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Predominance of malolactic fermentation over D-glucose utilization in *Lactobacillus plantarum* and *Lactobacillus curvatus* wine strains

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SUMMARY

Two selected wine strains of the genus *Lactobacillus* (*L. plantarum* 197 and *L. curvatus* 783) were tested for their ability to complete malolactic fermentation (MLF) in a synthetic medium (PBM-broth) supplemented with L-malic acid (7.5–74.6 mM) and D-glucose (5.5–55 mM). The 24 directed fermentation assays, 12 for each bacterial strain, were carried out at 20 °C and pH 3.5. MLF was completed (residual L-malic acid ≤0.2 mM) in eight days in 19 of the 24 fermentation assays, even in the presence of 74.6 mM L-malic acid or 55.5 mM D-glucose. D-Glucose utilization was generally simultaneous to MLF but was completed (residual concentrations ≤0.2 mM) only in 6 of the 24 fermentation assays. These results support the use of these strains in directed MLF assays at the very different L-malic acid and D-glucose concentrations tested.

INTRODUCTION

Malolactic fermentation (MLF) of wines consists of the bacterial conversion of L-malic acid into L-lactic acid and carbon dioxide [4]. This fermentation usually takes place in wine once alcoholic fermentation has been completed and results in a biological deacidification of the wines [9]. As a result of the cold and rainy weather in the vineyards in Northwestern Spain, the grapes often do not ripen properly, this resulting in grape juices and wines with high L-malate concentrations (up to 60 mM) [2,6]. Additionally, spontaneous or yeast-directed alcoholic fermentations are occasionally incomplete in these wines, yielding sugar (glucose + fructose) concentrations above 27 mM.

While members of the genera *Leuconostoc* or *Pediococcus* are seldom isolated, species of the genus *Lactobacillus* have been reported by us to lead to MLF in the wines from Northwestern Spain [1,12]. Among these species, *L. plantarum* and *L. curvatus* have a high isolation frequency and play an important role in the development of spontaneous MLF in these wines. Since our research has aimed at industrial employment of selected strains of *Lactobacillus* in wines that do not undergo spontaneous MLF (and consequently, preserve wine typicity in the vineyards of our region), it is necessary to evaluate the ability of selected wine strains to perform MLF over a wide range of L-malate concentrations and at very different concentrations of sugar present in the medium. With this aim, PBM-broth, a previously described medium for lactobacilli [15,16], was supplemented with L-malate and D-glucose (representing the total sugar content) at different ratios, these being related to the concentrations found in white wines of Albariño (Northwestern Spain) exactly at the end of alcoholic fermentations. The conclusions derived from these experiments and the enologic implications are discussed below.

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MATERIALS AND METHODS

Microorganisms

Lactobacillus curvatus (strain 783) and *Lactobacillus plantarum* (strain 197) wild strains belonged to the collection at our laboratory and were originally isolated, respectively, in spontaneous MLF and the slow period of alcoholic fermentation in wines from Albariño as described elsewhere [12,15]. The strains were selected considering the following parameters in MLF: a high L-malic acid fermentative rate, completion of MLF under habitual wine conditions and a low production of acetic acid [12].

Culture and fermentation conditions

MRS broth [3] was used for the maintenance of lactic acid bacteria. The composition of the physiologic basal medium (PBM-broth) for the fermentation assays was as follows: peptone (Difco), 5 mg ml^{-1}; D-glucose (Merck), 1 mg ml^{-1}; ammonium sulfate (Merck), 2 mg ml^{-1}; bipotass-

ium phosphate (Merck), 1.4 mg ml^{-1}; monopotassium phosphate (Merck), 0.6 mg ml^{-1}; citric acid (Sigma), 0.1 mg ml^{-1}; magnesium sulfate (Merck) 1 M, 1 μl ml^{-1}; sodium chloride (Merck), 5 mg ml^{-1}; calcium chloride (Merck), 0.7 mg ml^{-1} and manganese sulfate (Merck), 0.07 mg ml^{-1}. The fermentation assays were carried out at 20 °C in tubes containing 8 ml of PBM-broth, adequately supplemented with L-malic acid (Sigma) and D-glucose (Merck), as described in Table 1. Once supplementation of PBM-broth was completed, the initial pH of all media were adjusted to 3.5 with 1 N HCl since this was considered the best value for MLF in the lactic acid bacteria studied (data not shown) and coincided with that described by other authors [2,8]; this pH value was close to that found in wines from Albariño at the beginning of MLF [14].

Inoculation and growth determination

Twelve PBM-broth assays were carried out for each strain as described in Table 1. The initial bacterial density was adjusted to 10^6 bacteria ml^{-1} by counting in a Neubauer chamber. The evolution of bacterial growth during the fermentation assays was determined by measuring optical density at 550 nm in a Beckman DU-40 spectrophotometer and by direct counting as before. The assays were considered to be completed after 8 days, when bacterial sedimentation occurred, the concentrations of L-malate and D-glucose being below 0.1 mM or very near to those determined at the seventh day.

Analytical methods

For estimating L-malate, D-glucose and L-lactate concentrations, samples were collected under sterile conditions at 2, 4, 7 and 8 (end of MLF) days after the beginning of the experiment. Afterwards, they were filtered through 0.22 μm-Millipore filters. Estimations of L-malate, D-glucose, and L-lactate were carried out in duplicate using commercial enzymatic kits (Boehringer-Mannheim).

RESULTS

MLF and D-glucose utilization at a low D-glucose concentration (5.5 mM)

The main goal of these assays (a, b and c) was to study the comparative evolution of MLF and D-glucose utilization when this substrate was present in the medium at low concentrations and in the presence of three different concentrations of the substrate of MLF (L-malate); 5.5 mM D-glucose is equivalent to the most frequent sugar concentration at the end of complete spontaneous alcoholic fermentations (0.1%) [15]. Both *L. plantarum* and *L. curvatus* completed MLF within 48 h in the assays containing 7.5 mM and 22.4 mM initial L-malate while MLF was extended up to seven days in the assays with the highest L-malate initial concentration (74.6 mM) (Table 2). Nevertheless, MLF was completed by both lactobacilli in all cases and, in general, before all the D-glucose had been fermented. Thus, a higher affinity of *L. plantarum* for L-malate than for D-glucose was observed under these conditions. D-Glucose utilization by *L. plantarum* was simultaneous to MLF and was lower at a high concentration of L-malate (74.6 mM) than at 7.5 mM or 22.4 mM L-malate (Table 3); D-glucose utilization by *L. curvatus* was also continuous and simultaneous to MLF. D-Glucose final concentrations were below 0.1 mM in all cases.

MLF and D-glucose utilization at an intermediate D-glucose concentration (22 mM)

After partially incomplete alcoholic fermentations, sugar concentrations up to 0.4% (equivalent to 22 mM D-glucose) remain in wine. Our aim in these assays (d, e and f) was to determine MLF and D-glucose utilization under such conditions. MLF was predominant with regard to D-glucose utilization in both lactobacilli during the first two days of assay; MLF even seemed to be exclusive in *L. plantarum* during this period of time since the D-glucose concentrations remained unvaried or even increased (Tables 2 and 3), this probably being due to a positive balance between D-glucose utilization and a β-glucosidase activity of these strains elicited at this D-glucose concentration (data not shown). After the fifth day of assay the utilization of D-glucose by both lactobacilli increased, and yielded final D-glucose concentrations between 3.8 and 18.3 mM (Table 3). MLF was completed by *L. curvatus* only in the fermentation assays with the highest L-malate concentrations (22.4 and 74.6 mM). By contrast, rate of L-malate fermentation by *L. plantarum* decreased after the second day of assay and MLF was completed in only one assay (when the initial L-malate concentration was 7.5 mM) (Table 2). Therefore, an apparently inverse relationship between D-glucose utilization and MLF at these particular concentrations of both substrates was observed: a higher L-malate fermentative rate was paralleled by a lower utilization of D-glucose, this being more

TABLE 1

Scheme of supplementation of PBM-broth with L-malic acid and D-glucose

	Fermentation assay											
	a	b	c	d	e	f	g	h	i	j	k	l
L-malic acid*	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6
D-glucose*	5.5	5.5	5.5	22	22	22	38.8	38.8	38.8	55.5	55.5	55.5

*Concentrations expressed as millimolar (mM).

TABLE 2

Malolactic fermentation by *L. plantarum* 197 and *L. curvatus* 783

Initial D-glucose	7.5 mM			22 mM			38.8 mM			55.5 mM		
assay	a	b	c	d	e	f	g	h	i	j	k	l
	Residual L-malate (mM)											
L. plantarum 197												
Initial	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6
2 days	0.2	0.5	29.8	2.1	13.4	44.8	0.1	1.1	28.3	0.1	0.3	40.3
5 days	0.1	0.1	1.8	0.8	13.5	43.9	0.1	0.3	21.6	<0.1	0.1	25.4
7 days	<0.1	0.1	0.3	0.2	7.2	44.1	<0.1	0.1	0.4	<0.1	<0.1	0.4
Final (8 days)	<0.1	0.1	0.1	<0.1	6.9	44.0	<0.1	<0.1	0.2	<0.1	<0.1	0.2
L. curvatus 783												
Initial	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6	7.5	22.4	74.6
2 days	0.3	2.4	51.5	5.7	0.2	0.3	0.5	14.2	0.2	5.6	15.8	73.4
5 days	0.1	0.2	6.2	3.9	<0.1	0.1	<0.1	10.9	<0.1	3.9	6.0	57.6
7 days	<0.1	0.1	0.4	3.9	<0.1	<0.1	<0.1	11.3	<0.1	0.3	0.3	57.8
Final (8 days)	<0.1	0.1	<0.1	3.9	<0.1	<0.1	<0.1	11.2	<0.1	<0.1	<0.1	57.6

TABLE 3

D-Glucose fermentation by *L. plantarum* 197 and *L. curvatus* 783

Initial L-malate	7.5 mM				22.4 mM				74.6 mM			
assay	a	d	g	j	b	e	h	k	c	f	i	l
	Residual D-glucose (mM)											
L. plantarum												
Initial	5.5	22	38.8	55.5	5.5	22	38.8	55.5	5.5	22	38.8	55.5
2 days	0.5	25.7	28.7	38.0	0.6	25.1	24.6	33.6	1.9	24.7	22.2	44.6
5 days	0.1	21.3	22.5	18.2	0.2	25.2	10.9	22.6	1.7	25.0	17.9	40.1
7 days	<0.1	9.2	12.1	23.1	<0.1	13.1	4.5	7.7	0.1	4.2	13.3	21.4
Final (8 days)	<0.1	8.8	12.2	22.0	<0.1	12.5	4.1	6.5	<0.1	3.8	12.7	20.1
L. curvatus												
Initial	5.5	22	38.8	55.5	5.5	22	38.8	55.5	5.5	22	38.8	55.5
2 days	4.3	19.8	19.2	34.1	2.1	20.2	31.2	34.7	2.0	24.2	28.1	26.4
5 days	0.4	15.0	12.5	35.2	0.4	18.5	27.7	17.3	0.5	24.7	18.8	22.5
7 days	<0.1	13.0	4.7	15.4	0.1	6.2	29.2	18.8	<0.1	18.6	14.0	24.3
Final (8 days)	<0.1	12.5	4.3	14.8	<0.1	5.7	28.4	19.0	<0.1	18.3	13.4	24.5

important when the L-malate fermentative rate decreased and MLF was extended in time.

MLF and D-glucose utilization at a moderately high D-glucose concentration (38.8 mM)

Incomplete spontaneous alcoholic fermentations often yield sugar concentrations up to 0.7 mM (equivalent to 38.8 mM D-glucose) and therefore a study was made of the effect of this D-glucose concentration on MLF at different L-malate bioavailabilities (assays g, h and i).

Simultaneous utilization of L-malate and D-glucose by *L. plantarum* and *L. curvatus* was observed until MLF ceased (Tables 2 and 3). When MLF was completed before the fifth day of assay, additional D-glucose utilization by both lactobacilli was observed until the seventh day, when bacterial sedimentation occurred (Table 3). In the case of *L. plantarum*, MLF was extended beyond the third day when the initial L-malate bioavailability was 74.6 mM; the final D-glucose concentrations left by *L. plantarum* were between 4.1 and 12.7 mM (Table 3). Wide fluctuations in the D-glucose utilization were observed for *L. curvatus* as the initial bioavailability of L-malate varied (Table 3). Thus, the highest utilization of D-glucose corresponded to the assay with the lowest initial concentration of L-malate (7.5 mM) and final D-glucose concentrations between 4.3 and 28.4 mM were achieved, as can be observed in Table 3. MLF was

completed within 48 h by *L. curvatus* except in the assay with 22.4 mM L-malate, in which MLF was extended until the eighth day and was not completed (Table 2). In contrast to the assays with 22 mM D-glucose, the two assays in which MLF was completed by *L. curvatus* within the first two days coincided with the highest D-glucose utilization rates.

MLF and D-glucose utilization at a high D-glucose concentration (55.5 mM)

Sometimes, very incomplete spontaneous alcoholic fermentations occur, yielding residual sugar concentrations up to 1% (equivalent to 55.5 mM D-glucose). Therefore, MLF and D-glucose utilization by wine lactobacilli were evaluated under such conditions (assays j, k and l). In a similar way to the results found at 5.5 mM and 38.8 mM initial D-glucose concentrations, MLF was carried out by *L. plantarum* at a high rate (Table 2), this being simultaneous to D-glucose utilization (Table 3). In this sense, MLF was completed by this strain at any initial concentration of L-malate. D-Glucose utilization by *L. plantarum* was continuous during the eight days of the assays and yielded final concentrations between 6.5 and 22 mM (Table 3).

A significant D-glucose utilization by *L. curvatus* was observed within the first 48 h but decreased after that moment, thus yielding final D-glucose concentrations between 14.8 and 24.5 mM (Table 3). MLF was found to be unfavored in *L. curvatus* in the presence of 74.6 mM initial L-malate (more than 70% of the initial L-malate remained unfermented) but was completed in the assays with 7.5 and 22.4 mM initial L-malate (Table 2). This L-malate fermentative behavior was quite different from that shown by *L. plantarum*, although similar patterns of D-glucose utilization were observed for both strains (Table 3).

DISCUSSION

The development of MLF, either spontaneously or induced by the addition of a selected pure culture, is greatly affected by L-malic acid and sugar concentrations available in the wine at the end of the alcoholic fermentation [11]. In the present work, MLF was completed by both lactobacilli in almost all the fermentation assays, even in the presence of 74.6 mM L-malic acid and very different sugar concentrations (5.5–55 mM D-glucose). Other authors have described the relationship between the utilization of residual wine sugars by malolactic bacteria and the growth of these strains [10, 13]. The results reported in this work show that, in general, MLF was simultaneous to D-glucose utilization, the latter probably being aimed at fulfilling the energetic requirements of *L. plantarum* and *L. curvatus* while MLF was being carried out. According to this, significant decreases in D-glucose utilization were generally observed when MLF decreased or was completed. D-Glucose was completely utilized by *L. plantarum* and *L. curvatus* only at the 5.5 mM initial concentration but partially at the other initial D-glucose concentrations (22, 38.8 and 55.5 mM). D-Glucose utilization was in general simultaneous to MLF, the latter being more intense; further D-glucose utilization was observed until the seventh day, even when all the L-malic acid had been fermented. Nevertheless, D-glucose utilization was frequently lower than 70% of that observed initially. Except for the fermentation assays with 22 mM initial D-glucose, a direct relationship between MLF and D-glucose utilization was observed. L-lactate estimations (data not shown) confirmed the predictions of the MLF profiles based on the evolution of L-malic acid: the highest L-lactate yields were generally related to the highest L-malic acid fermentative rates, thus suggesting that utilization of L-malic acid was via malolactic enzyme rather than malic enzyme (which is glucose repressed). Determinations of the evolution of pH during fermentations evidenced no pH increase higher than 0.3 units, which suggests that this physico-chemical parameter implies a secondary effect in utilization of the sugar. Additionally, the MLF profile at pH 3.5 was similar to that observed at pH 3.9, as has been observed previously at our laboratory [2].

Unlike what has been described by other authors for *Leuconostoc oenos* [5], a diauxic growth pattern that could be related to an alternative utilization of L-malic acid and D-glucose was not observed, even in the assays (22 mM D-glucose) where apparent alternation was achieved. Plate bioassays evidenced D-glucose-regulated β-glucosidase activity in both *L. plantarum* and *L. curvatus* wine strains (data not shown) which might explain the results found at 22 mM initial D-glucose concentration: β-glucosidase was found to be active in these strains when D-glucose was below 27 mM. This might suggest that D-glucose was being utilized since the first day of assay, as in the rest of assays, although D-glucose concentrations apparently did not decrease due to D-glucose-generating β-glucosidase activity. In fact, the presence of terpenic β-glucosides in musts and wines has been described by other authors [7].

Fermentation assays in the presence of ethanol concentrations similar to those found in the wines of Northwestern Spain (12% v/v) have previously been carried out at our laboratory and evidenced no significant delay in MLF and disclosed a final fermentative balance higher than 80% of that found at 9% (v/v) ethanol, this being equivalent to the control assay in the absence of ethanol [2].

This work opens the way to the industrial employment of *L. plantarum* and *L. curvatus* wine strains described in this work since MLF was generally completed at the very different concentrations of L-malic acid and D-glucose tested. The fact that these strains were previously selected as exhibiting other optimum enologic properties [2,12] as well as their excellent adaptation to the musts of the vineyards of Northwestern Spain (much more than that observed for *Leuconostoc* spp., as derived from the isolation frequencies in spontaneous fermentations) [12] underlines their potential industrial application.

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REFERENCES

1 Agrelo, D., E. Longo, P. Combarro, M.J. Garrido and T.G. Villa. 1987. Estudio taxonómico en bacterias lácticas epifitas en uvas de Galicia. Microbiol. SEM 3: 213–216.

2 Calo, P., J. Cansado, J.B. Velázquez, C. Sieiro, E. Longo and T.G. Villa. 1991. Effect of different physico-chemical conditions on malolactic fermentation of four *Lactobacillus plantarum* wild strains isolated from wines of Northwestern Spain. Biotechnol. Lett. 13: 781–786.

3 de Man, J.C., M. Rogosa and M.E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23: 130–135.

4 Kunkee, R.E. 1967. Malo-lactic fermentation. Adv. Appl. Microbiol. 9: 235–279.

5 Lafon-Lafourcade, S. 1983. Wine and brandy. In: Biotechnology (Reed, G., ed.), Vol. V, pp. 81–163, Verlag-Chemie, Heidelberg.

6 Longo, E., J. Cansado, D. Agrelo and T.G. Villa. 1991. Effect of climatic conditions on yeast diversity in grape musts from Northwest Spain. Am. J. Enol. Vitic. 42: 141–144.

7 Maia, M.O. and J.M. Oliveira. 1992. Terpenols flavour in the vinification of "vinhos verdes". In: Profiles on Biotechnology (Villa, T.G. and J.E. Abalde, eds), pp. 603–614, University of Santiago Press, Santiago, Spain.

8 McCord, J.D. and D.D.Y. Ryu. 1985. Development of malolactic fermentation process using immobilized whole cells and enzymes. Am. J. Enol. Vitic. 36: 214–218.

9 Pilone, G.J. and R.E. Kunkee. 1966. Chemical characterization of wines fermented with various malo-lactic bacteria. Appl. Microbiol. 14: 608–618.

10 Radler, F. 1967. Étude microbiologique des bactéries de la fermentation malolactique. Conn. Vigne Vin 1: 73–91.

11 Riberéau-Gayon, J., E. Peynaud, P. Riberéau-Gayon and P. Sudraud. 1975. Traite d'Oenologie. Sciences et techniques du vin, Dunod, Paris.

12 Sieiro, C., J. Cansado, D. Agrelo, J.B. Velázquez and T.G. Villa. 1990. Isolation and enological characterization of lactic acid bacteria from the vineyards of NW Spain. Appl. Environ. Microbiol. 56: 2936–2938.

13 Silver, J. and T. Leighton. 1982. Control of malolactic fermentation in wine. In: Proceedings of the University of California, Davis Grape Wine Symposium (Webb, A.D., ed.), pp. 170–175.

14 Velázquez, J.B., D. Agrelo, P. Calo and T.G. Villa. 1990. Temporizing the malolactic fermentation. In: From Genes to Bioproducts (Iborra, J.L., ed.), pp. 391–395, University of Murcia Press, Murcia, Spain.

15 Velázquez, J.B., P. Calo, E. Longo, J. Cansado, C. Sieiro and T.G. Villa. 1991. Effect of L-malate, D-glucose and L-lactate on malolactic fermentation and growth of *Lactobacillus plantarum* and *Lactobacillus curvatus* wild strains isolated from wine. J. Ferment. Bioeng. 71: 363–366.

16 Velázquez, J.B., J. Cansado, E. Longo, C. Sieiro, P. Calo and T.G. Villa. 1992. Physiology of malolactic fermentation in wine lactobacilli. Optimization of a novel medium for plate differentiation. In: Profiles on Biotechnology (Villa, T.G. and J.E. Abalde, eds), pp. 615–626, University of Santiago Press, Santiago, Spain.