Journal International des Sciences de la Vigne et du Vin, 1991, **25**, N° 2, 99-108

EFFECT OF ORGANIC ACIDS ON THE GROWTH OF *LEUCONOSTOC ŒNOS* AND *LACTOBACILLUS HILGARDII* STRAINS ISOLATED FROM RED WINES

María Cristina MANCA DE NADRA[1*] and Ana María STRASSER DE SAAD[1]

Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán
and Centro de Referencia para Lactobacilos (CERELA),
Chacabuco 145, 4000 Tucumán, (Argentina)

Summary : *The effect of organic acids on the growth of Leuconostoc œnos and Lactobacillus hilgardii strains was investigated, and the interaction between organic acids, ethanol and inoculum level was determined. The four selected strains of Leuconostoc œnos : X_2L, E_2, ST_2 and L_2 and the two selected strains of Lactobacillus hilgardii : 7_j and X_1B are resistent to 10 p. cent ethanol, a typical concentration for a table wine.*

The effect of the additives depends on the strain, except for dodecanoic acid which produces a growth inhibition in the six strains. Dodecanoic acid is an effective inhibitor when added to grape juice and might be used as a substitute of SO_2, or rather in association with SO_2.

INTRODUCTION

Spoilage of different fruit juices and related products by lactic acid bacteria is not uncommon. Among the foods more susceptible to spoilage are those such as most wines, wine coolers, ciders and certain soft drinks, that have not undergone thermal treatment.

At present, there is a tendency to use less or to omit SO_2, an effective inhibitor of lactic acid bacteria at low pH level (LIU and GALLANDER, 1983; WITTER et *al*, 1958), in fruit products due to its allergenic role in sensitive individuals (STEVENSON and SIMON, 1981).

Some compounds were proposed as possible substitutes of SO_2 : fumaric acid (COFRAN and MEYER, 1970; WIBOWO et *al*, 1985), sorbic acid (EDINGER and SPLITTSTOESSER, 1986; PHILLIPS and MUNDT, 1950; SPLITTSTOESSER, 1982), and benzoic and propionic acids (CHICHESTER and TANNER, 1972).

---

[1]Members of the Scientific Researcher's Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina.
*To whom correspondence should be sent.

Certain secondary metabolites formed by yeast in the wine, during alcoholic fermentation such as fatty acids have a bactericidal and fungicidal effect (NIEMAN, 1954; KABARA, 1979). Other authors report that fatty acids play a role in the inhibition of the malolactic activity of *leuconostoc œnos* in wine (LONVAUD-FUNEL et *al*, 1988) but their inhibitory effect on bacterial growth is less obvious.

The lactic microflora of Argentine wines has been investigated since 1985, and the behavior of certain nutritional and physico-chemical factors was determined by MANCA DE NADRA and STRASSER DE SAAD (1987, 1987, 1989) and by STRASSER DE SAAD and MANCA DE NADRA (1987, 1989). For this study, *Leuconostoc œnos* and *lactobacillus hilgardii* strains, isolated from different wines, were selected for their technological properties, with the aim of determining the effect of different organic acids on their growth.

MATERIALS AND METHODS

I — MICRO-ORGANISMS

Leuconostoc œnos : X_2L, L_2, ST_2 and E_2 and *Lactobacillus hilgardii* : 7_J and X_1B were isolated from four differents red wines of Argentina's winemaking.

II — CULTURES

The strains were propagated in a 5° Brix, grape juice medium added with 0.5 p. cent yeast extract, pH 4.0.

The cells were harvested during the late logarithmic phase, usually after an incubation of 3 and 5 days at 30°C, for *Lactobacillus hilgardii* and *leuconostoc œnos*, respectively. The pellet was re-suspended in an appropriate volume of distilled water to obtain the needed cellular concentration. Two different initial concentrations of cells were tested : 10^5 and 10^7 cells/ml. Viable cells were determined by plate count using MRS agar added with 15 p. cent tomato juice, pH 4.8.

III — ADDITIVES

Commercial grape juice containing 0.5 p. cent yeast extract served as basal medium. The following organic acids were added to the medium : propionic, fumaric, sorbic and benzoic acids (300 mg/l) and octanoic, decanoic and dodecanoic acids (20 mg/l) were first disolved in 95 p. cent ethanol to facilitate their solution in the model juice. Sterilization was carried out for 10 min. at 118°C. Two levels of ethanol (5 and 10 p. cent) were assayed for each additive.

IV — BACTERIAL GROWTH

Duplicate 16 x 150 mm screwcap culture tubes, containing a given concentration of additive in 5 ml of basal medium, were incubated for 90 days at 30°C. During this time at intervals

of 24 heures, the growth was measured by reading the absorbance at 560 nm.

RESULTS AND DISCUSSION

The effect of the additives : propionic, fumaric, sorbic and benzoic acids (300 mg/l) and octanoic, decanoic and dodecanoic acids (20 mg/l) on the growth of *Leuconostoc œnos* strains, from 0 to 30 days of incubation at 30°C in presence of 10 p. cent ethanol, is showed in figure 1. The inoculum size was 10^5 cells/ml. The growth of *Leuconostoc œnos* X_2L (figure 1 a) was almost completely inhibited, from the beginning of incubation by sorbic and dodecanoic acids. The inhibition by benzoic and decanoic acids was lower than that observed with the precedent additives. Propionic and octanoic acids, failed to prevent growth after a lag period of 10 days. Fumaric acid had no detectable effect. The behavior of *Leuconostoc œnos* E_2 is shown in figure 1 b. On this strain, benzoic and dodecanoic acids had a significative inhibitory effect since the start of the experiment.

E_2 strain was more inhibited by decanoic acid and less inhibited by sorbic acid with respect to X_2L strain. In the first 10 days of incubation, propionic acid had a stimulatory effect on the growth on this strain, and octanoic acid failed to prevent growth after 10 days of incubation. Fumaric acid had a light inhibitory effect but to a higher extent with respect to X_2L strain. The effect of additives on ST_2 and L_2 strains is shown in figure 1 (c and d).

Propionic and fumaric acids have no detectable effect ; benzoic acid has a light inhibitory activity and dodecanoic acid produces a complete inhibition on the growth of both strains. Octanoic acid acts as inhibitor and as activator on the growth of ST_2 and L_2, respectively. The addition of decanoic acid increases the growth of ST_2 strain and the same effect is observed on the growth of L_2 strain after 20 days of incubation.

The influence of organic acids on the growth of *Lactobacillus hilgardii* strains is shown in figure 2 (a and b). Propionic, sorbic and octanoic acids acted as activators ; fumaric acid had no effect ; benzoic and decanoic acids produced partial inhibition and dodecanoic acid prevented the growth of *Lactobacillus hilgardii* 7_j completly (figure 2 a). However, the growth of *Lactobacillus hilgardii* X_1B was partialy inhibited by propionic, decanoic, sorbic, octanoic, fumaric and benzoic acids ; dodecanoic acid produced a total inhibition (figure 2 b).

From these results, it is important to know the effect of the tested additives on the growth of lactic acid bacteria strains in respect to ethanol concentration and the size of the inoculum. The effect on the growth of *Leuconostoc œnos* strains after 90 days of incubation at 30°C in the medium with different concentrations of ethanol : 0, 5 and 10 p. cent, and inoculated with 10^5 and 10^7 cells/ml are shown in table 1. Propionic acid had no detectable effect on the growth of the four strains of *Leuconostoc œnos* independently of the presence of 5 and 10 p. cent ethanol and the size of the inoculum. All strains were only slightly inhibited by fumaric acid when the juice contained ethanol. Inhibition by sorbic acid was observed in all strains even in absence of ethanol. In the juice containing 10 p. cent ethanol, the effect was higher, *Leuconostoc œnos* X_2L and E_2 being the most affected. Similar behavior

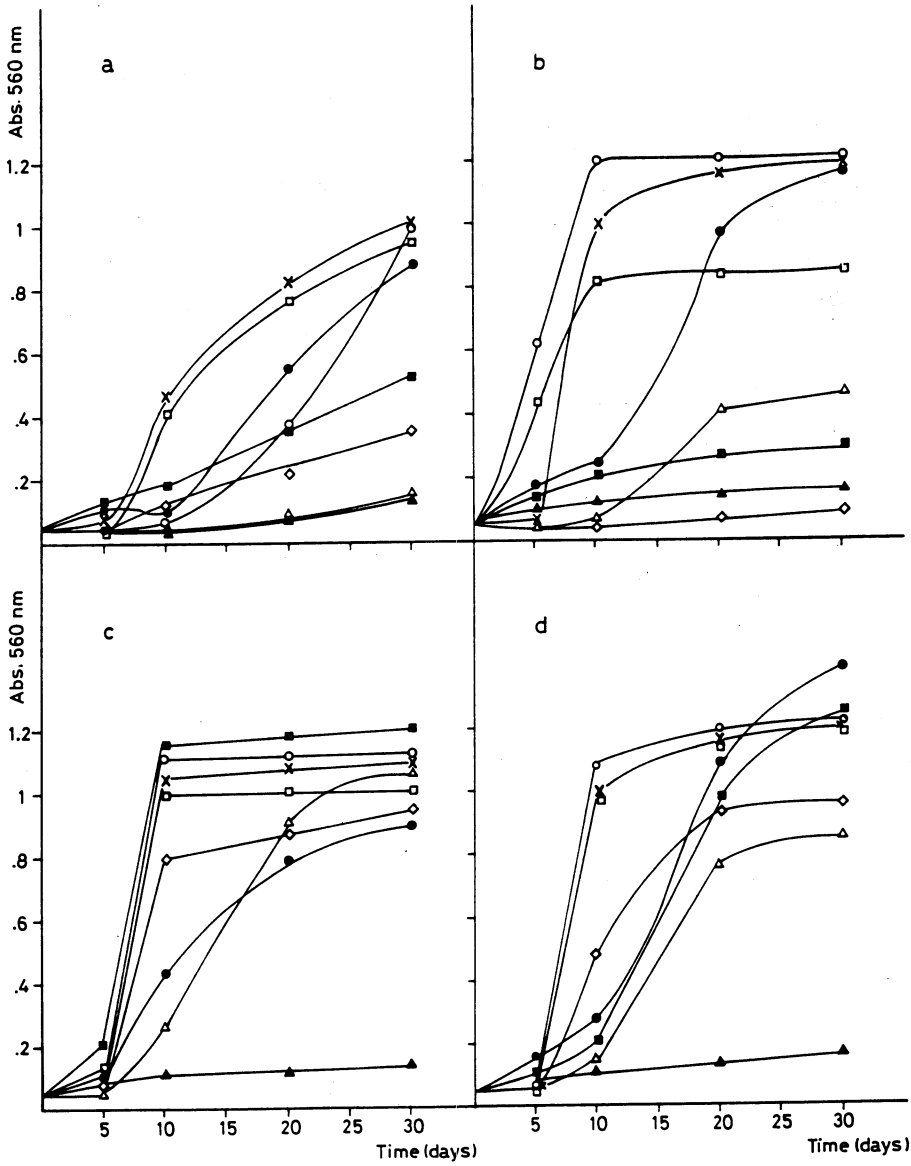


Fig.1 - Growth of *Leuconostoc œnos strains* on grape juice medium

x : control. In presence of 300 mg/ l : ○ , propionic ; □ , fumaric; △ , sorbic; and ◇ benzoic acids. In presence of 20 mg/l : ● , octanoic; ■ , decanoic; ▲ , dodecanoic acids.

Inoculum : 10^5 cell/ml - Ethanol concentration : 10%

Strains : a - X_2L ; b - E_2; c - ST_2, and d - L_2

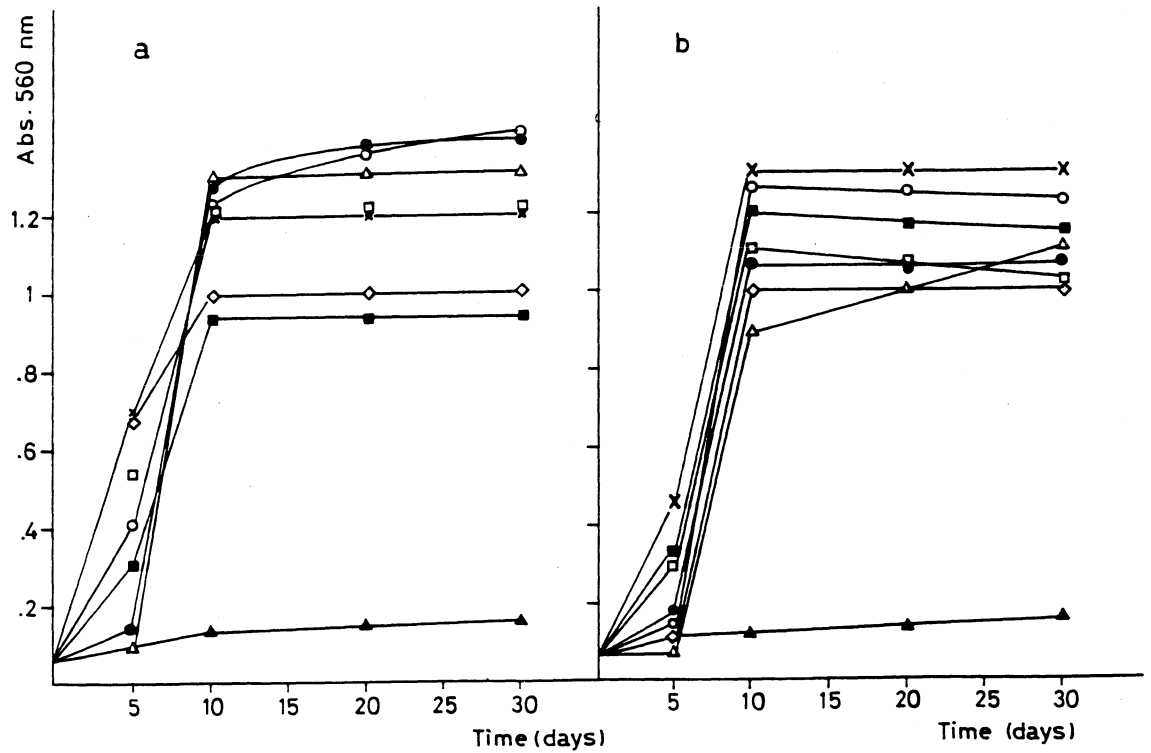


Fig.2 - Growth of *Lactobacillus hilgardii* strains on grape juice medium

x : control. In presence of 300 mg/l : ○ , propionic; □ , fumaric; △ , sorbic; and ◇ benzoic acids. In presence of 20 mg/l : ● , octanoic; ■ , decanoic; ▲ , dodecanoic acids.

Inoculum : 10^5 cell/ml - Ethanol concentration : 10%

Strains : a - 7_j ; b - X_1B

TABLE I

Influence of organic acids and inoculum size on the growth of *Leuconostoc œnos* strains in the presence of ethanol.

Organic acids	Inoculum cell/ml	STRAIN X_2L			E_2			ST_2			L_2		
		% Ethanol			% Ethanol			% Ethanol			% Ethanol		
		0	5	10	0	5	10	0	5	10	0	5	10
———	10^5	1.1*	1.1	1.1	1.3	1.3	1.3	1.1	1.2	1.3	1.2	1.2	1.2
	10^7	1.2	1.3	1.2	1.2	1.2	1.3	1.2	1.2	1.4	1.3	1.3	1.3
Propionic (300 mg/l)	10^5	1.2	1.2	1.1	1.2	1.2	1.3	1.1	1.2	1.2	1.2	1.2	1.2
	10^7	1.2	1.2	1.2	1.2	1.3	1.3	1.2	1.2	1.5	1.4	1.2	1.4
Fumaric (300 mg/l)	10^5	1.1	1.0	0.8	1.2	1.1	0.8	1.0	1.0	0.8	1.0	1.0	1.1
	10^7	1.2	1.2	0.9	1.2	1.0	1.0	1.1	1.1	1.1	1.2	1.2	1.2
Sorbic (300 mg/l)	10^5	1.0	0.9	0.3	0.9	0.6	0.5	1.0	0.9	0.7	1.0	1.0	0.7
	10^7	1.0	0.9	0.7	1.0	0.7	0.5	1.0	1.0	0.9	1.1	1.1	0.9
Benzoic (300 mg/l)	10^5	0.7	0.7	0.4	0.8	0.8	0.1	0.8	0.8	0.7	0.9	0.8	0.8
	10^7	1.1	0.9	0.4	1.0	0.9	0.3	1.0	1.0	0.7	1.1	1.1	1.1
Octanoic (20 mg/l)	10^5	1.1	1.2	1.0	1.5	1.4	1.3	1.5	1.5	1.3	1.5	1.5	1.4
	10^7	1.3	1.3	1.2	1.1	1.2	1.3	1.2	1.3	1.5	1.3	1.3	1.3
Decanoic (20 mg/l)	10^5	1.0	1.1	0.5	0.7	0.7	0.5	1.2	1.4	1.4	1.1	1.3	1.3
	10^7	1.2	1.2	0.7	0.9	0.7	0.5	1.2	1.5	1.5	1.5	1.5	1.5
Dodecanoic (20 mg/l)	10^5	0.22	0.17	0.17	0.25	0.17	0.15	0.21	0.16	0.14	0.34	0.28	0.14
	10^7	0.27	0.19	0.17	0.31	0.19	0.21	0.25	0.18	0.16	0.36	0.31	0.18

*Growth A_{560} - Incubation : 90 days at 30°C.

TABLE II

Influence of organic acids and inoculum size on the growth of *Lactobacillus hilgardii* strains in the presence of ethanol.

Organic acids	Inoculum cell/ml	STRAIN 7_j % Ethanol 0	7_j % Ethanol 5	7_j % Ethanol 10	X_1B % Ethanol 0	X_1B % Ethanol 5	X_1B % Ethanol 10
———	10^5	1,5*	1.5	1.4	1.5	1.5	1.4
	10^7	1.6	1.5	1.4	1.6	1.6	1.4
Propionic (300 mg/l)	10^5	1.5	1.5	1.4	1.7	1.8	1.5
	10^7	1.7	1.7	1.5	1.4	1.4	1.4
Fumaric (300 mg/l)	10^5	1.5	1.3	1.4	1.4	1.5	1.3
	10^7	1.1	1.4	1.3	1.1	1.5	1.3
Sorbic (300 mg/l)	10^5	1.6	1.6	1.5	1.2	1.3	1.4
	10^7	1.3	1.5	1.5	1.4	1.3	1.4
Benzoic (300 mg/l)	10^5	1.2	1.0	0.9	1.1	1.0	1.0
	10^7	1.3	1.2	1.0	1.2	1.1	1.1
Octanoic (20 mg/l)	10^5	1.5	1.2	1.5	1.4	1.5	1.3
	10^7	1.4	1.4	1.1	1.5	1.3	1.2
Decanoic (20 mg/l)	10^5	1.5	1.3	0.9	1.4	1.5	1.1
	10^7	1.4	1.3	1.2	1.4	1.2	1.3
Dodecanoic (20 mg/l)	10^5	0.17	0.17	0.08	0.18	0.16	0.12
	10^7	0.26	0.24	0.22	0.24	0.19	0.17

*Growth A_{560} - Incubation : 90 days at 30°C.

was observed with benzoic acid but in this case the inhibition of the growth of *Leuconostoc œnos* E_2 in the presence of 10 p. cent ethanol was higher than that observed with sorbic acid. The effect of fumaric, sorbic and benzoic acids was related to the size of inoculum.

The following fatty acids, octanoic, decanoic and dodecanoic are produced by yeasts and are usually present in fermented fruit juices (EDWARDS and BELLMAN, 1987; LAFON-LAFOURCADE et *al*, 1984). Octanoic acid had no significative effect on the growth of *Leuconostoc œnos* strains, even in presence of ethanol. The inhibitory activity of decanoic acid was observed in *Leuconostoc œnos* E_2 in the absence or presence of ethanol, being higher when the ethanol concentration increased. This inhibitory effect was also observed on the growth of *Leuconostoc œnos* X_2L but only in the presence of 10 p. cent ethanol. The growth of *Leuconostoc œnos* ST_2 and L_2 strains was not affected by the additon of 20 mg/l decanoic acid, even in presence of 10 p. cent ethanol. Thus, this concentration might not protect a table wine. From these results, it is possible to conclude that the sensitivity to decanoic acid depends on the strains.

All strains were almost completely inhibited by 20 mg/l dodecanoic acid and the inhibition was higher when the ethanol concentration was increased.

The results obtained for the *Lactobacillus hilgardii* strains are shown in table 2. After 90 days of incubation at 30°C, the addition of propionic, fumaric, sorbic and octanoic acids had no significative effect on the growth of *Lactobacillus hilgardii* 7_j and X_1B independently of the size of inoculum and the ethanol concentration. Similar behavior was observed on the growth of X_1B strain when the medium was added with decanoic acid, but *Lactobacillus hilgardii* 7_j was more inhibited in presence of 10 p. cent ethanol.

The *Lactobacillus hilgardii* growth, was slightly inhibited by the presence of benzoic acid and the inhibition increased when the ethanol concentration increased from 5 p. cent to 10 p. cent. The results were similar to the two inoculum tested. Both strains of *Lactobacillus* were inhibited by 20 mg/l dodecanoic acid and the inhibition was higher when the ethanol concentration was increased but total inhibition was not achieved.

It is possible to conclude, that *Leuconostoc œnos* strains are more sensitive than *Lactobacillus hilgardii* strains to the effect of organic acids. Among the additives tested, only dodecanoic acid acted to a significative degree upon the growth of *Leuconostoc œnos* and *Lactobacillus hilgardii* strains, even at 90 days of incubation. The effect of this fatty acid is also significant in the absence of ethanol ; indeed at a cellular concentration of 10^7 cells/ml.

The results of this study indicate that dodecanoic acid is an effective inhibitor when added to grape juice and might be used as a substitute for SO_2 or rather to decrease the SO_2 level. Further studies are necessary to adjust fatty acids to the optimal level for microbiological stability of wines, ciders and other beverages with respects to organoleptic quality.

Manuscrit reçu le 10 mai 1991; accepté pour publication le 6 juin 1991.

Acknowledgements

The authors wish to thank biochemist PLINIO TORRES for his technical assistance.

The study was partly supported by grants form CONICET and CIUNT.

BIBLIOGRAPHIC REFERENCES

COFRAN D.R and MEYER J., 1970. The effect of fumaric acid on malolatic fermentation. *Am. J. Enol. Vitic.*, **21**, 189-192.

CHICHESTER D.F. and TANNER F.W., 1972. Antimicrobial food additives. In handbook of Food Additives, 2nd Ed. (T.E. Furia ed.), 115-184. Chem. *Rubber Publ. Co.*, Cleveland, Ohio.

EDINGER W.D. and SPLITTSTOESSER D.F., 1986. Sorbate tolerance by lactic acid bacteria associated with grapes and wines. *J. Food Sci.*, **51**, 1077-1078.

EDWARDS C.G. and BEELMAN R.B., 1987. Inhibition of the malolactic bacterium *Leuconostoc œnos* (PSU-1) by decanoic acid and subsequent removal of the inhibition by yeast ghosts. *Am. J. Enol. Vitic.*, **38**, 239-242.

KABARA J.J., 1979. Toxicological, bactericidal and fungicidal properties of fatty acids and some derivatives. *J. Amer. Chem. Soc.*, **56**, 760A-767A.

LAFON-LAFOURCADE S., GENIX C. and RIBEREAU-GAYON P., 1984. Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeast and their elimination by yeast ghost. *Appl. Environ. Microbiol.*, **47**, 1246-1249.

LIU J.W.R. and GALLANDER J.F., 1983. Effects of pH and sulfur dioxide on the rate of malolactic fermentation in red table wines. *Am. J. Enol. Vitic.*, **34**, 44-46.

LONVAUD-FUNEL A., JOYEUX A; and DESENS C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolism. *J. Sci. Food. Agric.*, **44**, 183-191.

MANCA de NADRA M.C. and STRASSER de SAAD A.M., 1987. Evolution of lactic acid bacteria during the different stages of vinification of Cafayate (Argentina) wine. *Microbiol.-Aliments - Nutrition*, **5**, 235-240.

MANCA de NADRA M.C. and STRASSER de SAAD A.M., 1987. Effect of pH, sulfur dioxide and ethanol concentrations on the growth of lactic acid bacteria isolated from Cafayate (Argentina) wines. *Microbiol. - Aliments - Nutrition*, **5**, 241-245.

MANCA de NADRA M.C. and STRASSER de SAAD A.M., 1989. Characterization and properties of *Leuconostoc œnos* strains isolated from Amaicha (Argentina) wine. *Microbiol.- Aliments - Nutrition*, **7**, 377-382.

NIEMAN C., 1954. Influence of trace amounts of fatty acids on the growth of micro-organisms. *Bact. Rev.*, **18**, 147-163.

PHILLIPS G.F. and MUNDT J.O., 1950. Sorbic acid as an inhibitor of scum yeast in cucumber fermentation. *Food Technol.*, **4**, 291-293.

SPLITTSTOESSER D.F., 1982. Micro-organisms involved in the spoilage of fermented fruit juices. *J. Food. Prot.*, **45**, 874-877.

STEVENSON D.D. and SIMON R.A., 1981. Sensitivity to ingested metabisulfites in asthmatic subjects. *J. Allergy Clin. Immunol.*, **68**, 26-32.

STRASSER de SAAD A.M. and MANCA de NADRA M.C., 1987. Isolation and identification of lactic acid bacteria from Cafayate (Argentina) wines. *Microbiol. - Aliments-Nutrition*, **5**, 45-49.

STRASSER de SAAD A.M. and MANCA de NADRA M.C., 1989. Factors affecting the cellular growth and malolactic activity in *Leuconostoc œnos* strains isolated from Cafayate (Argentina) wines. *Microbiol. - Aliments-Nutrition*, **7**, 67-72.

WIBOWO D., ESCHENBRUCH R., DAVIS C.R., FLEET G.H. and LEE T.H., 1985. Ocurrence and growth of lactid acid bacteria in wine. A review. *Am. J. Enol. Vitic.*, **36**, 302-313.

WITTER L.D., BERRY J.M. and FOLINAZZO J.F., 1958. The viability of *Escherichia coli* and a spoilage yeast in carbonated beverages. *Food Res.*, **23**, 133-142.