



Comparative genomics and phenotypes of *Oenococcus alcoholitolerans* and *Oenococcus oeni*, two sister species adapted to ethanol-rich environments

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ABSTRACT

From the genus *Oenococcus*, the most studied species is *O. oeni* due to its role in malolactic fermentation (MLF) during the winemaking process. In 2014, a new member of the genus—alongside *O. kitaharae* and *O. siceræe*—was discovered: *O. alcoholitolerans*. Whereas *O. oeni* is found in oenological matrixes, from grapes to wine, *O. alcoholitolerans* is found in cachaça residues and bioethanol fermentation. Though these ecological niches are different, they share the presence of high ethanol concentrations, which could be related to their evolutionary adaptation. This study provides the first sequenced and circularized genome of *O. alcoholitolerans* (UFRJ-M7), which was used for a comparative genomic analysis with the species *O. oeni*. In addition, the physiological traits of both species were compared regarding malolactic fermentation performance and survival in conditions of increasing ethanol and acid. The results showed great genetic differences in genes related to defence mechanisms, transcriptional factors, and some aspects of metabolism, such as sugar catabolism, purine/pyrimidine, coenzyme and vitamin biosynthesis. All these variations can be attributed to the specific adaptations of *O. oeni* and *O. alcoholitolerans* to their respective ecological niches. Notably, *O. alcoholitolerans* has not been detected in analyzed wine samples. This observation aligns with laboratory-based physiological tests, which demonstrated that the combined stress of ethanol and low pH prevented the survival of *O. alcoholitolerans*, despite its ability to carry out the MLF.

1. Introduction

Oenococcus is a genus of lactic acid bacteria (LAB) that is rarely detected in the natural environment but becomes dominant in alcoholic fermented beverages. To date, four *Oenococcus* species specialized to different fermented beverages have been isolated: *O. alcoholitolerans*, isolated from cachaça residues and bioethanol fermentation vats (Badotti et al., 2014), *O. kitaharae* from a composting distilled shochu residue (Endo and Okada, 2006), *O. siceræe*, from cider fermentation, and *O. oeni*, the predominant species in wine, and also in cider and kombucha (Coton et al., 2017; Salih et al., 1988). All four species appear to be specialized to low pH, and to moderate to high ethanol concentrations found in their ecological niches.

The most studied species is *O. oeni*. Since the early 20th century, it was isolated from wines undergoing malolactic fermentation (MLF) in various regions of the world, but the species was not formally described until 1967 (Garvie, 1967). It was initially designated as *Leuconostoc oenos*, then reclassified and renamed *Oenococcus oeni* based on phylogenetic data (Dicks et al., 1995). Since then, this species, rare in the natural environment but dominant in all wines within the first days of winemaking, has been extensively studied (González-Arenzana et al., 2012; Portillo et al., 2016). Numerous strains have been isolated from all wine-growing regions and characterized using molecular methods or genome sequencing (Bilhère et al., 2009; Campbell-Sills et al., 2017; Contreras et al., 2018; Jamal et al., 2013; Romero et al., 2018; Sternes and Borneman, 2016). Current phylogenomic data show that *O. oeni*

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strains cluster into four major genetic lineages, referred to as phylogroups A, B, C, and D, based on differences in Average Nucleotide Identity (ANI) among their sequenced genomes (Lorentzen et al., 2019). The observed genomic differences are partly related to the ecological origin of the strains. Strains from phylogroups A, B, and C can be found together in grape must, although they differ in their ethanol tolerance. Thus, during alcoholic fermentation, the population of group A strains increases significantly, while strains from groups B and C eventually disappear (Balmaseda et al., 2023). In cider, strains from groups B and C become dominant, likely due to the lower ethanol content (Balmaseda et al., 2023). Strains from group D, on the other hand, have been found exclusively in kombucha (Lorentzen et al., 2019).

While several hundred genomes of *O. oeni* have been reported to date, much fewer sequences of the three other *Oenococcus* species are available: five of *O. kitaharae*, including 1 complete genome (Borneman et al., 2012; Campbell-Sills et al., 2015), two of *O. sicerae* – 1 complete – (Cousin et al., 2019), and a single draft genome of *O. alcoholitolerans*. A previous comparison of a complete *O. kitaharae* genome and 3 *O. oeni* genomes has revealed key functional variations of the species (Borneman et al., 2012). *O. kitaharae* carries restriction-modification systems and CRISPR elements to fight against foreign DNA invasion and bacteriophages as well as bacteriocins, which may provide a selective advantage over other bacteria in a mixed-species environment such as composting shochu residue. In contrast, *O. oeni* has no CRISPR system and no bacteriocin pathways were detected in the analyzed strains, which correlated with the lack of bacterial competitors in the harsh environment of wine. Genomic data also revealed variations in carbohydrates and amino acids metabolism that may be linked with the different metabolite compositions of their environments and, in *O. kitaharae*, an early stop mutation in the gene coding for the malolactic enzyme, which makes it unable to perform MLF (Badotti et al., 2014). The main phenotypic properties of *O. kitaharae* are consistent with genomic predictions as they show its inability to develop in wine because it not only lacks MLF activity, but its optimum pH (6.0) is incompatible with growth in wine and the bacterium does not survive in the presence of 10 % ethanol (Endo and Okada, 2006).

The only available genome of *O. alcoholitolerans* has a much smaller size than the three other species (1.2 Mb, versus 1.7 Mb for *O. sicerae*, and 1.8 Mb for *O. oeni* and *O. kitaharae*). It comprises only 22 RNA genes, which is insufficient to derive a full set of tRNAs and rRNAs, and it is made of 698 contigs, suggesting that many genes of this assembly are missing or truncated (Badotti et al., 2014). However, a part of the gene encoding the malolactic enzyme has been identified and it does not contain the early stop mutation detected in *O. kitaharae*, which means that *O. alcoholitolerans* might be able to perform MLF. In addition, the phenotypic properties of *O. alcoholitolerans* (growth at pH 4 or in presence of 12 % (v/v)) are compatible with life in wine (Badotti et al., 2014). It differs from most *O. oeni* strains by its capacity to ferment sucrose, although this property was detected in *O. oeni* strains isolated from cider and wine that belong to phylogroups B and C (Cibrario et al., 2016).

Although more than 240 genome sequences of *O. oeni* strains are available in public databases, the genetic determinants of its adaptation to wine are not fully elucidated. The aim of this study was to take advantage of the recent description of *O. alcoholitolerans* to revisit the genetic properties of *O. oeni* and compare these two sister species to better understand their respective ecological adaptations. To this end, we produced the first complete genome assembly of an *O. alcoholitolerans* strain and conducted a comparative analysis with publicly available *O. oeni* genomes. This approach not only provides insights into the evolutionary traits of both species, but also contributes to a better understanding of the molecular mechanisms underlying the resistance of *O. oeni* to wine stress conditions. Such knowledge is essential not only for improving the performance and control of *O. oeni* in wines where MLF is desired, but also for developing strategies to inhibit its activity in wines like some sparkling wines, where unwanted

post-bottling MLF due to adapted and still viable *O. oeni* cells can compromise product quality.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *O. alcoholitolerans* UFRJ-M7 (=DSM 17330) type strain was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. For phenotypic characteristic comparison, *O. oeni* VF (Vitulactic F, Martin Vialatte) strain was used.

Both strains were grown in liquid grape juice medium (1 L): 250 mL of red grape juice (Jafaden, Leclerc, France) –approximately 40 g/L of glucose + fructose–, 5 g of yeast extract, 1 mL of Tween 80, adjusted to pH 4.8. Cultures of 12 mL were incubated in plastic tubes at 25 °C for 5–7 days, until they reached the late exponential growth phase. Bacterial enumeration was performed by epifluorescence microscopy for inoculation, and by plating in solid grape juice medium during the experiments.

2.2. De novo sequencing

High molecular weight DNA of *O. alcoholitolerans* UFRJ-M7 was extracted with the MasterPure™ Complete DNA and RNA Purification Kit MasterPure DNA Purification kit from Lucigen Corp. (Middleton, WI, USA). DNA quantity and purity was evaluated with Qubit 2.0 fluorometer and NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing library for Nanopore was prepared with the Rapid Barcoding Sequencing kit (SQK-RBK004) and was sequenced on a MinION Mk 1 sequencer using a FLO-MIN106 R9 version flow cell. Nanopore reads were basecalled with Albacore (v2.3.0) and adapters were trimmed with Porechop (v.0.2.4). A DNA library was prepared with the Illumina Nextera Paired-End protocol (2x250 bp reads) and sequenced on a Miseq (Illumina, CA, USA). A hybrid genome assembly was performed with both Illumina paired-end reads and trimmed Nanopore reads using the Unicycler assembler (v.0.4.3) (Wick et al., 2017). *O. alcoholitolerans* UFRJ-M7 genome (BioProject: PRJNA1196163) is available under the accession numbers: CP176039 (Chromosome), CP176040 (pOALC.1), CP176041 (pOALC.2), CP176042 (pOALC.3). The genome was annotated with LABGeM's MicroScope service (Vallenet et al., 2017), followed by manual curation of every gene through the use of the comparative genomics tools and databases built into the platform (synteny with model/related species and sequence-based based predictions (SwissProt/TrEMBL alignment, InterProScan, FigFam, among others).

2.3. Genomic analysis

For genomic comparison with *O. alcoholitolerans* UFRJ-M7, *O. oeni* public genomes already assigned to a phylogroup (Lorentzen et al., 2019) were used: AWRIB429 (A), PSU-1 (A), 19 (A), ATCC BAA-1163 (B), AWRIB787 (B), CRBO_1381 (C), and UBACC-A-315001 (D).

The genome of *O. oeni* PSU-1 was used as reference on the RGPfinder tool of MicroScope (Vallenet et al., 2013) to find potentially horizontally transferred genes gathered in Regions of Genomic Plasticity (RGP). The sequences of the other *O. oeni* genomes and the one of *O. alcoholitolerans* were aligned to identify those genes present in these representant strains from the species *O. oeni*, but not in *O. alcoholitolerans*. Similarly, *O. alcoholitolerans* genome was used as references to find those genes present in *O. alcoholitolerans* and not on *O. oeni* genomes.

2.4. qPCR assays

The analyzed samples were collected from four different Bordeaux wineries at different stages of winemaking, from must to wine during MLF. DNA extraction was performed as in Balmaseda et al. (2023).

Species-specific primers for *O. oeni*, and *O. alcoholitolerans* targeted the *rpoB* or *rpoD* constitutive genes (Suppl. Table 1). iQ SYBR® Green Supermix (Bio-Rad, CA, USA) was used for qPCR run on a Bio-Rad CFX96 Real-Time PCR system with a first step of 30 s at 90 °C, followed by cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Serial dilutions of extracted DNA (0–100 ng/μL) were used as standard curve for quantification in each plate, and a negative control with water. Each reaction was done in triplicate. The amplification efficiencies obtained for *O. oeni* and *O. alcoholitolerans* were 81.7 % and 84.6 %, respectively.

2.5. Adaptation to wine stressors in culture medium

O. alcoholitolerans type strain and *O. oeni* VF strain were tested to assess their adaptation to pH and ethanol. Cells were inoculated at approximately 10⁴ CFU/mL into 20 mL of grape juice medium in sterile glass culture tubes. The culture medium was adjusted to pH 4.0, 3.5, 3.25, or 3.0, and 6, 8, 10, or 12 % v/v ethanol. The cultures were incubated at 25 °C. Bacterial populations were monitored for two weeks. All the assays were done in duplicate.

A similar approach was followed to evaluate the adaptation to pH and ethanol at the same time. Grape juice medium was adjusted to pH 3.5 and different ethanol concentrations (6, 8, 10, 12 % v/v) were added. Also, the standard grape juice medium containing 8 % of ethanol was adjusted to different pHs (4.0, 3.5, 3.25, 3.0). Each condition was done in duplicate.

2.6. MLF performance

The ability of *O. alcoholitolerans* to perform MLF (L-malic acid biotransformation) was tested in grape juice medium (initially containing around 0.6 g/L of L-malic acid and 40 g/L of glucose + fructose) supplemented with 2.5 g/L and 5.0 g/L of L-malic acid. 20 mL of these two media were inoculated with 10⁶ cell/mL of *O. alcoholitolerans* in sterile glass culture tubes. For comparison, the same procedure was performed with *O. oeni* VF. The bacterial cultures were incubated at 25 °C and performed in duplicate. Samples were collected 2, 7, and 14 days after inoculation for determining the bacterial population (CFU) and L-malic acid concentration using the L-Malic Acid Assay Kit (Megazyme, Wicklow, Ireland). All the assays were done in triplicate.

A similar protocol was employed to evaluate MLF ability in wine like medium (WLM), except that bacteria were inoculated in 10 mL WLM –containing 2 g/L of fructose and 2 g/L of L-malic acid (Balmaseda et al., 2023) filled in disposable syringes to limit air contact and incubated at 20 °C.

3. Results and discussion

3.1. Genomic analysis

With the purpose of comparison between *O. alcoholitolerans* and

O. oeni, the first complete genome of *O. alcoholitolerans* species was produced. This feature provides a better understanding of the *Oenococcus* genus, with particular interest in *O. oeni*, and its adaptation to wine conditions.

3.1.1. *Oenococcus alcoholitolerans* has less pseudogenes than *Oenococcus oeni*

For comparison, Table 1 shows the main features of the complete genome of *O. alcoholitolerans* with other public complete genomes belonging to the *Oenococcus* genus. The complete *O. alcoholitolerans* UFRJ-M7 genome is 1.6 Mb in size, which is significantly longer than the previously published 1.2 Mb draft genome. It also contains 3 plasmids of 18.4, 32.6, and 46.3 kb. It shows that, like the rest of *Oenococcus* species, *O. alcoholitolerans* has two rRNA operons, a full set of 43 tRNAs and a protein coding density like that of *O. kitaharae* (Table 1).

LAB have relatively high number of pseudogenes compared to other bacterial groups, and they are especially prevalent among several highly specialized species found in nutrient-rich environments of food products (Schroeter and Klaenhammer, 2009). The number of pseudogenes in *O. oeni* and the trend of genome reduction are not out of the ordinary; *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* have comparable levels or higher number of pseudogenes (182 and 270, respectively) with genome sizes of 1.8 and 2.2 Mb, respectively (Goh et al., 2011; Makarova et al., 2006; van de Guchte et al., 2016).

The number of pseudogenes is significantly higher in *O. oeni* strains, suggesting a greater degree of domestication to the wine environment. This pattern has been observed for LAB in general (Steensels et al., 2019). For example, wild *Lactococci* isolates exhibit a wide range of enzymatic activities involved in carbohydrate catabolism, whereas domesticated strains often lack these functions due to pseudogenization or transposon insertions (Kelly et al., 2010). Notably, strains from phylogroups A and B show a high number of pseudogenes, whereas those from phylogroups C and D have fewer, supporting the hypothesis of a lower degree of domestication. In particular, strains from phylogroup A systematically exhibit pseudogenization in genes related to the catabolism of alternative carbohydrates such as arabinose and maltose (Cibrario et al., 2016). More specifically, the Aw subgroup within phylogroup A harbors the *eps2* locus, which is composed exclusively of pseudogenes (Dimopoulou et al., 2014). Nevertheless, all *O. oeni* strains possess more pseudogenes than other *Oenococcus* species. This variation across *O. oeni* strains reflects differing levels of domestication, while the markedly lower number of pseudogenes in *O. alcoholitolerans*, *O. kitaharae*, and *O. sicerae* suggests that these species have undergone less specialization. However, *O. alcoholitolerans* has undergone a 200 kb reduction in genome size relative to other species, indicating that its genomic streamlining has progressed beyond gene inactivation to gene loss, as observed for *Lactobacillus bulgaricus* (Van de Guchte et al., 2006). This could suggest a more ancient form of specialization compared to the ongoing domestication process observed in *O. oeni*. Nevertheless, since this observation is based on a single

Table 1

Main features of complete *Oenococcus* genomes. Table shows the newly circularized *O. alcoholitolerans* genome, together with other public complete *Oenococcus* genomes.

Species	Strain	Sequence length	% GC	Conting nb	CDS	% Protein coding density	Pseudogenes
<i>O. alcoholitolerans</i>	UFRJ-M7	1610122	39.14	1	1657	91.2	8
<i>O. alcoholitolerans</i>	UFRJ-M7 plasmid 1	46274	41.45	1	53	74.07	0
<i>O. alcoholitolerans</i>	UFRJ-M7 plasmid 2	32644	38.75	1	42	79.57	0
<i>O. alcoholitolerans</i>	UFRJ-M7 plasmid 3	18361	37.39	1	16	82.53	0
<i>O. kitaharae</i>	DSM_17330	1835245	42.70	1	1908	90.16	22
<i>O. oeni</i> ^a (group A)	PSU-1	1780517	37.89	1	1859	83.13	206
<i>O. oeni</i> ^a (group B)	19	1826824	37.92	1	2037	84.4	163
<i>O. oeni</i> ^a (group C)	CRBO_1381	1834577	37.81	1	1923	87.1	60
<i>O. oeni</i> ^a (group D)	UBOCC-A-315001	1876981	37.73	1	1916	87.53	57
<i>O. sicerae</i>	UCMA15228	1664394	40.30	1	1712	95.21	25

^a For *O. oeni* only one representative genome of each phylogroup (A, B, C and D) is presented.

genome, and the link between gene loss and reduced pseudogene content remains to be confirmed, additional genomic data from other *O. alcoholitolerans* strains will be necessary to support this hypothesis.

Furthermore, we compare the distribution of the CDS of *O. alcoholitolerans* and three selected phylogroup A *O. oeni* strains in terms of Cluster of Orthologous Groups (COGs).

We have previously observed that this *O. alcoholitolerans* strain has smaller genome length and CDS number than *O. oeni* strains (Table 1). Interestingly, its COG distribution is different from that in *O. oeni* (Table 2). There is a general lower CDS in each category in *O. alcoholitolerans* genome, corresponding to the lower CDS number. Besides, this difference is more dramatic in some COGs, which appear to be crucial for *O. oeni* adaptation to wine: (i) defence mechanisms (V), (ii) transcription (K), (iii) nucleotide transport and metabolism (F), (iv) carbohydrate transport and metabolism (G), and (v) secondary metabolites biosynthesis, transport, and catabolism (Q). Indeed, these COGs are those that are always reported as the most regulated ones in wine related environments (Balmaseda et al., 2022; Margalef-Català et al., 2016; Olguín et al., 2015).

3.1.2. Regions of genomic plasticity

3.1.2.1. *Oenococcus alcoholitolerans* presents less defence mechanisms and alternative sugar related genes than *Oenococcus oeni*. We have further analyzed these differences between these two species by the Regions of Genomic Plasticity (RGP) tool on MicroScope (Vallenet et al., 2013). For this purpose, we first use the *O. oeni* PSU-1 genome as reference and aligned *O. alcoholitolerans* genome and all the public complete *O. oeni* genomes already assigned to a phylogroup: AWRIB429 (A), 19 (A), ATCC BAA-1163 (B), AWRIB787 (B), CRBO_1381 (C), and UBOCC-A-315001 (D). We find 36 different RGPs (Suppl. Table 2). We analyze them in terms of absence/presence of genes, as there is just one *O. alcoholitolerans* complete genome available.

From these 36 RGP, we investigate genes present in all *O. oeni* strains and absent in *O. alcoholitolerans*: identifying 355 genes and 26 pseudogenes (Suppl. Table 2). As expected, the main gene differences are observed in V (18), K (36), amino acid transport and metabolism (E)

(22), F (24) and G (39) COGs (Fig. 1). Moreover, we identify differences in cell wall/membrane/envelope biogenesis (M) (21) and inorganic ion transport and metabolism (P) (21) COGs, apart from function unknown (S) (124). There are also 26 pseudogenes present in *O. oeni* and not in *O. alcoholitolerans*. Generally, the presence of high number of pseudogenes is related with a high degree of domestication/specialization to a particular ecological niche (Lorentzen et al., 2019).

Defence mechanism genes (V) are one of the key tools for *O. oeni* adaptation in wine environment (Bech-Terkilsen et al., 2020). These genes are located in different RGPs (Suppl. Table 2), but most of them are related with an upregulated expression in wine-like conditions: for instance, five (out of eight differentially found genes) ABC-type multi-drug transport system components (OEOE_1711, OEOE_1712, OEOE_0722, OEOE_0723, OEOE_0761), a β -lactamase (OEOE_1067), and two peptide ABC transporter components (OEOE_0270, OEOE_0734) (Margalef-Català et al., 2016; Balmaseda et al., 2022). Indeed, OEOE_0734 (peptide ABC transporter permease) presents a very high protein abundance in early MLF stages (Balmaseda et al., 2022). Still, other stress mechanisms as the genes *dnaJ*, *dnaK*, *clpC*, *clpP*, *hsp20* or the complex Gro-ES-GroEL (Costantini et al., 2015; Grandvalet et al., 2005; Maitre et al., 2014) are also present in *O. alcoholitolerans* genome.

Some *O. oeni* phosphotransferase systems (PTS) are absent in *O. alcoholitolerans*. From those, the *manA* operon lacks *manY* (OEOE_0380) and *manXa* (OEOE_0382), related with sugar intake, mainly mannose (Jamal et al., 2013). Indeed, the expression of these genes is upregulated in wine-like conditions (Balmaseda et al., 2021; Margalef-Català et al., 2016). No differences are observed in genes related to uptake of glucose, fructose, trehalose or cellobiose, which are substrates that seem to be common to both ecological niches (Amorim et al., 2016; Ribéreau-Gayon et al., 2006).

The comparative genomics between these two species also reveals the absence of some genes related with the pentose phosphate pathway (Fig. 2A). The pentose phosphate pathway is an essential pathway in *O. oeni* to use the alternative carbon sources, for instance mannose, trehalose, ribose or cellobiose, found in wine (Cibrario et al., 2016). The absence of these enzymes (Suppl. Table 2) supports the phenotypic data

Table 2

Distribution of coding sequences (CDS) in Cluster of Orthologous Groups (COGs) in *O. alcoholitolerans* and three selected phylogroup A *O. oeni* strains.

Process	Class ID	Description	<i>O. oeni</i> PSU-1		<i>O. oeni</i> 19		<i>O. oeni</i> AWRIB429		<i>O. alcoholitolerans</i>	
			CDS	%	CDS	%	CDS	%	CDS	%
Cellular processes and signaling	D	Cell cycle control, cell division, chromosome partitioning	16	0.79	16	0.79	18	0.82	14	0.85
Cellular processes and signaling	M	Cell wall/membrane/envelope biogenesis	109	5.40	98	4.81	110	5.06	104	6.28
Cellular processes and signaling	N	Cell motility	4	0.20	4	0.20	4	0.18	3	0.18
Cellular processes and signaling	O	Posttranslational modification, protein turnover, chaperones	47	2.33	50	2.45	52	2.39	51	3.08
Cellular processes and signaling	T	Signal transduction mechanisms	43	2.13	39	1.91	43	1.98	32	1.93
Cellular processes and signaling	U	Intracellular trafficking, secretion, and vesicular transport	20	0.99	23	1.13	21	0.97	20	1.20
Cellular processes and signaling	V	Defense mechanisms	51	2.53	55	2.7	56	2.58	23	1.39
Information storage and processing	J	Translation, ribosomal structure, and biogenesis	132	6.54	134	6.58	132	6.07	126	7.6
Information storage and processing	K	Transcription	123	6.10	124	6.09	129	5.93	99	5.97
Information storage and processing	L	Replication, recombination and repair	102	5.05	101	4.96	114	5.24	102	6.15
Metabolism	C	Energy production and conversion	87	4.31	87	4.28	85	3.91	78	4.70
Metabolism	E	Amino acid transport and metabolism	119	5.90	129	6.33	136	6.26	107	6.46
Metabolism	F	Nucleotide transport and metabolism	73	3.62	70	3.44	74	3.40	47	2.84
Metabolism	G	Carbohydrate transport and metabolism	182	9.02	181	8.89	188	8.65	120	7.24
Metabolism	H	Coenzyme transport and metabolism	45	2.23	44	2.16	47	2.17	37	2.23
Metabolism	I	Lipid transport and metabolism	44	2.18	46	2.26	46	2.12	40	2.41
Metabolism	P	Inorganic ion transport and metabolism	92	4.56	84	4.12	87	4.0	87	5.25
Metabolism	Q	Secondary metabolites biosynthesis, transport, and catabolism	20	0.99	22	1.08	17	0.78	7	0.42
Poorly characterized	S	Function unknown	366	18.14	399	19.59	426	19.60	324	19.55

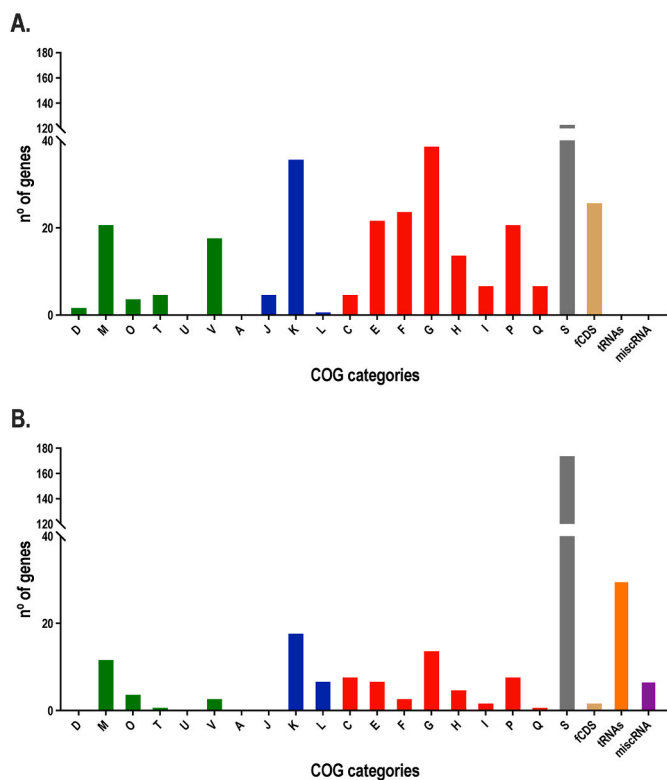


Fig. 1. Number of genes classified as COGs, also including pseudogenes, tRNAs, and miscellaneous RNA (miscRNA) different in (A) *O. alcoholitolerans* vs *O. oeni* and (B) *O. oeni* vs *O. alcoholitolerans*. For comparison *O. alcoholitolerans* UFRJ-M7 and selected *O. oeni* genomes were used. COG color code refers to their main categories: Cellular processing and signaling (green), information storage and processing (blue), metabolism (red), poorly characterized (grey). D: cell cycle control, cell division, chromosome partitioning. M: cell wall/membrane/envelope biogenesis. O: post-translational modifications, protein turnover, and chaperones. T: signal transduction mechanisms. U: intracellular trafficking, secretion, and vesicular transport. V: defense mechanisms. A: RNA processing and modification. J: translation, ribosomal structure, and biogenesis. K: transcription. L: replication, recombination, and repair. C: energy production, and conversion. E: amino acid transport and metabolism. F: nucleotide transport and metabolism. G: carbohydrate transport and metabolism. H: coenzyme transport and metabolism. I: lipid transport and metabolism. P: inorganic ion transport and metabolism. Q: secondary metabolites biosynthesis, transport, and catabolism. S: poorly characterized. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

showing that *O. alcoholitolerans* is less capable of catabolizing alternative carbohydrates (Badotti et al., 2014). In addition, it constitutes evidence of less fitness to a wine environment since these sugars are usually the only ones found in wine. On the contrary, also related with carbon metabolism, *treR* repressor (OEOE_1339) is also absent in *O. alcoholitolerans*. *treR* is present in other LAB found in wine, and in the two sister *Oenococcus* species. This could also be related with the absence of trehalose in *O. alcoholitolerans*' niche.

There are few operons missing in *O. alcoholitolerans* genome in comparison to *O. oeni* genomes. The complete *pur* operon (RGP23, OEOE_1133 – OEOE_1138) is absent in *O. alcoholitolerans* (Suppl. Table 2). This could lead to a completely different pathway in the biosynthesis of purines (Fig. 2B). *O. oeni* can produce them from other metabolites, such as D-ribose, by combining it with L-glutamine through the enzymatic reactions of the *pur* operon. Indeed, D-ribose appears to be an important metabolite, as it can also be used as growth substrate by the majority of *O. oeni* strains (Cibrario et al., 2016). D-ribose is a metabolite that can accumulate in wine as result of yeast autolysis, thus

stimulating *O. oeni* growth (Tudela et al., 2013). Also, it lacks an adenine deaminase (OEOE_1462) found on the RGP28. Interestingly, the other two *Oenococcus* species have this operon, which could be related to a specific adaptation to *O. alcoholitolerans*' ecological niche. In addition, the genetical environment found up- and down-stream seem to be conserved also in other LAB, suggesting a deletion of *pur* operon in *O. alcoholitolerans*. Close to this *pur* operon in *O. oeni* genome, *O. alcoholitolerans* lacks the OEOE_1140 gene, a cell division protein. OEOE_1140 is present in other wine LAB and in the other two *Oenococcus* species.

The *pyr* operon is another relevant genomic element found in *O. oeni*, is also missing in *O. alcoholitolerans*. As with the *pur* operon, the enzymes encoded in this operon enables *O. oeni* to produce pyrimidines from other metabolites (Fig. 2C). Found in other LAB, including other *Oenococcus* species, it is related with the biosynthesis of pyrimidines, and it is usually downregulated in wine conditions (Margalef-Català et al., 2016), where low bacterial growth is observed.

3.1.2.2. The genome of *Oenococcus alcoholitolerans* reflects its adaptation to nutrients in cachaça residues over wine. Similarly, we use the newly completed *O. alcoholitolerans* genome (CP176039) as reference to analyse RGP in comparison with the publicly available *O. oeni* genomes. Again, we analyse the absence/presence of genes, as there is just one *O. alcoholitolerans* genome available.

In this case, 29 RGPs are found (Suppl. Table 3), which comprise 268 genes, 2 pseudogenes, 30 tRNAs, and 8 miscellaneous RNAs (Fig. 1, Suppl. Table 3). No absent genes classified as cell cycle control, cell division, chromosome partitioning (D), U, and translation, ribosomal structure, and biogenesis (J) COGs are found. The main gene absent COGs are M (12), K (18), G (14), and S (175).

When comparing the presence/absence of genes of *O. alcoholitolerans* in *O. oeni* genome, some tRNAs are found, especially in the RGP9 (Suppl. Table 3). However, tRNA sequences are not conserved within species and their absence in the sum of the selected genomes are not remarkable. Besides, the comparison is done with just a genome of *O. alcoholitolerans*, thus, not considering the intra-species heterogeneity.

The RGP15 genetic environment represent an insertion of a prophage, which interestingly includes a gene encoding lysozyme (OEAL_v1_0817), which could confer greater bacterial dominance over other bacteria, especially Gram-positive ones, in cachaça residues. Interestingly, some regions of the inserted sequence are also found in some *O. oeni* strains and other LAB.

A complete CRISPR loci sequence is present in *O. alcoholitolerans*. It is known that hypermutable bacteria, as *O. oeni* are likely to not have or loss this phage-resistance mechanism. Recently, Barchi et al. (2023) have analyzed the presence of CRISPR loci in the four *Oenococcus* sister species, concluding that the presence of CRISPR was strain-rather than species-specific, since only few strains posed it.

The RGP3 sequence has little conservation within *Oenococcus* species and other LAB (Suppl. Table 3). The cluster of genes OEAL_v1_0110 - OEAL_v1_0114, related with the metabolism of pantothenate and subsequent synthesis of coenzyme-A is specific for *O. alcoholitolerans*. *coaA*, encoding a pantothenate kinase, is also absent in RGP15. Besides, genes OEAL_v1_0118 (putative aldehyde dehydrogenase) and OEAL_v1_0119 (aldose sugar dehydrogenase) related with sugar catabolism are absent in other LAB. Another alcohol dehydrogenase (RGP4, OEAL_v1_0147) is absent in *O. oeni* but present in other LAB.

Thiamine is a vitamin found in considerable concentration in wine, which could come from grape must or yeast lees autolysis (Evers et al., 2023a; Liddicoat et al., 2015), necessary for wine fermentations (Evers et al., 2023b; Labuschagne and Divol, 2021). This fact could support the no need of *O. oeni* to synthesize this vitamin. In this sense, it is found that the absence of some genes encoding enzymes related with thiamine biosynthesis, like the thiaminase (transcriptional activator *tenA*) (OEAL_v1_1008), a thiamine-phosphate synthase (*thiE*, OEAL_v1_1010),

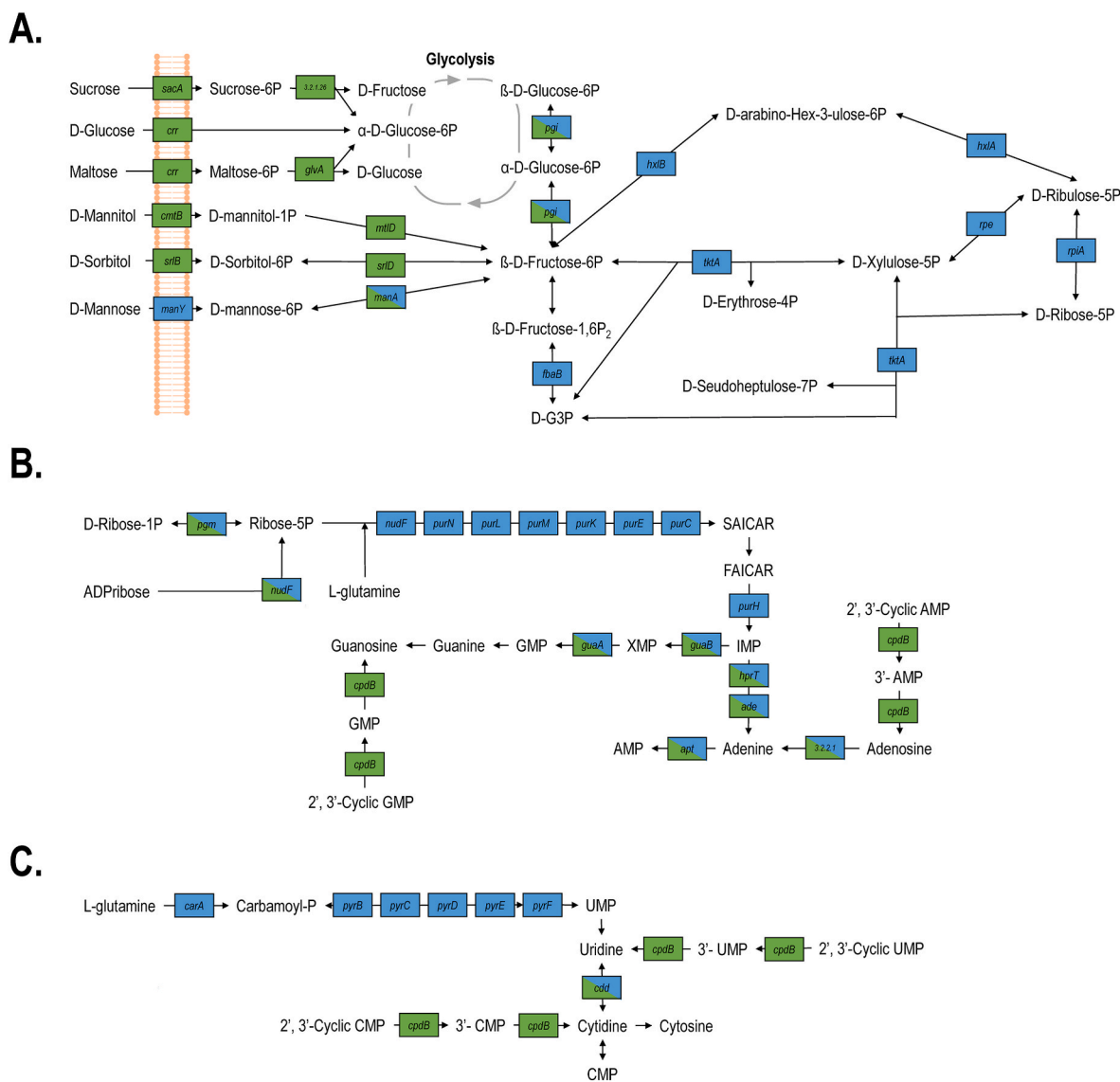


Fig. 2. Some important pathway differences in *O. alcoholitolerans* and *O. oeni*. A. Relevant pathway module differences in carbohydrate metabolism. B. Purine metabolism. C. Pyrimidine metabolism. Pathways are simplified to highlight the difference between the two species. Boxes represent enzymes; green means genes exclusively found in *O. alcoholitolerans*, blue means genes exclusively found in *O. oeni*, mixed green and blue means common enzymes between species. D-G3P: D-Glyceraldehyde-3P. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

or a hydroxyethylthiazole kinase (*thiM*, OEAL_v1_1011) could be related to the need of *O. alcoholitolerans* for synthesizing thiamine in its ecological niche.

Sucrose utilization is one of the main features of *O. alcoholitolerans* (Badotti et al., 2014). Indeed, sucrose is the main sugar found in sugarcane (45–56 g/L), followed by fructose (18–32 g/L), and glucose (10–20 g/L) (Amorim et al., 2016). Two genes associated with sucrose uptake are present in *O. alcoholitolerans*: a sucrose phosphorylase (OEAL_v1_0329) and a sucrose PTS system EIIBC or EIIBC component (OEAL_v1_1242) (Fig. 2). Both genes are not present in *O. oeni* PSU-1 and appear truncated in other strains, which could explain the general sucrose-negative phenotype of *O. oeni*. Interestingly, these genes appear also truncated in the other *Oenococcus* species and most LAB (data not shown).

The transcriptional repressor of the fructose operon, *deoR* family (OEAL_v1_0547) is absent in *O. oeni* and *O. kitaharae*, but present in *O. sicerae* and other LAB. The fructose 1-phosphate kinase (OEAL_v1_0548), absent in *O. oeni* and *O. kitaharae*, is present in *O. sicerae* and other LAB. This could also be related with the availability

of fructose in the different ecological niches; the abundance of fructose may be linked to the presence of this genomic features.

The presence of a mannitol-1-phosphate 5-dehydrogenase (OEAL_v1_1328), absent in *O. oeni*, may support the potential utilization of D-mannitol as a substrate (Fig. 2). However, the gene encoding the D-mannitol-specific phosphotransferase (OEAL_v1_1332), which is also missing in *O. oeni*, appears to be truncated—a feature commonly observed in other LAB. Although *O. oeni* does not consume mannitol (Cibrario et al., 2016), mannitol production is a general trait of the species. It is typically synthesized through fructose import coupled with mannitol export, contributing to redox balance. Notably, mannitol can act as a direct electron acceptor without entering the heterolactic fermentation pathway (Richter et al., 2003).

Overall, the differences observed between *O. alcoholitolerans* and *O. oeni* are primarily associated with sugar metabolism, reflecting adaptations to the distinct ecological niches they occupy. Other minor genetic divergences likely relate to niche-specific selective pressures. The presence or limited availability of certain carbon sources appears to be a key driver of these genomic changes, while adaptations to stress

factors, such as low pH tolerance, are less pronounced in *O. alcoholitolerans* due to the milder conditions in its typical environment.

3.2. qPCR analysis

Although found in cachaça residues and bioethanol fermentation, the genetic characteristics of *O. alcoholitolerans* suggest that this species could thrive in a wine environment, mainly in grapes or first fermentative stages in must, where the ethanol content is not high, as it happens with non-*O. oeni* LAB species in wine. In this sense, *O. alcoholitolerans* possesses some relevant stress related mechanisms found in *O. oeni*, which could allow the bacterium to face the moderate ethanol concentration found in cachaça residues (Badotti et al., 2014), similar to that in fermenting must. In this study, we evaluate the presence of *O. alcoholitolerans*, together with *O. oeni*, in grape musts and wines during the MLF- of four wineries by qPCR.

Results of the qPCR quantification are shown in Table 3. During the five wine fermentations studies, no *O. alcoholitolerans* is detected, regardless of the fermentative stage. On the contrary, *O. oeni* presence is detected and it increases in wine as result of MLF, with bacterial population increasing up to 10^9 cells/mL.

The ethanol concentration during wine fermentation is not far from the 12 % v/v that *O. alcoholitolerans* can tolerate (Badotti et al., 2014). However, the low pH of wine could also be the reason why *O. alcoholitolerans* is not detected during wine fermentation. *O. oeni* is the best adapted LAB to wine not only due to its high adaptation to ethanol concentration, but also to low pH. In this sense, Badotti et al. (2014) report weak growth at pH 4.0, and they do not test more acidic values. Nevertheless, the presence of the whole set of MLF genes and stress response mechanisms, suggest the eventual possibility to grow under wine-like conditions.

3.3. Adaptation to wine stressors in culture medium

To better understand the phenotypic response to low pH and moderate ethanol concentrations we study the growth of *O. alcoholitolerans* under different conditions. We test *O. alcoholitolerans* in grape juice medium under varying pH conditions within the wine range (3–4) and different ethanol concentrations ranging from 6 % to 12 % v/v. We also evaluate the growth of a well-known commercial MLF performer *O. oeni* VF strain. A control condition of grape juice medium without ethanol at pH 4.8 is used as reference.

Both species show similar maximal population, around 10^9 CFU/mL in grape juice medium (Fig. 3). In this sense, *O. alcoholitolerans* reach this maximal population before *O. oeni* at two and four days, respectively. The presence of any of the two stressors delay the development of *O. alcoholitolerans* in the medium. The bacterium is more affected by low pH than the alcoholic content. In fact, *O. alcoholitolerans* could adapt to all ethanol concentrations, also to the highest one of 12 %, even if it

Table 3

Presence of *O. oeni*, and *O. alcoholitolerans* in the studied musts and wines.

	Quantification ^a	
	<i>O. oeni</i>	<i>O. alcoholitolerans</i>
Must 1	+	–
Must 2	+	–
Must 3	+	–
Must 4	+	–
Must 5	+	–
Wine 1	++	–
Wine 2	++	–
Wine 3	+++	–
Wine 4	+	–
Wine 5	+++	–

^a < 10^2 cell/mL, 10^2 – 10^5 cell/mL, ++: 10^7 – 10^9 cell/mL, +++: > 10^9 cell/mL.

takes a long time to start growing (Fig. 3A), as previously reported by Badotti et al. (2014) in MRS/BHI broth medium. A pH lower than 3.5 is not compatible with *O. alcoholitolerans* growth. These results extend the lowest pH value that supports *O. alcoholitolerans* growth, compared to the previous report by Badotti et al. (2014), where no growth was detected at pH 4.0. On the contrary, *O. oeni*, which is the best adapted LAB species to wine environment, grows in all conditions. In the case of this bacterium, *O. oeni* is more affected by ethanol rather than low pH (Fig. 3C). Still, it shows a better adaptation performance to both stressors than *O. alcoholitolerans*.

The inhibition effect of pH and ethanol act in synergy on *O. oeni* in wine environments (Balmaseda et al., 2018). To evaluate the combined effect of ethanol and low pH, we test both stressors simultaneously in grape juice medium by keeping one parameter constant while varying the other—either ethanol concentration or pH. Specifically, pH is fixed at 3.5, a value compatible with *O. alcoholitolerans* growth, while ethanol is maintained at 8 %, slightly below the maximum concentration (10 %) that allows growth within two weeks (Fig. 3B).

Under these conditions, *O. oeni* display robust growth and is not significantly affected by the combination of stressors (Fig. 3D). Although growth is slower under the most extreme conditions—12 % ethanol at pH 3.5, and pH 3.0 with 8 % ethanol—the strain still reach a similar maximum population as in the presence of a single stressor.

In contrast, none of the tested combinations support active growth of *O. alcoholitolerans* (Fig. 3B). However, in milder conditions—6 % or 8 % ethanol at pH 3.5, and pH 4 with 8 % ethanol—the bacterium is able to maintain its initial population, suggesting survival without proliferation.

In conclusion, the synergistic effect of ethanol and low pH severely compromises the survival of *O. alcoholitolerans* in wine-related environments. The absence of a robust response to acid stress, as observed in *O. oeni*, is likely linked to the lack of key genes involved in maintaining membrane and cell wall integrity—genes that are known to be up-regulated in *O. oeni* under wine conditions (Margalef-Català et al., 2016; Olgún et al., 2015). Low pH, in combination with moderate to high ethanol levels, increases membrane fluidity (Grandvalet et al., 2008; Silveira et al., 2003), a condition that appears particularly detrimental to *O. alcoholitolerans*.

Nevertheless, even *O. oeni* can show impaired growth under more extreme conditions—such as pH values below 2.8 or ethanol concentrations above 16 % (v/v)—(Franquès et al., 2018) demonstrating that the synergistic inhibition by pH and ethanol is a general to both LAB, though with differing levels of tolerance. Genomic analysis further supports this interpretation: *O. alcoholitolerans* lacks several antiporters involved in intracellular pH regulation, such as ABC-type multidrug transporters (OEOE_1711, OEOE_1712, OEOE_0722, OEOE_0723, OEOE_0761) and peptide ABC transporters (OEOE_0270, OEOE_0734), which are present in *O. oeni* and likely contribute to its superior stress resistance.

3.4. MLF performance

We test the ability of consuming L-malic acid by *O. alcoholitolerans* in grape juice medium supplemented with 2.5 and 5 g/L of L-malic acid, to a final concentration of around 3 and 5.6 g/L, respectively. For comparison, *O. oeni* VF is used as positive control. Under the studied conditions, the initial inoculum of 10^6 CFU/mL, rapidly reaches a population of 10^9 CFU/mL after two days (Fig. 4A and B). Interestingly, the *O. alcoholitolerans* population in the grape juice medium supplemented with 5 g/L of L-malic acid is higher than that in the 2.5 g/L, 4.4×10^9 and 1.5×10^9 CFU/mL respectively (Fig. 4A and B).

The L-malic acid consumption is very fast. Regardless of the initial concentration, *O. alcoholitolerans* is able to deplete it in two days (Fig. 4A and B). Under the same conditions, *O. oeni* consumes just the 20–35 % of the available L-malic acid (Fig. 4A and B). Even if *O. oeni* is the most adapted LAB to wine conditions and it is a very good MLF performer,

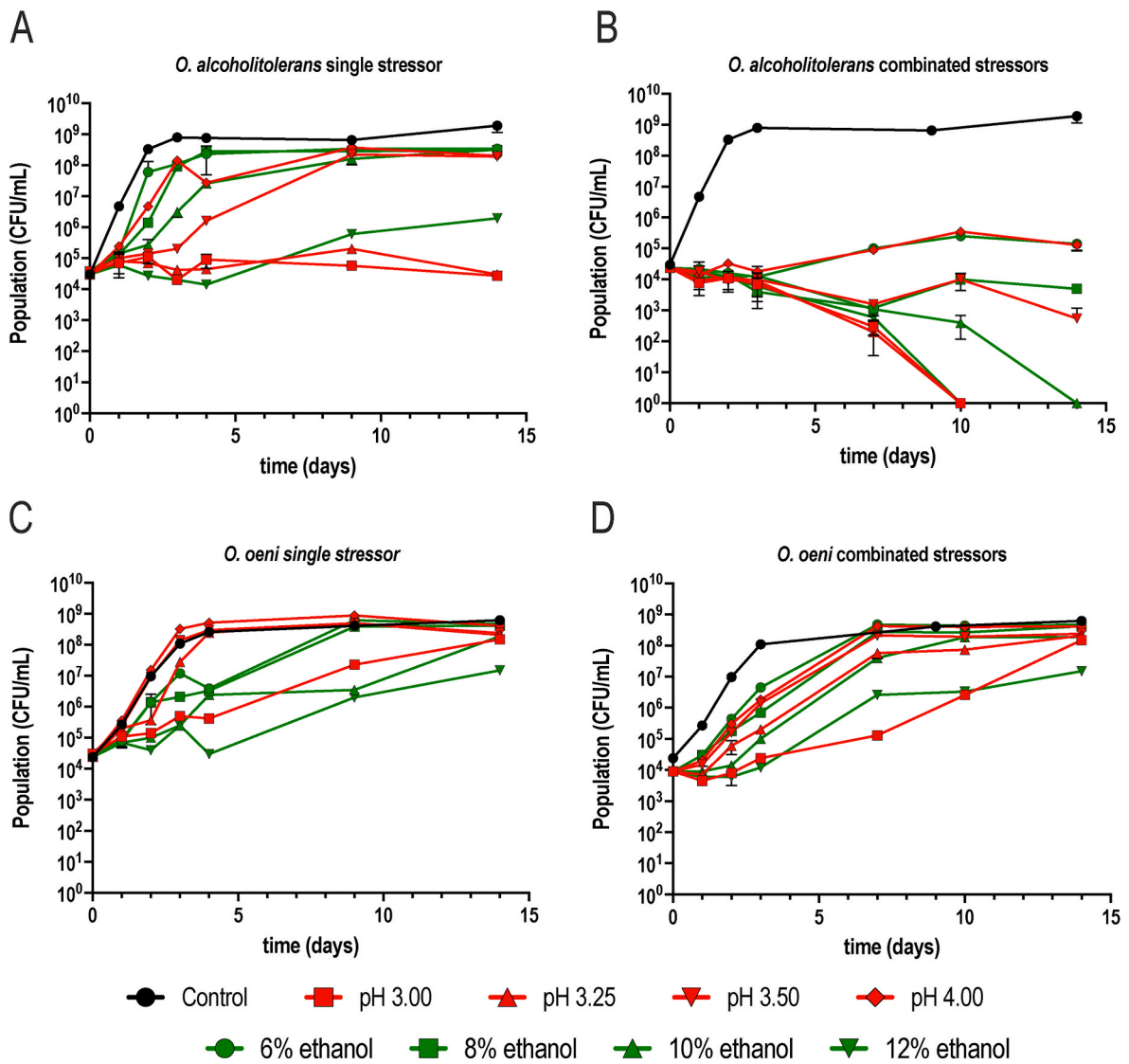


Fig. 3. Growth of *O. alcoholitolerans* and *O. oeni* in grape juice medium with different pH and ethanol concentrations. A: *O. alcoholitolerans* in grape juice medium with different ethanol concentrations (standard pH at 4.8), or pH values (with no ethanol). B: *O. alcoholitolerans* in grape juice medium with different ethanol concentrations (at pH 3.5), or pH values (with 8 % vol/vol of ethanol). C: *O. oeni* in grape juice medium with different ethanol concentrations (standard pH at 4.8), or pH values (with no ethanol). D: *O. oeni* in grape juice medium with different ethanol concentrations (at pH 3.5), or pH values (with 8 % vol/vol of ethanol). Each value represents the mean of duplicates \pm SD.

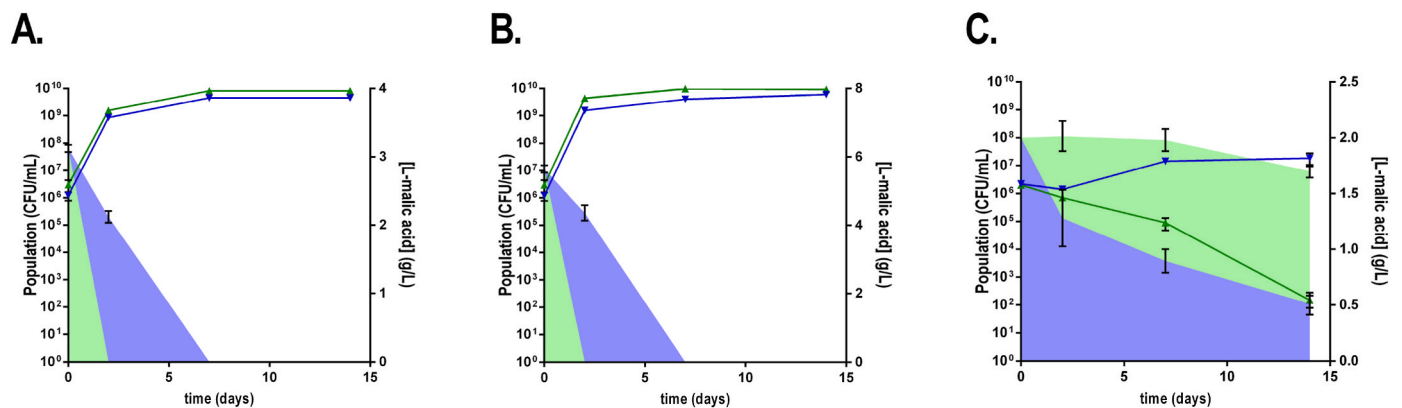


Fig. 4. Malolactic fermentation performance of *O. alcoholitolerans* and *O. oeni* in different media. A: grape juice medium supplemented with 2.5 g/L of L-malic acid. B: grape juice medium supplemented with 5 g/L of L-malic. C: WLM. Green lines represent *O. alcoholitolerans* population. Blue lines represent *O. oeni* population. Shaded green areas represent L-malic acid concentration in *O. alcoholitolerans*. Shaded blue areas represent L-malic acid concentration in *O. oeni*. Each value is the mean \pm SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

O. oeni shows a fast metabolic activity in this culture medium. Still, *O. oeni* deplete L-malic acid in the next sampling point (Fig. 4A and B).

As expected, the MLF of *O. oeni* in WLM is not completed (Fig. 4C). The harsh conditions found in WLM do not enable the survival of the bacterium, and thus, L-malic consumption. L-malic acid is not consumed during the experiment, and at the same time, bacterial viability decreases dramatically down to 10^2 CFU/mL. Also, the control fermentation with *O. oeni* shows a significant increase in bacterial population, reaching 10^7 CFU/mL, and consuming almost all the available L-malic acid.

Overall, the ability of *O. oeni* to degrade L-malic acid is confirmed. When inoculated into grape juice medium, *O. oeni* rapidly deplete L-malic acid, indicating functional malolactic activity under favorable conditions. Compared to *O. oeni*, it exhibits faster growth in low-stress environments (Fig. 3). However, no L-malic acid consumption is observed in WLM, likely due to a rapid decline in cell viability. L-malic acid plays a dual biological role: it serves as an alternative energy source and contributes to cytoplasmic pH homeostasis under acidic stress. *O. oeni* appears less tolerant than *O. oeni* to low pH, which limits its ability to carry out MLF in wine, despite harboring the complete MLF gene cluster (*mleR*, *mleA*, *mleP*). This physiological limitation may also explain why *O. oeni* is not typically found in wine.

4. Final remarks

We here report the first complete *O. oeni* genome from the UFRJ-M7 strain. The comparison of general genomic features, such as genome length and pseudogenes, among sister species of the *Oenococcus* genus suggests that *O. oeni* is genetically closer to *O. sicerae* and *O. kitaharae* than to *O. oeni*. The higher number of pseudogenes in *O. oeni*, reflects ongoing domestication to the wine, whereas the lower pseudogene content and genome reduction in *O. oeni* suggest an earlier and distinct domestication process. In addition, comparative genomic analysis of *O. oeni* with some selected *O. oeni* strains, including all four phylogroups, allows us to identify the main genetic differences between these two species. *O. oeni* has a generally lower number of CDS in some COGs than *O. oeni*. These include (i) defence mechanisms (e.g. ethanol and acidic stress survival); (ii) transcriptional factors, which trigger the stress response; or (iii) some metabolism pathways, such as sugar catabolism, or purine/pyrimidine, and coenzyme- and vitamin biosynthesis. All these differences are likely related to the specific adaptation of *O. oeni* and *O. oeni* to their respective ecological niches. Indeed, *O. oeni* is not detected in the wine samples analyzed in this study. This absence is further supported by physiological assays, which show that the combination of ethanol and low pH stress prevents the survival of *O. oeni*, despite its ability to perform malolactic fermentation (MLF) under less stressful, early fermentative conditions. Altogether, the public availability of this genome will help to better understand the evolutionary pathway of *Oenococcus* species. Understanding the adaptations of species closely related to *O. oeni* may also contribute to improving the management of this key bacterium in winemaking. In particular, identifying stress resistance determinants could help enhance its performance in controlled fermentations or prevent its activity in cases where MLF is not desirable.

CRedit authorship contribution statement

Aitor Balmaseda: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marc Lorentzen:** Software, Methodology, Investigation, Formal analysis, Data curation. **Lucie Duthil:** Investigation. **Cécile Miot-Sertier:** Resources, Methodology, Investigation. **Olivier Claisse:** Software, Resources, Investigation. **Tue K. Nielsen:**

Writing – review & editing, Software, Resources, Methodology. **Lars Hansen:** Supervision, Software, Resources, Methodology. **Patrick Lucas:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2025.104886>.

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