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2 # Phage-host interactions as a driver of population dynamics during wine
3 fermentation: betting on underdogs

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12

13 ## Abstract

14 Winemaking is a complex process in which numerous microorganisms, mainly yeasts and
15 lactic acid bacteria (LAB), play important roles. After alcoholic fermentation (AF), most
16 wines undergo malolactic fermentation (MLF) to improve their organoleptic properties and
17 microbiological stability. *Oenococcus oeni* is mainly responsible for this crucial process where
18 L-malic acid (MA) in wine converts to softer L-lactic acid. The bacterium is better adapted to
19 the limiting conditions imposed by the wine matrix and performs MLF under regular
20 winemaking conditions, especially in wines with a pH below 3.5. Traditionally, this process
21 has been conducted by the natural microbiota present within the winery. However, the start,
22 duration and qualitative impact of spontaneous MLF are unpredictable, which prompts
23 winemakers to use pure starter cultures of selected bacteria to promote a more reliable,
24 simple, fast and efficient fermentation. Yet, their use does not always ensure a problem-free
25 fermentation. Spontaneous initiation of the process may prove very difficult or does not

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26 occur at all. Such difficulties arise from a combination of factors found in some wines upon
27 the completion of AF (high ethanol concentration, low temperature and pH, low nutrient
28 concentrations, presence of free and bound SO_2). Alongside these well documented facts,
29 research has also provided evidence that negative interactions between *O. oeni* and other
30 biological entities such as yeasts may also impact MLF. Another insufficiently described, but
31 highly significant factor inhibiting bacterial growth is connected to the presence of
32 bacteriophages of *O. oeni* which are frequently associated to musts and wines. The purpose
33 of this review is to summarize the current knowledge about the phage life cycles and
34 possible impacts on the trajectory of the microbiota during winemaking.

35

36 **Highlights**

37 ► Up to date inventory of lytic and temperate oenophages infecting *O. oeni* ► Interactions
38 between MLF bacteria and phages over fermentation and impact on wine quality ►
39 Lysogeny and selection of commercial starters ►Future prospects

40

41 **Keywords**

42 Malolactic Fermentation, Phage diversity, Lysogeny, Predation, Evolution, Starter selection

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44

45 ## 1. Introduction

46 Musts and wines are harsh environments sheltering a diverse microbial population
47 inherited from grape berries, which undergo a succession of microbial community shifts
48 during the winemaking process. The alcoholic fermentation (AF) is first driven by yeasts and
49 is usually followed by the malolactic fermentation (MLF). The lactic acid bacterium (LAB)
50 *Oenococcus oeni* is mostly involved in this transformation, which consists in the
51 decarboxylation of L-malic acid (MA) into L-lactic acid and CO_2. De-acidification will
52 increase microbial stability though the utilization of remaining substrates and/or the
53 production of antimicrobial components. MLF will also influence sensory properties and the
54 wine color profile (Lorentzen and Lucas, 2019; Virdis et al., 2021).

55 Traditionally, MLF has been conducted by the natural microbiota present within the
56 winery, and starts when the LAB population reaches 10^6 CFU/mL (Bartowsky, 2005;
57 Lorentzen and Lucas, 2019). The MLF step is indeed a capricious step and slow or stuck MLF
58 are frequent. Such issues can increase the risk of spoilage by other microorganisms and then
59 decline the quality of wines. To better control MLF, commercial starters have been
60 progressively selected. Despite great progress in the selection of robust strains, their
61 stabilization through freeze-drying and acclimatization before inoculation, and finally use of
62 different inoculation strategies (sequential versus co-inoculation), some wine types are still
63 difficult to ferment. In the same time, natural solutions are searched for and should include
64 environmentally friendly tools to improve the sustainability aspects of winemaking all along
65 the production chain (Nardi, 2020).

66 The most frequently studied causes of problematic MLF have been identified and are well
67 documented (Malherbe et al., 2007). They arise from a combination of factors found in some
68 wines upon the completion of AF (high ethanol concentration, low temperature and pH, low

69 nutrient concentrations, presence of SO_2). Aside from these physicochemical factors, little is
70 known about the involvement of microbiological interactions. Antagonistic interactions
71 between wine yeasts and LAB have been reported (Balmaseda et al., 2018; Virdis et al., 2021)
72 showing that inhibitory metabolites produced by yeasts may also have an important role in
73 the correct development of MLF. As natural predators of bacteria, bacteriophages may also
74 impact the population of *O. oeni* over winemaking. High viral lysis pressure on the host may
75 exert a major impact on bacterial diversity and the community structure (Braga et al., 2020).
76 More importantly, for most phages infecting *O. oeni* (also named oenophages), lysis is not the
77 only possible infection outcome. Most *O. oeni* strains are known to bear one or more
78 prophages, which are released in wine upon their spontaneous induction from lysogens
79 (Claisse et al., 2021).

80 Evidences about the crucial role of phages on ecosystem functions start to accumulate
81 from other environments such as oceans, animal gut, or fermented foods (Braga et al., 2020;
82 Paillet and Dugat-Bony, 2021). This may be time to look back on the knowledge gleaned on
83 phage-host interactions in wine over the last 40 years. The objective of this review is to
84 provide an update on the current knowledge and research on phage-host interactions in the
85 wine LAB *O. oeni* and outline significant questions for future research. An analysis of the
86 main structural and genomic characteristics of oenophages characterized during past
87 decades will be given. Next the phage life cycles will be outlined, with a focus on the lytic-
88 lysogeny decisions and co-evolution between the host and its phages. Finaly, a perspective
89 on the impact of lysogeny in the area of starter selection is discussed.

90

91 2. Isolation and life cycles of oenophages

92 *2.1. The isolation of oenophages from the enological environment*

93 Transmission electron microscopy (TEM) provided the earliest evidence of viral
94 abundance in diverse red and white grape varieties in Switzerland (Cazelle and Gnaegi,
95 1982; Gnaegi, 1983; Gnaegi et al., 1984; Sozzi et al., 1976, 1982), Australia (Henick-Kling et al.,
96 1986a) and Germany (Arendt and Hammes, 1992). Although most wines under study had
97 been inoculated by *O. oeni* specific strains, additional LAB were also part of the indigenous
98 microbiota, such as pediococci, which are considered undesirable bacteria because of flavor
99 depreciation and ropiness. An essential step was therefore to establish that the observed viral
100 particles corresponded indeed to oenophages. To this aim, researchers exploited their own
101 strain collections and wines in order to find the key sensitive strains of *O. oeni* that would
102 serve as a host indicator to propagate the phages (Henick-Kling et al., 1986a). Most teams
103 successfully isolated oenophages from wines by plating filtered samples directly with a
104 sensitive host and looking for spot clearing or plaque formation using the double-agar plate
105 (DAP) assay (Gratia, 1936). Philippe et al. (2017) later published an alternative enrichment
106 culture method that might be useful when phage concentrations in samples are very low.
107 Unfiltered samples were diluted into MRS broth containing pimaricin, an antimycotic
108 compound killing yeasts, molds and other fungi and incubated during 5 days prior to
109 plating. Phage lytic growth during this initial step was expected to be enhanced, while
110 natural lysogens were also likely to release prophages.

111 Since the 80's, the DAP technique has been the gold standard method to detect
112 oenophages over winemaking, in crushed fruits, musts, as well as over AF and MLF. A list of
113 major phage biodiversity surveys in enological environment is given in **Table S1**. Trials to
114 isolate oenophages from grape berries and vine leaves have been so far unsuccessful
115 (Jaomanjaka et al., 2013; Nel et al., 1987), possibly due to low concentrations of metabolically
116 active bacteria at these steps of the winemaking process. Of note, oenophages have been

117 isolated from materials such as second hand barrels (Berthelot, 2000). This may guide future
118 surveillance in this neglected reservoir where *O. oeni* forms biofilms which confer specific
119 technological properties to wine associated with the chemical transfers occurring at the
120 wood/wine interface (Coelho et al., 2019). More importantly the DAP method also gave first
121 insights into the viral community of wines. Plaques with distinct sizes, aspects and turbidity
122 morphotypes could be repeatedly observed, reflecting phage diversity in wine samples
123 (Philippe et al., 2017; Sozzi et al., 1976, 1982). Upon excision, several rounds of purification
124 using the streak plating purification method were necessary to obtain a pure lysate for each
125 individual phage, before subsequent characterization.
126
127 *2.2. Strains as a reservoir for oenophages.*
128 Most references related to the isolation of oenophages constantly reported the observation
129 of turbid plaques on bacterial host lawns, and turbid plaques are the typical plaque
130 morphology of temperate phages. It was therefore logical to screen bacterial isolates for
131 lysogeny. Accordingly, cultivation-based assessment of lysogeny in the *O. oeni* species
132 mobilized the community of microbiologists from all wine producing countries. The first
133 demonstration of lysogeny amongst MLF bacteria was provided by Lee (1978) using
134 mitomycin C (MC) as the inducing agent. This alkylating agent forms adducts with DNA
135 and triggers prophage induction through bacterial DNA damage SOS response. Induction
136 was carried out on early exponential cultures grown in MRS broth (OD 0.3-0.5) (**Table 1**).
137 The test was considered as positive when lysis in MC-treated cultures was higher than in the
138 control without MC. Chemical treatments of lysogenic strains with 0.5 to 1 µg/mL were the
139 most effective at inducing phage production in *O. oeni* (Arendt et al., 1990, 1991; Cavin et al.,
140 1991; Doria et al., 2013; Huang et al., 1996; Jaomanjaka et al., 2013; Poblet-Icart et al., 1998;

141 Tenreiro et al., 1993). Additional experimental verifications consisted in checking that the
142 MC-treated culture supernatants formed lysis plaques on a sensitive strain, and not on the
143 original lysogenic strain due to immunity to superinfection (Arendt and Hammes, 1992;
144 Boizet et al., 1992; Huang et al., 1996; Tenreiro et al., 1993). A better efficacy of prophage
145 release during UV treatment was reported by Doria et al. (2013) (**Table 1**).

146 Overall, there was a good correlation between lysis under the inducing agent and plaque
147 forming capacity on a sensitive bacterial lawn. A few exceptions were observed including
148 data by Tenreiro et al. (1993), who observed no MC-induced lysis for some strains, even
149 though the supernatants produced lysis plaques on a host. In contrast, other authors
150 observed lysis in MC-treated cultures without the concomitant detection of phages,
151 suggesting the presence of defective phages and/or the lack of indicator strains in the panel
152 used (Arendt et al., 1991). Finding a host to propagate a given induced phage is not as easy,
153 and an alternative was proposed by Cavin et al. (1991) who successfully tested a MC-based
154 curing treatment to isolate sensitive strains of *O. oeni*.

155 Convincing arguments for a high incidence of lysogeny in the species were provided by
156 independant research groups, with frequencies ranging from 45% (Huang et al., 1996; Poblet-
157 Icart et al., 1998) to 66% (Tenreiro et al., 1993) in different sets of strains. Molecular biological
158 tools such as genetic mapping of strains (Zé-Zé et al., 2008), genomic substractive
159 hybridization (Bon et al., 2009) and finally high throughput genome comparisons (Borneman
160 et al., 2010; Claisse et al., 2021) later confirmed that bacterial genomes are replete with
161 prophages. Fast-sequencing throughput methods have increased the number of completely
162 and publically available sequenced bacterial genomes to about 247 by february 2022, with the
163 number increasing rapidly. Sequence-based explorations reported rates of 64%, 78% and 58%
164 using sets of 14 (Borneman et al., 2010, 2012), 28 (Jaomanjaka et al., 2013) and 231 strains

165 (Claisse et al., 2021), respectively. In the later study, lysogens mostly harbored a single
166 prophage (64.2%). Yet, a total of 48 poly-lysogens were identified and contained two (n=43)
167 or three distinct prophages (n=5). Last, a bacteriophage sequence was shown to be tandemly
168 duplicated at the integration site (Borneman et al., 2012).
169
170 *2.3. Other phage life cycles*
171 The existence of virulent oenophages was first suggested by Arendt et al. (1990) during
172 the preparation of starter cultures. Strain 58N exhibited an unusual lysis and either
173 spontaneous and MC-inductions of the culture resulted in the isolation of two nearly
174 identical phages, namely P58I and P58II. No homology was observed between both phage
175 DNAs and the chromosomal DNA. In addition, phage-free cultures of strain 58N could be
176 isolated upon successive single-colony isolations. Both observations suggested unstable
177 lysogeny in the cultures. Of note, *O. oeni* 58N strain was not immune any more to phage
178 P58I. Its virulent behavior was proposed to result from a mutational event occurring into the
179 prophage and leading to a poor repressor/operator affinity. Santos et al. (1996) and
180 Jaomanjaka et al. (2016) made similar observations, and OE33PA was the first ex-temperate
181 oenophages (phages which lost their ability to initiate and/or maintain lysogeny) to be
182 characterized (Jaomanjaka et al., 2016).
183 These data also raised a number of questions regarding whether oenophages can establish
184 a relationship with their hosts where bacteria and phages remain associated in equilibrium.
185 Such persistent forms of infection have been described in virulent phages, and include the
186 carrier state life cycle, which is also often observed during nutrient-limited conditions. Under
187 these circumstances, phages can remain associated with a compatible host and continue to
188 produce free virions to prospect for new hosts. The possible implications of this specific

189 phage-host interaction, both with respect to phage evolution and phage adaptation to new
190 hosts have been recently discussed in the dairy LAB *Lc. lactis* (Marcelli et al., 2020) and
191 deserve more work in *O. oeni*.

192 While microbial communities seemed to be shaped through ecological interactions
193 between *O. oeni*, temperate and ex-temperate phages, the lack of strictly lytic oenophages
194 was intriguing, and could be interpreted as an indication of an insufficiently sampled
195 environment. In 2013, the whole oenological reservoir was explored for this specific phage
196 hunt (Philippe et al., 2017). The strategy was to sample all wine-types and essential steps of
197 the winemaking process (crushed grapes before fermentation, also named must; AF; MLF
198 and aging wine in bottle) and to achieve a faster processing of the 166 samples as possible. A
199 set of 30 phages producing clear plaques were isolated from the early steps of winemaking,
200 including the representative strictly lytic Vinitor 162 phage (Philippe et al., 2021).

201

202 **3. Main characteristics of oenophages**

203

204 *3.1. Oenophages electron microscopy and protein profiles*

205 Transmission electron microscopy (TEM*)* was used to observe more than 45 oenophages
206 isolated from the Northern and Southern Hemispheres. They all possess icosahedral heads
207 and long non-contractile tails (**Fig. 1; Table S2**) and are morphologically very similar to other
208 characterized LAB phages, bearing in mind that most are of dairy origin (White et al., 2022).
209 Phages able to infect other wine-associated LAB species such as *Lactiplantibacillus plantarum,*
210 as well as *Leuconostoc* and *Weisella* species associated to various vegetable fermentations are
211 more diverse and include tailless particles as well as phages with contractile tails (Kot et al.,
212 2014; Kyrkou et al., 2019, 2020).

213 Head and tail structures vary in size among oenophages. Up to a twofold difference in
214 phage-particle size was observed in lysates. LTH33P (head 75 ± 5 nm and tail 303 ± 20 nm)
215 isolated in Germany (Schwarzriesling) is the larger oenophage observed so far (Arendt and
216 Hammes, 1992). Santos et al. (1996) observed the smaller phage fogPSU-1 (head 34-43 nm
217 and tail 228-243 nm) upon the MC treatment of a commercial starter (Tenreiro et al., 1993)
218 (**Table S2**). The dimensions of the recently characterized strictly lytic phage Vinitor 162
219 (head 55 ± 3 nm and tail 205 ± 8 nm) fell in this range. Of note, Vinitor 162 differed from
220 other oenophages by presence of an extended unique thin tail fiber at the extremity of its tail
221 (Philippe et al., 2021). The authors suggested that the tail extension harbors the receptor
222 binding modules (carbohydrate binding modules) and constitutes therefore the *bona fide*
223 receptor binding protein of the phage.

224 Consistent with the results generated using TEM, oenophages could also be
225 distinguished based on the number and sizes of major structural proteins. Using one-
226 dimensional SDS gels, particles were shown to contain 1 to 5 major proteins, in the molecular
227 weight range of 14 to 46.5 kDa (**Table 2**). Phages were divided into 3 (Arendt and Hammes,
228 1992; Arendt et al., 1991) or 4 groups (Santos et al., 1996).

229

230 *3.2. Host ranges and burst sizes*

231 The host range of a bacteriophage is defined by what bacterial genera, species and strains
232 it can infect. As seen in **Table 2**, the host ranges of oenophages encompassed just certain
233 strains of *O. oeni*. Phages 84, Φ1002, fog30, OE33PA and Vinitor162 could not infect any
234 strains of the related *O. kitaharae* species, nor bacteria from different LAB genera such as
235 Pediococci, Lactiplantibacilli, Lactococci and *Leuconostoc* (**Table 3**).

236 The quantitative study of the life cycle of oenophages was characterized by one-step
237 growth experiments. Data were obtained for 6 oenophages, namely P58I, P58II, Φ1002,
238 LcO23, S1.1S and OE33PA. The most rapid phage growth in MRS broth was observed with
239 Φ1002 which had a relatively short latent period (60 min), followed by a rise period of 100
240 min. The burst sizes all 6 tested oenophages were calculated to be approximately 16-20 to 55
241 phage particles per infected bacterial cell (**Table 3**).

242

243 *3.3. Oenophages and their genomes*

244 As from 2005, NGS technologies combined with sophisticated bioinformatics changed the
245 field of phage studies in *O. oeni* (Mills et al., 2005). Such innovations enabled a drastic
246 increase in available sequence data (prophages and virulent phages) and paved the way to
247 comparative genomic analyses. When processing these data, we and others could measure
248 the importance and accuracy of the work carried out over previous decades using more time-
249 consuming and technically-demanding strategies.

250 During the pre-genomic era, restriction enzymes proved to be useful for the physical
251 dissection of the double-stranded DNA genomes of around fifteen oenophages. Restriction
252 patterns of the genomes with various endonucleases (*Bam*HI, *Blg*I, *Eco*RI, *Eco*RV, *Hin*dIII,
253 *Hinf*I, *Nde*I, *Stu*I) showed unambiguous differences between the temperate phages infecting
254 *O. oeni*, which distributed into 3 to 6 groups (**Table 2**). The sizes of the phage genomes could
255 also be deduced from the sum of the restriction fragments and ranged from 25.3 kb (phage
256 P58I) to 40 kb (phage fog30) (Arendt et al., 1990; Santos et al., 1998). In 2021, the full genomes
257 of 189 temperate and lytic oenophages, of various geographical origins and types of beverage
258 (red, dry and sweet white wines, sparkling wines, cider and kombucha tea) have become
259 available (Claisse et al., 2021; Jaomanjaka et al., 2018; Philippe et al., 2021). Their comparison

260 confirmed the limited variation in genome size (ranging from 35 to 46.2 kb) amongst
261 oenophages and their uniform distribution across this interval. Of note, no phage holding a
262 genome of about 25 kb resembling those earlier observed by Arendt et al. (1990) was
263 identified. As the set of genomes analyzed contained most exclusively temperate phages, it is
264 therefore likely that efforts are still needed to expand the repertoire of lytic phages infecting
265 *O. oeni*.

266 All sequenced oenophages (lytic, temperate and ex-temperate) share the same synteny in
267 terms of genome organization, with te replication module followed by modules for DNA
268 packaging, morphogenesis, and lysis (Borneman et al., 2012; Chaïb et al., 2019; Jaomanjaka et
269 al., 2016; Philippe et al., 2021) (**Fig. 2**). Host lysis relies on the viral endolysin and holin
270 activities (Nascimento et al., 2008; São-José et al., 2000). Of note, the Lys44 lysin encoded by
271 phage fOg44 has brought to light a new mechanism for endolysin targeting the
272 peptidoglycan since it contains a peptide signal which allows its secretion to the periplasm.
273 The peptide was later shown to be exported by the Sec machinery from the moment of its
274 synthesis, and kept in an inactive state in the murein layer. Local conditions may inhibit the
275 catalytic site of the enzyme, until the proper moment for lysis occurs (São-José et al., 2000).
276 Oenophage gene sequences specifying endolysins are polymorphic (Borneman et al., 2012;
277 Doria et al., 2013). The substrate recognition and optimum activity of such enzymes are not
278 known.

279 Most temperate phages harbor two additional modules (**Fig. 2**). First prophages harbor
280 unique complements of morons ("more DNA") organized in a specific region downstream of
281 the lysis module. Accretion of such elements is suggested to increase host fitness by
282 encoding novel genes that make the bacteria more competitive in an old niche or allow them
283 to exploit a new niche (Cumby et al., 2012).

A module at the left end of the genome is dedicated to lysogeny establishment and maintenance of temperate phages (**Fig. 2**). To carry out the site- specific recombination (SSR), such phages utilize an integrase of the tysosine integrase family, of which *Escherichia coli* phage λ is the prototypical example. The two major constituents of the SSR unit, corresponding to the phage integrases and attachment sites (*attB*), have been studied in detail and this resulted in the clustering of prophages into six major groups (Int$_A$ to Int$_F$), which are related to the integration site in the host chromosome (Claisse et al., 2021; Gindreau et al., 1997; Jaomanjaka et al., 2013; Parreira et al., 1999). All known members of a group included: 1) high identity of the integrase sequences (> 98% at the amino acid level) and 2) tropism for a specific bacterial attachment site corresponding to the 3′ end of a tRNA gene. All identified integrases were observed to preferentially drive prophage integration into their expected cognate site, except some Int$_B$ prophages (22%) which become integrated at a secondary *att* site (Claisse et al., 2021; São-José et al., 2004).

Prophage genomes in wine strains belong to four groups (Int$_A$ to Int$_D$) while Int$_E$ and Int$_F$ phages are likely to be associated with cider and kombucha strains, respectively. All prophage genomes are architecturally mosaics. In particular, temperate phages of the Int$_A$, Int$_B$ and Int$_C$ groups are uniquely interconnected from an evolutionary perspective and undergo multiple events of genetic exchange, possibly in response to the selective pressure of their hosts, which drives their diversity (**Fig. 3**). Such homologies had been earlier observed during hybridization experiments between labeled phage DNA and the restriction fragments obtained from other phage genomes (Arendt and Hammes, 1992; Boizet et al., 1992; Nel, et al., 1987; Santos et al., 1996; Tenreiro et al., 1993).

The dominance of Int$_A$ prophages has been reported in wine-related strains (Chaïb et al., 2022; Claisse et al., 2021). They also represent most of the free-replicating phages isolated

from the oenological environment (Philippe et al., 2017). The intra-group relatedness reveals a continuum of genetic diversity which is shown in **Fig. 4 A**.

The reconstructed phylogeny of temperate oenophages showed that the sequences of Int$_D$ phages segregated into a distinct and well-supported cluster (**Fig. 3**). This particular cluster is homogeneous, and its members show few variable loci on their genomes. They have been recently assigned to the new genus Sozzivirus (Walker et al., 2021). A third cluster is represented by the lytic Vinitor phages (**Fig. 4 B**), a possible hybrid group, such as recently observed amongst dairy phages (see for review Romero et al., 2020). The phylogenetic trees constructed using their whole nucleic acid sequences as well as the MCP and TerL proteins indicated that Vinitor phages and a prophage in *C. intestini* (a gut-associated LAB from the bumble bee) are more closely related to each other than to any other sequenced LAB phages (Philippe et al., 2020).

Such clustering of oenophages was also later supported by the analyses of the mechanisms of encapsidation. Indeed, NGS data contain all the information necessary to deduce the genome end types and locations by using programs such as Phage Term (Garneau et al., 2017). A joint branching of the *cos*-containing oenophages of the Int$_A$-Int$_B$-Int$_C$ group was observed while Int$_D$ as well as Vinitor clusters harbor terminally redundant and circularly permuted phages due to headful packaging (*pac*-phages) (Claisse et al., 2021; Jaomanjaka et al., 2013).

4. Presence of oenophages during winemaking and impact on MLF

During the 80's, a body of information from industrial dairy fermentations suggested that presence of phages resulted in reduced factory productivity and product quality worldwide. These elements highly challenged the development of dairy starter cultures (Romero et al.,

332 2020). Accordingly, first studies on oenophages also questioned the relationships between
333 phage abundance and MLF defects in wines (Henick-Kling et al., 1986b; Gnaegi and Sozzi,
334 1983). The first reported oenophages were indeed successfully isolated from wines with
335 sluggish or delayed MLF at the Swiss Federal Research Institute Changins. Cazelles and
336 Gnaegi (1982) monitored 30 wines in swiss cellars which all exhibited a delay in their
337 spontaneous MLF and phages were detected in 16 tanks. Sozzi et al. (1982) inoculated 4
338 wines with a single strain of *O. oeni*. All assays showed irregular or stuck MLF and phages
339 were also readily observed. The same trend was later observed by Arendt and Hammes
340 (1992), and oenophages were detected in 11 of 34 wines with problematic MLF. Lastly Davies
341 et al. (1985) observed loss of viability of *O. oeni* in a Cabernet-Sauvignon wine and a
342 concomitant interruption of MA degradation. This was not seen with Shiraz wines.
343 Interestingly, the cellars which suffered viral attacks did not have any difficulties during the
344 next vintage (Gnaegi, 1983).

345 These experimental data provided evidence that physicochemical parameters over
346 winemaking (such as low pH and SO_2) were impacting MLF. For the first time, they also
347 suggested that phages could be responsible for irregular MLF and/or failure of starter
348 activity upon inoculation. Hence, the observations made in cellars provided a major input
349 into the possible impact of phage-host dynamics on the kinetics and efficiency of MA
350 degradation in wines. Presence of phages was proposed to delay MLF (thereby increasing
351 the time of storage) and produce wines with loss of freshness and residual MA content,
352 allowing other undesirable LAB to develop, even in bottled wine (Davies et al., 1985; Gnaegi
353 et al., 1984).

354 At this point, the observations from the cellars also left researchers with many
355 unanswered questions. As an example, most studies included a qualitative assessment of the

356 presence of phage over MLF, and no continuous monitoring of the phage population was
357 carried out over the whole process. Additional avenues for further research focused on (i) the
358 nature of the mechanisms producing phages *in vino* (phage predation of sensitive cells *versus*
359 prophage induction and lysis of lysogens), (ii) the possible permissiveness of some wine
360 compositions towards phage growth and (iii) the ability of the ecosystem to readily evolve
361 resistance to phage attack. Lastly, some technical challenges could also be pointed out in
362 relation with the sampling design and striking limitations of the DAP method, due to
363 possible adverse effects of the food matrix on phage particles. All these points are discussed
364 below.

365

366 *4.1. Limitations of the DAP method and wine factors determining phage activity and stability*

367 The DAP method requires a permissive strain to detect/enumerate oenophages and there
368 is little chance that all phages present in a given wine will infect that specific strain. To date,
369 few highly sensitive strains have been identified. As an example, Poblet-Icart et al. (1998)
370 identified an isolate that proved to be sensitive to 75% of the strains of the FOEB collection
371 (Faculty of Oenologie, Bordeaux, France). With this caveat, there is evidence that phage
372 numbers in wines are likely to be underestimated when using the DAP method.

373 Plaque assay is restricted to the viruses that induce cell lysis or death and is therefore
374 used to count infectious particles. Yet, phage infectivity may be lost upon prolonged
375 incubation in wine and/or during storage of the collected samples prior to their processing in
376 the laboratory. Phage lysates in MRS broth are stable during several months at 4°C
377 (Jaomanjaka et al., 2013). In contrast, irreversible inactivation of particles was observed when
378 the pH of the lysate was lowered from 4.8 to 3.5, resulting in a drastic decrease in titer of 1.6

379 and 4 $\log_{10}$ after 22 h and 45 h of incubation, respectively. Oenophages loose their ability to
380 form plaques in low pH wines below 3.5 (Davies et al., 1985; Henick-Kling et al.,1986a).

381 Ethanol is another compound that may alter phage infectivity. Its concentration in wines
382 is most often ranging from 12.5% to 14.5% (vol/vol). Scarcely any reduction of the OE33PA
383 phage titer was observed after 72 h of storage in the presence of 15% ethanol, and similar
384 trends have been observed with most LAB phages (Jaomanjaka et al., 2013). Robustness of
385 particles to alcohol is supported in the data by Liu et al. (2015) who successfully used phages
386 to control production losses due to spoiling *Lb. plantarum* strains in the ethanol fermentation
387 industry.

388 Different treatments such as the addition of sulfites (SO_2 >50 mg/mL) and bentonite also
389 inactivate phage particles during the storage of red and white wines (Davies et al.,1985;
390 Henick-Kling et al., 1986a). Bentonite is used for fining, clarification, settling and stabilization
391 of white or rosé wines.

392 As a conclusion, there's a complexity of wine styles and chemical compositions
393 worldwide. Phage inactivation can be due to a number of factors of which some are arguably
394 little understood, and yet unspecified ones. Taking this into account, the design of surveys
395 using the DAP method should encourage the frequent collect of samples in cellars and their
396 rapid processing back to the laboratory. Future developments based on qPCR, digital PCR or
397 new imaging strategies are also needed to help in circumventing the current issues during
398 phage studies on wine (Jaomanjaka et al., 2013; Perlemoine et al., 2021; Philippe, 2017).

399

400 *4.3. Phage populations in wine reflect the spontaneous induction from lysogens during MLF*

401 Presence of prophages has been progressively shown to be prevalent amongst commercial
402 strains (Borneman et al., 2010; Jaomanjaka et al., 2013; Onetto et al., 2021; Patel, 1989) as well

as in the dominating indigenous population isolated from red wines upon completion of MLF (Chaïb et al., 2022). During the successful spontaneous MLF of red wines, prophages were shown to be excised from the chromosome, replicated, packaged, and then released from the cells in susbtantial amounts. Parallel growth curves of bacteria and phages were observed and the bacteria to phage ratio, as assessed by the DAP method, kept constant (about 10^3-10^4) (Gindreau, 1998). Later Jaomanjaka et al. (2013) compared the spontaneous phage induction (SPI) of strains harboring Int_A, Int_B or Int_D type prophages. Strains were inoculated in a filtered wine at the initial concentration of $5x10^7$ CFU/mL. Lower phage concentrations were measured for strains carrying an Int_D prophage compared to others, suggesting a higher stability of the Int_D prophage or a higher sensitivity of Int_D particles to wine (Jaomanjaka, 2014). Such differences ~~in the lysis-lysogeny decision amongst different types of prophages~~ were also observed in MRS and Red Grape Juice (RGJ) broths (Chaïb et al., 2019; Jaomanjaka et al., 2013). Intriguingly, the induced release of prophages did not seem to result in massive cell lysis under laboratory conditions. It has been long thought that when ratios of phage to bacteria are high, phages would enter a lysogenic life cycle to ensure persistence in the community. However, Knowles et al. (2016) proposed an opposing view point. With increased bacterial abundances, phages would make a transition from lysis to lysogeny allowing them to take advantage of the success of their host in “Piggy-Back-The-Winner” dynamics.

4.4. Does phage predation occur during winemaking ?

Another important question to better understand phage-host dynamics “in the wild” has been to assess whether oenophages could prey on bacteria under regular wine conditions during MLF. Phage infection requires actively metabolic bacterial cells. Also as a first step in

427 the process, the recognition and binding of the phage to its bacterial host requires the
428 presence of functional and accessible receptors and anti-receptors on the host and the phage,
429 respectively. It was therefore anticipated that growth in wine could not only determine
430 phage infectivity and stability but also impact the bacterial partner in this interaction. To
431 study phage predation under wine conditions, the indigenous microbiota was removed by
432 filtration offering simpler wine models in which bacteria and phages were added.

433 A first trial by Davies et al. (1985) reported on the inoculation of a filter-sterilized Shiraz
434 wine (pH 3.5; no SO_2) with two *O. oeni* sensitive strains, in conjunction with 10^3 PFU of
435 phage. Infection did not modify the trajectory of the culture and MLF was completed. In
436 another study, filtered red and white wines were inoculated with a single phage and its
437 respective host for propagation, and a phage-free culture served as a control (Henick-Kling
438 et al., 1986b). Two phages were tested: Lco23 from a Chasselas white wine from Switzerland
439 and S1.1s from an australian red wine. In red wine, phage S1.1s inhibited MLF at pH 3.25; 3.5
440 and 4. The onset and completion of MLF were delayed by 8 and 2 days in the infected assays
441 at pH 3.25. In contrast, phage Lco23 did not modify the trajectory of the culture in the same
442 wines. It was further shown in laboratory media that S1.1s and Lco23 were the most active at
443 low (3.5) and high pH (5.5), respectively.

444 More importantly, this elegant study also showed that MLF in the white wine tested was
445 not inhibited by either phage. This pointed to the possible distinct level of permissiveness of
446 wines to phage infection and attention was drawn to their phenolic compounds. Monomeric
447 phenolic compounds such as caffeic acid, present at high concentration in white wines, were
448 shown to protect *Lacticaseibacillus casei* strain from the attack by phage PL-1 (Lee et al., 1985).
449 Supporting data were later obtained by Philippe et al. (2017). Quercetin, myricetin and *p*-
450 coumaric acid, significantly reduced the phage predation of *O. oeni* by phage OE33PA when

provided as individual pure molecules, as also did other structurally related compounds such as cinnamic acid. Their presence was correlated with a reduced adsorption rate of the phage on its host. Strikingly, none of the identified compounds affected the killing activity of the distantly related lytic phage Vinitor162. OE33PA and Vinitor162 were recently shown to exhibit different entry mechanisms to penetrate into bacterial cells (Goulet and Cambillau, 2021; Philippe et al., 2021). The authors proposed that ligand-receptor interactions that mediate phage adsorption to the cell surface are diverse in *O. oeni* and are subject to differential interference by phenolic compounds. Their presence did not induce any modifications in the cell surface as visualized by TEM. In contrast, docking analyses suggest that quercetin and cinnamic acid may interact with the tail of OE33PA and compete with host recognition.

4.5. Resilience of the ecosystem to phage pressure

Some of the different studies described above highlighted the possible resilience of the wine ecosystem upon phage attack during MLF. In phage-containing wines, a succession of decrease and regrowth of the bacterial population (although at a slower rate than initially) was observed (Davis et al., 1985; Henick-Kling et al., 1986a). It was early proposed that wines contain a population of distinct *O. oeni* strains which perform MLF, and this has been recently confirmed through progress in genotyping (Chaïb et al., 2022; El Khoury et al., 2017). A diversity of the indigenous microflora regarding to phage sensitivity (~~was expected to lead to the rapid emergence of resistant clones which would then outgrow the other strains (Davis et al., 1985; Henick-Kling et al., 1986a). Thus, initial bacterial diversity post AF in wines)~~ would ensure the production of a sufficient biomass to degrade MA in the presence of phages. ~~The hypothesis that phage selection can maintain steady-state bacterial~~

475 ~~community composition via classic predator-prey dynamics was confirmed by)~~ Recent *in*
476 *silico* analyses of bacterial genomes ~~which~~ highlighted the presence of phage resistant
477 determinants such as Sie (superexclusion), Abi (abortive infection) and RM (restriction-
478 modification) which may serve to limit phage host range in *O. oeni* (Claisse et al., 2021;
479 Jaomanjaka et al., 2013). Other types of innate immunity mechanisms in bacteria have been
480 recently reviewed and may be present and prove to be functional in the *O. oeni* species (see
481 for review Isaev et al., 2021). The possible contribution of mobile genetic elements to
482 extensive horizontal gene transfer of such defense systems and their turnover in bacterial
483 populations also deserve further work in *O. oeni* (Claisse et al., 2021; Hussain et al., 2021).
484 Recent advances now suggest to integrate ecological interactions into a co-evolutionary
485 framework. The Red Queen hypothesis, also termed the evolutionary arms-race hypothesis,
486 posits that competitive environmental interactions, such as those displayed by hosts and
487 parasites, will lead to continuous variation and selection towards adaptation of the host, and
488 counter-adaptations on the side of the parasite (Stern and Sorek, 2011; Van Valen, 1973).
489 Bacterium-phage coevolution produces consecutive rounds of mutations whereby bacteria
490 evolve resistance to initial adsorption or infection, followed by phage host range mutations
491 to overcome resistance. In agreement with the hypermutator status of the species (Marcobal
492 et al., 2008), Bacterial Insensitive Mutants (BIMs) have been readily produced with different
493 two oenophages (Vinitor162 and OE33PA) under laboratory conditions (Philippe et al., 2020).
494 Sequencing of BIMs derivatives should bring important information on the bacterial genes
495 that are necessary for these phages to complete their lytic cycle in *O. oeni*. In contrast to the
496 dairy LAB *Streptococcus thermophilus,* the emergence of BIMs will not be dependent on
497 CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas system, as the

498 later system is absent in the *O. oeni* species (Borneman et al., 2010; Marcobal et al., 2008; Mills
499 et al., 2005).
500
501 **5. Lysogeny during winemaking: part of the problem or the solution ?**
502 *5 .1. Lysogens are detected upon completion of MLF in wines*
503 A simple and effective tool to provide genetic, ecological, and functional insights into
504 lysogeny in the *O. oeni* species was proposed by Chaïb et al. (2019). Lysogens were observed
505 to undergo white-red morphotypic switching when grown on agar media containing red
506 grape juice, providing a simple and inexpensive method to distinguish between lysogenic
507 and non lysogenic derivatives. The method was further used to assess the incidence of
508 lysogeny during winemaking in 3 wineries (Chaïb et al., 2022). The dominant LAB
509 population present in eleven red wines was collected at the end of the MLF step and
510 enumerated on RGJ agar. Using VNTR and prophage typing analyses, the authors showed
511 the prevalence of lysogeny in the *O. oeni* population driving the spontaneous MLF in red
512 wines (58.5%, n= 94 strains). The rate of lysogeny was not homogeneous across wineries
513 (24%, 65% and 86%). Yet, a few prophage-types belonging to the Int_A and Int_B groups
514 dominated amongst the mono- and double-lysogens isolated, as well as amongst the free
515 replicating particles found in the tested wines. Interestingly, some bacterial genetic
516 backgrounds were particularly prone to lysogenization. This would suggest that lysogens
517 may release prophages which may infect and convert other sensitive strains to lysogeny
518 (Chaïb et al., 2022).
519
520 *5 .2. Lysogeny amongst MLF starters*

521 Today, more than 50 different strains are commercialized and the market is highly
522 competitive. Cultures were first available in liquid forms, and direct inoculation of freeze-
523 dried cultures have been progressively developped. They contain a minimum of 10^{11} CFU/g
524 and are inoculated to ensure an initial population of 10^{6} CFU/mL, which is required to start
525 MLF. Winemakers have the possibility to inoculate wine LAB into the grape must together
526 with the yeast or sequentially at the end or post AF.

527 The early studies on oenophages questioned the need to take proactive strategies to phage
528 management in the enological sector, especially during the development of MLF cultures. In
529 particular, the question was raised as to select several strains with different sensitivity to
530 phages and use them to design mixed and possibly rotational mixtures (Sozzi et al., 1982).
531 Yet, the majority of the selected wine LAB starter cultures are currently the pure single strain
532 cultures. An exception is the use of a mixed culture consisting of one selected
533 *Lactoplantibacillus plantarum* and one selected *O. oeni* strain (Krieger-Weber et al., 2020).
534 Multi-strain starters are not currently favored because no scientific data proved any synergy
535 between strains, resulting in a higher performance compared to pure strains.

536 Guidelines for selecting MLF starters were proposed by Torriani et al. (2011) and included
537 the absence of prophages in selected strains. From the perspective of a supplier of
538 commercial MLF starter cultures, the instability of prophages was expected to impose a huge
539 metabolic burden on the cells by phage particle production. Alternately, prophages may also
540 recombine with other lysogenic or lytic phages in wines, resulting in the release of new
541 phages with modified host range and virulence. Yet, recent surveys of prophages among *O.*
542 *oeni* commercial strains highlighted a contrasted situation with prophage-free strains, as well
543 as lysogens harboring one or two prophages (Jaomanjaka et al., 2013; Onetto et al., 2021).
544 This might suggest that lysogeny is not detrimental during the production and

545 implementation of these strains in wines. Most commercial starters have been isolated from
546 wines that exhibited rapid and efficient MLF. The question is then to know whether the
547 selection of lysogens is a pure coincidence reflecting the high prevalence of lysogeny during
548 spontaneous MLF, or whether true benefits can be due to prophages at some stages.

549 First insights were provided by Chaïb et al. (2019) who observed that temperate
550 oenophages do not impose a cost to cellular growth in RGJ broth and may even favor
551 growth. The authors used a sensitive strain that was lysogenized by distinct phages
552 ($\Phi CiNe_{IntA}$, $\Phi 10MC_{IntB}$ or $\Phi OE33SAG_{IntD}$). The growth profiles and quantification of phage
553 particle release of the original strain and its three derivatives were monitored
554 simultaneously. Strains had similar growth patterns in RGJ broth but the lag time duration
555 was significantly lower for the derivative harboring $\Phi 10MC_{IntB}$. In another study, lysogens
556 isolated from wines were observed to be the most phage-resistant strains (Costantini et al.,
557 2017) suggesting that prophages may therefore provide protection to the bacterial host
558 against other phages.

559 Ongoing research and analysis will shed more light on the interactions between starters
560 and the indigenous flora, which may change over winemaking, in relation with population
561 levels and composition of the medium. Prophage may supply beneficial functions contained
562 in morons and/or control expression of essential bacterial genes under stressfull conditions.
563 Alternately, prophages may also constitute weapons in bacterial competition (Alexeeva et al.,
564 2018). The release of active phage particles by SPI in this context may therefore facilitate
565 starter implantation (Harrison and Brockhurst, 2017).

566

567 **6. Future prospects and conclusive remarks**

Investigations in the last four decades show that *O. oeni* co-exists with a diversity of temperate as well as strictly lytic phages. However, the infection strategies are more intricate than previously postulated. It is likely that bacteriophage predation regulates microbial abundance and diversity and more efforts are needed to expand the repertoire of lytic oenophages. Yet, the impact and ecology of temperate oenophages have been underestimated and they may be "double-edged swords" in bacterial warfare (Gama et al., 2013).

These complex interactions probably reflect the oenological parameters of the wines and the overall fluctuating resources resulting from competition with other microorganisms. Data found in the literature point to the importance of a broader understanding of the spatiotemporal dynamics of oenophages and their hosts. Future work is needed to better understand how evolved and intrinsic bacterial phage resistance drives antagonistic coevolution that can shape phage and bacterial host genomes.

Phage-host interactions offer a comprehensive way of viewing the biology of *O. oeni*. They may provide insights into cell wall dynamics, mobile elements with regard to phage defense systems and their horizontal transfer through transduction, architecture of the genomes, and domestication to specific niches. Research advances may also give interesting clues as to the selection of MLF starters and fitness trade-offs resulting from bacteriophage resistance, and open the way for novel biocontrol strategies in the sector.

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595

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