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Influence of pH on *Oenococcus oeni* metabolism: Can the slowdown of citrate consumption improve its acid tolerance?

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ABSTRACT

Oenococcus oeni is the lactic acid bacteria most suited to carry out malolactic fermentation in wine, converting L-malic acid into L-lactic acid and carbon dioxide, thereby deacidifying wines. Indeed, wine is a harsh environment for microbial growth, partly because of its low pH. By metabolizing citrate, *O. oeni* maintains its homeostasis under acid conditions. Indeed, citrate consumption activates the proton motive force, helps to maintain intracellular pH, and enhances bacterial growth when it is co-metabolized with sugars. In addition, citrate metabolism is responsible for diacetyl production, an aromatic compound which bestows a buttery character to wine. However, an inhibitory effect of citrate on *O. oeni* growth at low pH has been highlighted in recent years. In order to understand how citrate metabolism can be linked to the acid tolerance of this bacterium, consumption of citrate was investigated in eleven *O. oeni* strains. In addition, malate and sugar consumptions were also monitored, as they can be impacted by citrate metabolism. This experiment highlighted the huge diversity of metabolisms between strains depending on their origin. It also showed the capacity of *O. oeni* to *de novo* metabolize certain end-products such as L-lactate and mannitol, a phenomenon never before demonstrated. It also enabled drawing hypotheses concerning the two positive effects that the slowing down of citrate metabolism could have on biomass production and malolactic fermentation occurring under low pH conditions.

1. Introduction

Malolactic fermentation (MLF) is the second fermentation step occurring in wine. This fermentation, conducted by lactic acid bacteria (LAB), consists mainly of the decarboxylation of L-malic acid into L-lactic acid and CO_2. As a result, the total acidity of wines is reduced and pH increases by 0.1–0.2 units (Bartowsky, 2005). During this step, bacterial activity will also improve the microbial stability of the beverage/or wine as the bacteria will consume residual carbon sources. Furthermore, their metabolism will enhance the organoleptic quality of wine, contributing buttery, nutty, fruity notes. Wines which have undergone MLF are described as being rounder and fuller in taste (Lerm et al., 2010). One of the main aromatic compounds that can be produced during MLF is diacetyl, which bestows a buttery character to wine (Lonvaud-Funel, 1999; Bartowsky and Henschke, 2004). This C4 compound is mainly derived from LAB citrate metabolism (Fornachon and Lloyd, 1965; McKay and Baldwin, 1990; Ramos et al., 1995). Diacetyl production is wanted in only limited quantities in wine (<4 mg/L), otherwise the buttery note is too pronounced and becomes undesirable (Bartowsky and Henschke, 2004).

LAB associated with wine belong to the *Lactobacillus, Latilactobacillus, Lentilactobacillus, Laticaseibacillus, Fructilactobacillus, Liquorilactobacillus, Apilactobacillus, Lactiplantibacillus, Limosilactobacillus, Levilactobacillus, Oenococcus, Leuconostoc, Pediococcus* and *Weissella*, genera (Dicks et al., 1995; Bartowsky, 2005; Zheng et al., 2020). At the end of alcoholic fermentation, most species will not survive the harsh environmental conditions, i.e. low pH, high ethanol and SO_2 concentrations, low temperature and the depletion of available nutrients due to yeast activity. Thus, it is not rare to observe stuck or delayed MLF due to extreme environmental parameters that can affect growth or LAB metabolism (Bech-Terkilsen et al., 2020). To ensure the completion of MLF, winemakers may inoculate musts or wines with commercial starter cultures of *O. oeni* strains. Indeed, *O. oeni* is the most suitable species to conduct malolactic fermentation in wine because of its considerable

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ability to withstand the stresses associated with wine. (Lonvaud-Funel et al., 1991; Bartowsky, 2005; Lerm et al., 2010). This Gram-positive bacterium shows a high genetic diversity correlated with its ecological niche, as it is also found in other beverages such as cider and kombucha (Campbell-Sills et al., 2015; Lorentzen et al., 2019). To date, four phylogenetic groups have been identified, named A, B, C and D. In wine, the most common phylogenetic group represented is group A, although strains from groups B and C can also be found. Known strains from group B and C have often been isolated from cider while, finally, strains from group D essentially originate from Kombucha (Campbell-Sills et al., 2015; Lorentzen and Lucas, 2019; Lorentzen et al., 2019; Balmaseda et al., 2023).

The pH of wine ranges between 2.9 and 3.9 (Mills et al., 2005). Under conditions of high acidity (pH lower than 3.2), the survival of *O. oeni* is strongly compromised (Henick-Kling, 1993). The selection of more acid tolerant strains is required to ensure the success of MLF in complicated environmental conditions such as those found in Champagne wine, where pH can reach 2.9 after alcoholic fermentation.

In an attempt to understand the acid tolerance mechanism, an adaptive evolution was performed with the ATCC BAA-1163 *O. oeni* strain (Julliat et al., 2023). At the end of this process, the genome of 4 evolved populations was sequenced and demonstrated the systematic acquisition of mutations into citrate operon. This operon is composed of 9 different genes (Mills et al., 2005). Indeed, citrate is internalized under its anionic form through a uniport encoded by *maeP* (Ramos et al., 1994). Citrate is then catalyzed into oxaloacetate and acetate by citrate lyase which is composed of 3 subunits, encoded and activated by the expression of 6 genes (i.e. *citC, D, E, F, X* and *G*) (García-Quintáns et al., 2008). Finally, *mae* encodes a putative oxaloacetate decarboxylase enzyme, converting oxaloacetate into pyruvate and CO_2 (Sender et al., 2004; García-Quintáns et al., 2008). An extra gene, *citR*, is positioned in divergent direction in the *cit* locus and encodes a putative transcriptional regulator but its effect on citrate locus expression has not yet been established in *O. oeni*. Citrate metabolism has been reported to have a beneficial effect on *O. oeni* physiology and particularly on its acid tolerance. Indeed, as with malate metabolism, citrate metabolism is able to activate Proton Motive Force (PMF): $\Delta\psi$ and ΔpH are generated respectively by citrate uptake under its anionic form and when oxaloacetate is converted to pyruvate, leading to the consumption of one scalar proton (Poolman, 1993; Ramos et al., 1994; Lolkema et al., 1995). The consumption of protons during citrate catabolism also helps the cell to maintain its intracellular pH (Ramos et al., 1994). In addition, it has been demonstrated that the co-metabolism of citrate and sugar enhance *O. oeni* growth (Salou et al., 1994; Ramos and Santos, 1996). Nevertheless, a previous study has shown that the addition of citrate in pH 3.2 FT80 medium totally impaired the growth of ATCC BAA-1163 strain (Augagneur et al., 2007b). The same experiment was conducted by Julliat and collaborators (Julliat et al., 2023) on the evolved *O. oeni* populations obtained and demonstrated that slowing down the citrate consumption rate suppressed the growth inhibition observed in the parental strain (Julliat et al., 2023). It is therefore possible that a change in the rate of citrate metabolism may lead to improved acid tolerance in *O. oeni.*

The aim of this study was to gain insight on the influence of low pH on *O. oeni* metabolism (mainly malate, citrate and sugar consumptions) with a focus on citrate metabolism. A genomic comparison was performed between several *O. oeni* strains to identify genetic variabilities in their *cit* loci. Genotype was linked to phenotype by monitoring growth along with citrate, malate and sugar metabolism in 11 *O. oeni* strains originating from wine and kombucha and cultivated in GEMO medium under different pH conditions. The production of acetate, lactate, diacetyl, acetoin, ethanol and mannitol was also monitored to ascertain metabolism pathways. Thus, several hypotheses could be drawn concerning the positive effects that slowing down citrate metabolism in *O. oeni* could have on its acid tolerance.

2. Material and method

2.1. Bacterial strains and growth conditions

Eleven *O. oeni* strains were used in this study: two laboratory strains which are ATCC BAA-1163 (isolated from Bordeaux red wine) and PSU-1 (isolated from wine made in Pennsylvania, USA); three commercial strains IOEB_S450 (red wine, Laffort, France), B16 (white wine, Laffort, France) and NB_1858 (Cabernet Sauvignon, Laffort, France); five isolated strains: NB_1911 (isolated by l'Institut Français de la Vigne et du Vin (IFV), France), 13.3 (from Puligny 2013, isolated by Unité Mixte de Recherche Procédés Alimentaires et Microbiologiques (UMR PAM), France), 23.1 (from St Romain 2013, UMR PAM, France), 26.1 (from Les forêts 2013, UMR PAM, France), 32.2 (from Hautes Côtes 2013, UMR PAM, France) and one strain isolated from Black tea Kombucha (produced from 1 % (m/v) pure black tea infusion and 50 % (m/v) sucrose, inoculated with 12 % (v/v) of kombucha culture) named TTK (Biomère, France) (Tran et al., 2022). These strains were chosen according to their genetic characteristics to study their citrate metabolism and the impact on acid tolerance.

Cell growth was performed in 50 mL tubes (Greiner Bio-one, Austria) filled with 50 mL of GEMO medium (Growth Effective Medium for *Oenococcus oeni*) (Julliat et al., 2023) pH adjusted to 4.8, 3.6 or 3.2.

2.2. Analytical method for monitoring substrate consumption and product synthesis

Every 4 hours, 800 μL of the medium were sampled from each culture, centrifuged at 8,000 g for 3 min and supernatants were frozen at −20 °C for later analysis. Glucose and fructose (ref: 12800), malate (ref: 12803), citrate (ref: 12825), L-lactate (ref:12802), D-lactate (ref: 12801), acetate (ref: 12930) and glycerol (ref: 12812) were measured by enzymatic assays using the automatic sequential analyzer Y15 (Biosystems, Spain). Mannitol (ref: 15) and ethanol (ref: 26) were quantified using enzymatic kits from Biosentec (France) following the manufacturer's instructions.

Diacetyl and acetoin were simultaneously measured using the colorimetric reaction developed by Mattessich (Mattessich and Cooper, 1989) as follows: 125 μL of a filtered 1 % α-Naphtol solution in 2.5 N NaOH and 125 μL of 0.2 % creatine solutions were added to 20 μL of samples. The reaction was performed in 96 well plates and absorbance at 525 nm was measured using the Infinity 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland) after 30 min of incubation. A standard curve was made with a 5 g/L diacetyl solution diluted in GEMO medium to obtain a final concentration of 10, 50, 100, 150 and 200 mg/L.

Erythritol was quantified by HPLC using Agilent 1260 Infinity II (Vial Sampler G71291, Quat Pump G7111B, DAD WR G7115A and RID G7162A) with an Aminex HPX-87H ion exchange column (Bio-Rad, the United States). Mobile phase was H_2SO_4 0.065 mM with a flow of 0.45 $mL.min^{-1}$ and oven at 30 °C. Detection was performed by refractometry using the 1620 RID G7162A module. Standard curve was created using *meso*-Erythritol (purity $\geq$ 99 %, ref: E7500, Sigma-Aldrich).

2.3. Final carbon balance calculation

For final carbon balance calculation, the quantity (in millimolar (mM)) of each substrate consumed and product synthesized was multiplied by the number of carbons composing the molecule. As the quantity of CO_2 produced could not be neglected but was not dosed, it was assumed that one mole of CO_2 is produced for one mole of citrate, malate, glucose and fructose (minus the quantity of mannitol produced) consumed and one mole of diacetyl or acetoin the origin of the production of two moles of CO_2. Then, total carbon from products was divided by total carbon from substrates and the result was multiplied by 100. To resume, the equation below (1) was applied:

$$Final\,carbon\,balance\,(\%) = \frac{\sum (Products\,(mM)\,\times\,carbon)}{\sum (Substrates\,(mM)\,\times\,carbon)} \times 100 \qquad (1)$$

Final carbon balance calculation.

2.4. Citrate locus sequencing and genomic comparison

The *cit* loci from 13.1, 23.1, 26.1, 32.2, NB_1858, NB_1911 and TTK strains were sequenced by Sanger sequencing performed by Genewiz from Azenta Life Sciences (Leipzig, Germany). Twelve pairs of primers were designed to amplify DNA fragments from 542 to 1007 bp covering all the *O. oeni cit* locus (Table S1). The PCR products were purified using NucleoSpin Gel and PCR Clean-up kit following the manufacturer's instructions (Macherey-Nagel, Germany) and sent to Genewiz for the sequencing. Sequences were then submitted to GenBank and the accession numbers are: OR754044 (NB_1911), OR754045 (13.3), OR754046 (23.1), OR754047 (32.2), OR754048 (NB_1858), OR754049 (26.1) and OR754050 (TTK).

The 30 *cit* locus sequences were aligned and compared using Clustal Omega program version 1.2.4 (Sievers & Higgins, 2021). The evolutionary history was inferred by using the Maximum Likelihood method (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 31 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There was a total of 10,865 positions in the final dataset. Evolutionary analyses were conducted using the MEGA11 software (Tamura et al., 2021).

2.5. RNA extraction and qPCR analysis

A RT-qPCR analysis was performed on some strains. Total RNA was extracted after 16 hours of growth in pH 4.8 GEMO medium (end of exponential growth phase). RNA extraction, DNase treatment and reverse transcription were performed as previously described (Julliat et al., 2023). cDNA obtained after reverse transcription were quantified by qPCR using Real Time PCR System CFX96™ (Biorad, USA) using SsoAdvanced Universal SYBR Green Supermix (Biorad, USA) following manufacturer's instructions. The amplifications were carried out with an initial step at 98 °C for 3 min followed by 40 cycles of 98 °C for 10 s and 53.5 °C for 30 s. Specific primers were designed with Primer 3® 4.1.0 software (table S1) and *ldh* and *gyrA* were used as the housekeeping genes (Desroche et al., 2005). Standard curves for each pair of primers were run to check qPCR efficiency. Samples of total RNA which did not undergo reverse transcription were used as controls of the absence of residual genomic DNA.

3. Results

3.1. Genetic diversity of O. oeni cit loci between strains

The *cit* locus sequence of 30 *O. oeni* strains was compared to see whether an emergence of genetic variability could be observed over the years on the *cit* locus of strains whose original environment is wine or very acid beverages (Table 1 and Fig. 1). These strains were selected on the basis of the availability of genomes in the genomic databases and to ensure that the four genetic lineages A, B, C and D was represented. A majority of commercial strains were used as they were selected for their good capacity to resist and conduct malolactic fermentation in any circumstance. Five strains from Champagne and sparkling white wine were also added to the panel as they were isolated from very acid wines. Among the 30 strains, 7 have been recently isolated from wine (13.3, 23.1, 26.1, 32.2, NB_1858 and NB_1911) or kombucha, a fermented beverage made from sweet tea (TTK). The 30 citrate loci were all aligned to define a consensus sequence from which genetic variability could be identified for each strain.

A phylogenetic tree was drawn with the *cit* locus sequences of the 30 strains to obtain an idea of the evolutionary history (Fig. 1). The closest species *Leuconostoc mesenteroides* subsp. *cremosis,* possessing a citrate locus, was used to root the tree. This representation clearly emphasizes the four *O. oeni* phylogroups A, B, C and D. It is thus possible that the citrate locus evolved over time to adapt to the environmental conditions encountered by each strain. Indeed, all these strains belonging to the four known phylogroups were isolated from different beverages, i.e. wine, cider and kombucha, which represent very different physicochemical growth conditions for the bacteria. It can also be noted that the four strains isolated from acid wines (B16, Viniflora CH35, IOEB_0205, BL-01 and S22) are close to each other on this phylogenetic tree.

Table 1 shows the change in amino acid sequence (or DNA sequence for the two intergenic regions) caused by genetic variability for each strain. This comparison shows that there is a high genetic diversity between the strains. Indeed, only two out of the 30 strains do not possess any Single Nucleotide Polymorphisms (SNP) or amino acid substitution and only 9 of the remaining 28 strains have substitutions that could not be assigned as non-conservative. It can be observed that *citR*, *mae, maeP* and *citC* are the main genes targeted by genetic variations and, in many cases, a proline is substituted. Interestingly, in *citR*, all the non-conservative substitutions detected involve a threonine (hydrophile with an uncharged side chain) and an isoleucine (hydrophobic side chain) or a proline (hydrophobic with a bended geometry that could affect the protein secondary structure). As CitR is a putative regulator of *cit* locus expression, the genetic variations carried by 14 out of the 30 strains on *citR* could strongly impact the *cit* locus expression.

This genetic comparison also showed a divergent evolution of the *cit* locus from phylogroup A strains and phylogroups B, C and D. In phylogroup A, 10 out of the 22 strains possess missense mutations leading to a non-conservative substitution. In addition, CiNe and BL-01 possess nonsense mutations (annotated "STOP" in Table 1) resulting in the inactivation of MaeP which would clearly affect citrate metabolism. Indeed, these strains are commercial strains known to not metabolize citrate at all (stated in the product information sheet by Chr. Hansen and Martin Vialatte). Among the 10 strains mentioned above, 5 of them (Viniflora CH35, IOEB_205, BL-01, B16 and S22) are strains isolated from wines whose pH is very low (2.9), thus they probably have a better tolerance to low pH than the other strains. In phylogroups B, C and D, many more genetic variations can be observed, showing that citrate metabolism is probably different in these strains than in phylogroup A strains. It can also be noted that one strain (ATCC BAA-1163) out of the 4 phylogroup B strains studied possesses a nonsense mutation in *citR,* leading to the synthesis of a truncated protein.

3.2. Citrate metabolism: From genes to phenotype

The genomic comparison demonstrated a considerable genetic variability between the 30 *O. oeni* strains studied. To relate genomic typicity to phenotype, citrate uptake was monitored in 11 out of the 30 *O. oeni* strains. These strains, marked with the footnote "c" in Table 1 and with a star in Fig. 1, were deliberately chosen from different origins and belong to phylogroups A (9 strains), B (1) and D (1). The focus was on phylogroup A strains because of the interest in wine strains. Five out of the 11 strains were selected as they possess genetic variations that could influence citrate metabolism: 23.3 and 32.2 which possess nucleotide substitutions in the two intergenic regions, and the one located between *mae* and *maeP* could be on the RBS of *maeP*; NB_1858 which possesses an amino acid non-conservative substitution I7T on CitR; B16 which possesses an amino acid non-conservative substitution T8I on CitR; finally ATCC BAA-1163 (later abbreviated to ATCC) which possesses a nonsense mutation on *citR* (L256STOP). The 6 other strains are IOEB_S450, NB_1911, 26.1, 13.3, PSU-1 and TTK.

The citrate uptake of these strains was monitored in GEMO medium

Table 1
Genetic comparison of *cit* locus from 30 different *O. oeni* strains.

Strain name	Origin	Accession number	Phylogenetic Group	*citR*[a]	Intergenic (*citR* → *mae*) 149 p[b]	*mae*[a]	Intergenic (*mae* → *maeP*) 95 pb[b]	*maeP*[a]	*citC*[a]	*citD*[a]	*citE*[a]	*citF*[a]	*citX*[a]	*citG*[a]
VF	Commercial	AZLM00000000	A	–	–	–	–	–	–	–	–	–	–	–
Lalvin EQ54	Commercial	ALAF00000000	A	–	–	–	–	–	–	–	–	–	–	–
S19	Red wine	AZLK00000000	A	**T172I**	–	–	–	–	–	–	–	–	–	–
NB_1858[c]	Commercial	OR754048	A	**I7T**	–	–	–	–	–	–	–	–	–	–
PSU-I[c]	Commercial	CP000411	A	–	–	K53R A267G	–	–	–	–	–	A420V	–	–
13.3[c]	White wine	OR754045	A	–	–	K53R A267G	–	–	–	–	–	A420V	–	–
26.1[c]	Red wine	OR754049	A	–	–	–	–	–	–	–	–	V35I	–	A88T
VP41	Commercial	CP084701	A	–	–	–	–	–	–	–	–	V35I	–	A88T
S28	Red wine	AZJY00000000	A	–	–	–	–	–	–	–	–	V35I	–	A88T
S23	White wine	AZLL00000000	A	–	–	–	–	–	–	–	–	V35I	–	A88T
NB_1911[c]	Wine	OR754044	A	–	–	–	–	–	–	–	–	V35I	–	A88T
SB3	Commercial	MEHP00000000	A	Q254K	–	–	–	–	–	–	–	–	–	A88T
O37	White wine	CP053280	A	S42N	–	–	C → T (7)	–	–	–	–	–	–	A88T
23.1[c]	Red wine	OR754046	A	S259A	G → A (65)	–	**G → A (84)**	–	–	–	–	–	–	–
32.2[c]	White wine	OR754047	A	S259A	G → A (65)	–	**G → A (84)**	–	–	–	–	–	–	–
IOEB_S450[c]	Commercial	AZLT00000000	A	–	–	–	C → T (7)/C → T (59)	–	N75D	–	–	**P432S**	–	A88T
CiNe	Commercial	AZJV00000000	A	–	–	E356K	–	**G245STOP**	–	–	–	V35I	**G49R**	A88T
Viniflora CH35	Commercial	ALAG00000000	A	**T8I**/A154T/S259A	–	**L315P**	T → C (73)	–	T52A	–	–	–	E11K/**S155P**	A235V
IOEB_0205	Champagne wine	AZHH00000000	A	**T8I**/A154T/S259A	–	**L315P**	T → C (73)	–	T52A	–	–	–	E11K/**S155P**	A235V
BL-01	Commercial	ALAH00000000	A	**T8I**/A154T/S259A	–	**L315P**	T → C (73)	**K177STOP**	–	–	–	–	E11K/**S155P**	A235V
B16[c]	Commercial	AZKC00000000	A	**T8I**/A154T/S259A	–	**L315P**	T → C (73)	–	T52A	–	–	–	E11K/**S155P**	A235V
S22	Sparkling white wine	AZKA00000000	A	**T3I**/D5N/**T173I**/S259A/R318Q	–	N253D/**S279F**/**L315P**/V355I	A → C (60)/T → C (73)	R25Q /L132F/**S158I**	RAS	–	H128N	–	**S155P**	–
SD-2a	Chinese wine	CP038451	B	**T77P**/K191R/S194A /S259A	G → C (89)	N253D/T301V/**L315P**/G326S/V346A/V374A	C → T (20)/A → C (60)/T → C (73)	A19V/R25Q/V142I/**P270S**	**S7L**/N118T/G130D/**P289S**	–	A2V/**S211R**/**P233S**	–	**S155P**	**G92R**
IOEB_C23	Cider	AZJU00000000	B	**T77P**/K191R/S194A /S259A	G → C (89)	N253D/T301V/**L315P**/V346A/V374A	C → T (20)/A → C (60)/T → C (73)	A19V/R25Q/V142I/**P270S**	**S7L**/N118T/G130D/**P289S**	–	A2V/**S211R**/**P233S**	**P465T**	**S155P**	T123A
S12	White wine	AZLH00000000	B	**T77P**/K191R/S194A /S259A	G → C (89)/C → A (117)	N253D/T301V/**L315P**/V346A/V374A	C → T (20)/A → C (60)/T → C (73)	A19V/R25Q/V142I/**P270S**	**S7L**/N118T/G130D/**P289S**	–	A2V/**S211R** /**P233S**	V422I	**S155P**	–
ATCC BAA-1163[c]	Red wine	AAUV00000000	B	**T77P**/A154T/K191R/S194A/**L256STOP**	G → C (89)	N253D/T301V/**L315P**/V346A/V374A	A → C (60)/T → C (73)	A19V/R25Q/V142I/**P270S**/A284T	**S7L**/N118T/G130D/**P289S**	–	A2V/**S211R**/**P233S**	–	**S155P**	–
CRBO_1381	Cider	LR031358	C	**T77P**/K191R/S194A/S259A	A → G (62)	N253D/T301A/Q305E	A → C (60)/T → C (73)	R25Q/V142I	**S7L**/N118T/V283M	–	**S211R**/Q213K	R239K	**S155P**	–

(*continued on next page*)

Table 1 (*continued*)

Strain name	Origin	Accession number	Phylogenetic Group	*citR*[a]	Intergenic (*citR* → *mae*) 149 p[b]	*mae*[a]	Intergenic (*mae* → *maeP*) 95 pb[b]	*maeP*[a]	*citC*[a]	*citD*[a]	*citE*[a]	*citF*[a]	*citX*[a]	*citG*[a]
						/**<u>L315P</u>**/ V374A								
CRBO_1384	Cider	ULGC00000000	C	**<u>T77P</u>**/K191R/ S194A/S259A	A → G (62)	N253D/ T301A/Q305E /**<u>L315P</u>**/ V374A	A → C (60)/T → C (73)	R25Q/V142I	**<u>S7L</u>**/ N118T/ V283M	–	**<u>S211R</u>**/ Q213K	R239K	**<u>S155P</u>**	–
UBOCC-A-315001	Kombucha	CP014324	D	**<u>T77P</u>**/S194A/ S259A	G → C (89)	N253D/ **<u>L315P</u>**/V374A	G → A (28)/A → C (60)/T → C (73)	R25Q/V142I	T227A	–	**<u>S211R</u>**/ **<u>P233S</u>**	V493I	**<u>S155P</u>**	–
TTK[c]	Kombucha	OR754050	D	**<u>T77P</u>**/A153T/ S194A/S259A	G → C (89)	**<u>P116S</u>**/ N253D/ **<u>L315P</u>**/V374A	A → C (60)/T → C (73)	R25Q/V142I/ V305I	T227A	–	**<u>S43Y</u>** / **<u>S211R</u>**	L210I / V493I	**<u>S155P</u>**	–

[a] Substitutions in amino acid sequences cause by a genetic variation are indicated. Genetic variations that may have an impact on the function of the protein (i.e. residues with quite different properties, in terms of hydrophobicity, charges and structures) are underlined and in bold and those wich cause the appearance of a premature stop codon are marked with a "STOP".

[b] Single Nucleotide Polymorphisms (SNP) are indicated and the nucleotide position. Genetic variations that may have an impact on the locus expression are underlined and in bold.

[c] Strains used in this study.

(Julliat et al., 2023). This medium was specially created for optimizing *O. oeni* growth, taking into account several studies on the nutritional requirements of LAB, especially *O. oeni* (Snell, 1945; MacLeod and Snell, 1947; Raccach, 1985; Cavin et al., 1989; Salou et al., 1994; Dicks et al., 1995; Hébert et al., 2004; Zhang and Lovitt, 2005). This medium offers a better growth rate than several other known media such as FT80 or MRS modified or not, and all *O. oeni* strains can grow on it when the pH is adjusted to 4.8, which is optimal for most strains. Therefore, this makes it easier to compare the growth of different strains even under more acidic conditions. The medium is supplemented with malate and citrate as the main organic acid sources and with grape juice to get closer to oenological conditions. The experiment was conducted at two pH values: pH 4.8 (optimal pH) and pH 3.6, which represents a more acidic condition but still favorable for bacterial growth. In addition to citrate, malate uptake was also monitored to know if a modification of the citrate consumption rate could influence malolactic fermentation. Finally, glucose and fructose consumption was also monitored as it is well established that this metabolism is closely related to citrate metabolism in LAB (Salou et al., 1994; Ramos and Santos, 1996; Zaunmüller et al., 2006). The results are represented in Fig. 2 and Table 2.

At pH 4.8 (Fig. 2A, B, C and D), the growth and malate consumption kinetics are quite similar between the 11 strains. ATCC and TTK stood-out at this pH range, as they consumed citrate and hexoses much faster than the other strains. In the same way, they grew faster and achieved the highest final biomass (Table 2). Note that B16 stopped consuming hexoses after 52 h of growth, leaving a quarter of residual sugar (4 g/L of glucose/fructose) in the medium. This fact was already reported by the past in different Champagne strains (Cibrario et al., 2016).

Growth and metabolism were affected for all strains when pH was decreased to pH 3.6 (Fig. 2E, F, G & H). The first thing of note was that 13.3 was not able to grow, making it the least acid tolerant strain in the group. For the others, while malate consumption rate did not seem to have been strongly affected by the pH decrease, citrate and hexose uptake was slower than at pH 4.8. Indeed, while all the sugars were consumed at pH 4.8 in less than 60 h, only 33 % of them disappeared from the medium at pH 3.6 (around 12 g/L left out of 18 g/L at the beginning, except for ATCC and TTK). As seen at pH 4.8, both ATCC and TTK had much faster citrate and hexose metabolisms than the other strains and achieved a higher final biomass (Table 2).

This experiment demonstrated a high diversity of citrate metabolism between the strains. To analyze the results in greater depth, the time required to consume half the citrate was determined for each strain under both pH conditions (Fig. 2, I & J). NB_1911 and 26.1 were used to define the reference of the citrate consumption rate as they show less genetic variation among the 11 strains on their citrate locus (Table 1), and none of them involve major changes in protein sequence or in intergenic regions. Statistical analysis enabled classifying the strains into 3 groups depending on their citrate consumption rate: the slowed down strains (4 at pH 4.8 and 3 at pH 3.6, blue boxes in Fig. 2I & J), the standard strains (PSU-1, NB_1911, IOEB_S450, 13.3 and 26.1, uncolored boxes in Fig. 2I & J) and the fast-consuming strains (ATCC and TTK, pink boxes in Fig. 2I & J). Interestingly, the 9 phylogroup A strains studied, which are wine-related strains, are all divided in the standard and the slowed down group, while the two strains belonging to other phylogroups always consumed citrate faster. In addition, it can be noted that the slowed down strains were able to reach a higher final biomass compared to the standard group at pH 3.6, while this was not the case at optimal pH (Table 2).

A link between citrate consumption and gene expression was established by performing a RT-qPCR analysis in some of the 11 strains grown at pH 4.8 (i.e. PSU-1, IOEB_S450, B16, NB_1858, 32.2 and ATCC, Figure S1). The ΔΔCt method was applied using the PSU-1 strain as a reference (Livak and Schmittgen, 2001). It shows that the number of transcripts quantified for the *citR, mae, maeP* and *citE* genes is not significantly different between standard strains PSU-1, IOEB_S450 and B16, with the exception of *maeP* in B16 which is slightly downregulated

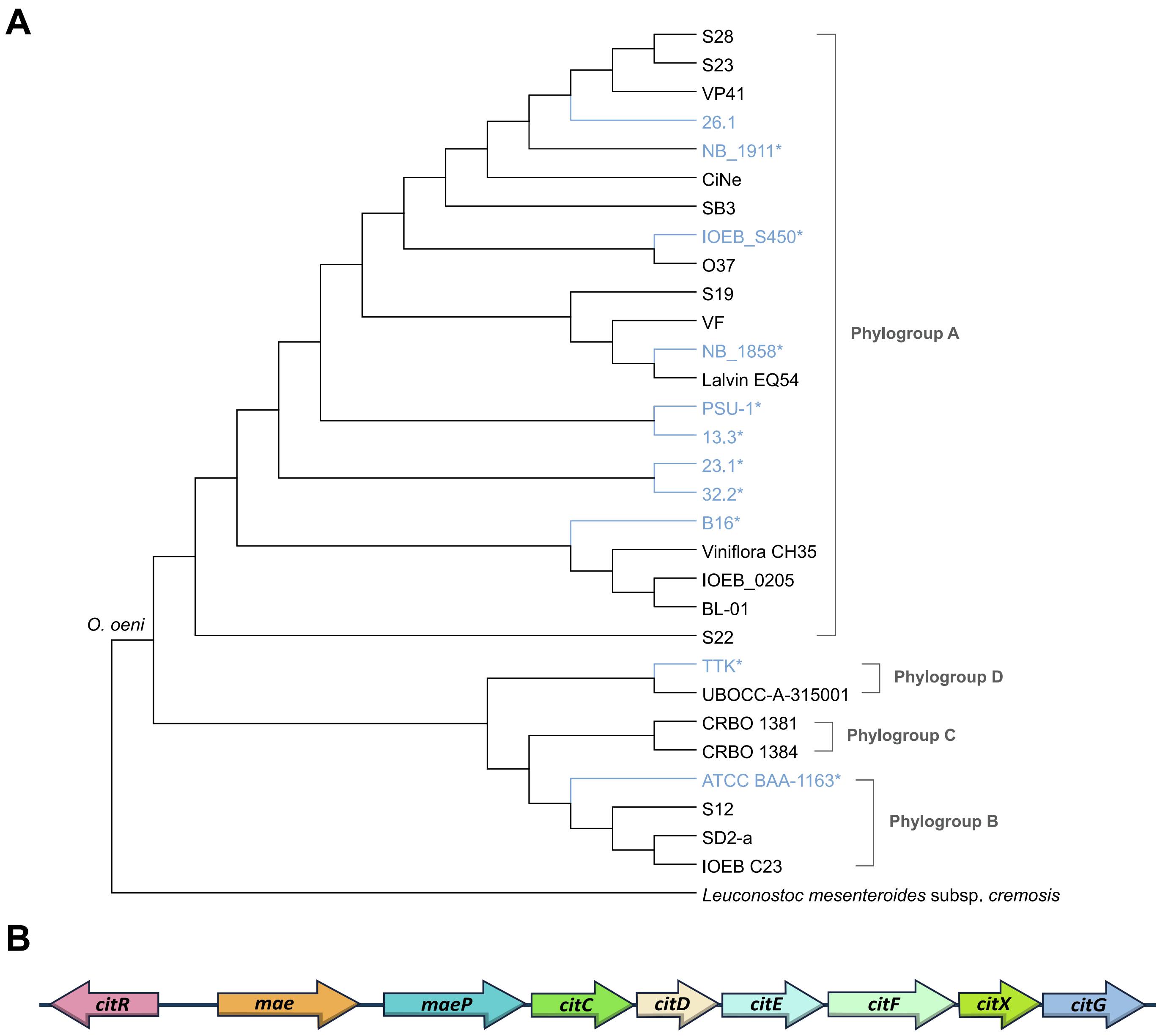


Fig. 1. Maximum likelihood tree of the *O. oeni* strains studied. A: Phylogenetic tree. strains used for the metabolism study are written in blue marked with a star. The closest species *Leuconostoc mesenteroides* subsp. *cremosis* was used to root the tree. **B:** *cit* locus of *O. oeni*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in this strain (calculated relative expression = 0.348). This correlates with the slower citrate consumption reported at pH 4.8 for this strain. On the other hand, the number of transcripts quantified in NB_1858 is much lower than that quantified for PSU-1 (with a calculated relative expression ranging from 0.002 to 0.271 depending on the gene), which explains why this strain consumed citrate slower. Unexpectedly, the number of transcripts quantified for 32.2 is not significantly different from PSU-1, which does not correlate with citrate consumption of this strain. 32.2 possesses three genetic variations, including one substitution in the putative RBS before *maeP* (Table 1). Thus, it may be possible that the deregulation of the *cit* locus expression occurs at the level of translation in this strain. Finally, about 2–9 times more transcripts were quantified for the *cit* genes in ATCC. Therefore, with the exception of 32.2, the rate of citrate consumption correlates with the level of transcription of the *cit* locus in each strain.

3.3. *Slowing down of the citrate consumption rate is correlated with a modification of the final carbon balance under low pH*

One question raised was whether the higher final biomass production observed in the slowed down strains could be the consequence of a reorientation of carbon fluxes which optimize energy production. To answer this question, the final production of acetate, D/L-lactate and mannitol was quantified (Fig. 3, C & D) along with the total consumption of malate, citrate and hexoses (Fig. 3, A & B) for each strain grown at both pH values.

At pH 4.8, the strains consumed all the substrates except for B16 which did not consume all hexoses (Fig. 2D). Globally, the strains belonging to the standard or the slowed down group, except B16, produced the same amount of L/D-lactate, mannitol and acetate, i.e. 20 % L-lactate, 26 % mannitol, 33 % D-lactate and 20 % acetate. On the other hand, B16 produced significantly less L/D-lactate and mannitol. Lower

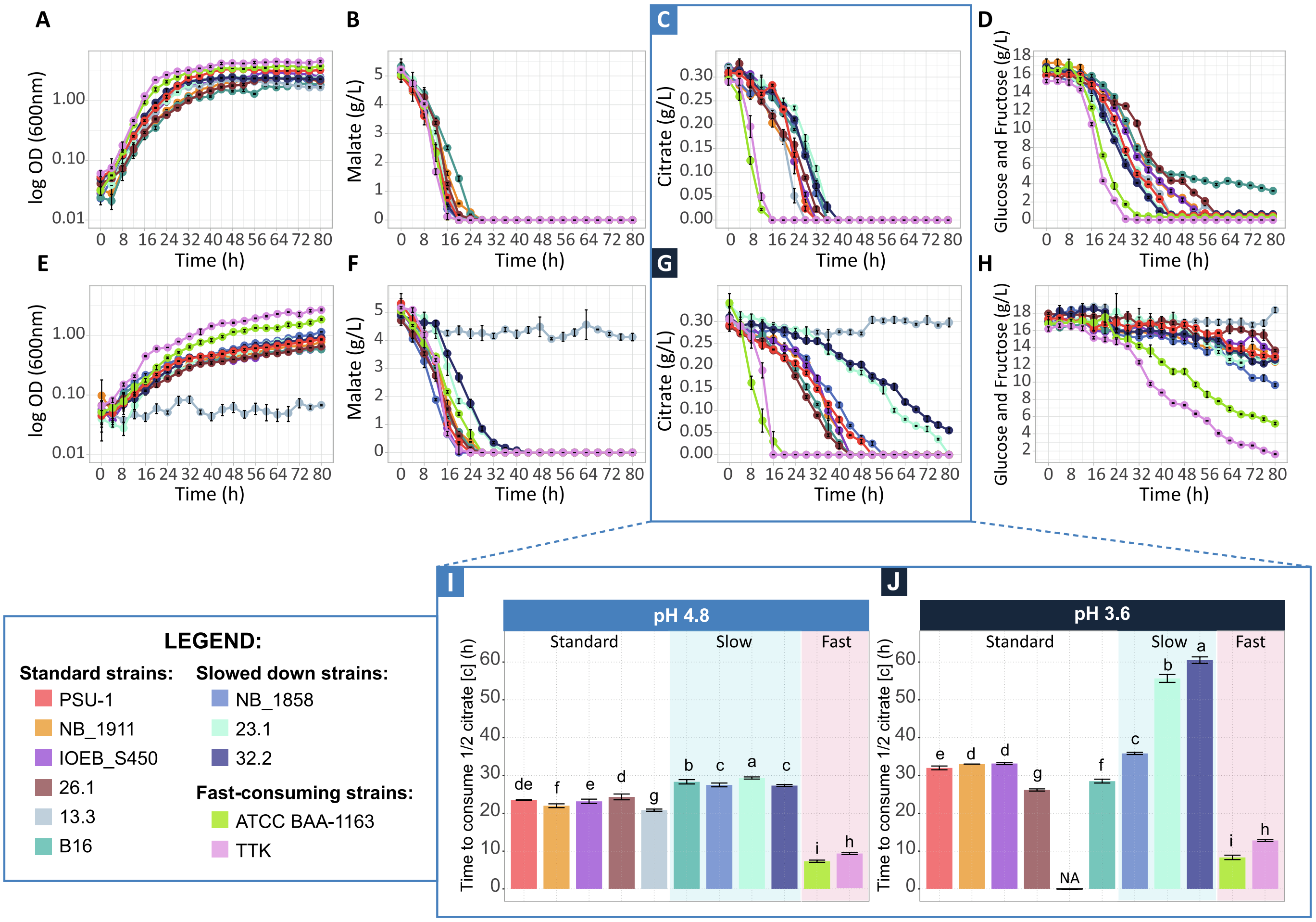


Fig. 2. Growth, citrate, malate and hexose uptake for the 11 strains as a function of pH. The growth (A, E), consumption of malate (B, F), citrate (C, G) and glucose/fructose (D, H) were monitored for the 11 *O. oeni* strains in GEMO medium, with pH adjusted to 4.8 (A, B, C and D) or 3.6 (E, F, G and H). To discriminate the strains, the remaining time to consume half of the citrate (0.15 g/L) contained in the GEMO medium was determined for each strain under pH 4.8 (**I**) and pH 3.6 (**J**). As 13.3 was not able to grow at pH 3.6, no value could be defined for this strain in this condition (NA). Color boxes frame the 3 consumption groups: “Standard” (uncolored) which are strains consuming citrate as fast as the two reference strains NB_1911 and 26.1, “Slow” (blue) for strains that consume citrate significantly more slowly, and “Fast” (pink) for strain that consume citrate significantly faster. Data represent the means of three independent biological replicates. Significant differences are based on the Kruskal-Wallis test ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

D-lactate and mannitol production correlates with the strain’s lower hexose intake. However, this did not explain why this strain produced less L-lactate from malate than the others. The same behavior was observed for ATCC and TTK. While 92 % of L-malate was converted into L-lactate in the other strains, it seemed that only 73, 71 and 76 % of malate was metabolized to L-lactate in B16, ATCC and TTK, respectively. Regarding ATCC and TTK, the behavior of these strains was completely different from the others. Indeed, they produced less L-lactate, mannitol and acetate in favor of D-lactate production (50 % of the metabolites produced were D-lactate for ATCC *vs.* 59 % in TTK). It is therefore possible that carbons from the substrates were oriented differently in these two strains compared to the others.

At pH 3.6, 13.3 stood out, as it consumed only 6.8 mM of malate and a very small amount of citrate (0.2 mM) and thus produced only small amounts of L-lactate and acetate. The fast-consuming strains produced more mannitol, acetate and D-lactate than the other strains, since they consumed almost all the hexoses of the medium (71.1 and 90.9 out of 99.9 mM for ATCC and TTK, respectively). On the one hand, the quantity of end product synthesized was approximately the same for the standard strains, i.e. 47 % L-lactate, 19 % mannitol, 18 % D-lactate (except for B16 which produced less D-lactate) and 16 % acetate. On the other hand, the 3 slowed down strains produced significantly more mannitol (22.8, 18.8 and 18.4 mM for NB_1858, 23.1 and 32.2, respectively, *vs.* an average of 13.6 mM for the other strains) and to a lesser extent D-lactate (24.4, 15.4 and 16.1 mM for NB_1858, 23.1 and 32.2 *vs.* an average of 12.9 mM for the other strains). This could be partly explained by the fact that NB_1858 was able to consume more hexoses than the other strains (46.2 mM *versus* between 24.1 and 31.2 mM for the others). However, 23.3 and 32.2 consumed the same quantity of sugar as the standard strains; they were expected to produce the same amount of mannitol and D-lactate. Therefore, this experiment highlighted a modification of the final carbon balance in the strains that consumed citrate slower.

3.4. Impact of pH on O. oeni metabolism pathway: The role of citrate

To understand how the modification of citrate consumption could trigger a modification of carbon fluxes, carbon substrates (citrate, malate and sugar) and metabolites (L/D-lactate, mannitol, ethanol, glycerol, erythritol and acetoin/diacetyl) were monitored throughout growth (metabolic pathways are presented in Fig. 7). This experiment was monitored for 6 strains out of the 11 characterized before: PSU-1 and NB_1911 as the standard strains, NB_1858 and 23.1 as the slowed down strains and ATCC plus TTK for their capacity to consume citrate faster. This experiment aimed at analyzing the consumption and

Table 2
The growth parameters of strains according to the pH of the medium.

Strains	μmax h^{-1} (pH 4.8)	Maximum OD (pH 4.8)	μmax h^{-1} (pH 3.6)	Maximum OD (pH 3.6)
STANDARD				
PSU-1	0.155 ± 0.000^{de}	3.360 ± 0.105^{bc}	0.101 ± 0.014^{bc}	0.852 ± 0.019^{f}
NB_1911	0.179 ± 0.017^{bcd}	2.393 ± 0.064^{e}	0.082 ± 0.004^{de}	0.772 ± 0.060^{g}
IOEB_S450	0.166 ± 0.031^{cde}	3.008 ± 0.068^{cd}	0.108 ± 0.010^{ab}	0.624 ± 0.009^{i}
26.1	0.149 ± 0.001^{e}	2.385 ± 0.055	0.056 ± 0.006^{ef}	0.652 ± 0.013^{h}
13.3	0.159 ± 0.012^{cde}	2.164 ± 0.050^{f}	NA	0.084 ± 0.012^{k}
B16	0.185 ± 0.019^{bc}	1.925 ± 0.073^{f}	0.090 ± 0.003^{cd}	0.586 ± 0.011^{j}
SLOWED DOWN				
NB_1858	0.190 ± 0.014^{ab}	2.433 ± 0.028^{e}	0.106 ± 0.010^{ab}	1.141 ± 0.008^{c}
23.1	0.179 ± 0.021^{bcd}	2.557 ± 0.054^{d}	0.118 ± 0.012^{ab}	0.878 ± 0.006^{e}
32.2	0.170 ± 0.013^{bcd}	2.423 ± 0.056^{e}	0.082 ± 0.008^{de}	0.924 ± 0.017^{d}
FAST-CONSUMING				
ATCC BAA-1163	0.213 ± 0.013^{a}	3.821 ± 0.033^{ab}	0.117 ± 0.018^{a}	1.845 ± 0.054^{b}
TTK	0.217 ± 0.002^{a}	4.579 ± 0.157^{a}	0.123 ± 0.008^{a}	2.663 ± 0.090^{a}

Significant differences are based on the Kruskal-Wallis test ($p < 0.05$).

production kinetics more precisely in order to identify potential changes due to the slowing citrate consumption rate.

3.4.1. *Sequential consumption of carbon sources by O. oeni strains*

Three consumption phases could be determined for the standard and the slowed down strains, as they did not simultaneously consume the substrates (Fig. 4): phase I corresponding to malate consumption, phase II corresponding to citrate consumption, and phase III corresponding to hexose (glucose/fructose) consumption. The end of each phase was established when the substrate concerned is completely consumed by the bacteria (i.e. when less than 0.1 g/L of L-malate, less than 0.05 g/L for citrate and less than 0.2 g/L for sugars remained in the medium). On the other hand, ATCC and TTK consumed citrate and malate simultaneously in both pH conditions. Thus, for these strains, phase I and phase II were simultaneous (Fig. 4). The growth parameters (μmax and maximal OD achieved) were calculated for each phase and the data are given in Table 3. Whatever the pH, the fast-consuming strains, TTK and ATCC, obtained the highest growth parameters at the end of each consumption phase, except for phase III at pH 4.8. Interestingly, for these two strains, a fourth consumption phase could be defined at pH 4.8, as they stayed metabolically active after the end of hexose consumption, unlike the other strains. Indeed, a significant amount of mannitol and L-lactate was *de novo* uptaken during this period (Fig. 5). This fourth consumption phase is represented by yellow boxes in Fig. 4.

From a more general point of view, these results demonstrated bacterial growth in several stages punctuated by the sequential consumption of the different substrates. It was particularly pronounced at pH 3.6, where diauxic-type growth curves were observed. It can be noted that 23.1 underwent a latency phase of 8 h at pH 3.6 before starting to consume any substrate. In addition, this strain finished consuming citrate at 80 h. Therefore, no phase III was defined for it (Fig. 4). Whatever the pH, the best μmax values were obtained for each strain during phase I associated with malate consumption. Thus, malate is the substrate that best supports the growth of these bacteria, which could explain why its consumption is prioritized. In addition, the maximal OD reached during phase II by NB_1858 and 23.1 was significantly higher than that obtained by the two standard strains (Table 3). A correlation may be established between the citrate consumption rate and hexose metabolism, as more glucose/fructose was consumed than the standard strains whatever the pH condition.

3.4.2. *At optimal pH: O. oeni, the recycling bacterium*

Final carbon balance was calculated for each strain to verify that all the products from the three metabolisms studied were dosed (Equation (1) and Table S2). This final carbon balance was comprised between 96.7 and 102.2 % at pH 4.8 and between 95.0 and 101.2 % at pH 3.6, according to the strain (except for TTK at pH 3.6, for which only 85.1 % could be achieved). This confirms that all the major products from malate, citrate and glucose/fructosderlined and in bold and those which cause the appearance of a premature e metabolism were targeted.

The consumption/production balance sheet per phase for each strain was represented on radar charts (Figs. 5 and 6). During this experiment, no glycerol could be detected in any of the conditions tested, which is why it is not represented in the graphics. As the metabolism of ATCC and TTK is quite different from the other strains, their balance sheets are represented in separate graphics (Figs. 5 and 6B).

At optimal pH, the standard and slowed down strains (Fig. 5A) consumed all the malate during phase I along with a small amount of citrate (between 0 and 0.8 mM according to the strain) and hexoses. Consistently, they produced mainly L-lactate, but also mannitol, D-lactate, and small amounts of ethanol (around 1.5 mM). Diacetyl/acetoin could be detected only for NB_1911 (0.6 mM). At the end of phase II, all the remaining citrate was consumed, along with glucose and fructose. The two slowed down strains were able to consume more hexoses, particularly glucose, during this phase compared to PSU-1 and NB_1911 (on average 24.3 mM whereas PSU-1 and NB_1911 consumed only 12.3 and 8 mM). During phase II and III, bacteria produced D-lactate, mannitol, acetate and ethanol (with a final production of around 28 mM for the standard and the slowed down strains). No diacetyl/acetoin could be detected during phase II, whereas a small production was dosed during phase III (between 0.3 and 0.9 mM according to the strain). Unexpectedly, *de novo* uptake of L-lactate was observed for all these four strains, as the disappearance of 0.3 to 2.1 mM of L-lactate could be quantified during phases II and III.

Regarding TTK and ATCC, along with citrate and malate consumption, these strains were able to consume more hexoses than the other strains during phase I/II, which explains the higher growth parameters measured (Table 3). Consequently, they produced large amounts of D-lactate, acetate and mannitol, in addition to the production of L-lactate (Fig. 5B). TTK also produced 14.9 mM of ethanol while only 3.1 mM were synthesized by ATCC, in which a very small production of diacetyl/acetoin could also be detected (0.2 mM). The remaining sugars were metabolized by these strains during phase III, producing D-lactate, mannitol, acetate and ethanol. The *de novo* uptake of L-lactate was also observed in these two strains, which were also able to consume mannitol. Indeed, for these strains, a supplementary consumption phase was defined, named phase IV, during which the cells metabolized only products synthesized earlier, i.e., mannitol and L-lactate (Fig. 5B). This “recycling” phase resulted in the production of D-lactate, acetate, ethanol, and a small amount of erythritol for TTK (4.1 mM). It can be hypothesized that mannitol was mostly reoriented through the phosphoketolase pathway to produce additional ATP, which explains how TTK and ATCC reached a higher final biomass than the other strains, although they all consumed the same quantity of initial energy sources (Table 3).

3.4.3. *Decrease in pH induces modifications of O. oeni metabolism*

The same experiment was performed at pH 3.6 (Fig. 6). In these conditions, the strains mostly consumed only malate during phase I, even though a small quantity of hexose was also metabolized. In addition, a citrate uptake of 0.5 and 0.6 mM was observed for PSU-1 and NB_1911, respectively. On the other hand, TTK and ATCC consumed the entire pool of citrate (1.6 mM) while 23.1 and NB_1858 had barely begun to consume it, since the disappearance of only 0.2 and 0.1 mM in

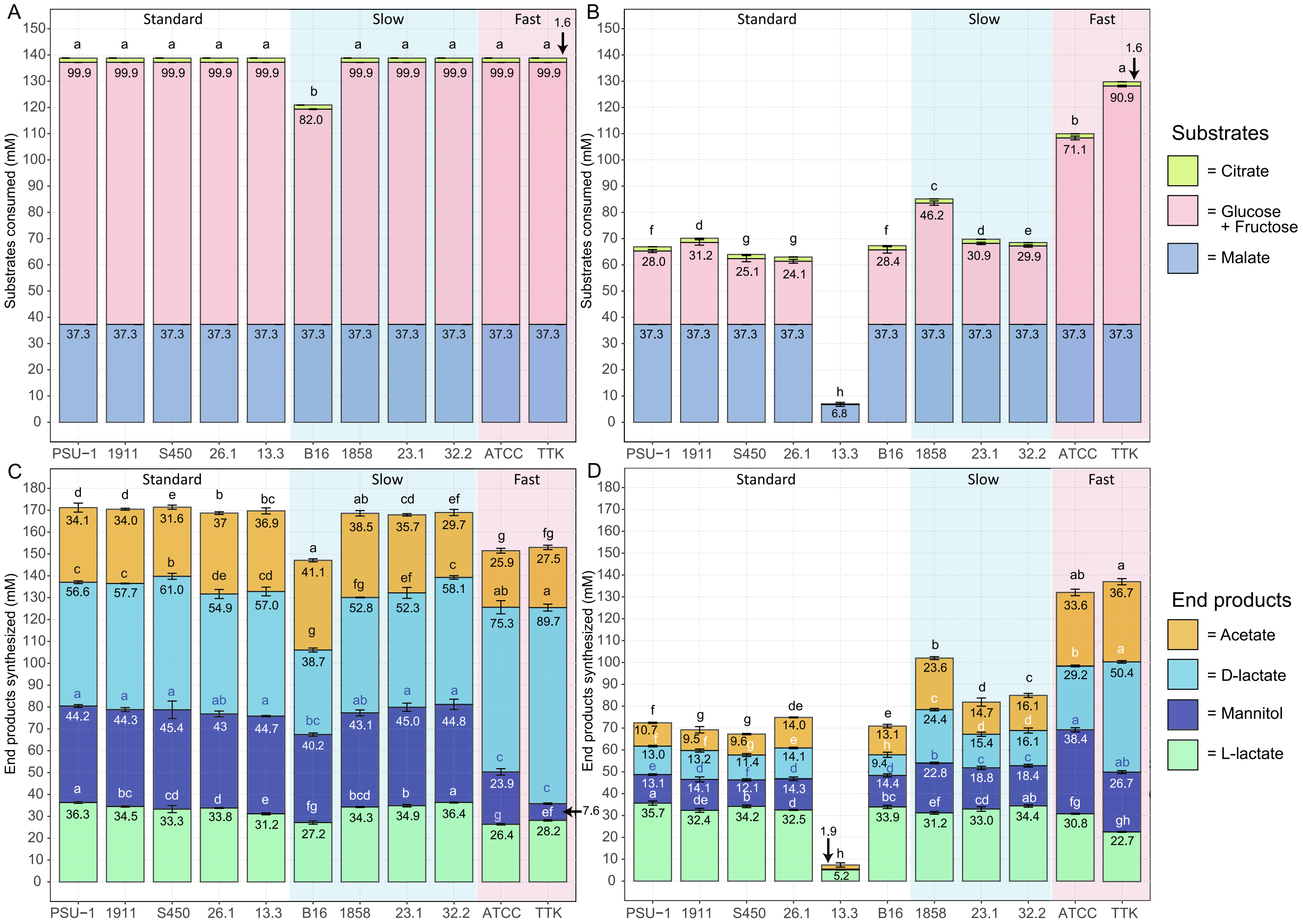


Fig. 3. Substrates consumed and end products synthesized by each strain after 80 h of growth at pH 4.8 and pH 3.6 in GEMO medium. Bar plots represent the concentration (in mM) of malate, citrate and glucose/fructose consumed (A & B) or the concentration of L-lactate, mannitol, D-lactate, and acetate produced (C & D) during growth at pH 4.8 (A & C) or 3.6 (B & D). Color boxes frame the 3 consumption groups: “Standard” (uncolored) which are strains that consume citrate as fast as the two reference strains NB_1911 and 26.1, “Slow” (blue) for strains that consume citrate significantly more slowly, and “Fast” (pink) for strain that consume citrate significantly faster. Data represent the means of three independent biological replicates ± SD and the value are indicated inside the bars. Significant differences are based on the Kruskal-Wallis test ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the medium could be quantified, respectively. Therefore, the major end-products dosed during this consumption phase was L-lactate, completed by a small amount of D-lactate (between 3 and 6.7 mM), acetate (between 1.5 and 10.6 mM) and mannitol (between 2.1 and 6.6 mM). Approximately 0.2 mM of diacetyl/acetoin was dosed for each strain. During this consumption phase, no major difference could be highlighted between the strains in terms of metabolism or growth rate, but ATCC and TTK were able to reach a higher final biomass.

During phase II (which concerns only PSU-1, NB_1858, NB_1911 and 23.1), it is interesting to note that a higher consumption of hexoses, especially fructose, could be detected for the two slowed down strains (6.5 and 16.3 mM of fructose consumed for NB_1858 and 23.1 *vs.* 3.3 and 3.9 mM for PSU-1 and NB_1911). In addition, they produced much more D-lactate (14.5 and 8.6 mM of D-lactate produced for 23.1 and NB_1858 *vs.* 2.5 and 2.4 mM for PSU-1 and NB_1911), as well as mannitol, acetate and a small amount of ethanol (1 mM). A higher OD was also measured for these two strains compared to PSU-1 and NB_1911 (Table 3). As for diacetyl/acetoin production, some differences could be noted between the two groups (Table S3). Indeed, between 20 and 52 h of growth, a significant production was quantified for PSU-1 and NB_1911, with maximum concentrations of 0.4 mM for PSU-1 and 0.7 for NB_1911. These maximum values were dosed at the end of citrate consumption for both strains. As two moles of pyruvate are necessary to form one mole of diacetyl/acetoin and that the initial concentration of citrate was 1.6 mM, most of the pyruvate from citrate metabolism was probably oriented through the C4 pathway in these strains. On the contrary, almost no diacetyl/acetoin could be quantified for the two slowed down strains during this period. Therefore, it may possible that in these slowed down strains, pyruvate from citrate metabolism was oriented differently, which would be beneficial for hexose uptake and metabolism, helping cells to achieve a higher final biomass. A significant amount of diacetyl/acetoin could be dosed after 56 h in both slowed down strains. However, this production probably stemmed from hexose metabolism, as comparable quantities were also produced at pH 4.8 during the phase III.

During phase III, the strains continued to consume hexoses (12.1 mM of glucose and 17.7 mM of fructose for NB_1858, while PSU-1 and NB_1911 consumed only 2.9 mM of glucose each added to 6.0 mM fructose for PSU-1 and 12.0 mM for NB_1911). They *de novo* metabolized a small amount of L-lactate (with a maximal consumption of 4.5 mM for PSU-1, the other strains consumed less than 1 mM). The dosed end-products for this phase were D-lactate (7.3 to 11.1 mM according to the strain), acetate (8.6 to 11.1 mM), mannitol (8.5 to 15.5 mM), ethanol (around 1 mM) and diacetyl/acetoin (between 0.3 and 0.7 mM according to the strain). Regarding TTK and ATCC, they consumed more hexoses than the others (28.5 mM glucose and 40.1 fructose for ATCC *vs.* 34.2 mM glucose and 39.8 mM fructose for TTK) as well as L-lactate (1.7 mM for ATCC and 14.7 mM for TTK). The proportion of end-products synthesized by these two strains was quite different, with mannitol

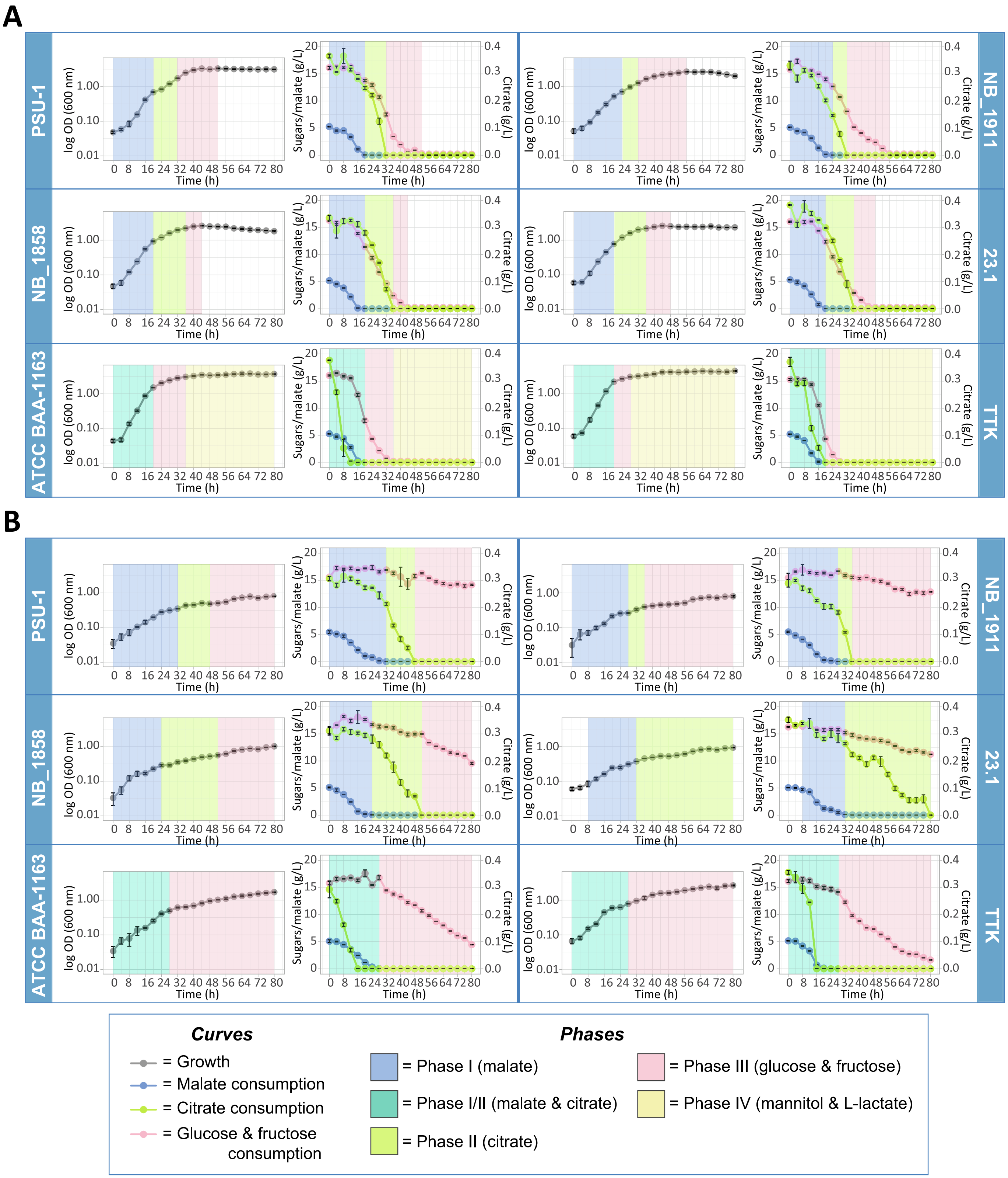


Fig. 4. Determination of consumption phases for each strain grown in pH 4.8 or pH 3.6 GEMO medium. Growth (graphs on the left, grey curves) and the substrate consumption (graphs on the right), i.e. malate (blue curves), citrate (green curves), and glucose/fructose (pink curves) are represented for each strain depending on the pH (A = pH 4.8, B = pH 3.6). The durations of each consumption phase are represented by coloured boxes (blue for malate consumption = phase I, blue green for malate + citrate consumption = phase I/II (TTK and ATCC only), pink for hexose consumption = phase III, yellow for mannitol and L-lactate consumption = phase IV). Data represent means of three independent biological replicates ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

The growth parameters per consumption phase for each strain grown in GEMO medium according to pH.

Strain	Citrate consumption rate	Phase duration h (pH 4.8)	μmax of phase h^{-1} (pH 4.8)	maximum OD per phase (pH 4.8)	Phase duration h (pH 3.6)	μmax of phase h^{-1} (pH 3.6)	maximum OD per phase (pH 3.6)
PHASE I (MALATE)							
PSU-1	Standard	20	0.154 ± 0.003^{c}	0.688 ± 0.020^{e}	32	0.075 ± 0.010^{NS}	0.377 ± 0.006^{c}
NB_1911	Standard	24	0.122 ± 0.007^{c}	0.708 ± 0.002^{e}	28	0.082 ± 0.027^{NS}	0.266 ± 0.011^{d}
NB_1858	Slowed down	20	0.173 ± 0.010^{b}	0.924 ± 0.027^{c}	24	0.092 ± 0.014^{NS}	0.287 ± 0.015^{d}
23.1	Slowed down	20	0.173 ± 0.010^{b}	0.778 ± 0.019^{d}	32	0.062 ± 0.008^{NS}	0.374 ± 0.005^{c}
PHASE I/II (MALATE & CITRATE)							
ATCC BAA-1163	Fast-consuming	20	0.218 ± 0.009^{a}	1.515 ± 0.030^{b}	28	0.097 ± 0.013^{NS}	$0,502 \pm 0.021^{b}$
TTK	Fast-consuming	20	0.212 ± 0.003^{a}	2.231 ± 0.020^{a}	20	0.111 ± 0.008^{NS}	$0,595 \pm 0.017^{a}$
PHASE II (CITRATE)							
PSU-1	Standard	12	0.077 ± 0.001^{a}	1.741 ± 0.026^{b}	16	0.016 ± 0.004^{c}	0.489 ± 0.020^{c}
NB_1911	Standard	8	0.074 ± 0.005^{a}	1.280 ± 0.051^{c}	8	0.050 ± 0.008^{a}	0.396 ± 0.009^{d}
NB_1858	Slowed down	16	0.055 ± 0.002^{c}	2.212 ± 0.045^{a}	28	0.024 ± 0.002^{b}	0.564 ± 0.026^{b}
23.1	Slowed down	16	0.064 ± 0.002^{b}	2.175 ± 0.044^{a}	48	0.064 ± 0.002^{c}	0.933 ± 0.009^{a}
PHASE III (GLUCOSE & FRUCTOSE)							
PSU-1	Standard	20	0.054 ± 0.001^{a}	3.332 ± 0.030^{a}	+32	0.015 ± 0.002^{d}	0.805 ± 0.005^{d}
NB_1911	Standard	24	0.031 ± 0.001^{d}	2.701 ± 0.060^{c}	+ 44	0.016 ± 0.002^{d}	0.802 ± 0.038^{d}
NB_1858	Slowed down	8	0.020 ± 0.001^{e}	2.635 ± 0.037^{cd}	+28	0.021 ± 0.001^{c}	1.020 ± 0.012^{c}
23.1	Slowed down	12	0.021 ± 0.001^{e}	2.609 ± 0.037^{d}	NA	NA	NA
ATCC BAA-1163	Fast-consuming	16	0.044 ± 0.002^{b}	3.071 ± 0.023^{b}	+52	0.023 ± 0.001^{b}	1.706 ± 0.010^{b}
TTK	Fast-consuming	8	0.040 ± 0.001^{c}	3.071 ± 0.032^{b}	+60	0.037 ± 0.001^{a}	2.663 ± 0.090^{a}
PHASE III (GLUCOSE & FRUCTOSE)							
ATCC BAA-1163	Fast-consuming	+ 44	0.004 ± 0.001^{b}	3.725 ± 0.037^{b}	NA	NA	NA
TTK	Fast-consuming	+ 52	0.007 ± 0.001^{a}	4.579 ± 0.157^{a}	NA	NA	NA

A "+" means that substrate consumption did not end after 80 h of growth, the end of the kinetics. Significant differences are based on the Kruskal-Wallis test ($p < 0.05$).

production predominating (38.7 mM), then acetate (23.9 mM) and D-lactate (21.2 mM) for ATCC, while TTK produced a higher amount of D-lactate (43.7 mM) than acetate (33.3 mM), mannitol (23.3 mM) and ethanol (15.5 mM). These results highlight the differences in metabolic networks between *O. oeni* strains, while the availability of carbon sources was the same.

3.5. What happens under more drastic pH conditions?

To ascertain the effect of very acid conditions on *O. oeni* metabolism, the experiment was repeated in pH 3.2 GEMO medium. It showed that the growth of all the strains was strongly affected by the decrease in pH, with a maximal OD of between 0.210 and 0.305 for the standard and the slowed down strains, while both ATCC and TTK, the fast-consuming strains, were able to produce more biomass, as they obtained a final OD of 0.570 and 0.828, respectively (Table 4). It took on average twice as long as at pH 3.6 to consume all the malate for each strain, except for NB_1858 which was able to consume the whole malate pool in only 28 h (Table 4 and Figure S2). On the other hand, 23.1 was not able to finish malate consumption in 80 h and showed a latency phase of 32 h before starting to consume it (Figure S2). None of the reference and slowed down strains were able to finish citrate consumption in 80 h, which is why no phase III was defined for these strains (Figure S2).

During phase I, malate was the preferred substrate and almost the only one to have been consumed (Figure S3). The major end-product quantified was L-lactate. On the other hand, ATCC and TTK consumed all the citrate during phase I/II and larger quantities of glucose/fructose. Thus, larger amounts of D-lactate, acetate and mannitol could be dosed for these strains. During phase II, the metabolism of PSU-1, NB_1858 and NB_1911 was almost stopped, while TTK and ATCC once again consumed more sugars, especially TTK.

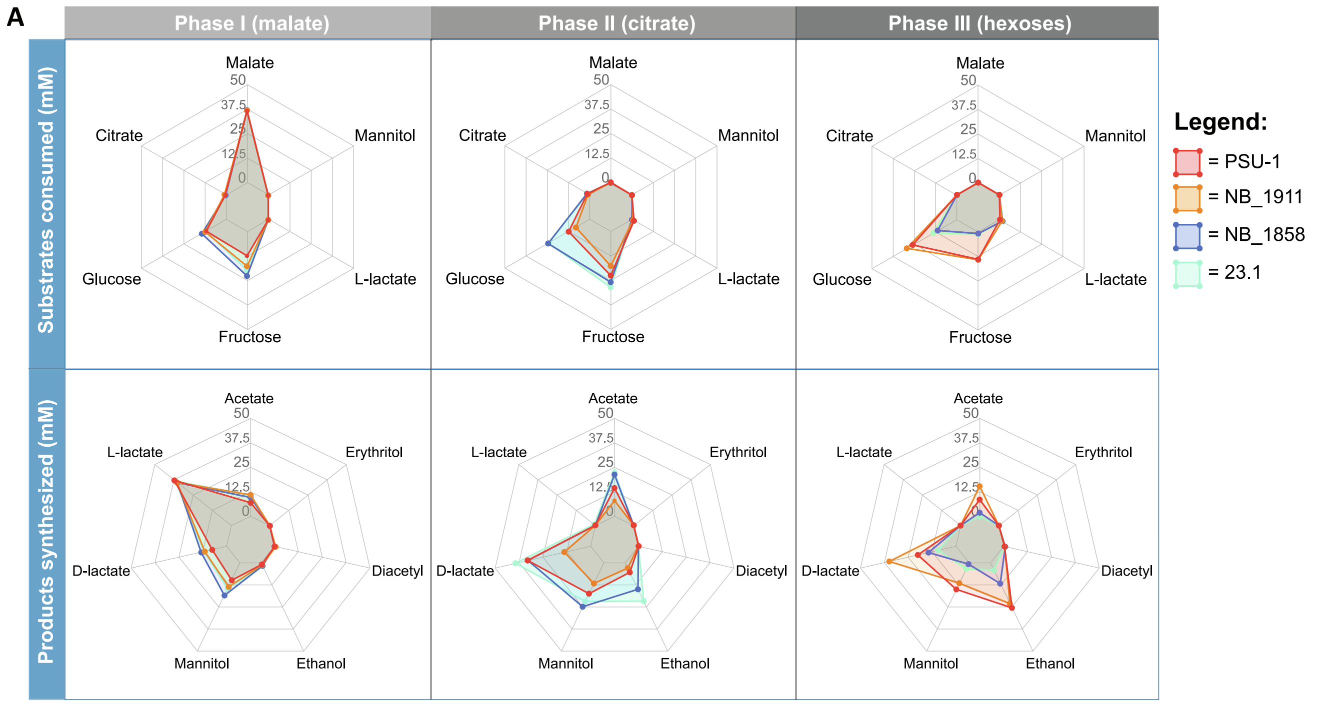
This experiment showed that acidity strongly affected the *O. oeni* metabolic pathways. Interestingly, while citrate metabolism of PSU-1, NB_1858, NB_1911 and 23.1 was slowed down with the decrease in pH, this metabolism was not at all affected in TTK and ATCC. Indeed, it ended after 16 to 24 h of growth whatever the pH condition, while both malate and hexose metabolism were at least twice as slow at pH 3.2 as at pH 3.6. On the other hand, NB_1858 differed from the other strains in its ability to consume malate quickly under very acid conditions. Interestingly, this strain was one of those that consumed citrate more slowly than the average and was the only strain that did not consume any citrate during malate consumption at pH 3.6 and 3.2.

4. Discussion

Citrate metabolism is well known for its positive effects on LAB physiology, i.e. activation of PMF, the consumption of protons and the reoxidation of co-factors from the phosphoketolase pathway (Poolman, 1993; Ramos et al., 1994; Salou et al., 1994; Ramos and Santos, 1996). Nevertheless, some controversial effects of citrate on *O. oeni* acid tolerance have been underlined (Augagneur et al., 2007b; Julliat et al., 2023). These findings raised questions as to the true impact of citrate metabolism on *O. oeni* acid tolerance.

4.1. The great diversity of the O.oeni cit locus seems to be related to life in different ecosystems

A genomic comparison was performed on 30 *O. oeni* strains and showed considerable genetic diversity between the strains. Several genetic variations were identified in the different strains that could strongly affect citrate metabolism in these bacteria. It appeared that CitR is one of the proteins whose amino acid sequence varies greatly from one strain to another. This gene encodes the putative transcriptional regulator of *cit* operon in *O. oeni* but it has not yet been characterized. In other species like *Lactococcus lactis* subsp. *lactis* bv. diacetylactis or *Weissella paramesenteroides,* the CitR ortholog protein CitI encodes for a transcriptional activator of the citrate operon (Martín et al., 2000, 2004; Martin et al., 2005). In *W. paramesenteroides,* two operator sites for CitI have been identified in the intergenic region before *citM,* the ortholog of *mae* (Martin et al., 2005). Sequences similar to these two operators could be found in other LAB, notably *O. oeni*. Thus, the authors suggested that the regulation mechanism of the citrate operon identified in *W. paramesenteroides* could be the same in the majority of LAB (Martin et al., 2005). The SNP identified in the intergenic regions between *citR*



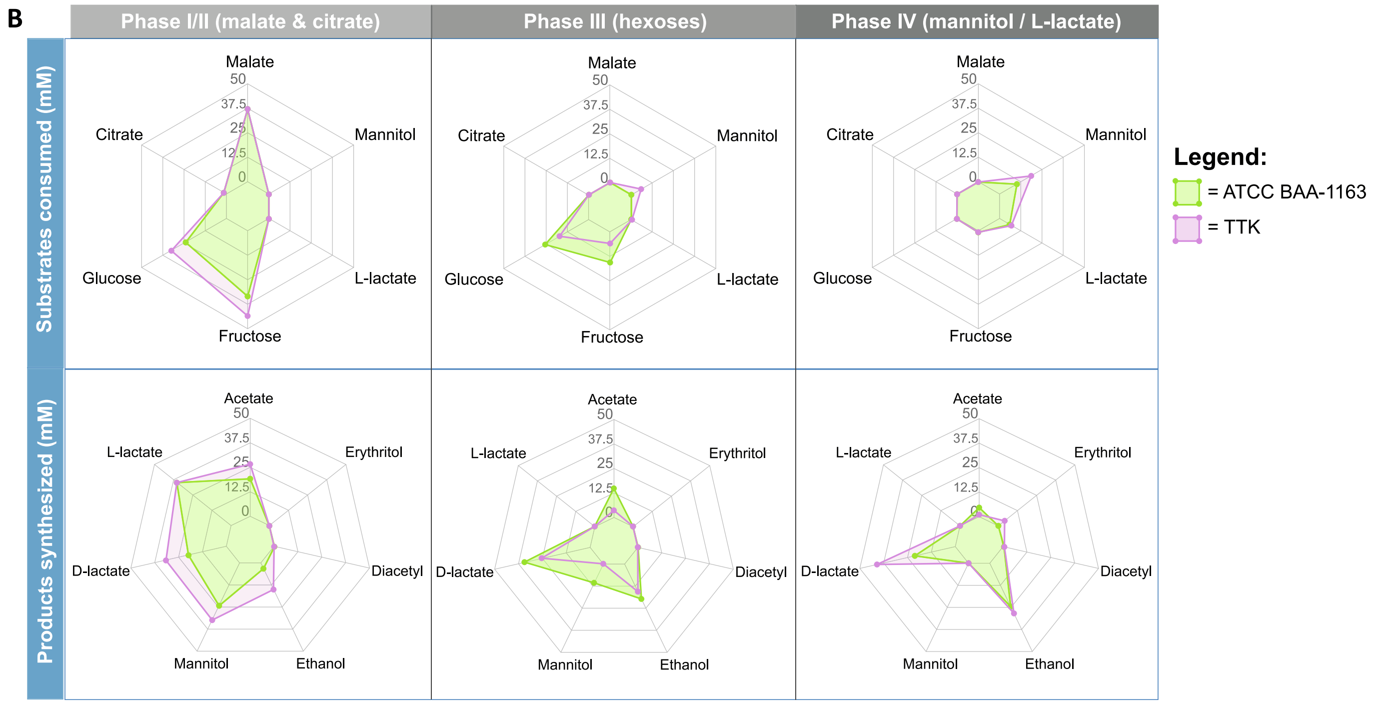


Fig. 5. Consumption/production balance sheet for each strain grown at pH 4.8. Radar charts represent the quantity (in mM) of substrates consumed and the products synthesized per consumption phase by standard and slowed down strains (A) or fast-consuming strains (B) grown in pH 4.8 GEMO medium. Data represent means of three independent biological replicates.

and *mae* of some strains could be located on one of these operator sites or on transcription regulatory sequences such as −10 and −35 sequences and thus may affect the expression of whole *cit* operon. The non-conservative amino acid substitutions observed in 14 out of the 30 strains always implies a threonine, replaced by an isoleucine or a proline (except for NB_1858, for which an isoleucine is replaced by a threonine). Threonine is a hydrophilic amino acid with an uncharged side chain, while proline and isoleucine are both hydrophobic, with a bended geometry for proline. Thus, these changes could have an impact on the tertiary structure of the protein and, consequently, on its activity. All these mutations could modulate citrate metabolism according to the strain. More knowledge is needed on the regulation of *cit* locus in *O. oeni*.

A phylogenetic tree was drawn using alignments of the *cit* loci from the 30 strains (Fig. 1). The four phylogenetic groups, determined by phylogenetic studies based on Multilocus Sequence Typing (Bilhère et al., 2009; Bridier et al., 2010; Campbell-Sills et al., 2015; Lorentzen et al., 2019), are well separated, meaning that the *cit* locus evolved with the strains according to their ecosystem. Indeed, most of the known strains have been isolated from wine. Nevertheless, *O. oeni* can also be found in cider and kombucha (Lorentzen and Lucas, 2019). Phylogroup

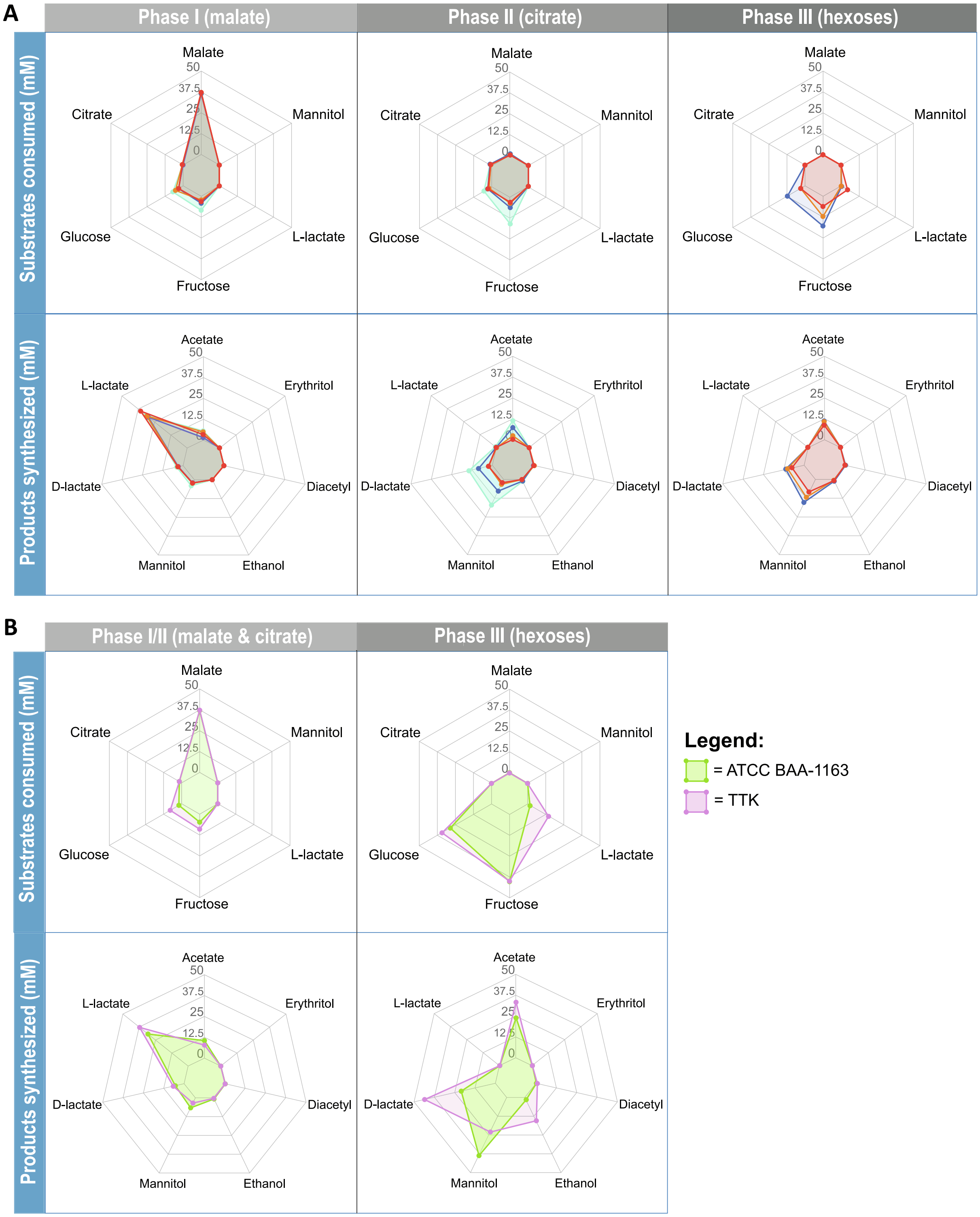


Fig. 6. Consumption/production balance sheet for each strain grown at pH 3.6. Radar charts represent the quantity (in mM) of substrates consumed and products synthesized per consumption phase by standard and slowed down strains (A) or fast-consuming strains (B) grown in pH 3.6 GEMO medium. Data represent the means of three independent biological replicates.

A strains are the most represented in wine, although phylogroup B strains can also be found. More recently, strains from phylogroup C could also be detected in Bordeaux wines during the FML (Balmaseda et al., 2023). Strains from phylogroup B & C were isolated from cider and finally all the known phylogroup D strains have come from kombucha so far (Lorentzen and Lucas, 2019; Lorentzen et al., 2019). Thus, the ecosystems of these strains belonging to the four phylogroups are very different and *cit* locus may evolve to adapt to different environmental conditions.

Moving from genomics to phenotype, the monitoring of citrate, malate and glucose/fructose consumption along with growth obtained in GEMO medium in different pH conditions demonstrated a huge difference of citrate and sugar metabolism between the phylogroup A strains (PSU-1, NB_1858, NB_1911, 23.1, B16, IOEB_S450, 26.1, 32.2,

Table 4
The growth parameters per consumption phase for each strain grown in pH 3.2 GEMO medium.

Strain	Citrate consumption rate	Phase duration h (pH 3.2)	μmax of phase h^{-1} (pH 3.2)	maximum OD per phase (pH 3.2)
		PHASE I (MALATE)		
PSU-1	Standard	44	0.039 ± 0.005^{b}	0.255 ± 0.005^{b}
NB_1911	Standard	56	0.037 ± 0.004^{b}	0.173 ± 0.006^{e}
NB_1858	Slowed down	28	0.072 ± 0.001^{a}	0.190 ± 0.005^{d}
23.1	Slowed down	+80	0.025 ± 0.005^{c}	0.210 ± 0.011^{c}
		PHASE I/II (MALATE & CITRATE)		
ATCC BAA-1163	Fast-consuming	60	0.041 ± 0.001^{b}	0.473 ± 0.012^{a}
TTK	Fast-consuming	44	0.048 ± 0.005^{a}	$0{,}497 \pm 0.026^{a}$
		PHASE II (CITRATE)		
PSU-1	Standard	+36	0.005 ± 0.000^{b}	0.305 ± 0.006^{a}
NB_1911	Standard	+24	0.009 ± 0.002^{a}	0.213 ± 0.005^{b}
NB_1858	Slowed down	+52	0.003 ± 0.001^{c}	0.219 ± 0.005^{b}
		PHASE III (GLUCOSE & FRUCTOSE)		
ATCC BAA-1163	Fast-consuming	+20	0.009 ± 0.002^{b}	0.570 ± 0.012^{b}
TTK	Fast-consuming	+36	0.014 ± 0.002^{a}	0.828 ± 0.008^{a}

A "+" means that substrate consumption did not end after 80 h of growth, the end of the kinetics. Significant differences are based on the Kruskal-Wallis test ($p < 0.05$).

13.3) and the phylogroup B (ATCC) and D (TTK) strains. Indeed, these last two strains consumed both citrate and hexoses much faster than all the other strains, whatever the pH conditions (Figs. 2 & 4). Furthermore, the consumption of malate is not favored in these two strains as they both consumed citrate first. Recently, it has been demonstrated that the different *O. oeni* phylogroups are "built" to survive and develop in their respective ecological niches (Balmaseda et al., 2023). Indeed, while phylogroups A, B and C are represented in grape must and during the alcoholic fermentation of wines, phylogroup B and C populations declined at the end of alcoholic fermentation and could not be detected during MLF. These three phylogroups are also present in cider, in which alcoholic fermentation does not lead to the selection of one phylogroup. However, phylogroups B and C strains were able to develop more than phylogroup A strains whose populations remained stable or decreased slightly. Wine, cider and kombucha represent completely different growth conditions for the bacteria. Indeed, ethanol content is much lower in cider (1.5-8 %) and in kombucha (approximatively 0.5 %) than in wine (10–16 %). pH also differs from one matrix to another (the pH of cider comprises between 3.3 and 4.2, while the pH of wine is between 3 and 3.8, and kombucha close to 3) and the biggest difference lies in the quantity of carbon sources available. Indeed, while citrate and malate could be found in comparable concentrations in the three beverages (around 0.3 g/L of citric acid for all beverages and 5 g/L of malic acid for wine and cider, while in kombucha a lower amount of malic acid could be found, up to 1 g/L), the amount of sugars available for bacteria was much higher in cider (80–170 g/L, mainly fructose) than in wine (less than 0.5 g/L). Regarding traditional kombucha, it contains near 100 g/L saccharose which can be cleaved by yeast thanks to an invertase activity in glucose and fructose, but the hydrolysis kinetics is highly dependent on the microbial community and environmental parameters (Bauer and Dicks, 2004; Ye et al., 2014; Coton et al., 2016; Neffe-Skocińska et al., 2017; Tran et al., 2020; De Miranda et al., 2022). Indeed, while *O. oeni* grows during alcoholic fermentation in cider and kombucha along with yeasts and other bacteria, it intervened mostly after alcoholic fermentation in wine, when all the nutrients were metabolized by yeasts. Thus, bacteria are confronted to two very different ecosystems: a deserted environment with low nutrients and a high content of ethanol and two richer and competitive environments because of the presence of other microorganisms. In an environment where competition for carbon sources is fierce, it is important for the bacterium to quickly consume the available nutrient sources. On the other hand, in wine, the slowing down of energy-consuming metabolism such as the glucose/fructose metabolism is of interest to concentrate all the energy on resisting stresses, thanks to F_0F_1-ATPase functioning, Heat Shock Protein synthesis, maintaining membrane integrity (Poolman, 1993; Guzzo et al., 2000), etc. This explains why TTK has a faster metabolism than phylogroup A strains. Concerning ATCC, although it has been isolated from wine, its metabolism is more in phase with cider growth conditions which could explain why the populations of phylogroup B strains decline after alcoholic fermentation in wine (Balmaseda et al., 2023). The same experiment needs to be performed on other strains from phylogroup B, C & D to back this hypothesis. It could also provide an explanation concerning the appearance of systematic mutation on the *cit* locus of ATCC during the experimental evolution to adapt cells to low pH (Julliat et al., 2023). It is likely that the slower rate of citrate metabolism in this strain provides advantages in terms of resistance to highly acidic conditions.

4.2. *O. oeni general metabolism and its specificities*

Malate, citrate and glucose/fructose uptake was monitored first in 11 *O. oeni* strains. It was demonstrated that these three carbon sources are sequentially consumed by all the strains, and that malate consumption is favored, which is in accordance with previous studies (Saguir and Manca De Nadra, 1996; Viljakainen and Laakso, 2000; Contreras et al., 2018). While the metabolism of phylogroup B and D strains differs greatly from that of phylogroup A strains, these latter are fairly similar, except for B16. Indeed, this strain was not able to consume all hexoses at optimal pH and produced significantly less D-lactate than the other strains at pH 3.6 (Fig. 3). In *O. oeni,* glucose is metabolized through the phosphoketolase pathway, producing pyruvate, acetyl phosphate and CO_2 (Fig. 7) (Kandler, 1983; Cibrario et al., 2016). This pathway leads to the synthesis of three $NAD(P)H + H^+$ that must be reoxidized. Several pathways are employed by the bacterium to help this reoxidation: the conversion of pyruvate to D-lactate thanks to D-lactate dehydrogenase, the production of ethanol from acetyl-P, the production of glycerol from glyceraldehyde-3-P and finally the production of mannitol from fructose and erythritol from fructose-6-P (Maicas, Ferrer, & Pardo, 2002; Richter, Hamann, & Unden, 2003; Unden & Zaunmüller, 2009; Zaunmüller, Eichert, Richter, & Unden, 2006). In addition, citrate metabolism could also facilitate NADH reoxidation when citrate-derived pyruvate is converted into D-lactate (Ramos and Santos, 1996; Zaunmüller et al., 2006). In *O. oeni*, 6 genes encode for putative D-lactate dehydrogenases (locus tag OEOE_0025, OEOE_0413, OEOE_0701, OEOE_1182, OEOE_1672 and OEOE_1709 from PSU-1 genome). It appears that B16 has genetic variations leading to a premature stop codon in both paralogous genes OEOE_0701 and OEOE_1709. This important pathway for NADH reoxidation could thus be affected in this strain and could explain the lower quantity of D-lactate synthesized compared to the other strains at pH 3.6 and the early ceasing of glucose/fructose consumption at pH 4.8 due to intracellular redox imbalance (Fig. 4). Another study already highlighted the specificity of sugar metabolism in several Champagne strains which lack certain sugar transporters and grow slowly compared to other strains (Cibrario et al., 2016). These strains were isolated from very acid wines and are thus well adapted to resist to low pH.

On the other hand, the 13.3 strain was the least acid tolerant strain of the group, as it was not able to develop at pH 3.6. The dosage of final compounds in the medium revealed that this strain consumed 6.8 mM of malate and 0.1 mM of citrate and produced 5.2 mM of L-lactate and 1.9

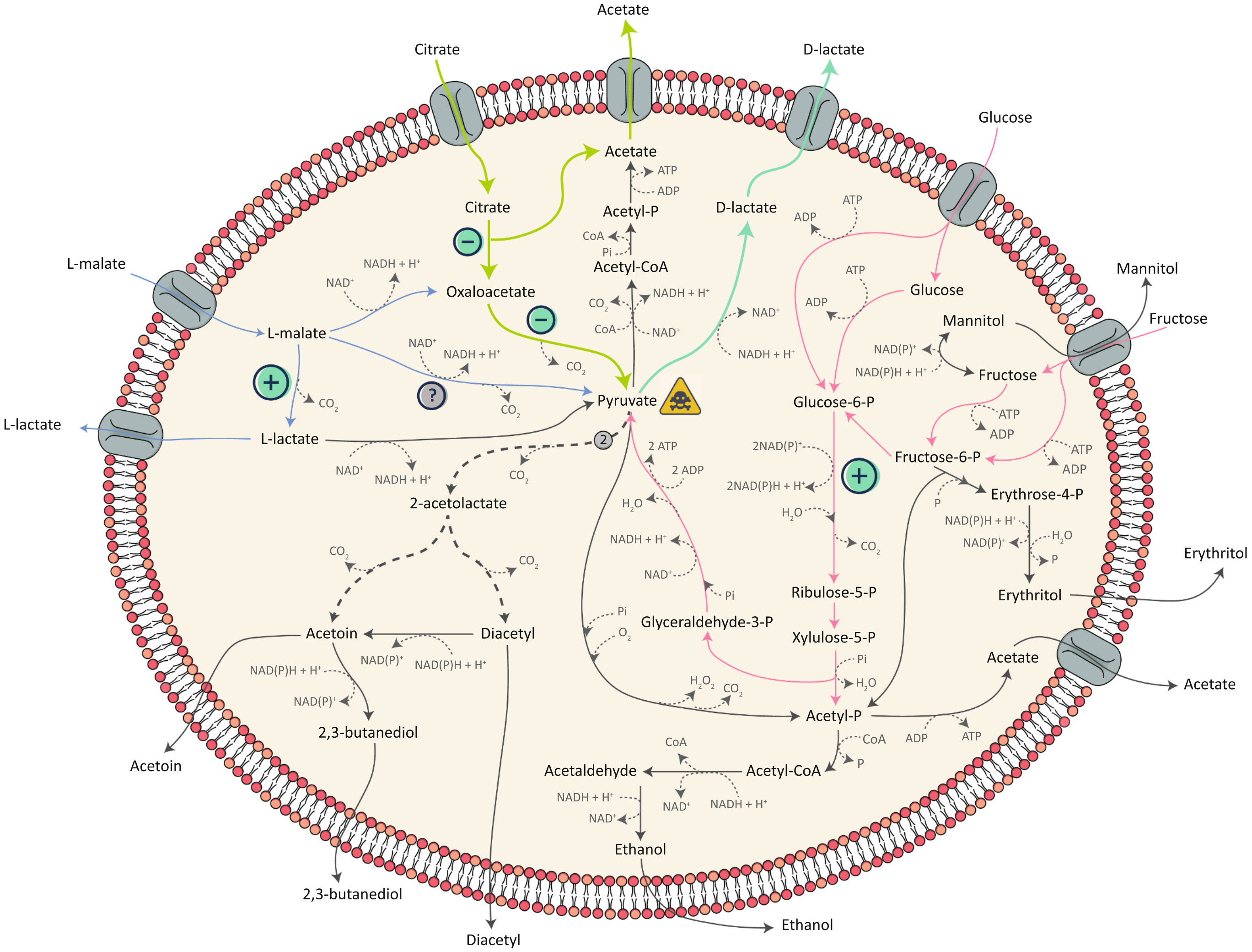


Fig. 7. Metabolism pathways in *O. oeni* and the possible beneficial effects of a slowing down of citrate metabolism on the cellular machinery. The pathways linked to malate (blue arrows), citrate (green arrows) and glucose/fructose (pentose-P pathway, pink arrows) metabolism in *O. oeni* are indicated. When citrate metabolism is slowed down (represented by a minus symbol) it reduces the amount of pyruvate produced; its accumulation can be toxic for growth under low pH (Harvey and Collins, 1963; Augagneur et al., 2007a; Pudlik & Lolkema, 2011; Zuljan et al., 2014). In addition, when cells with slowed citrate metabolism are cultured in acidic conditions, pyruvate derived from citrate metabolism could be oriented to D-lactate synthesis (represented by blue green arrows) instead of diacetyl/acetoin production (represented by dashed grey arrows), enabling the reoxidation of NADH, which would promote the pentose-P pathway (symbolized by a plus) and thus ATP production. Finally, the down-regulation of *mae* expression encoding an oxaloacetate decarboxylase which catalyzes the conversion of oxaloacetate into pyruvate would favor the conversion of L-malate into L-lactate, representing the main desired MLF reaction but also the major energy source for *O. oeni* when it is grown under very acidic conditions. In parallel, it prevents a potential redox imbalance caused by the conversion of L-malate into pyruvate. The question mark means that it is not certain that this pathway exists in *O. oeni*, as no gene has been linked to a malic enzyme in this species. This representation of metabolic pathways in *O. oeni* is based on several other studies (Veiga-da-Cunha et al., 1993; Maicas et al., 2002; Richter et al., 2003; Mills et al., 2005; Zaunmüller et al., 2006; Olguín et al., 2009; Cibrario et al., 2016; Mendoza et al., 2017; Contreras et al., 2018) and the KEGG database/Pathway tools V26.0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mM of acetate. In *O. oeni*, L-malate will mainly be metabolized to L-lactate, representing the main desired reaction of MLF, but can also be redirected to produce pyruvate (Fig. 7) (Kunkee, 1991; Alexandre et al., 2008). Two different pathways could be implicated in this reaction: the first one is the conversion of L-malate to oxaloacetate by a malate dehydrogenase. In the PSU-1 genome, only one gene coding for a malate dehydrogenase has been identified, OEOE_1552. Then, oxaloacetate is converted into pyruvate *via* the oxaloacetate decarboxylase encoded by the *mae* gene included in *cit* locus. The other enzyme that could be implicated in the direct transformation of L-malate to pyruvate is a malic enzyme, but no gene in the PSU-1 genome could be related to the production of this protein. In both cases, the conversion of L-malate into pyruvate will lead to the production of NADH + H^+ that must be reoxidized. However, in 13.3, it seems that the 1.6 mM of L-malate that was not transformed into L-lactate was redirected for the production of acetate through the pyruvate pathway, probably for ATP production. Nevertheless, none of the pathway leading to the production of acetate leads to the regeneration of NAD^+. Thus, the metabolic stop in this strain could be explained by an intracellular redox imbalance, but also because NAD^+ is required as a cofactor for malolactic enzyme activity whereas NADH has a strong inhibitory effect on it (Miranda et al., 1997). This confirms the importance of maintaining an intracellular redox balance for growth.

The monitoring of the carbon source used and the metabolite produced enabled calculating the final carbon balance for each strain grown under the three pH conditions (pH 3.2, 3.6 and 4.8). A carbon imbalance could mostly be highlighted for TTK at pH 3.6 (85.1 %) and ATCC at pH 3.2 (86.5 %) (Table S2). This could be explained by the incorporation of glucose into biomass or by the production of an unidentified product derived from acetyl coenzyme A, as has been suggested in previous studies (Salou et al., 1994; Saguir and Manca De Nadra, 1996). In addition, an accumulation of metabolites into the cell could also happen as the stationary phase had not been reached at the end of the monitoring (80 h) when cells were grown at pH 3.6 and 3.2. A light carbon imbalance in favor of the metabolic products could also be noted for PSU-1 and NB_1858 at pH 4.8 (102 and 102.2 %, respectively), NB_1911 at pH 3.6 (101.2 %) and NB_1858 at pH 3.2 (102.2 %). These imbalances were probably due to the estimation of CO_2 and, to a lesser extent, to other metabolic pathways that were not taken into account.

The stoichiometric equation of glucose, fructose, malate and citrate conversion was calculated at pH 4.8. For phylogroup A strains, i.e. PSU-1, NB_1858, NB_1911 and 23.1, the following equation was obtained:

$$1 \text{ glucose} + 1 \text{ fructose} + 0.76 \text{ malate} + 0.03 \text{ citrate} \rightarrow 0.83 \text{ mannitol} + 1.20 \text{ D-lactate} + 0.60 \text{ EtOH} + 0.66 \text{ acetate} + 0.70 \text{ L-lactate} + 2\, CO_2.$$

Stoichiometric equation of glucose, fructose, malate and citrate conversion in PSU-1, NB_1858, NB_1911 and 23.1 grown at pH 4.8 (2).

This equation is very close to that calculated by Salou and collaborators from an experiment performed in similar growth conditions (Salou et al., 1994). The small differences observed were due to the presence of both malate and citrate in the medium added in our study. On the other hand, as TTK and ATCC possess a very different metabolism, these strains cannot satisfy the equation. This is largely due to the capacity to *de novo* metabolize two end-products: L-lactate and mannitol.

Mannitol was only reused by TTK and ATCC under optimal pH conditions. It is first formed by bacteria through the conversion of fructose into mannitol by mannitol dehydrogenase (Fig. 7). It represents an important pathway for NAD(P)H reoxidation in *O. oeni* (Richter et al., 2003). In these two strains, fructose was quickly metabolized into mannitol at pH 4.8. Immediately afterwards, mannitol was *de novo* uptaken by bacteria until it was almost completely consumed at the end of the kinetics (Fig. 4). As D-lactate and ethanol production could be quantified in these two strains during the consumption of mannitol (Fig. 5B), it may have been reoriented to the phosphoketolase pathway for the production of ATP. A previous study suggested that mannitol could be transported through a PEP-dependent phosphotransferase systems (PTS) and then converted to fructose-6-phosphate by mannitol-1-P dehydrogenase (Cibrario et al., 2016). This could explain how these two bacteria were able to achieve a higher final biomass compared to the others while they consumed the same quantity of substrates and represented an ingenious way to optimize energy production. This phenomenon has never been described in *O. oeni* and poorly studied in other heterofermentative LAB (Wisselink et al., 2002). However, this phenomenon comes close to the "make-accumulate-consume" strategy described in certain yeasts such as *Saccharomyces cerevisiae* or *Dekkera bruxellensis*, in which yeasts are capable to change their metabolism to consume ethanol and acetate produced previously. This enables them to hold their own in a fiercely competitive environment (Pronk et al., 1996; Piskur et al., 2006; Rozpędowska et al., 2011).

L-lactate was also *de novo* metabolized by the cells whatever the pH condition. The disappearance of L-lactate from the medium could be observed for almost all the strains whatever the pH condition (from 0.1 to 14.5 mM, depending on the strain and the pH condition). The question of the presence of a putative L-lactate dehydrogenase in *O. oeni* enabling the conversion of L-lactate into pyruvate has been raised only once. Sternes and collaborators indeed suggested that this pathway could lead to diacetyl production. The gene that may encode this enzyme is OEOE_1325 but does not seem to be present in all the *O. oeni* strains (Sternes et al., 2017). It can also be hypothesized that L-lactate is used by *O. oeni* to produce additional ATP by converting L-lactate into pyruvate then acetate. In addition, it has been reported that the presence of L-lactate inhibits the growth of *O. oeni* at a concentration higher than 3 g/L (Bauer and Dicks, 2004). Thus, the re-metabolization of L-lactate would help to prevent the potential toxic effect of L-lactate on *O. oeni* growth.

4.3. Could citrate metabolism have an impact on O. oeni acid tolerance?

A high diversity of citrate metabolism was observed between the 11 strains studied, which correlated with the *cit* locus expression in some of the strains tested (Figure S1). It is difficult to compare TTK and ATCC to the other strains as this study showed huge differences in general metabolism between these two strains and the phylogroup A strains. Nevertheless, the latter could be divided into two citrate consumption groups: the slowed down strains (23.1, NB_1858 and 32.2) and the standard strains (PSU-1, 13.3, 26.1, NB_1911, B16 and IOEB_S450). A difference in final carbon distribution was highlighted between the two consumption groups at pH 3.6 correlated with a higher production of final biomass by the slowed down strains (Fig. 3 and Table 2). To go further, the consumption and production of metabolites was monitored in two strains from each group: PSU-1 and NB_1911, representing the standard strains, and NB_1858 and 23.1 the slowed down strains. This experiment highlighted a faster glucose/fructose uptake from the two slowed down strains whatever the pH condition (Fig. 4). The consumption phase during which citrate is the main substrate metabolized seems to demonstrate the difference, since NB_1858 and 23.1 produced more biomass during this period compared to PSU-1 and NB_1911 correlated with a higher consumption of sugars in all the conditions tested (Figs. 5 and 6A and Table 3). During this phase, NB_1858 and 23.1 both produced a higher amount of D-lactate. Furthermore, a difference in diacetyl/acetoin production between the two groups could be highlighted (Table S3). As citrate metabolism in *O. oeni* produces pyruvate that could then be converted to D-lactate, acetate, or diacetyl/acetoin, it can be hypothesized that pyruvate from citrate metabolism may be divergently oriented in the slowed down strain compared to the reference strain. Therefore, 23.1 and NB_1858 would produce D-lactate from citrate-derived pyruvate while PSU-1 and NB_1911 produce diacetyl/acetoin. The conversion of pyruvate into D-lactate instead of diacetyl/acetoin will facilitate cofactor reoxidation from the phosphoketolase pathway, thus promoting sugar metabolism in these strains. This hypothesis, illustrated in Fig. 7, must be confirmed by using C-13 labelled citrate.

The experiment was repeated under more drastic pH conditions (3.2). While 23.1 had difficulties in developing and consuming malate, NB_1858 was the fastest to perform MLF in only 28 h (Figure S2). This strain was the only one which did not consume any citrate during malate consumption. It has been demonstrated that an accumulation of pyruvate could become toxic at low pH for bacterium (Harvey and Collins, 1963; Augagneur et al., 2007a; Pudlik and Lolkema, 2011; Zuljan et al., 2014). In these conditions, the slowdown of citrate metabolism and thus the production of pyruvate would be of interest. When comparing the malate consumption kinetic at pH 3.2 of the reference strain NB_1911 to the slowed down strain NB_1858, it is interesting to note that NB_1858 metabolized L-lactic acid from L-malate faster than NB_1911 from 4 h of growth (Fig. 8). The same observation can be made by comparing strain PSU-1. In NB_1858, it has been proven by RT-qPCR analysis that the genetic variability identified in *citR* (I7T) impacts *cit* locus expression which causes the slowdown of citrate metabolism highlighted by these experiments (Figure S1). The oxaloacetate decarboxylase enzyme, encoded by *mae*, plays a role in both citrate and malate metabolism, as it triggers the conversion of oxaloacetate to pyruvate (Fig. 7). If this protein is produced less, it could affect the conversion of L-malate into pyruvate and thus favor the conversion of L-malate into L-lactate. The production of pyruvate from L-malate is associated with the conversion of NAD^+ to $NADH + H^+$ which can cause an intracellular redox imbalance, as the pathways contributing to NADH reoxidation (i.e. ethanol, mannitol, erythritol and D-lactate production pathways) are limited by the drop in pH. In this way, the reduction in the conversion of L-malate to pyruvate due to the down regulation of *mae* expression could bestow an advantage to the bacterium. This hypothesis may also explain the

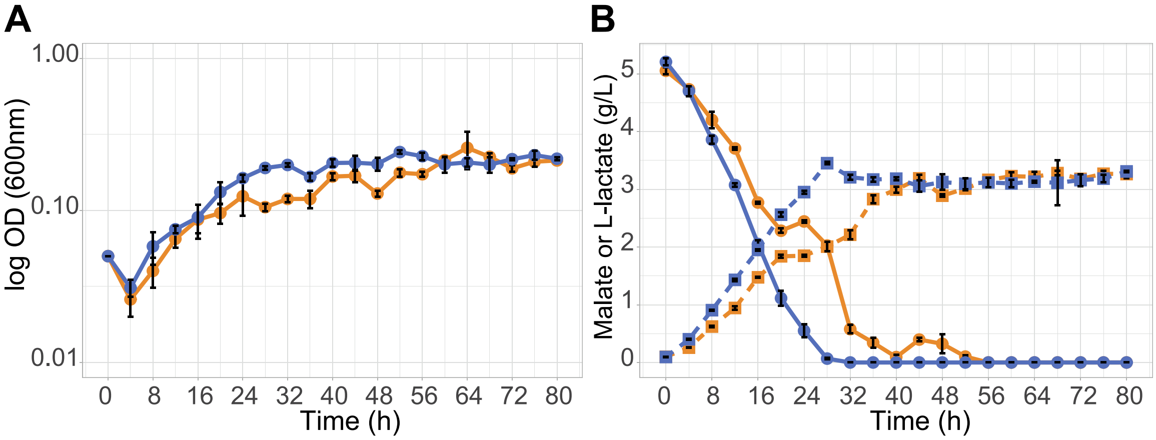


Fig. 8. Malate consumption and L-lactate production from NB_1858 and NB_1911 at pH 3.2. The growth (A) along with malate consumption (B, represented by solid lines and round points) and L-lactate production (B, dashed lines and square points) was monitored during growth of NB_1858 (blue lines) and NB_1911 (orange lines) in GEMO medium at pH 3.2. Data represent the means of three independent biological replicates. Standard deviations are indicated as error bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appearance of mutations on ATCC *cit* locus during an adaptive evolution to low pH (Julliat et al., 2023). These mutations have become fixed in the genome of evolved populations and are located in the intergenic region before *mae* or inside the coding sequence of this gene. The down regulation of the *mae* gene thus could help to favor the conversion of L-malate into L-lactate at the expense of pyruvate production, which is one of the main reactions for producing energy at low pH levels in *O. oeni*.

5. Conclusion

This study aimed at exploring the metabolism of *O. oeni* grown under different pH conditions with a focus on citrate metabolism. This represents the first comparative study of the *O. oeni* metabolism performed on several strains. It is also the first time that the metabolism of an *O. oeni* Kombucha strain has been characterized and showed considerable differences compared to the metabolism of wine strains, except for ATCC, a phylogroup B strain, with which similarities in behavior have been observed. This difference in metabolism must be related to the growth conditions encountered by each strain in their natural environment.

This study also highlighted for the first time the capacity of *O. oeni* to *de novo* uptake and metabolize two end-products of malate and sugar metabolisms: L-lactate and mannitol. These additional pathways may help the bacteria to produce extra ATP or to avoid a growth-inhibiting effect caused by an accumulation of L-lactate in the extracellular medium (Bauer and Dicks, 2004).

One of the main objectives of this study was to clarify the role of citrate metabolism on *O. oeni* acid tolerance. *O. oeni* is recalcitrant to all known genetic modification techniques, and its transformation is laborious (Grandvalet, 2017). For the moment, it is therefore not possible to knockout genes of interest in this bacterium to characterize their role in specific conditions, here the role of *cit* locus expression in the *O. oeni* acid tolerance. To overcome this issue, several *O. oeni* strains were screened to select those with different citrate consumption profiles, and 11 strains were chosen. Their citrate, malate and sugar metabolisms were compared and demonstrated a huge diversity between them. It appeared that strains in which citrate metabolism is significantly slower underwent a modification of their final carbon balance, which translates a modification of metabolic fluxes compared to the strains considered as the reference. These modifications would enable the bacteria to produce more biomass at low pH due to an enhancement of sugar consumption. This study led to the formulation of hypotheses regarding the effect of slowed citrate consumption on the reorientation of metabolic pathways in *O. oeni,* thus helping to improve ATP production. In addition, the down regulation of the *mae* gene encoding for an oxaloacetate decarboxylase could promote malolactic fermentation, i.e. the conversion of L-malate into L-lactate under very acid conditions. Nevertheless, it cannot be excluded that the differences in acid tolerance are due to other genetic variations. More research is in progress to test the hypotheses formulated, notably through RNA silencing and genetic complementation. Furthermore, the medium used in this study was created to optimize *O. oeni* growth, and is therefore richer in nutrients than wine. This can lead to a bias in acidic stress response of the cells compared with what might happen in wine. Nevertheless, a similar behavior in terms of organic acid consumption and acid tolerance was observed for the strains that were inoculated in wine (data not shown). To comfort this work, a similar experiment could be reproduced in a synthetic wine with or without citrate to confirm the link between acid tolerance and citrate metabolism in conditions closer to the field. Screening a larger number of strains with different citrate consumption profiles would also help to confirm the hypotheses.

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CRediT authorship contribution statement

Camille Eicher: Conceptualization, Methodology, Investigation, Project administration, Validation, Writing-original draft, Writing –review & editing. **Thierry Tran:** Investigation, Ressources, Writing –review & editing. **Edouard Munier:** Investigation, Writing –review & editing. **Joana Coulon:** Conceptualization, Ressources, Writing –review & editing. **Marion Favier:** Conceptualization, Ressources, Writing –review & editing. **Hervé Alexandre:** Writing –review & editing, Supervision. **Cristina Reguant:** Writing – review & editing, Supervision. **Cosette Grandvalet:** Conceptualization, Methodology, Validation, Writing –review & editing, Supervision.

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.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114027.

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