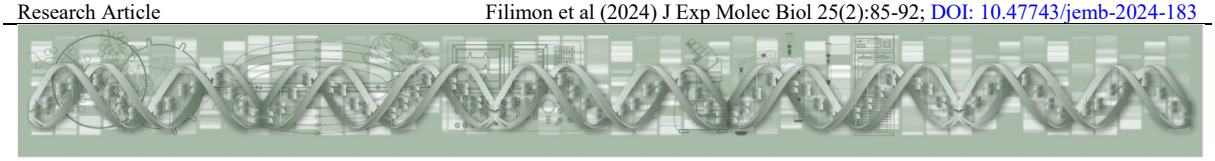

STUDIES ON THE CARBON CATABOLITE REPRESSION IN LACTIC ACID BACTERIA ISOLATED FROM WINE

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Abstract

In wine, lactic acid bacteria (LAB) are responsible for the bioconversion of malic acid to lactic acid, malolactic fermentation that mainly aims at reducing wine acidity. Two LAB strains isolated from the red wine microbiota (*Oenococcus oeni* 13-7 and *Lactobacillus plantarum* R1-1), were tested for their ability to exhibit the carbon catabolite repression (CCR) mechanism, that allows the rapid use of certain carbohydrates, over other carbon sources. Bacterial cells were inoculated in 0.1 M glycine buffer (pH 3.5), incubated at 30°C, with different carbohydrates (45 mM) and malic acid (45 mM). For both strains, the presence of glucose significantly inhibited malic acid metabolization (−60%), a similar effect being observed for galactose, mannose and maltose. The highest rate of malic acid conversion was shown in fructose/malate medium. Obtained results showed that malolactic strains can control the utilization of carbon sources via CCR, further studies being necessary to elucidate the mechanisms underlying this process.
Keywords: carbohydrate metabolism, *Lactobacillus plantarum*, malolactic fermentation, *Oenococcus oeni*, wine

Introduction

Malolactic fermentation (MLF) is defined as the enzymatic bioconversion of malic acid to lactic acid, a process performed by lactic acid bacteria (LAB), that aims at the reduction of wine acidity and to enhance the aromatic profile (Filimon et al. 2022). Performed in a controlled manner, with selected starter cultures, MLF has a significant influence on wine quality: balances the acidity, slightly increases the pH, ensure the biological stability of the wine by avoiding subsequent uncontrolled fermentations, and improves the aroma and taste of the wine, increasing its complexity (Capozzi et al. 2021, Lerm et al. 2010).
LAB species involved in the winemaking process belongs to the *Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus* genera. For conducting a controlled MLF, the starter cultures are obtained from strains belonging mainly to two species: *Oenococcus oeni* and *Lactobacillus plantarum* (Filimon 2023). To be used as starter cultures, after isolation from indigenous microbiota LAB isolates are subjected to various screening procedures, testing of the yield of malic acid bioconversion and the ability to produce undesirable or even toxic by-products (acetic acid, ethyl carbamate, biogenic amines, acetoin or diacetyl, acrolein, mannitol).
LAB are Gram-positive, catalase-negative, immobile and non-sporulated, anaerobic microorganisms, tolerant to high concentrations of acids, that assimilate carbohydrates both in the homofermentative and heterofermentative pathways (De Vos et al. 2009). Malolactic

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bacteria exhibit a chemoorganotrophic metabolism, requiring media rich in nutrients and fermentable sugars, presenting a high phenotypic variability (Coelho et al. 2022).
Carbon catabolite repression (CCR) is a mechanism utilized by various species of bacteria and fungi to accommodate changes in the environment, allowing for the rapid use of certain substrates like glucose over other carbon sources (Nair and Sarma 2021). CCR has a universal function in the regulatory system, which ensures efficient utilization of preferred carbon sources and prevents the activation of unnecessary metabolic pathways to save energy (Vinuselvi et al. 2012). For example, *E. coli* in a medium with a mixture of glucose and lactose uses the glucose completely first, then stops growing while the genes for degradation of lactose are induced, a phenomenon that enable bacteria to make a hierarchical choice between different sources of carbon (Plumbridge 2009). Also, catabolite repression is an important process for biotechnological applications. In winemaking, there are concerns that the MLF can be delayed, slowed down or even blocked in wines with high concentrations of specific carbohydrates (semi-sweet and sweet wines). Reidler (1967) showed that in the absence of carbohydrates, the biological conversion of malic acid was not possible. The main sugars in wine are glucose and fructose, LAB species being able to use both carbon sources (Cibrario et al. 2016). Also, most LAB are able to use other monosaccharides present in wine in lower concentrations (arabinose, mannose, galactose, xylose etc.), as well as polysaccharides or glycosylated compounds (Déléris-Bou and Krieger-Weber 2014). The carbohydrates metabolism is the main way of obtaining the energy necessary for LAB growth and development, fructose being preferentially used, compared to glucose. According to Nonomura (1983), the lack of fructose does not always allow the growth of LAB cells, although there were some cases when pyruvic acid, pentoses, ascorbic acid or cysteine can replace fructose. Also, was reported that MLF can be inhibited in wines where the sum of glucose and fructose is less than 0.2 g/L (Krieger 2005). Studies on malate-carbohydrate co-fermentation suggested that malate metabolism significantly influences carbohydrate metabolism (Henick-Kling 1993), but the opposite has also been reported (Salou et al. 1991). Moreover, Miranda et al. (1997) showed that carbohydrate-malate co-fermentation seems to depend largely on the strain involved. Considering these aspects, the purpose of the study was to highlight the CCR phenomenon in indigenous LAB strains responsible for the malolactic fermentation of wines. For a more rigorous control of the bioconversion process, is necessary to understand the interactions that occur in the competitive use of different carbon sources present in wine.

Materials and Methods

Tested LAB strains were isolated from red wines (Merlot, Cabernet Sauvignon, Arcaş), obtained by classic winemaking technology (grape crushing and destemming) at the Research - Development Station for Viticulture and Winemaking Iasi, NE of Romania. After preliminary selection, identification and characterization, the two strains R1-1 *Oenococcus oeni* and 13-7 *Lactobacilus plantarum* were preserved in De Man-Rogosa-Sharpe (MRS) broth medium supplemented with 30% glycerol, at −20 °C, a procedure that ensured their stability of during storage (minimum 6 months) (Filimon et al. 2022). Bacterial isolates were subsequently inoculated in FT80 broth medium (Cavin et al., 1989), at a cell density of 10^8 CFU/mL, and incubated in anaerobiosis (GENbag anaerobic®; BioMérieux, France), 72 hours, at 28°C. The bacterial cell biomass was separated by centrifugation (4000 rpm, 15 min.), washed twice in physiological serum and inoculated (25 mg dry biomass/mL) in 0.1 M glycine buffer solution (pH 3.5), supplemented with 45 mM malic acid and 45 mM of each tested carbohydrate (glucose, fructose, galactose, mannose, ribose and maltose), at 30°C, for 72 hours, according to the protocol presented by Miranda *et al*. (1997). Glycine is one of the 20 proteinogenic amino acids, used as nitrogen source. To highlight the CCR sensibility, the ability of the LAB strains

to metabolize malic acid was tested in the presence of different concentrations of glucose: 1, 5, 10, 30, 45 and 50 mM.
Monitoring of the MLF process was performed by thin layer chromatography (TLC), using cellulose plates 20×20 cm (Merck, Germany) and a mixture of solvents as mobile phase: n-butanol : distilled water : acetic acid : bromophenol blue (100:20:20:0.1 v/v/v/w). For the quantitative determination of malic acid was used the enzyme kit produced by Biosystems, Spain (Ref. 12803). Malic acid generates NADH when transformed by L-malate dehydrogenase, its concentration (g/L) being measured spectrophotometrically at 340 nm.
The reported data are mean values obtained in three independent experiments (n=3), with standard deviation (±). Analysis of variance ANOVA test (XLSTAT 2021.5.1 for Microsoft® Excel) was initiated to investigate significant differences between data, *p*-values ≤ 0.05 were considered significant. The method used to discriminate among the means was Tukey's test at 95% confidence level.

Results and discussions

Bacteria use CCR through different mechanisms to achieve different physiological goals required to their survivability and development (Nair and Sarma 2021). The pH of the incubation medium was low (3.5), similar to wine, due to the fact that at low pH malic acid is metabolized at a higher rate, while carbohydrate metabolism proceeds very slow (Firme et al. 1994). During the fermentation process, the increase in pH induced by the metabolism of malate allows the subsequent utilization of carbohydrates.
After 72 hours, the presence of malic and lactic acids was assessed by thin layer chromatography (TLC), the corresponding spots being observed (Figure 1). The spots of malic acid were visible on the plate, indicating its partial conversion.

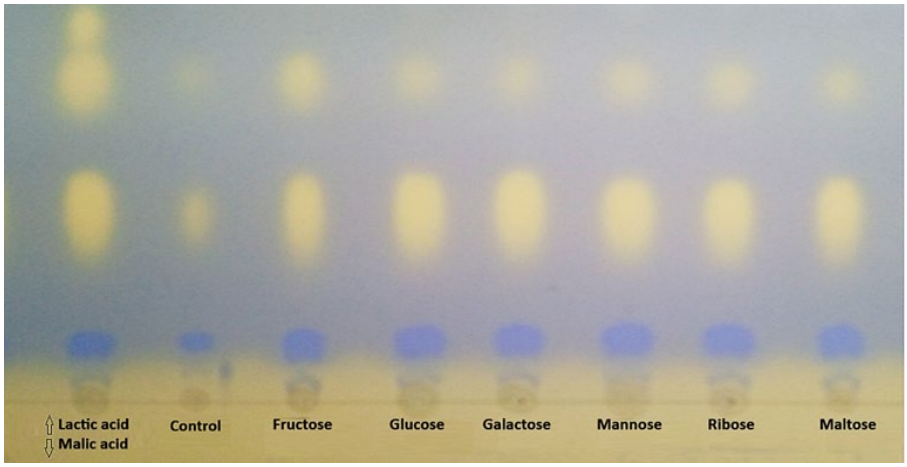

Figure 1. TLC chromatogram of the 0.1 M glycine buffer solution (pH 3.5) inoculated with *Oenococcus oeni* 13-7, supplemented with malic acid (45 mM) and different carbohydrates (45 mM) (72 h, 30°C). Control sample represent the glycine buffer solution (pH 3.5) with malic acid, without carbohydrate.

The retention factors (Rf), calculated as the ratio between the migration distance of each compound (to the center of the spot) and the total migration distance of the solvent (7.5 cm), were 0.48 for malic acid, respectively, 0.75 for lactic acid.

The experimental results obtained indicated that for both LAB species the presence of glucose in the medium at concentrations of 45 mM significantly inhibited the malolactic bioconversion. Residual malic acid in the presence of glucose was between 4.32-4.39 g/L from the initial quantity of 6.0 g/L (45 mM), while in the case of fructose were determined the lowest

residual amounts of malic acid for both strains (1.03-1.10 g/L) (Table 1). For both galactose and mannose, the concentration of malic acid determined in the medium after the incubation period varied non-significantly from 4.05±0.31 to 4.22±0.22 g/L. Excluding glucose and galactose, *O. oeni* strain showed a more pronounced CCR phenomenon compared to *L. plantarum.*

Table1. Residual malic acid (g/L) determined in the glycine buffer solution with carbohydrates

Species/ strain code	Carbohydrates						
	Control	Fructose	Glucose	Galactose	Mannose	Ribose	Maltose
O. oeni 13-7	1.55±0.12^{b}	1.03±0.09^{d}	4.39±0.19^{a}	4.22±0.22^{a}	4.05±0.31^{a}	1.90±0.24^{bc}	4.13±0.21^{a}
L. p. R1-1	1.59±0.16^{b}	1.10±0.15^{d}	4.32±0.14^{a}	4.16±0.19^{a}	4.09±0.41^{a}	2.01±0.14^{c}	4.17±0.19^{a}

The disaccharide maltose showed an effect similar to glucose. Although several studies showed that some LAB strains are unable to metabolize maltose (De Vos et al. 2009), this sugar can be used totally or partially by some strains of *O. oeni* or *L. plantarum* as a source of carbon and energy (Izquierdo et al. 2004). Cibrario et al. (2016) reported that from 41 *O. oeni* strains more than 75% were able to use glucose, ribose and mannose; fructose and L-arabinose were used by about half the strains, while 25% of the strains were able to use maltose.
However, for both LAB species, the presence of glucose in the medium reduced malic acid bioconversion by about 45% compared to the control sample (without carbohydrates). A negative effect on malic acid bioconversion, similar to glucose, was showed in the case of the hexoses galactose and mannose, the percentage of malic acid degraded being about 33% (Figure 2). A lower inhibition of the malolactic process was observed in the case of the pentose ribose, the malic acid being metabolized in a proportion of 68.57% by the strain 13-7 *O. oeni* and 66.74% by the bacterial strains R1-1 *L. plantarum*.

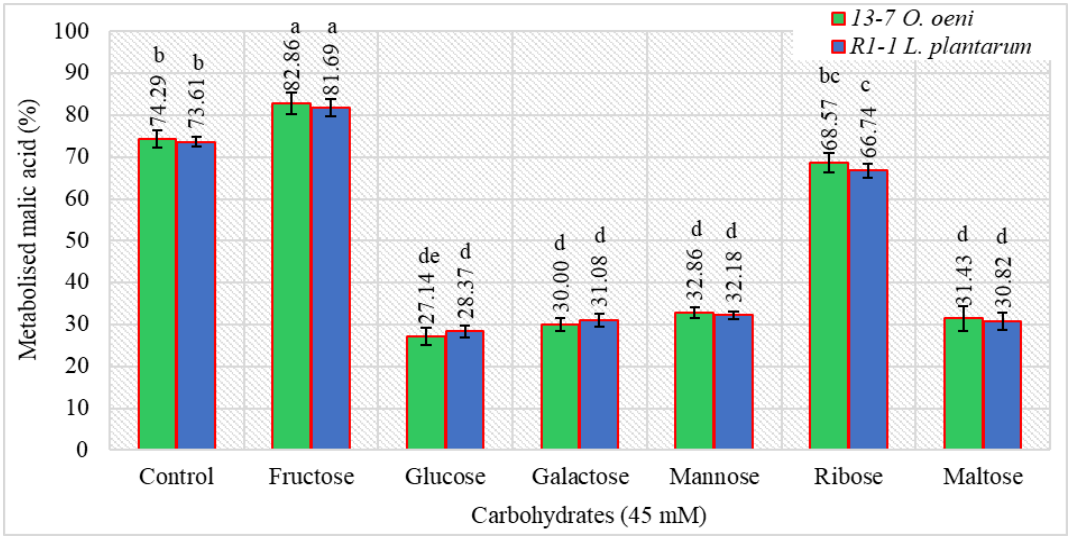

Figure 2. The effect of various carbon sources on the malolactic activity of indigenous LAB strains. Data represent the mean values (n=3), while error bars indicate standard deviation (±). Different letters within the same figure indicate significant differences in Tukey test ($p \leq 0.05$).

The highest rate of malic acid metabolization was registered in the medium with 45 mM fructose (>80%), the percentage of malic acid consumed being higher compared to the control by up to 10%, for both bacterial strains. According to Maicas et al. (1999), glucose is used as the carbon and energy source by all strains of *O. oeni*, but was reported that fructose is the most rapidly and efficiently metabolized sugar. The use of fructose as an electron acceptor has usually been seen as beneficial for most LAB strains. Also, should be mentioned that non-

significant differences were found between the two analyzed strains regarding the CCR process, their behavior being similar in the experimental conditions.
Because glucose induced the lowest rates of malic acid metabolization, the second part of the experiment aimed at highlighting the capacity of the indigenous LAB strains to metabolize malic acid at different concentrations of glucose (1, 5, 10, 30, 45 and 50 mM) (Figure 3).

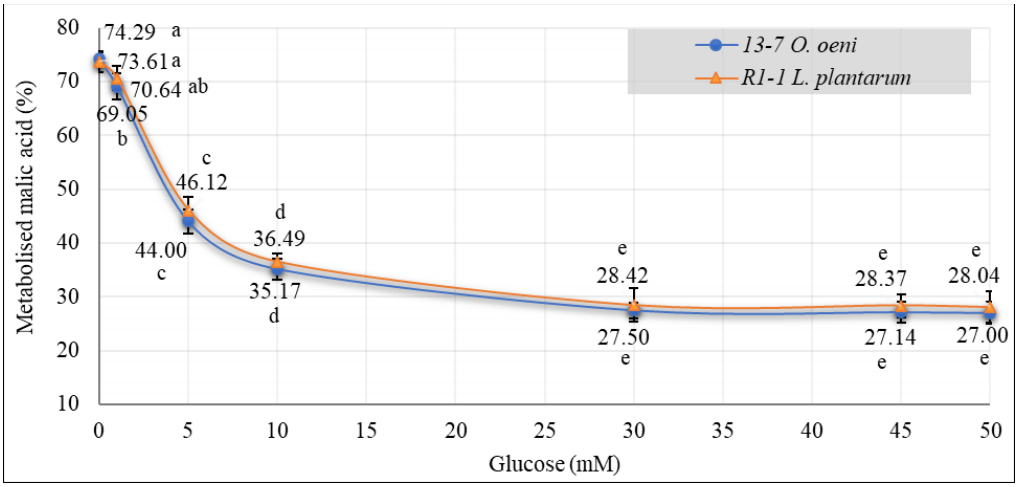

Figure 3. Effect of glucose concentration on malic acid metabolism by indigenous LAB strains. Data represent the mean values (n=3), while error bars indicate standard deviation (±). Different letters indicate significant differences in Tukey test ($p \leq 0.05$).

For both bacterial strains, a glucose concentration of 1 mM in the medium reduced the amount of malic acid metabolized with an average value of 7% compared to the control variant (without carbohydrates). Increasing the glucose concentration to 5 and 10 mM, resulted in a corresponding decrease in the amount of malic acid transformed by the LAB strains by 37 and 52%, respectively. At glucose concentrations above 30 mM, the inhibition of malic acid metabolism was very high, the percentage of malic acid metabolized being between 27 and 28%, lower by about 62% compared to the control variant. It should be noted that the *O. oeni* 13-7 strain showed the lowest values of malate bioconversion in the presence of glucose regardless of its concentration.
The results obtained are consistent with those reported by Miranda et al. (1997), except that the CCR phenomenon in the case of the studied bacterial strains 13-7 and R1-1 was not of the same intensity. Previous research showed that for *O. oeni* strains a concentration of 2 mM glucose in the medium inhibited the malolactic fermentation by up to 50%, while concentrations of 5 mM determined an inhibitory effect of about 70% (Miranda et al. 1997).

Conclusions

The indigenous malolactic bacteria strains isolated from wine microbiota (*Oenococcus oeni* 13-7 and *Lactobacillus plantarum* R1-1), showed a high ability to exhibit the carbon catabolite repression mechanism. Cultivated in glycine buffer medium with different carbohydrates (hexoses and pentoses) and malic acid, the strains reduced the malolactic bioconversion process by up to 60% in the presence of glucose, a similar effect being observed for galactose, mannose or maltose. The highest rate of malic acid metabolization was shown in the presence of fructose. At glucose concentrations above 30 mM, the inhibition of malic acid metabolism was very high, the percentage of malic acid metabolized being between 27 and 28%, which means by up to 62% lower compared to the control variant. However, *Oenococcus oeni* strain showed lower values of malic acid bioconversion in the presence of glucose regardless of concentration. The

experimental results indicate that the addition of fructose in the medium may cancel the repression of malic acid metabolism, further studies being necessary to elucidate the mechanisms underlying this process.

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