified in the genome. The genome shares no DNA identity with any of the phages or prophage elements in the current NCBI database. Out of its 78 predicted proteins, it shares 40 proteins with several *Pseudomonas* phages including phage vB_Pae_SG_WM_Sew_P3 (PP740684.1), BL5 (PP791527.1), JG012 (KX898399), PaMx25 (JQ067084.3), vB_PaeS_PAJD-1 (NC_072500.1) and Quinobequin-P09 (MN504636.1), and shares 39 proteins with the *Xanthomonas* phage vB_Xar_IVIA-DoCa3 (ON911540.2), and 36 proteins with *Acinetobacter* phage HD01 (PQ437329.1). One notable feature of the phage A2 genome is that it has a gene encoding GTP cyclohydrolase I, as well as genes QueCDE encoding enzymes involved in tRNA-modifying queuosine biosynthesis (6-pyruvoyl tetrahydropterin synthase DueD, 7-cyano-7-deazaguanine synthases QueC and QueE).

Efficacy of phage inhibition of LAB and AAB in acidic and ethanol environments

Phage efficacy in inhibiting LAB and AAB growth was evaluated both in MRS media as well as in grape juice and wine supplemented with MRS (at a ratio of 8:2, juice/wine to MRS) to evaluate how phage could perform in simulated wine fermentation conditions. Juice/wine was supplemented with MRS to ensure the detectable growth of the bacterial strains under laboratory conditions during the experimental time frames. The strains of *L. brevis*, *A. pasteurianus* and *A. fabarum* were inoculated at a starting bacterial levels of ~ 10^5 cfu/ml, and phages Indaco_L1 (targeting L. brevis), Vinphasol_A2 (targeting *A. fabarum*) and Vinphasol_A1 (targeting *A. pasteurianus*) were added to cultures at a target MOI of 100 (Fig. 5A, 5B, and 5C, respectively). Given the similarity of Indaco_L2 to Indaco_L1, only Indaco_L1 was assayed. During the 90-h static incubation at ambient temperature of 21.1°C to approximate wine fermentation conditions, significant phage inhibition of the bacterial growth was evident compared to the non-phage treated control. In MRS media (pH 6.7), all three phages reliably inhibited their host growth compared to the no-phage control cultures. Phage Indaco_L1 inhibited *L. brevis* growth in MRS for up to 48 h, followed by a rapid rise in bacterial growth, although never reaching that of the no-phage control (**Fig. 5A**). There was a gradual increase of the *Acetobacter* growth in Vinphasol_A1 and A2 treated cultures and variation amongst replicates increased in the later stage of the experiment (reflected by increasing standard deviation values) (**Fig. 5B, 5C**). A similar effect was observed in the grape juice + MRS condition (pH 5.2), where compared to the no-phage control, bacterial growth was effectively inhibited for a minimum of ~ 48 hours. This was followed by a gradual increase in bacterial growth for *L. brevis* and *A. pasteurianus*, with the exception of Vinphasol_A2, which was capable of nearly completely

inhibiting *A. fabarum* growth throughout the experiment. Finally, despite supplementation with MRS media, the tested strains showed little growth in the wine condition (pH 4.5, 10.8% ethanol, v/v) from either the phage-treated and no-phage treated control cultures, likely due to the high ethanol content and added sulfites from the commercial wine sample used to set up the assay. Regardless of this limited growth, a trend can be seen for a reduction in bacterial culture OD in the phage-treated samples, particularly near the end of the experiment (**Fig. 5**).

It was found that the *A. pasteurianus* and *A. fabarum* strains used in this study could grow reliably in white grape juice even without MRS supplementation. Therefore, as a final evaluation of phages' potential as an antimicrobial in a wine production setting, phages Vinphasol_A1 and Vinphasol_A2 were tested for their bacterial inhibition efficacy over a one-week period in pure white grape juice (**Fig. 6**). The *L. brevis* strain could not grow reliably in grape juice without MRS supplementation; therefore, it was excluded from this assay. *A. pasteurianus* and *A. fabarum* were inoculated at 10^2 – 10^3 cfu/ml to pure grape juice (pH 4.2), followed by phage addition. As expected, no-phage controls achieved detectable growth after one day and significant growth of both *Acetobacter* cultures were evident by day 4, with *A. fabarum* reaching a maximum OD of ~ 0.5 and *A. pasteurianus* only reaching a max OD reading of ~ 0.2 by day 7. Similar to the phage inhibition effect seen in **Fig. 5B and 5C**, the bacterial growth in phage treated cultures was effectively inhibited for up to 4 days, after which there was a gradual rise in culture growth OD, although never to the level of the no-phage controls in the duration of the experiment. Together, results from **Fig. 5** and **Fig. 6** suggest that addition of single phages at the start of the experiments can effectively suppress the growth of LAB and AAB over long periods of time (up to a week) across a variety of conditions associated with wine manufacturing.

Figure 5. Phage efficacy in inhibiting LAB and AAB in acidic and ethanol environments. *L. brevis*, *A. pasteurianus* and *A. fabarum* were inoculated to MRS, white grape juice and white wine supplemented with MRS (at a ratio of 8:2, wine/juice to MRS, v/v) at a starting levels of ~ 10^5 cfu/ml, followed by addition of phages Indaco_L1, Vinphasol_A1, and Vinphasol_A2 to their respective host culture at an MOI of 100. Cultures without phage are included as controls. The cultures were incubated statically at 21°C for 90 h, and the bacterial growth (OD550nm) was monitored using a plate reader. Experiments were set up in triplicate and results are plotted as means with standard deviations.

Discussion

The main aim of this study was to investigate the longitudinal persistence and diversity of LAB and AAB throughout the wine production stages, and to assess the potential of using phages as a form of targeted biocontrol of LAB/AAB species in wine manufacturing as a supplement/alternative to current wine additives and preservatives. Enrichments of LAB and AAB in MRS and GYC (supplemented with acetic acid and ethanol), respectively, for up to 7 days were carried out using the collected wine samples in order to obtain sufficient amounts of bacterial DNA for 16S amplicon-based population composition analysis, as well as to capture a greater diversity of species by allowing proliferation of low-abundance species. It could be argued that the population composition revealed in **Fig. 1B** is biased towards strains

that had a growth advantage under these enrichment conditions, rather than representing the *de novo* population. However, the innate wine microbiome makeup is not the focus of this study, as the study was not designed to be an epidemiologically representative survey nor to monitor the bacterial-associated spoilage quality of wine batches derived from these samples and comparing them to unspoiled batches overtime. Enrichment of microaerophilic LAB species and AAB capable of thriving in acidic enrichment media allowed us to detect potential spoilage contributors at each stage of the process. In fact, the LAB and AAB species identified in the enriched samples are consistent with those frequently reported in the literature associated with wine spoilage (18, 19).

Figure 6. Inhibition of Acetobacter growth using phages in white grape juice. A. pasteurianus and A. fabarum were inoculated at 102–103 cfu/ml to white grape juice (pH4.2), and phages Vinphasol_A1 and Vinphasol_A2 and were added at an MOI of 1000 to their respective host cultures. The cultures were incubated at 21.1°C and the bacterial growth (OD600nm) was monitored over 7 days. Bacterial cultures without phage additions were included as controls (dashed lines) for the phage added cultures (solid lines). Experiments were set up in triplicate and results are plotted as means with standard deviations.

Bacterial populations in both LAB and AAB enrichments were dominated by *Oenococcus oeni* at the end of the malolactic fermentation (**Fig. 1B**), this is due to its deliberate inoculation by winemakers at this stage to improve the organoleptic properties of wine, as it has the capacity to deacidifies wine through its secondary metabolisms (4–6) (55). Despite the heavy dominance of *O. oeni* at this stage, the detection of other species that were also present in the earlier steps (wine/wash and mast/juice steps) were possible, revealing two key LAB species (*L. plantarum* and *L. brevis*) and two AAB species (*A. oryzoeni* and *A. pasteurianus*) in particular. The identification of the persistence of *A. pasteurianus* throughout wine fermentation is not surprising as it is naturally selected as an important vinegar fermentation bacterium due to its abilities to oxidize ethanol, sugar, or sugar alcohol, under the presence of oxygen, to organic acids such as acetic acid and gluconic acid (56).

Using the isolates of the key species persistent through the wine-making steps, phages targeting *L. brevis* and *Acetobacter* were isolated and characterized. During the phage hunts, we identified the temperate phage Brevisa, which was spontaneously induced from the prophage on its *L. brevis* host bacterial genome. Phage Brevisa is featured in this study as its genome is highly novel (not related to any phages in the current database on the nucleotide level), and it encodes two Asp23-like alkaline shock proteins, which was shown to be important in *Staphylococcus aureus* for controlling the response to environmental stress such as alkaline shock (51, 52), and in *Lactobacillus casei* for regulating the antibiotic-induced stress (53, 54). The possession of this protein in Brevisa as a prophage on its host genome may aid in the host bacteria's resistance to environmental stress including the low pH wine-making environment. Among the isolated *L. brevis* phages, while Indaco_L1 and L2 are related to phages in the ICTV's *Tybeckvirus* genus of the *Herelleviridae* family, they are distinct enough to form two novel species (less than 95% overall DNA identity to each other and to any phage in the current *Tybeckvirus* genus). Most LAB phages that are previously reported are isolated using dairy isolates. Results from this study highlight that phages targeting wine fermentation isolates are under studied.

The two *Acetobacter* phages reported in this study cannot be classified taxonomically in the current *Caudoviricetes* class as they share little DNA sequence similarity to other phages in the NCBI database. This study therefore contributed to the limited number of *Acetobacter* phages reported (30), by characterizing two novel *Acetobacter* phages, including to our knowledge, a first report of a jumbo phage of *Acetobacter*. Phages that have genomes over 200 kb are classified as “jumbo” phages, and phage Vinphasol_A1 in this study has a genome of 397 kb, larger than most of the jumbo phages reported (57). This jumbo phage exhibits some remarkable features previously reported for other jumbo phages (57, 58). For example, its genome is non-modular with genes of similar functions scattered rather than forming clusters. It has genes that exist in duplications and it is not known if all copies are functional. For example, it has three copies of terminase large subunits and five copies of DNA polymerase. One remarkable feature of this jumbo phage is that it encodes 14 copies of the RNA-guided DNA endonuclease sharing high homology to CRISPR–Cas12 effectors (such as PDB 8H1J_A and 7ODF_A, > 99% Hhpred probability) evolved from the transposon-associated TnpB proteins (59, 60). Acquisition of these CRISPR-Cas proteins spreading throughput this jumbo phage’s large genome highlights the evolutionary arms race between jumbo phages with host bacteria and competing viruses. Another novel *Acetobacter* phage reported in this study, Vinphasol_A2, presents a noticeable feature which is the possession of genes encoding enzymes involved in queuosine biosynthesis (GTP cyclohydrolase I, and QueD, QueC, and QueE)(61). Queuosine is a hyper-modified guanosine analogue that modifies tRNAs at position 34 (62). It was suggested some phages carry these genes to modify DNA as a protection mechanism against host restriction endonucleases (63, 64).

Except for phage Brevisa, which was induced from the prophage on the *L. brevis* host bacterial genome, the rest of the phages described in this study are predicted to have a lytic lifestyle as no lysogenic associated genes (such as integrase and repressors) were identified in their genomes. These traits make them effective bacterial control agents as no lysogens will be formed upon phage infection which will confer rapid development of phage resistance. The efficacy of phages in inhibiting LAB/AAB host growth under conditions simulating wine fermentation was demonstrated in this study. We first tested using grape juice or wine supplemented with MRS to promote bacterial growth. The phage host strains of *L. brevis*, *A. pasteurianus* and *A. fabarum* were inoculated at ~ 10^5 cfu/ml (**Fig. 5**), to simulate the level of LAB/AAB that might be expected to be present at various stages of manufacturing (65–67). Juice supplemented with MRS simulates the application of phage to the juice/must stage of the winemaking process to inhibit or knock down LAB/AAB present at the time of harvest; while the wine supplemented with MRS condition simulates application of phage to the late-stage process of such as aging or bottling. Compared to the control cultures (bacteria only without phage addition), the phage treated cultures in MRS or juice supplemented with MRS conditions showed inhibited growth throughout the experiments. Except for Vinphasol_A2 treated cultures where bacteria growth was completely inhibited throughout the experiment duration (90 h), the phage inhibition effect in the rest cultures was more prominent in the first half of the experiment (up to ~ 48 h), followed by an increase of the bacterial growth, as well as an increase of the variations amongst replicates (reflected by increasing standard deviation values) in phage treated cultures in the later stage of the experiment. This is likely due to

resistance founder effects, in that each replicate culture is likely to have a different number of naturally occurring spontaneous phage resistant mutants at the start of the experiment. In a real-world application, the probability of resistance could be reduced significantly by use of a “phage cocktail” consisting of several phages targeting separate receptors on the host bacterium. The wine supplemented MRS condition used in this study failed to support good growth of the bacterial strains, likely due to the ethanol content (10.8%, v/v) and the sulfites present in the commercial wine sample used to set up the assay. Despite this, there is a small growth of the bacteria towards the end of the experiment, which was absent in the phage treated cultures, highlighting the usefulness of phage as a preventative measure.

We then tested phage efficacy in an experiment using pure grape juice without MRS supplementation to mimic a real world scenario. *A. pasteurianus* and *A. fabarum* were inoculated at 10^2 – 10^3 cfu/ml, consistent with a very low-level AAB presence, but not yet considered to be substantially contributing to spoilage (67) (68). In this proof-of-concept study, a single application of phage at the start of the experiment was sufficient to inhibit the growth of its host in acidic conditions (pH 4.2) for at least 7 days. Based on the applications of phage in other clinical and food safety settings (11, 13), it is expected that this inhibitory effect could be enhanced by using multiple phages (i.e. a phage cocktail) that target different receptors of the host. Phage efficacy demonstrated in this study in low-pH conditions (pH 4.5 in wine plus MRS and pH 4.2 in pure grape juice) over a prolonged period (up to 7 days) highlights their potential for controlling unwanted LAB/AAB in the complex wine microbiome. Even the inhibition of undesirable AAB/LAB targets for a few days could be significant, as it would allow yeasts to begin or finish primary fermentation without competing for nutrients. Additionally, inhibiting undesirable LAB/AAB species with phages during secondary fermentation could allow this process to proceed more quickly without collateral damage to desirable *O. oeni* species.

In conclusion, our results identified the key LAB and AAB species that persist through the wine processing steps and established the earliest stage at which a particular spoilage microbe can be targeted by a phage for precise control. We also isolated and characterized novel phages that target wine-associated strains of *L. brevis*, *A. pasteurianus* and *A. fabarum*. The efficacy demonstrated by the phages to inhibit growth of *L. brevis* and *Acetobacter* over a long period of time (up to 7 days) in conditions simulating wine fermentation suggests their potential application in controlling microbial spoilage during winemaking.

Declarations

Additional Information

Competing Interest Statement

Justin Boeckman, the corresponding author, is an officer and shareholder of Inphasol Inc, a biotechnology company developing phage-based antimicrobial technologies. The research described in

this manuscript relates to technology that may have potential commercial applications for Inphasol Inc.

Acknowledgments

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Data availability statement

Bacteriophage genomes generated as a part of this work are available under NCBI GenBank accession numbers: PZ840260, PZ840261, PZ840259, QB031960, and PZ836639.

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Figures

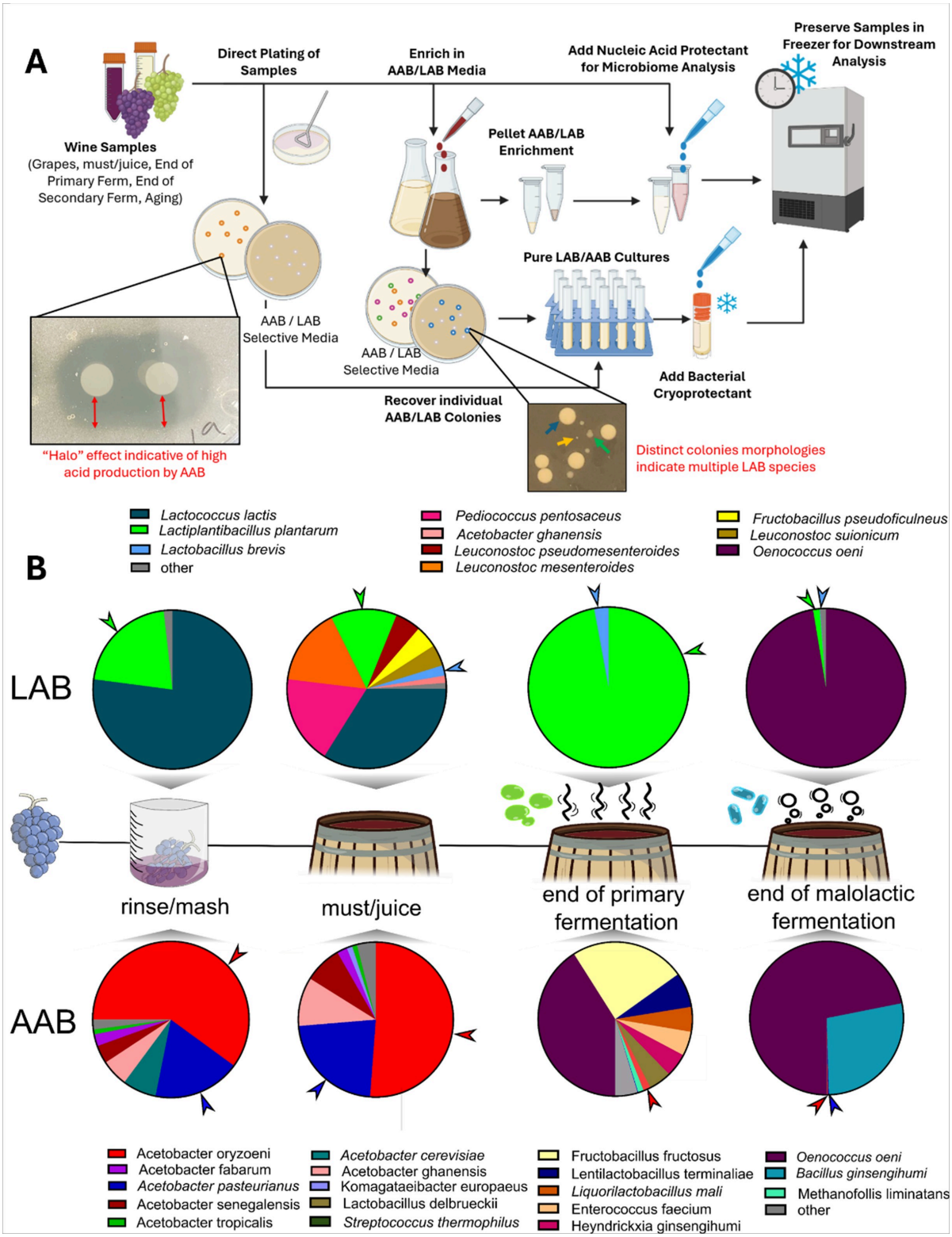


Figure 1

Sample collection, identification and isolation of LAB and AAB from different wine processing stages. **(A)** Processing steps of isolating and characterizing LAB and AAB. **(B)** Microbiome compositions of the LAB and AAB enrichment samples determined by 16s amplicon sequencing. Each pie chart represents the average population percentage of 2-3 independently processed samples for each sample type.

Arrows indicate the species present from early to end of the processing stages, and other species present at <1% of total population are grouped together as “other”.

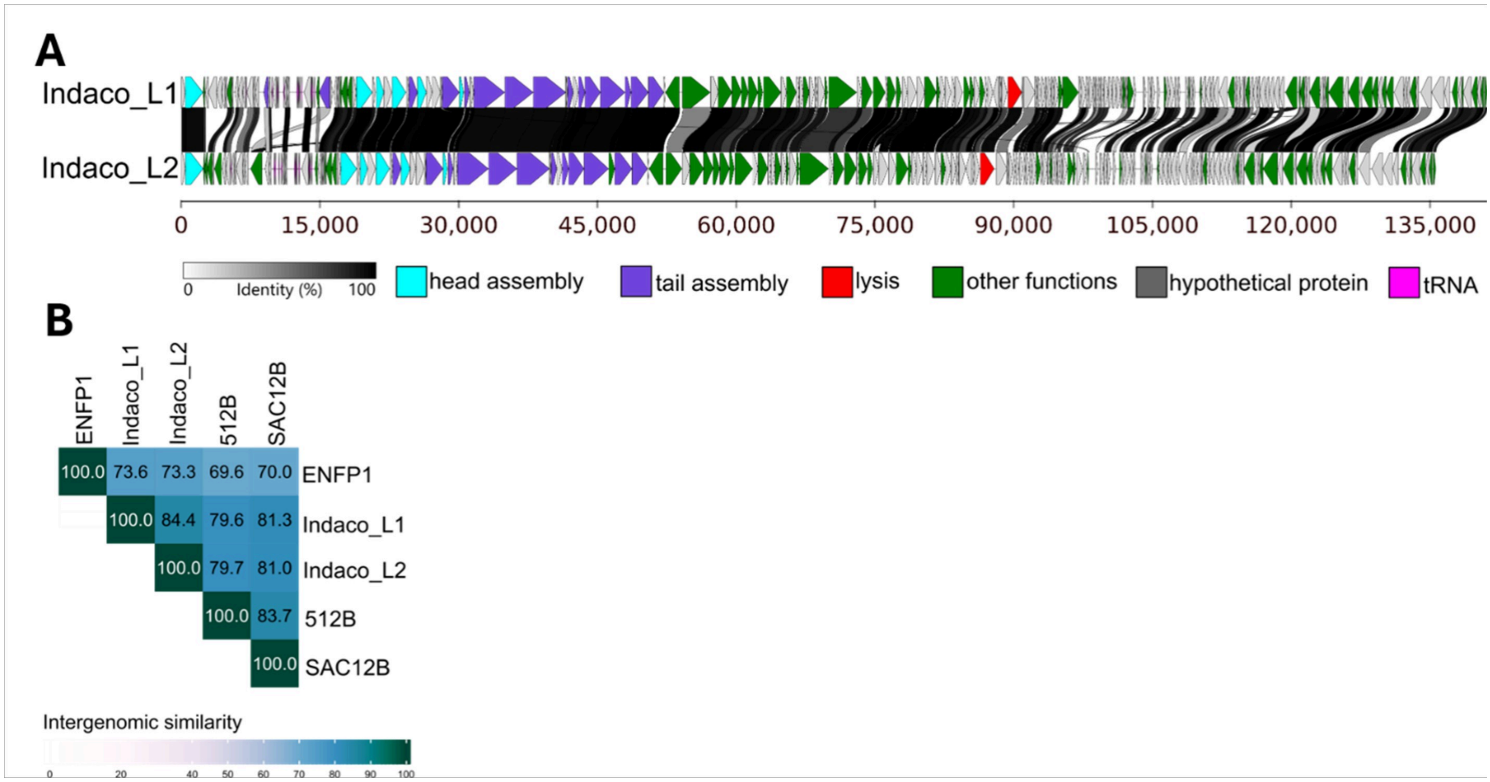


Figure 2

Genomic comparison of LAB phages Indaco_L1, Indaco_L2, 512B, SAC12B, and ENFP1. (A) comparative genome maps of phage Indaco_L1 and L2. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend. Shared protein between two genomes are linked and the percent identifies are illustrated. **(B) Pairwise intergenomic similarity analysis of the LAB phages with DNA sequence similarity calculated by VIRIDIC.**

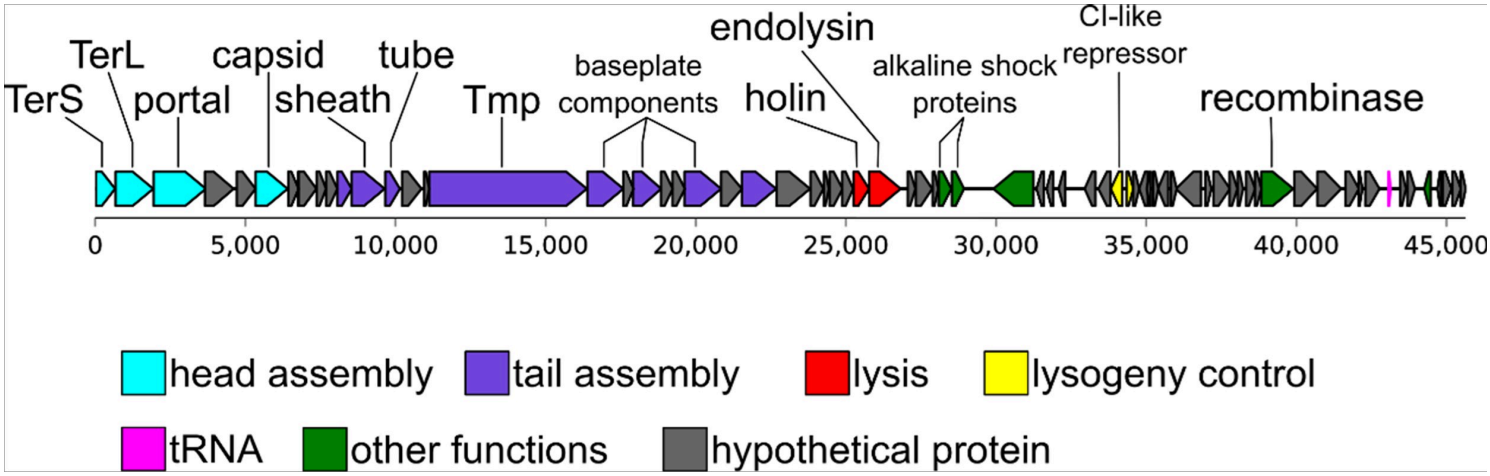


Figure 3

Genome map of phage Brevisa. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend.

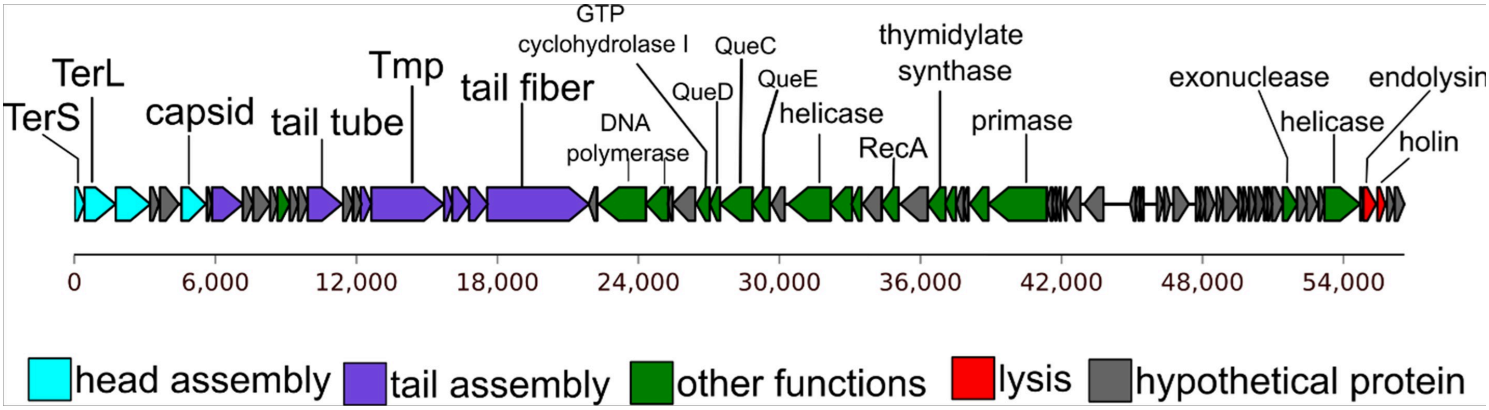


Figure 4

Genome map of phage Vinphasol_A2. Predicted genes are represented by arrows with directions indicating the forward and reverse coding strands, respectively. The ruler below the genomes indicates the scale (in bp). Genome features are color coded according to the legend.

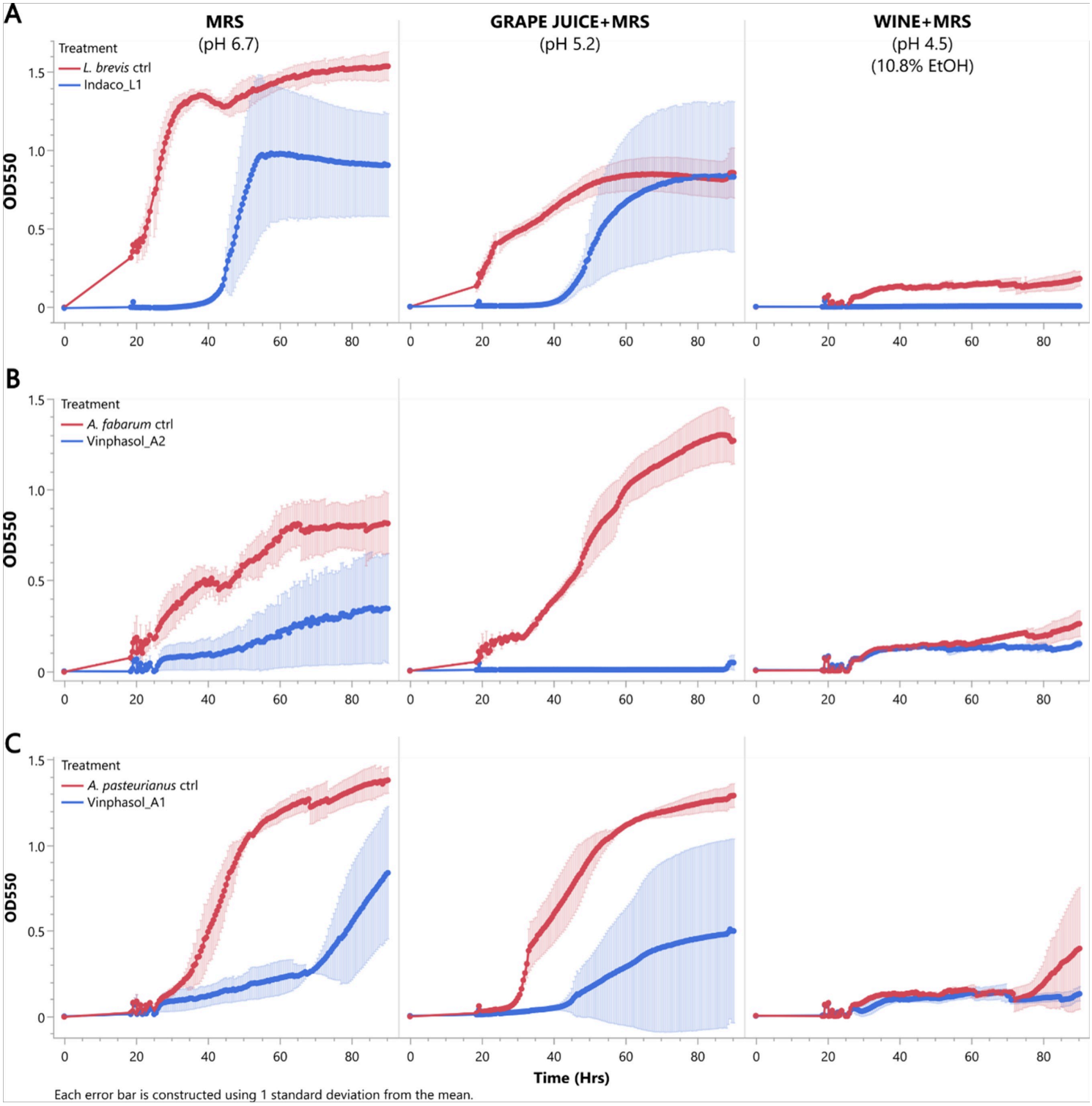


Figure 5

Phage efficacy in inhibiting LAB and AAB in acidic and ethanol environments. *L. brevis*, *A. pasteurianus* and *A. fabarum* were inoculated to MRS, white grape juice and white wine supplemented with MRS (at a ratio of 8:2, wine/juice to MRS, v/v) at a starting levels of ~10^5 cfu/ml, followed by addition of phages Indaco_L1, Vinphasol_A1, and Vinphasol_A2 to their respective host culture at an MOI of 100. Cultures without phage are included as controls. The cultures were incubated statically at 21°C for 90 h, and the

bacterial growth (OD550nm) was monitored using a plate reader. Experiments were set up in triplicate and results are plotted as means with standard deviations.

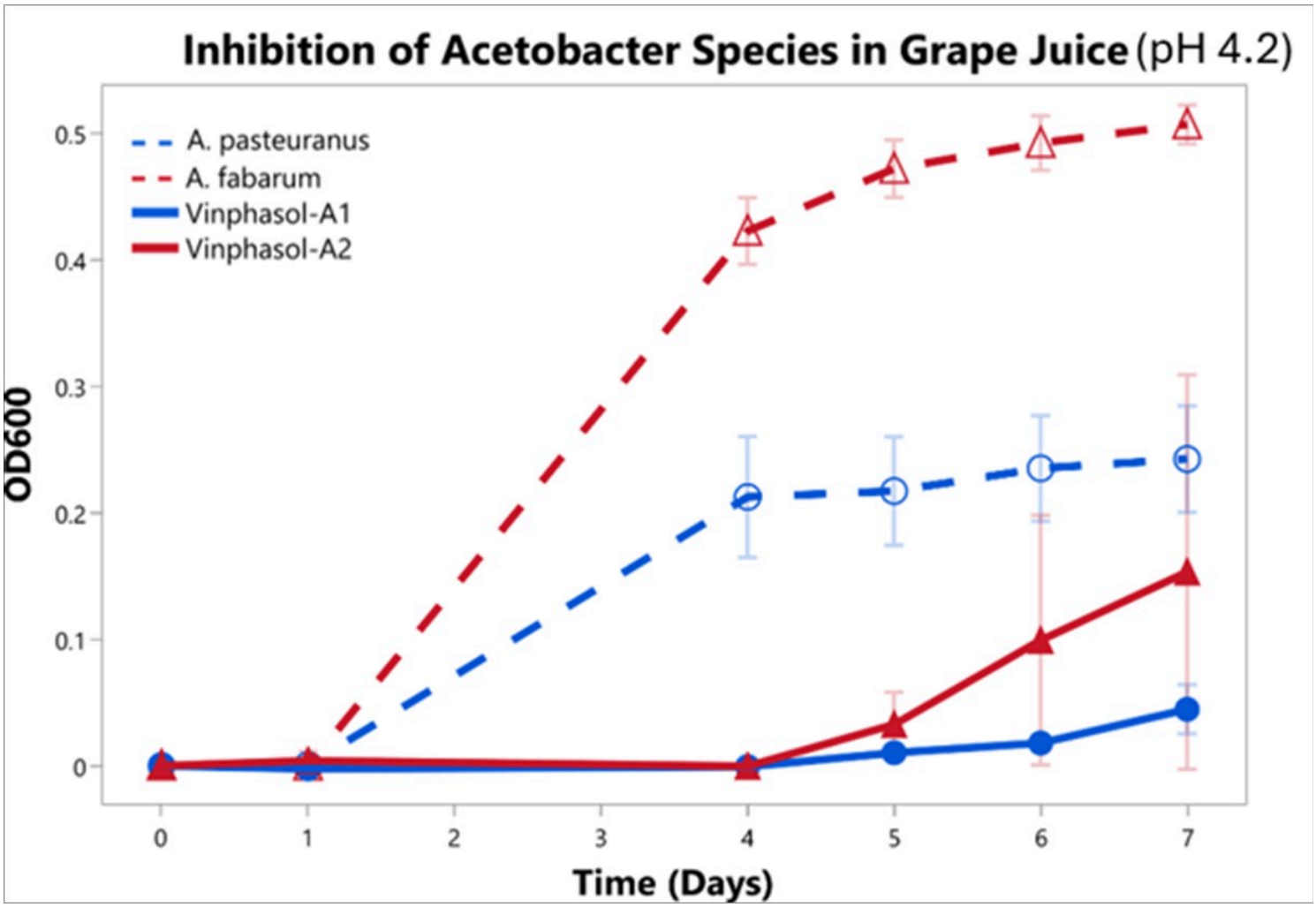


Figure 6

Inhibition of Acetobacter growth using phages in white grape juice. A. pasteurianus and A. fabarum were inoculated at 102 -103 cfu/ml to white grape juice (pH4.2), and phages Vinphasol_A1 and Vinphasol_A2 and were added at an MOI of 1000 to their respective host cultures. The cultures were incubated at 21.1 °C and the bacterial growth (OD600nm) was monitored over 7 days. Bacterial cultures without phage additions were included as controls (dashed lines) for the phage added cultures (solid lines). Experiments were set up in triplicate and results are plotted as means with standard deviations.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure1.pdf
- Table1.png