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Growth Response and Modifications of Organic Nitrogen Compounds in Pure and Mixed Cultures of Lactic Acid Bacteria from Wine

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Abstract. The interactions between the proteolytic X_2L strain of *Oenococcus oeni* and the non-proteolytic 12p strain of *Pediococcus pentosaceus* were assayed. The characteristics of cell growth, protein degradation, and amino acid production of both strains were determined in pure and mixed cultures. *O. oeni* showed poor cell growth and greater ability in the release of amino acids to the extracellular medium, whereas *P. pentosaceus* showed a higher yield in cell production with a decrease in the amino acid concentration in the medium. *P. pentosaceus* especially consumed essential amino acids for growth, and *O. oeni* released several of the essential amino acids important for growth of *P. pentosaceus*. In the mixed culture, mutualism was observed. The higher activity of the proteolytic system of *O. oeni* in mixed culture produced an increase in cell growth and in the amount of essential amino acids released. These findings provide new knowledge about the metabolic interactions between lactic acid bacteria isolated from wine when proteins are degraded in mixed bacterial populations.

Proteins are an important carbon and nitrogen source for bacterial communities in the presence of proteolytic enzymes in extracellular medium, because only small peptides and free amino acids can be transported across the bacterial cell membrane [6, 13]. Considering the amino acid requirements of *O. oeni* X_2L and *P. pentosaceus* 12p [3, 4] isolated from wines, the limited amino acid contents in the natural environment [2, 7] could restrict bacterial growth. Therefore, the peptides and amino acids generated from protein hydrolysis may be of great nutritional value for bacterial growth [12]. Manca de Nadra et al. [14–16] have reported that the proteolytic activity of *O. oeni* X_2L on the nitrogenous macromolecular fraction of white and red wines favored the release of peptides and amino acids.

The proteolytic system of *O. oeni* X_2L plays an important role in the release of peptides and essential amino acids due to its demanding requirement for several amino acids. In addition, proteolysis could be important for the reduction in protein precipitation, which produces a visible haze in wines. The ability of *O. oeni* to degrade proteins may provide beneficial conditions for the growth of non-proteolytic bacteria in the same medium. Interactions between proteolytic and non-proteolytic bacteria have been documented in several environments [11, 18, 23, 24].

At present, there is little information about the significance of interactions between proteolytic and non-proteolytic lactic acid bacteria from wine with respect to the metabolism of organic nitrogen compounds. The complexity of the wine ecosystem makes it difficult to analyze these interactions in the natural habitat.

This report describes the growth and nitrogen metabolism of *O. oeni* X_2L and *P. pentosaceus* 12p strains isolated from red wines, in pure and mixed cultures. Proteolytic activity and modification of proteins and free amino acids were examined in order to get a better understanding of interactions between populations.

Materials and Methods

Microorganisms. *Oenococcus oeni* X_2L and *Pediococcus pentosaceus* 12p were isolated from Argentinean red wines [17, 21].

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Table 1. Growth parameters in pure and mixed cultures

	P. pentosaceus 12p		*O. oeni* X_2L	
	μ [h^{-1}]	Growth extent[a]	μ [h^{-1}]	Growth extent
Pure culture	0.196 A[b]	2.121 A	0.134 A	1.547 A
Mixed culture	0.245 B	2.840 B	0.163 B	2.198 B

[a]Difference in cell concentration [log cfu/ml] between stationary phase and inocula.
[b]Values with different letter in the same column are significantly different ($p < 0.05$).

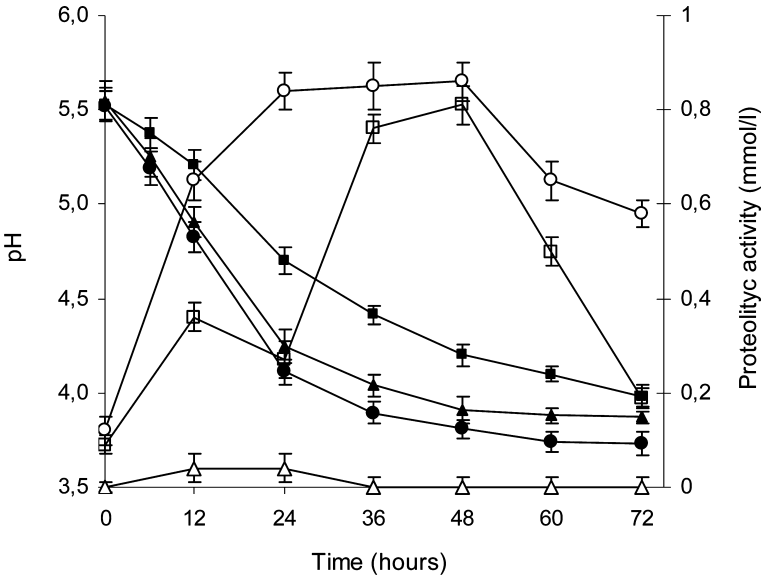

Fig. 1. pH modification in pure cultures of *P. pentosaceus* 12p (▲) and *O. oeni* X_2L (■), and mixed culture (●). Extracellular proteinase activity in pure cultures of *Pediococcus pentosaceus* 12p (Δ) and *Oenococcus oeni* X_2L (□), and in the mixed culture (○).

Culture medium and growth conditions. The basal medium used for the growth contained the following (g or ml) in 1 L distilled water: yeast extract, 10.0; Tween 80, 1.0; and natural grape juice, 170.0. The solution of yeast extract and Tween 80 was sterilized (autoclave) at 121°C, 20 min. Natural grape juice was sterilized (autoclave) with heating stopped immediately on reaching 121°C. Both fractions were pooled and the pH was adjusted at 5.5 with sterile 1M NaOH solution. The microorganisms were grown at 30°C. For mixed cultures, the inoculation rate was 1:1.

Bacterial growth and enumeration of microorganisms. The growth was measured by changes in the optical density to 560 nm. The growth rate and the growth extent (difference in cell concentration between stationary phase and inocula) were determined [4].

Enumeration in mixed cultures was performed on the basis of colonies morphology and growth rate [19].

Analytic determinations

Proteolytic activity

At different incubation times, cells were centrifuged, filtered through a 0.45-μm pore size membrane, and supernatant solutions were assayed for proteolytic activity. The proteolytic activities were determined in the supernatant by the method described by Farías and Manca de Nadra [10].

Proteins

The samples were taken from the culture flask at different times and were centrifuged to 3000 rpm by 20 min. The supernatant fluid was filtered through a 0.45-μm pore size membrane. Proteins were determined in the supernatant by the reaction with Coomassie brilliant blue G-250 [5]. Calibration was carried out using bovine serum albumin (Sigma, St. Louis, MO).

Amino acids measurements

Total free amino acids were quantified in supernatants using the cadmium ninhydrin reagent by the method of Doi et al. [8], using L-leucine as standard. Samples were filtered through a 0.45-μm filter. Amino acids were also analyzed by reverse phase HPLC (RP-HPLC) using an ISCO liquid chromatograph (ISCO, Lincoln, NE) [1]. Samples were injected in triplicate onto the column, after being filtered through a 0.22-μm filter.

Statistical analysis

Experimental data were analyzed by ANOVA with repeated measures. Variable means were compared using Scheffe test (Statistica for Windows Release 5.0). For growth experiments, the growth parameters means were compared using Student's *t*-test.

Results

Growth of pure and mixed cultures. Table 1 shows the growth parameters of *P. pentosaceus* 12p (homofermentative) and *O. oeni* X_2L (heterofermentative) in pure and mixed cultures. In pure culture, the growth parameters of *P. pentosaceus* 12p were higher

than that observed in *O. oeni* X_2L ($p < 0.05$). In a mixed culture, the growth rates increased 24% and 21% for *P. pentosaceus* 12p and *O. oeni* X_2L, respectively. In the same condition, the growth extent increased 34% for *P. pentosaceus* 12p and 42% for *O. oeni* X_2L.

Figure 1 shows that in pure cultures, after 24 h of incubation the pH changed from 5.55 to 4.25 (*P. pentosaceus* 12p) and 5.53 to 4.70 (*O. oeni* X_2L). At 48 h incubation, the pH dropped to 3.91 and 4.20 for *P. pentosaceus* 12p and *O. oeni* X_2L, respectively. In a mixed culture, the pH diminished more actively, reaching 4.11 and 3.81 at 24 and 48 h incubation, respectively.

Proteolytic activity. Figure 1 shows the proteolytic activity of both strains. No exoprotease activity was detected in the pure culture of *P. pentosaceus* and two peaks of proteolytic activity could be observed after 12 and 48 h of incubation in pure culture of *O. oeni*. In mixed culture, the activity was significantly higher than that observed in pure culture of the proteolytic microorganism at 12 and 24 h incubation ($p < 0.01$).

Modification of proteins and total free amino acids in pure and mixed cultures. Figure 2 shows the changes in proteins and total free amino acids in pure cultures of *P. pentosaceus* (Fig. 2A) and *O. oeni* (Fig. 2B) and in the mixed culture (Fig. 2C). No modifications in protein concentration could be detected during growth of *P. pentosaceus*. After 24 h of incubation of *P. pentosaceus*, the amount of total free amino acids in the culture medium dropped 1.69 mmol/l. Thereafter, no modifications were observed. In pure culture of *O. oeni*, initial protein hydrolysis became noticeable after 12 h of incubation (protein consumption was 6.42 mg/l) with an increase of 1.85 mmol/l in total free amino acids. Then, from 12 to 36 h, no significant variations in protein consumption and total free amino acids were detected. From 36 to 48 h of incubation, a protein consumption of 14.57 mg/l was observed. In this period, a release of 3.20 mmol/l of total free amino acids was detected.

In the mixed culture, protein consumption (11.55 mg/l) after 12 h of incubation was 80% higher than that observed in the pure culture of *O. oeni*. During this period, the release of total free amino acids (3.30 mmol/l) was 78% higher than that observed in pure culture of *O. oeni.* Proteins and free amino acids in the medium remained invariable between 12 and 36 h of incubation. After 48 h of incubation, protein consumption was 23.32 mg/l, 60% higher than that detected in pure culture of *O. oeni.*

Changes in the individual amino acid composition. Figure 3 shows the variation of amino acid concentrations throughout the experiment in pure cultures of *P. pentosaceus* 12p (Fig. 3A) and *O. oeni* X_2L

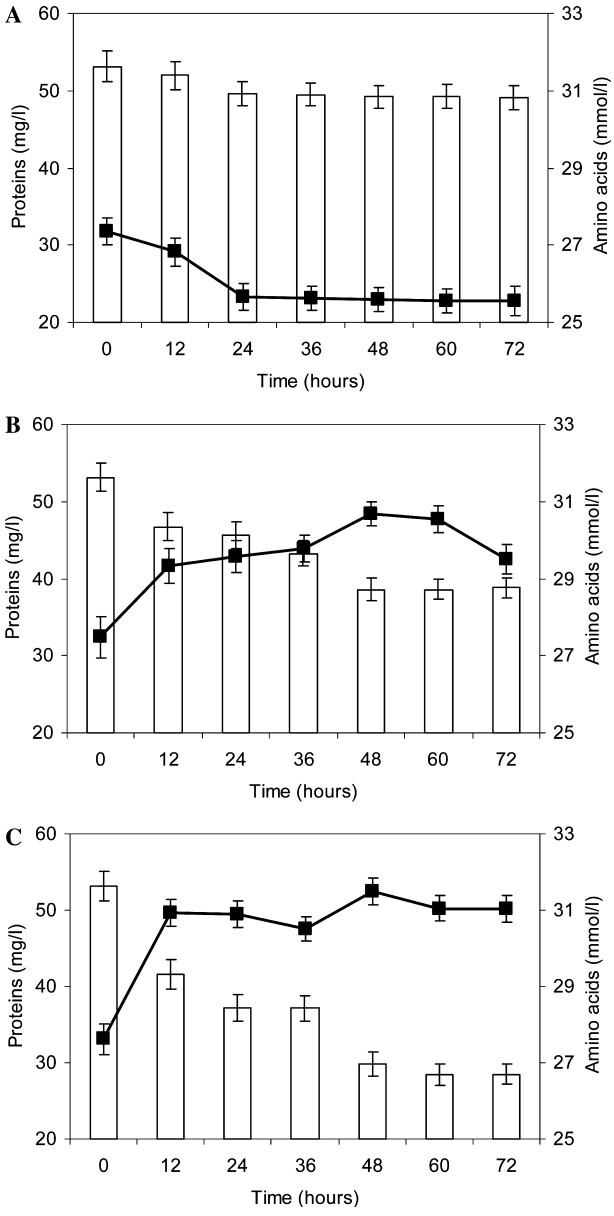

Fig. 2. Changes in protein hydrolysis (bars) and total free amino acids (lines) during 72 h of incubation with pure cultures of *Pediococcus pentosaceus* 12p (A), *Oenococcus oeni* X_2L (B), and with mixed culture (C). Values are means of three independent determinations.

(Fig. 3B) and in mixed culture (Fig. 3C). At the end of the exponential growth phase (48 h), *P. pentosaceus* mainly consumed the non-essential amino acid serine in addition to phenylalanine, threonine, arginine, methionine, histidine, glutamic acid, glycine, tyrosine, tryptophane, and isoleucine, which are essential for its growth. *O. oeni*, at the end of the exponential growth phase (48 h), released lysine, leucine, valine, alanine, glycine, glutamic acid, aspartic acid, arginine, serine, isoleucine, and threonine, all of which are non-essential

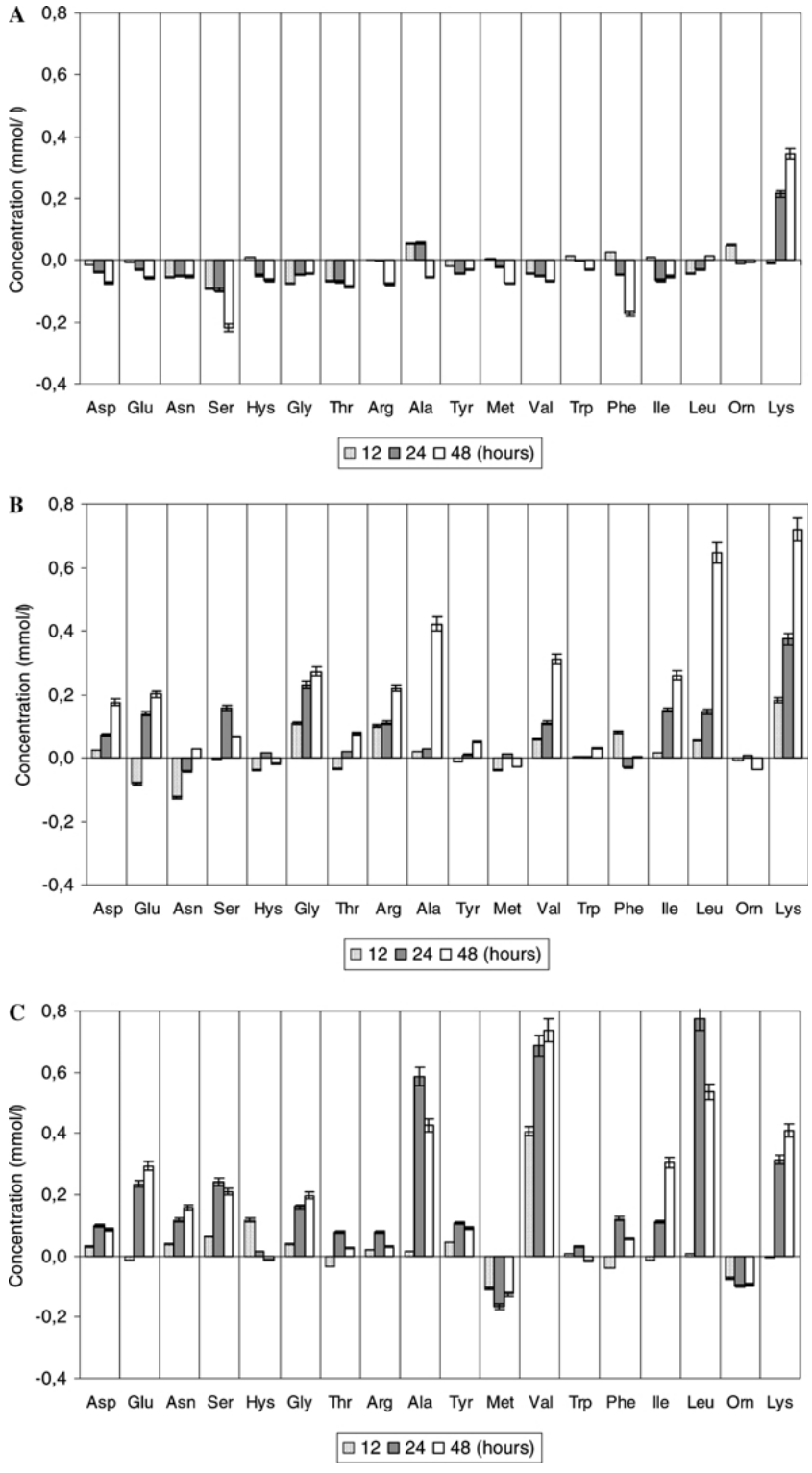

Fig. 3. Comparison between pure cultures of *Pediococcus pentosaceus* 12p (A) and *Oenococcus oeni* X_2L (B) and mixed culture (C) in the time-course (12, 24, and 48 h) with respect to production or utilization of each amino acid. Production or consumption of amino acids was determined measuring the increase or decrease in each concentration with respect to values at T_0 (Concentration $T_0 = 0$).

for this bacterium growth. The essential amino acid asparagine was consumed during the first 12 h of incubation, and no changes were observed for the other essential amino acids, phenylalanine, histidine, and methionine. The heterofermentative microorganism released the following essential amino acids for *P. pentosaceus* growth: glutamic acid, isoleucine, leucine, threonine, valine, glycine, and arginine.

In mixed culture, there was an increase mainly in the release of glutamic acid, asparagine, serine, histidine, alanine, tyrosine, valine, phenylalanine, leucine, and isoleucine with respect to pure culture of *Oenococcus oeni.*

Discussion

Although the concentration of free amino acids during bacterial growth may be influenced by several factors like consumption, cell release, and proteolysis, this last process plays the most important role in the accumulation of free amino acids in a pure culture of *O. oeni* X_2L. When this strain was co-cultured with *P. pentosaceus* 12p, it caused a substantial increase in total free amino acids compared with that obtained in a pure culture of *O. oeni.*

Farías and Manca de Nadra and their colleagues [10, 14–16] have reported that *O. oeni* X_2L is a proteolytic strain acting on wine and grape juice proteins. In this work, proteins were hydrolyzed when the extracellular proteinase produced by *O. oeni* accumulated in the medium in pure or mixed culture of this strain. Protein hydrolysates were utilized by both strains in the mixed culture. Consequently, growth of the non-proteolytic bacterium *P. pentosaceus* was stimulated by the presence of *O. oeni*. Growth stimulation has been observed for *Pseudomonas* and *Bacillus* strains producing extracellular proteinases [18, 23, 24], as well as for *Lactococcus* producing a proteinase bound to the cell wall [11].

In pure culture, strains 12p and X_2L showed a different behavior concerning growth kinetics, protein consumption, and extracellular amino acid production. The proteolytic *O. oeni* strain was efficient in production of amino acids, but showed lower growth parameters than *P. pentosaceus*. The abundant amino acids in the *O. oeni* culture nearly corresponded to the amino acids that were consumed in the culture of *P pentosaceus* 12p. Stimulation of the proteolytic system in mixed culture increased the release of essential amino acids for growth of both microorganisms. Therefore, co-culturing of both strains would enhance the growth yield of both bacteria (mutualism).

The higher rates of proteolysis and release of amino acids in mixed culture were due to an increment in proteolytic activity. Taking into account that the optimum pH for exoprotease activity of *O. oeni* X_2L is 4.5 [10], the rapid decrease in pH under the given conditions could produce a stimulation of the proteolytic system of *O. oeni* in co-culture with a homofermentative strain. The cultures reached a pH of 4.5 after 12 h and 24 h of incubation in mixed and pure culture of *O. oeni*, respectively.

Additionally, modification of certain nutritional factors in the medium could stimulate the expression of exoproteases. Farías et al. [9] have reported that exogenous peptide sources such as tryptone, peptone, gelatin, or casein hydrolysate reduce the formation of exoproteases by *O. oeni* X_2L. Strasser de Saad et al. [22] have reported that peptone and tryptone reduce the formation of proteolytic enzymes in *Lactobacillus murinus* CNRZ 313. On the other hand, in *Clostridium*, an increase in peptone enhanced protease production [13]. Hence, modification of the organic nitrogen composition of the medium during the symbiosis could stimulate the expression of proteolytic activity.

These findings can be of great technological value in terms of a starter selection, considering that the association in a mixed culture of *P. pentosaceus* 12p and *O. oeni* X_2L has been found to be beneficial; it avoids wine spoilage, because their association produces lower amounts of mannitol, even in the presence of sulfur dioxide [19, 20].

Additionally, despite the low concentrations of proteins in musts and wines, a greater proteolytic activity in mixed culture may have an important function in the stability of wines. It possibly reduces protein precipitation causing unacceptable haze or sediment in wine. Precipitation may occur at high temperatures and can also be provoked by trace metals.

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Literature Cited

1. Alberto MR, Arena ME, Manca de Nadra MC (2002) A comparative survey of two analytical methods for identification and quantification of biogenic amines. Food Control 13:125–129
2. Amerine MA, Ough CS (1980) Methods for analysis of must and wines. New York: John Wiley and Sons
3. Aredes Fernández PA, Saguir FM, Manca de Nadra MC (2003) Effect of amino acids and peptides on growth of *Pediococcus pentosaceus* from wine. Lat Am Appl Res 33:225–229
4. Aredes Fernández PA, Saguir FM, Manca de Nadra MC (2004) Effect of dipeptides on the growth of *Oenococcus oeni* in synthetic medium deprived of amino acids. Curr Microbiol 49:361–365
5. Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of proteins using the principle of protein dye-binding. Anal Biochem 72:248–252
6. Christensen JE, Dudley EG, Pederson JA, Steele JL (1999) Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 76:217–246
7. Colagrande O, Silva A, Casoli A (1984) Acides aminés dans les vins mousseux. Conn Vigne Vin 18:27–47
8. Doi E, Shibata D, Matoba (1981) Modified colorimetric ninhydrin methods for peptidase assay. Anal Biochem 118:173–184

9. Farías ME, Rollan GC, Manca de Nadra MC (1996) Influence of nutritional factors on the protease production by *Leuconostoc oenos* from wine. J Appl Bacteriol 81:398–402
10. Farías ME, Manca de Nadra MC (2000) Purification and partial characterization of *Oenococcus oeni* exoprotease. FEMS Microbiol Lett 185:263–266
11. Juillard V, Furlan S, Foucaud C, Richard J (1996) Mixed cultures of protease positive and protease-negative strains of *Lactococcus lactis* in milk. J Dairy Sci 79:964–970
12. Kunji ERS, Mireau I, Hagting A, Poolman B, Konings WN (1996) The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70:187–221
13. Macfarlane GT, Macfarlane S (1992) Physiological and nutritional factors affecting synthesis of extracellular metalloproteases by *Clostridium bifermentans* NCTC 2914. Appl Environ Microbiol 58:1195–1200
14. Manca de Nadra MC, Farías ME, Moreno-Arribas MV, Pueyo E, Polo MC (1997) Proteolytic activity of *Leuconostoc oenos*. Effect of proteins and polypeptides from white wine. FEMS Microbiol Lett 150:135–139
15. Manca de Nadra MC, Farías ME, Moreno-Arribas MV, Pueyo E, Polo MC (1999) A proteolytic effect of *Oenococcus oeni* on the nitrogenous macromolecular fraction of red wine. FEMS Microbiol Lett 174:41–47
16. Manca de Nadra MC, Farías ME, Pueyo E, Polo MC (2005) Protease activity of *Oenococcus oeni* viable cells on red wine nitrogenous macromolecular fraction in presence of SO_2 and ethanol. Food Control 16:851–854
17. Manca de Nadra MC, Strasser de Saad AM (1987) Evolution of lactic acid bacteria during the different stages of vinification of Cafayate (Argentine) wines. Micr Alim Nutr 5:235–240
18. Marshall DL, Schmidt RH (1991) Physiological evaluation of stimulated growth of *Listeria monocytogenes* by *Pseudomonas* species in milk. Can J Microbiol 37:594–599
19. Rodríguez AV, Manca de Nadra MC (1994) Sugar and organic acid metabolism in mixed cultures of *Pediococcus pentosaceus* and *Leuconostoc oenos* isolated from wine. J Appl Bacteriol 77:61–66
20. Rodriguez AV, Manca de Nadra MC (1997) Mixed culture of *Pediococcus pentosaceus* and *Leuconostoc oenos*: Effect of sulphur dioxide on growth and metabolism. Micr Alim Nutr 15:227–235
21. Strasser de Saad AM, Manca de Nadra MC (1987) Isolation and identification of lactic acid bacteria from Cafayate (Argentina) wines. Micr Alim Nutr 5:45–49
22. Strasser de Saad AM, Manca de Nadra MC, de Pesce Ruiz Holgado AA, Oliver G (1987) Effect of cultivation conditions on proteinase production by *Lactobacillus murinus*. Folia Microbiol 32:85–88
23. Verheul A, Rombouts FM, Abee T (1998) Utilization of oligopeptides by *Listeria monocytogenes*. Appl Environ Microbiol 64:1059–1065
24. Worm J, Jensen LE, Thomas SH, Søndergaard M, Nybroe O (2000) Interactions between proteolytic and non-proteolytic *Pseudomonas fluorescens* affect protein degradation in a model community. FEMS Microbiol Ecol 32:103–109