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STUDY OF GENES RELATED WITH THE ETHYL CARBAMATE PRECURSORS IN WINE LACTIC ACID BACTERIA

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INTRODUCTION

Wine, like most fermented foods and beverages, can contain trace amounts of ethyl carbamate (EC), which has been described as a potential carcinogenic compound (Mirvish 1968). It can be produced from the reaction of ethanol with urea produced by yeasts, but also from the reaction of citrulline with ethanol (Liu et al 1994). Citrulline can be generated by lactic acid bacteria (LAB) from arginine degradation by the arginine deiminase (ADI) pathway (Liu et al 1995, Mira de Orduña et al 2000) (**Figure 1**). So, this is one of the possibly negative aspects of LAB for the consumer health, in contrast with the known main positive role of LAB (mainly *Oenococcus oeni*) in wine, the malolactic fermentation (MLF).

Although the amounts of EC in wine are usually negligible, over the last few years there has been a slight but steady increase, as climate change has increased temperatures and alcohol levels have become proportionately higher (Mira de Orduña 2010), both of which favor EC formation (Ough et al 1988).

Genes encoding the three enzymes of ADI pathway (*arc* genes, **Figure 1**) in most LAB are clustered in an operon (Tonon et al 2001, Spano et al 2004) that includes the *arcD* gene for arginine/ornithine transporter. In this study, the ability to generate EC precursors by the ADI pathway was evaluated in several strains of wine LAB. Although *O. oeni* is the main species responsible for MLF, a large portion of LAB present in winemaking are strains of different species of *Lactobacillus*, *Pediococcus* and *Leuconostoc*. Therefore, their potential role as producers of EC precursors may be relevant.

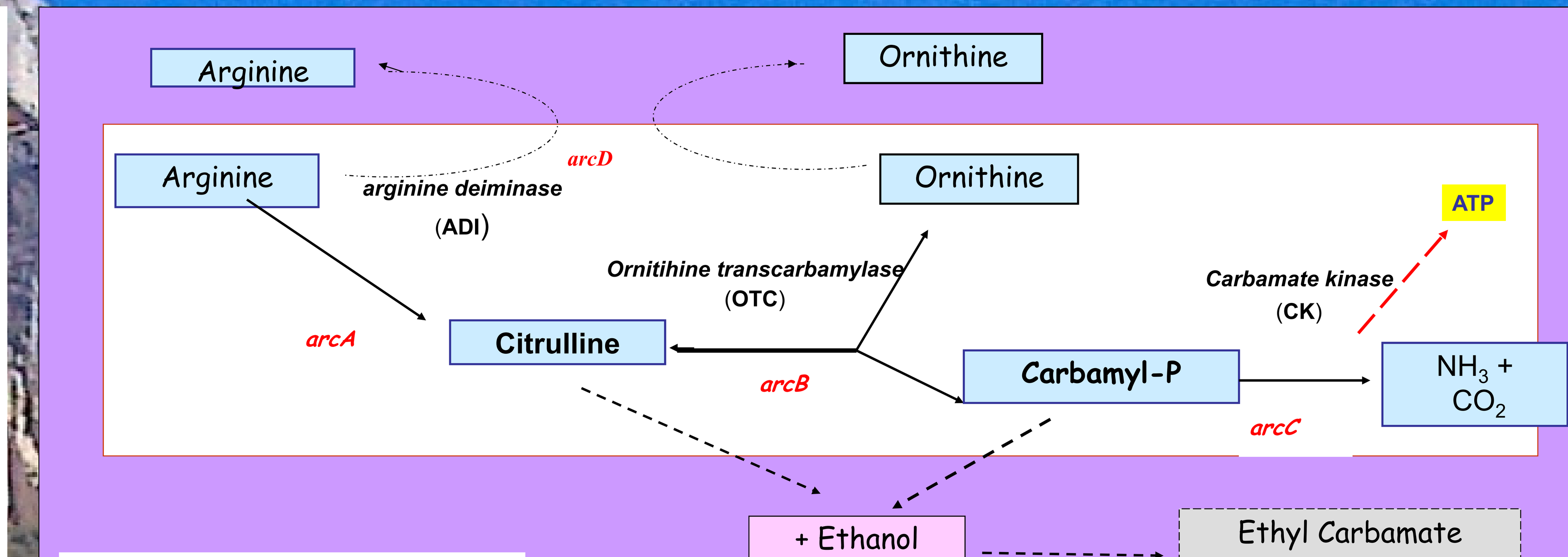

Figure 1. The ADI pathway in LAB

PRESENCE OF *arc* GENES IN WINE LAB

Several strains of different species of LAB were used (Araque et al 2009) (**Table 1**). The ability to degrade arginine in MRS medium (De Man et al 1960) was also studied, by quantifying arginine with the method of Sakaguchi (Gilboe & Williams 1956). To detect the presence of *arc* genes, degenerate primers were designed from the multiple alignment of conserved regions of protein sequences in already sequenced LAB. The usefulness of these degenerate primers was proven by sequencing some of the amplified PCR fragments and finding homologies higher than 90% with published sequences of the same species and related ones.

Correlation was found between the presence of genes (**Figure 2**) and the ability to degrade arginine (**Table 1**). Degrading strains included all heterofermentative lactobacilli, *O. oeni*, *P. pentosaceus*, and some strains of *Le. mesenteroides* and *Lb. plantarum*. This is the first time where strains of *P. pentosaceus* from wine have been found to degrade arginine. On the other hand, as known previously, no any *P. parvulus* can degrade it. Besides confirming that some *Lb. plantarum* of wine can degrade arginine, as found previously (Spano et al 2004), we saw that other *Lb. plantarum* (strain ATCC 8014) is non-arginine consuming and that it has an *arcB* gene. We found the same for some non-degrading strains of *Le. mesenteroides*. Nevertheless, surprisingly we observed some strains (isolated from wine) of this species that consume arginine, which presented the *arcA* gene but not the other two. In consequence, they cannot catabolize the citrulline produced, and it can be excreted. As confirmation, we found a higher ratio of citrulline/arginine (30%) for these *Le. mesenteroides* that for any other species. So, strains of *Le. mesenteroides* present in wine require special consideration because they would be able to accumulate citrulline, increasing the potential risk of EC formation.

Table 1. Degradation of arginine (5 g/l) in MRS medium at pH 4.5 and 27ºC, and presence of *arc* genes in strains of different LAB.

Species	n. strains	Degradation of arginine (%)	Presence of *arc* genes *arcA*	*arcB*	*arcC*
Lactobacillus hilgardii	3	80-90	+	+	+
Lactobacillus brevis	8	70-100	+	+	+
Lactobacillus buchneri	2	60-90	+	+	+
Lactobacillus plantarum	1	90	+	+	+
" "	1	0	-	+	-
Oenococcus oeni	8	10-100	+	+	+
Pediococcus pentosaceus	8	80-100	+	+	+
Pediococcus parvulus	4	0	-	-	-
Leuconostoc mesenteroides	2	0	-	+	-
" "	2	100	+	-	-

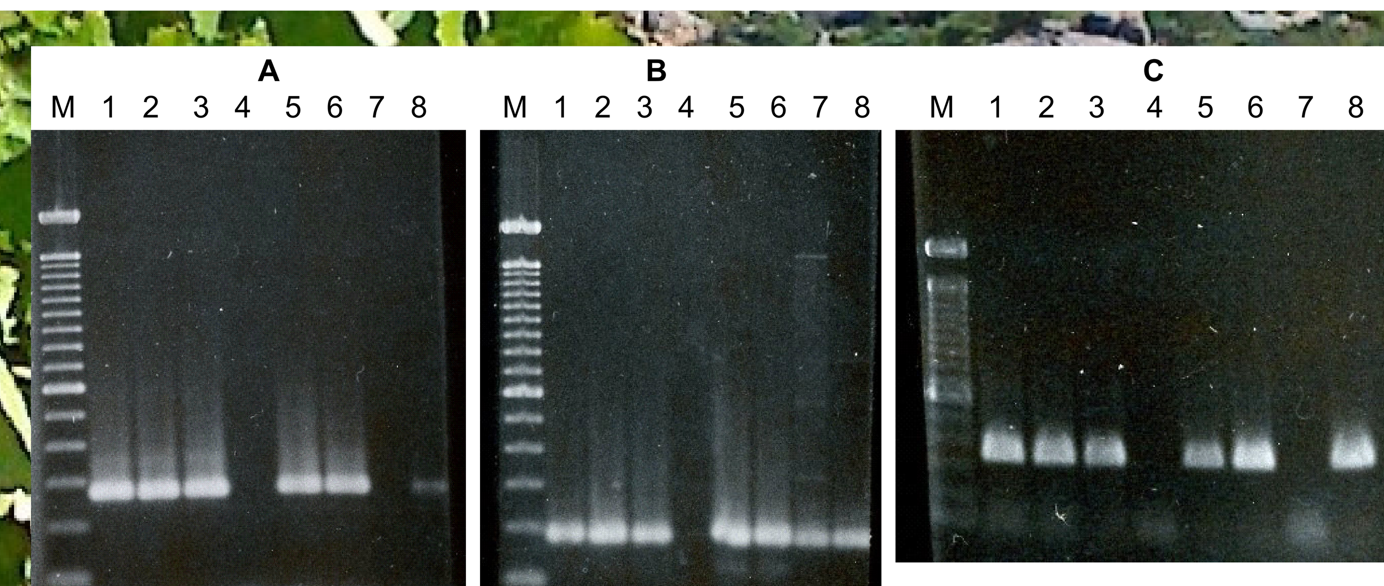

Figure 2. PCR-amplified fragments of *arcA* (**A**), *arcB* (**B**)and *arcC* (**C**) genes (266, 181 and 343 bp, respectively) of different LAB obtained with degenerate primers. Lanes 1 to 8 are respectively *Lb. hilgardii* 4786T, *O. oeni* 217T, *Lb. brevis* 4246, *P. parvulus* 813T, *Lb. buchneri* 4111T, *P. pentosaceus* 4695T, *Lb. plantarum* 220 and *Lb. plantarum* CB21. Lane M is a ladder DNA of 250 bp.

INFLUENCE OF WINE-LIKE CONDITIONS ON ARGININE UTILIZATION BY LAB

In base of the study above, some strains of arginine-consuming wine LAB species were used to evaluate the effects of ethanol, glucose, malic acid, and low pH on the ability to degrade arginine and excrete citrulline (Araque et al 2011). In order to avoid other interfering compounds and to do it in a short time, these assays were carried out with resting cells, growing them previously in MRS with 5 g/l arginine, harvesting them, and resuspending the cell pellets in resting-cell buffer, following Mira de Orduña et al 2000.

Malic acid was found to clearly inhibit arginine consumption in all strains (**Figure 3**), confirming that malic acid consumption is a priority over arginine in LAB. The relation between citrulline produced and arginine consumed was clearly higher in the presence of ethanol (10–12 %) and at low pH (3.0) (**Figure 4**). In *Lb. brevis* (CECT3824) and *Lb. buchneri* (CECT4674) isolated from wine and beer respectively, the ratio citrulline / arginine was higher than in the other type strains (isolated in other food), which suggests that strains accustomed to ethanol environments could have an increased ability to produce citrulline.

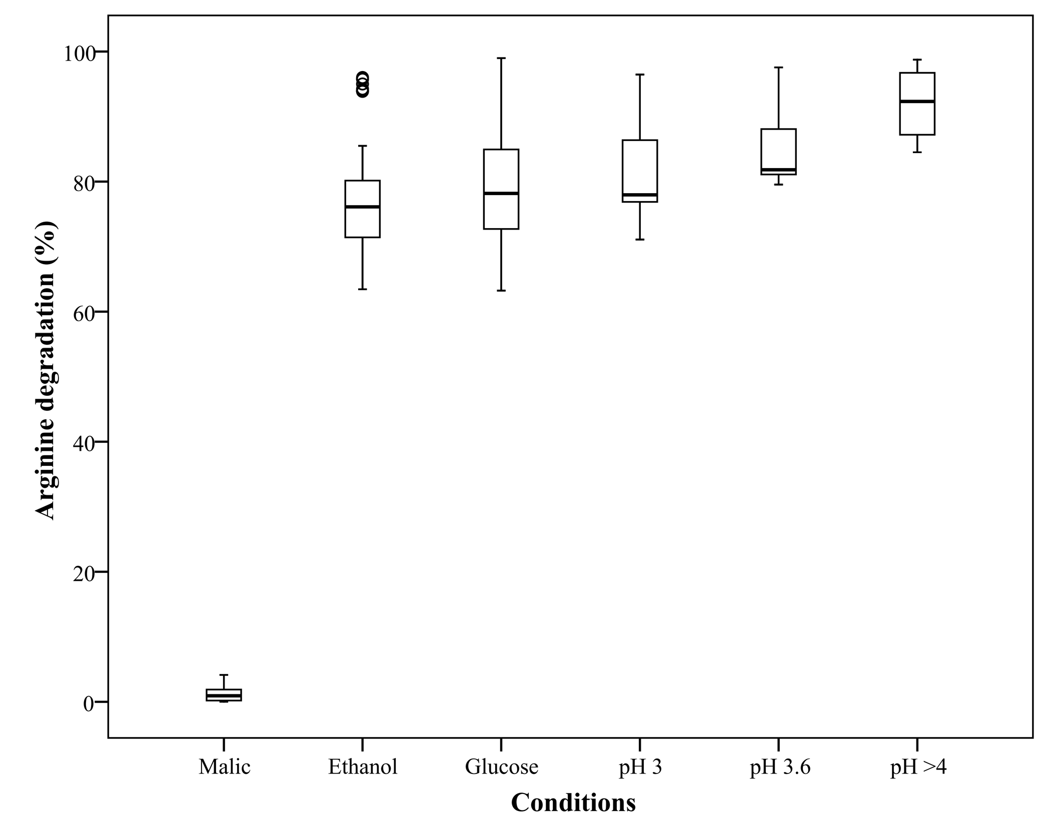

Figure 3. Effect of conditions on the degradation of arginine (0.5 g/l) for different LAB strains (*Lb. brevis* 4121[T], *Lb. hilgardii* 4786[T], *Lb. buchneri* 4111[T], *P. pentosaceus* 4695[T], *Lb. brevis* 3824, *Lb. hilgardii* 4681, *P. pentosaceus* 4214 and *Lb. buchneri* 4674) . Control assay was at pH 3.6. Malic: L-malic acid, 0.5 and 2.0 g/l. Ethanol: 5, 10 and 12 %. Glucose: 0.5, 3.0 and 5.0 g/l. The assays plotted at pH 3 and pH > 4 (4 and 4.5) were without ethanol or glucose or malic acid. Circles are the outliers which have values > 1.5 times the spread outside. Strains numbers are those from Colección Española de Cultivos Tipo (CECT, València, Spain).

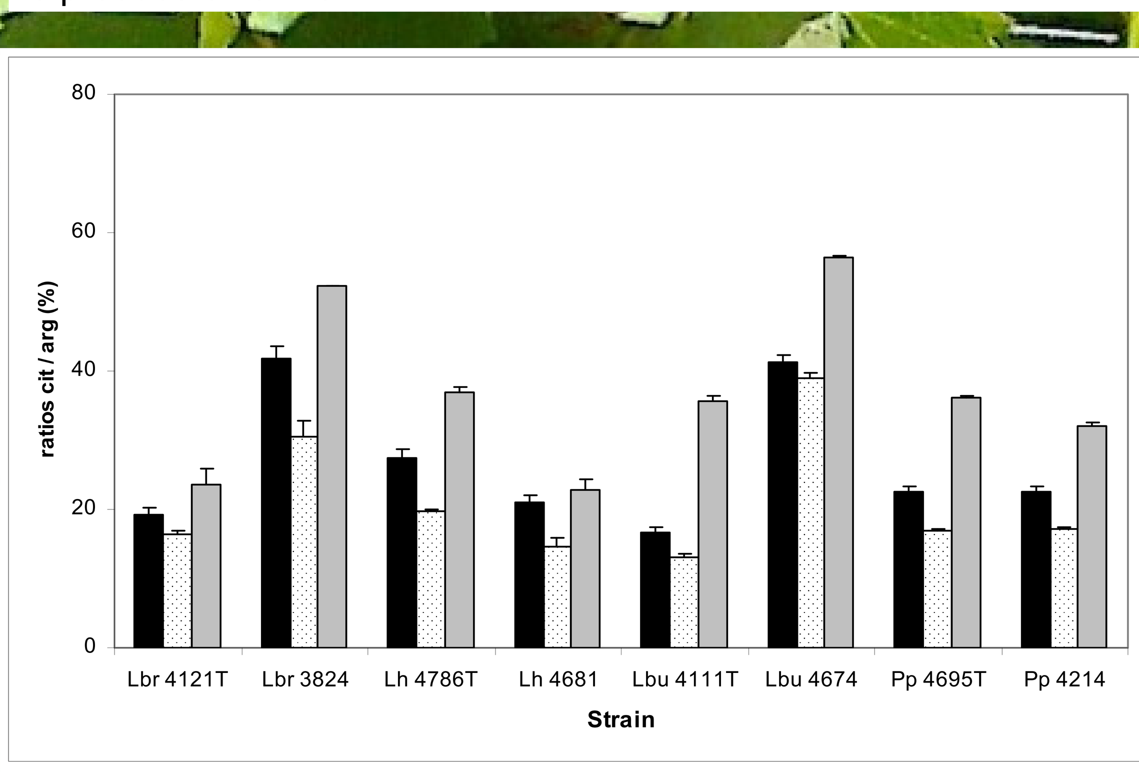

Figure 4. Ratios of citrulline produced from arginine by strains of LAB. Control without ethanol and pH 3.6 (dotted columns); pH 3.0 (black); ethanol 12 % (grey).

EFFECT OF ETHANOL AND LOW pH ON *arc* GENE EXPRESSION

Strains *Lb. brevis* CECT3824 and *P. pentosaceus* CECT4214, isolated from wines, were grown until Abs 0.4 in modified MRS with ethanol (0, 5 and 10%, v/v), then arginine (5 g/l) was added, and samples were taken for 24 h (Araque et al 2013). Total RNA was extracted, purified and reverse-transcribed to cDNA. Real-time quantitative PCR was performed with it, using primers designed in base of sequences obtained before (Araque et al 2009) and those of NCBI GenBank database. The qPCR analyses were done in an Applied Biosystems RT PCR Systems thermocycler using SYBR-green staining and quantification was based on the comparative critical threshold ($\Delta\Delta C_T$) following Livak & Schmittgen 2001.

We found that all *arc* genes of both bacteria are overexpressed at pH 4.5, with values of relative expression of 30-40 (results not shown). But at pH 3.6 (**Figure 5**) there was a general underexpression for *Lb. brevis* and a slight overexpression for *P. pentosaceus*, higher for the *arcC* gene. This was correlated with a found lower excretion of citrulline in *P. pentosaceus* than in *Lb. brevis*, suggesting that *Lb. brevis* is more likely to produce this amino acid precursor of ethyl carbamate.

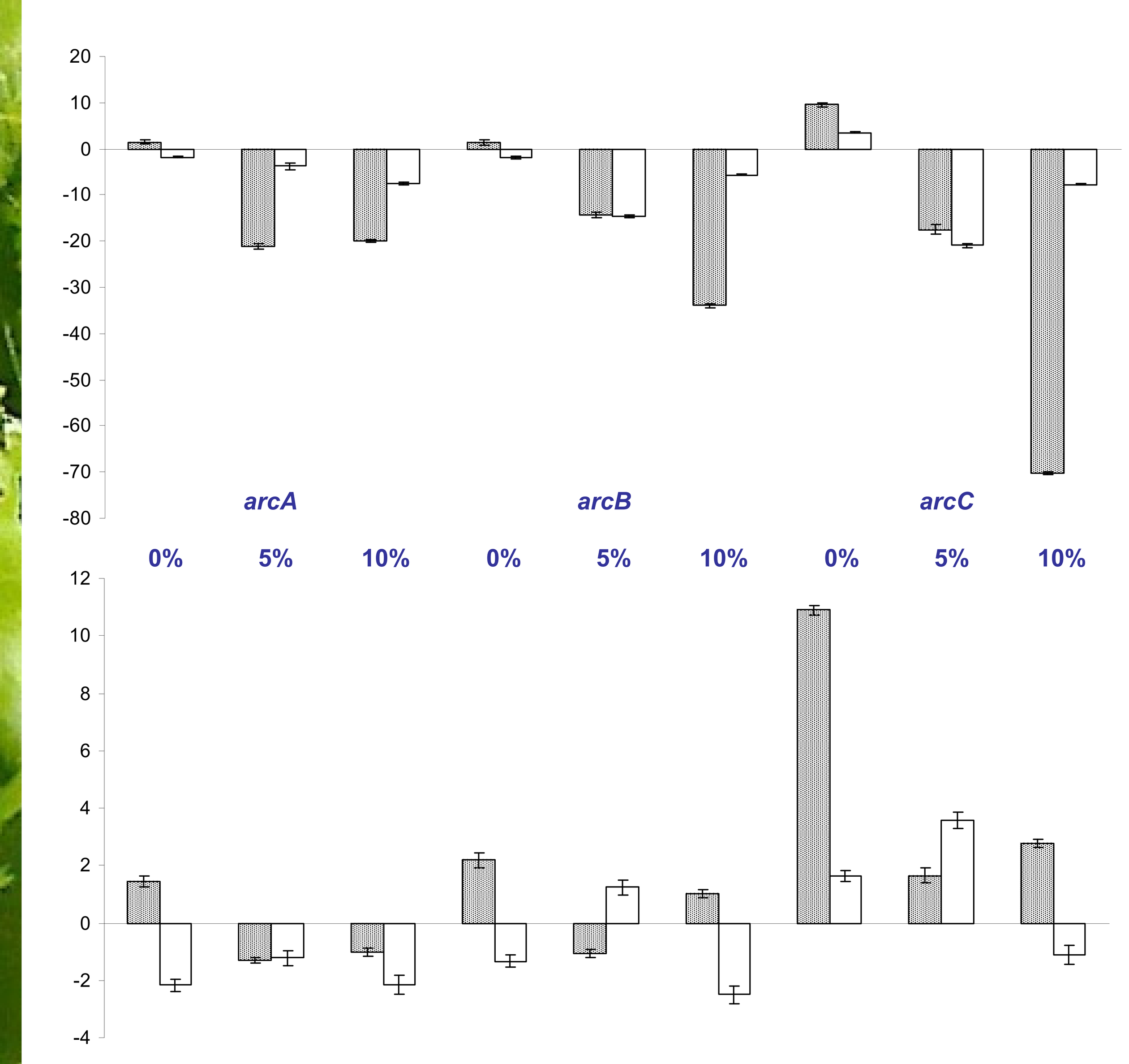

Figure 5. Relative expression of *arcA*, *arcB* and *arcC* genes at pH 3.6 after 6 h (dotted) and 24 h (plain) of arginine additions in *Lb. brevis* (top) *and P. pentosaceus* (bottom*)*. The percentages below the gene names indicate ethanol content.

CONCLUSIONS

1. The presence of *arc* genes in several LAB was correlated with the production of citrulline, precursor of ethyl carbamate.
2. Wine strains of *P. pentosaceus* were found to degrade arginine.
3. The ratio citrulline produced / arginine consumed is higher in the presence of ethanol and low pH in *Lb. brevis* and *Lb. buchneri*.
4. Expression of *arc* genes in *Lb. brevis* and *P. pentosaceus* is higher at pH 4.5 than at pH 3.6.
5. The higher expression of *arc* gene found at pH 3.6 in *P. pentosaceus* than in *Lb. brevis* suggests that this *Lb.* species is more likely to produce citrulline.
6. Since these non-oenococcal species are mainly related to wine spoilage, possible contaminations must be prevented with well-controlled aseptic processes and through the use of starters that ensure correct alcoholic fermentation and of MLF. This should prevent the appearance of citrulline, and thus a potential source of ethyl carbamate.

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