

# Utilization at high pH of starter cultures of lactobacilli for Spanish-style green olive fermentation

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## Abstract

Inoculation at alkaline pH (above 9) of lye-treated green olives with starter cultures of *Lactobacillus pentosus* CECT 5138 was studied. Despite an initial loss of viability in the order of 1–2 log cycles on average, depending mainly on time of application, cultures grew and initiated an accelerated fermentation process. Inoculation reduced the population of *Enterobacteriaceae*, and thereby potential spoilage, and produced a quicker acidification of brines and decrease of pH, when compared with control uninoculated batches. Results obtained throughout three consecutive seasons demonstrated that utilization at high pH of starter cultures of lactobacilli is feasible, provided that the inoculum size takes into account the initial low survival. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Green table olive; High pH; *L. pentosus*; *L. plantarum*; Lactic acid fermentation

## 1. Introduction

The production of table olives is 1,180,000 tons a year (Anonymous, 1999), and the fruit is the major fermented vegetable in western countries (Garrido-Fernández, 1997). Spain is the main producer, with 360,000 tons, of which nearly 250,000 tons are Spanish-style green olives (IOOC, 1999). Preparation of Spanish-style green olives includes an alkaline treatment with NaOH to hydrolyze the bitter glucoside oleuropein, a washing step to reduce the lye inside the olives, and a stage in brine, where the

fruits undergo the typical fermentation by lactic acid bacteria (Fernández-Díez et al., 1985). As is the case with other fermented vegetable products (cucumbers, sauerkraut, etc.), olive processing, despite its economic importance, has remained empirical and far from being controlled and modernized. Currently, pure lactic starter cultures are not very common in European vegetable fermentations, although preparations are available on the market (Buckenhüskes, 1993). However, interest in developing effective starter cultures to be used in table olives is increasing, since industrial experience suggests that an appropriate inoculation reduces the probability of spoilage and helps to achieve an improved and more predictable fermentation process. Research has been done on specific traits that are thought to be advanta-

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geous, such as oleuropein-splitting capability (Ciardini et al., 1994), bacteriocin production (Ruiz-Barba et al., 1994), growth in ripe olive brines (Durán-Quintana et al., 1994), and fermentation at low temperature (Durán-Quintana et al., 1999). Results in these subjects are promising, but no work has been done, as far as we know, on the optimization of starter culture use for Spanish-style green olive fermentation, in particular regarding the minimum inoculum size for significant improvement of the fermentation process, and the best time after brining to carry out the inoculation. For other fermented vegetable products, the inocula are around  $10^7$  CFU ml<sup>-1</sup> (or g<sup>-1</sup>) for sauerkraut or wine (Buckenhüskes, 1993), cocoa (Schwan, 1998), cabbage juice broth (Breidt et al., 1993), or lower ( $10^5$ – $10^6$  CFU ml<sup>-1</sup> for cucumbers; Daeschel et al., 1988; Etchells et al., 1964). In the case of olives, figures range from  $10^5$  CFU ml<sup>-1</sup> (Ruiz-Barba et al., 1994) to ca.  $10^7$  CFU ml<sup>-1</sup> (Etchells et al., 1966) via  $10^6$  CFU ml<sup>-1</sup> (Deiana et al., 1992), indicating that not enough research has been done on this topic. The same can be said regarding the optimum time for inoculation of green olives after brining. As a consequence of the alkaline treatment of the fruits, brine pH values during the first days are well above neutrality—more than 10 in some cases. This has led to the recommendation of decreasing the pH artificially by bubbling CO<sub>2</sub> (Borbolla y Alcalá et al., 1969), before inoculation, adding other acids or waiting until the pH is around 6–7 as a result of natural microbial growth, with the risk of spoilage during the days before the lactobacilli have grown. However, *Lactobacillus plantarum* and *L. pentosus*, the species which are responsible of the fermentation of olives (Vaughn, 1982; van den Berg et al., 1993), can withstand or even grow at pH values as high as 8.5 (Balatsouras, 1985) and 9.0–9.5 (Tanasupawat et al., 1992). The objectives of the present work were: (i) to study the behaviour of starter cultures of *L. plantarum* or *L. pentosus* when applied at high pH on the first days of brining Spanish-style green olives; (ii) to determine the minimum inoculum size for a significant improvement of the fermentation process; and (iii) to analyze the effects of inoculation on the growth of spoiling *Enterobacteriaceae*, on substrates consumed, and on products formed, in comparison with natural uninoculated processes.

## 2. Materials and methods

### 2.1. Brining procedure

Experiments were carried out with green olives of Manzanilla variety in three consecutive seasons. Alkaline treatment, the washing step, and brining were as specified in Table 1. In all cases, treated olives were divided into equal portions and placed into PVC fermenters with a fruit-to-brine ratio of 4.8 kg:4.0 l. Two duplicate fermenters per trial were studied. Experiments were carried out at ambient temperature (23–25°C).

### 2.2. Strains and preparation of inocula

Most fermentations were carried out using *L. pentosus* CECT 5138, a strain originally isolated in our laboratory from a natural Spanish-style green olive fermentation. For comparison, *L. pentosus* ATCC 8041 and *L. plantarum* ATCC 14917, type strains obtained from the American Type Culture Collection (Rockville, MD), were used in some instances. For propagation of inocula, MRS broth (Oxoid, Basingstoke, Hampshire, England), with 4.5% (w/v) NaCl to allow adaptation to the saline environment, was used. Incubation was at  $30 \pm 2^\circ\text{C}$ , and special care was taken to get the different inoculum concentrations from the same early stationary growth phase, since we have verified that survival decreases if cultures in log phase are used (unpublished information). To achieve the different inoculum concentrations, cultures were centrifuged, washed in saline, and the volume calculated to get the desired population was resuspended in brine from the fermenter and used as inoculum.

### 2.3. Microbial growth

Brine samples and appropriate decimal dilutions were plated using a Spiral System model DS (Inter-

Table 1  
Characteristics of initial treatments

Season	Lye treatment		Washing time (h:min)	Brine % NaCl (w/v)
	% NaOH (w/v)	Time (h:min)		
First	2.10	6:45	14:45	10.4
Second	1.96	6:30	17:30	10.7
Third	1.96	6:30	17:00	12.5

science, Saint Nom La Breteche, France). *Enterobacteriaceae* were counted on Crystal-violet neutral-red bile glucose (VRBD) agar (Merck, Darmstadt, Germany), lactic acid bacteria on MRS agar (Oxoid) and MRS agar with 0.02% (w/v) sodium azide (Sigma, St. Louis, USA), and yeasts on OGYE agar (Oxoid). Plates were incubated at  $30 \pm 2^\circ\text{C}$  for 48 or 72 h. Randomly selected colonies of lactobacilli from the highest dilution from each day were inoculated into tubes with 5 ml modified MRS broth prepared with L-sorbose (Sigma) or glycerol (Merck) at 1% (w/v) instead of glucose, and bromocresol purple as indicator. The capability of *L. pentosus* CECT 5138 to produce acid from both substrates, whereas *L. plantarum* ATCC 14917 does not ferment them and *L. pentosus* ATCC 8041 produces acid only from glycerol, allows us to confirm that the predominant strains isolated from each fermenter correspond to the inoculum applied.

#### 2.4. Chemical analyses

Brine samples were routinely analyzed for pH, titratable acidity, and NaCl concentration following the procedures described previously (Fernández-Díez et al., 1985). Organic acids and sugars were measured by HPLC using the methods described by Montañó et al. (1993). Ethanol was analyzed using the headspace method described by Montañó et al. (1990).

#### 2.5. Data analysis

Mean values of the different parameters from duplicate fermentations were calculated and plotted but, for clarity, error bars were not included. Instead, in order to give estimates of variability, coefficients of variation were obtained at each time, and average values were given in each figure caption.

### 3. Results and discussion

More than 40 fermentations were performed in three consecutive seasons. Most inoculation experiments were carried out the same day of brining or the second day of fermentation, when the brine pH was markedly alkaline. When the experiments were

performed in the same conditions, results were essentially the same; however, as a consequence of slight variations between seasons, and due to the large quantity of data, averaging tended to obscure peaks and troughs. In consequence, the results from one representative set of fermentations are shown below.

An example of inoculation of *L. pentosus* CECT 5138 at different days (0.5, 2, 3.5, and 5) is presented in Fig. 1. A marked decrease in the number of lactobacilli occurred when inoculation was on the first days of brining, this decrease being smaller the later the inoculation (Fig. 1a). Despite the initial loss of viability, a clear beneficial effect could be seen on both the microbial growth and the chemical charac-

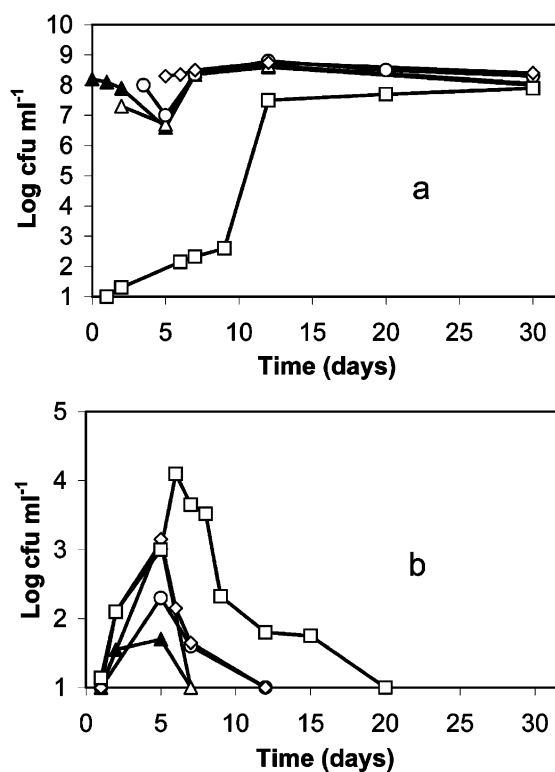


Fig. 1. Changes in population of (a) lactobacilli and (b) *Enterobacteriaceae* in brine during fermentation of green olives by an *L. pentosus* CECT 5138 starter culture added at day 0.5 ( $\blacktriangle$ ), day 2 ( $\triangle$ ), day 3.5 ( $\circ$ ), and day 5 ( $\diamond$ ), and in uninoculated control ( $\square$ ). Points are means of duplicate fermentations. Coefficients of variation (%) were: day 0.5—39.4 and 88.1, day 2—45.0 and 86.4, day 3.5—20.2 and 111.6, day 5—16.1 and 94.3, and control—78.0 and 122.3, for lactobacilli and *Enterobacteriaceae*, respectively.

teristics of the brines. The population of viable lactobacilli was always significantly higher when cultures were used than in uninoculated controls, and this difference had a noticeable effect on *Enterobacteriaceae* (Fig. 1b), which were inactivated faster in inoculated fermenters than in controls. This effect has been observed previously (Ruiz-Barba et al., 1994) and confirms the advantage of using starter cultures of lactobacilli to reduce the likelihood of spoilage during the first stage of Spanish-style green olive fermentation, when the risk of gas pocket (or bloater) and butyric acid spoilages is highest (Fernández-Díez et al., 1985). With regard to yeast growth, no significant differences between treatments could be detected. An early increase in yeast growth seemed to occur when inoculation was performed, but it soon disappeared, and the population in both inoculated and uninoculated fermenters oscillated around  $10^5$  CFU ml<sup>-1</sup> throughout the fermentation process (data not shown). Confirmation of the reisolated species of lactobacilli showed that the strain used in the different inoculation experiments was the only one detectable until natural growth was taking place. From that point on—around 15 days of fermentation on average—mixed populations of lactobacilli grew in all cases, as is normal in typical processes (Balatsouras, 1985; van den Berg et al., 1993; Ruiz-Barba et al., 1994).

Acid production and pH decrease are presented in Fig. 2. The effects of starter culture use on these fundamental parameters are clearly seen as a more rapid rate of the fermentation process. For pH (Fig. 2b), inoculation at days 0 or 2 (pH above 9) meant that brine pH at day 5 was one unit below the other fermenters. This difference, precisely when the population of *Enterobacteriaceae* is at its peak (Fig. 1b), is considered crucial, and contributes to the lesser growth of these spoilage microorganisms. When *L. pentosus* cultures were added at days 3 and 5, an immediate pH decrease took place, and again a marked difference with the control pH curve was noticed. This difference between inoculated brines and uninoculated control brines lasted as long as lactobacilli in the latter were in low numbers, and tended to disappear from day 12 on (Fig. 2b), when the population of lactobacilli reached more than  $10^7$  CFU ml<sup>-1</sup> in all brines (Fig. 1a). Titratable acidity (Fig. 2a) follows pH curves. No acidity was de-

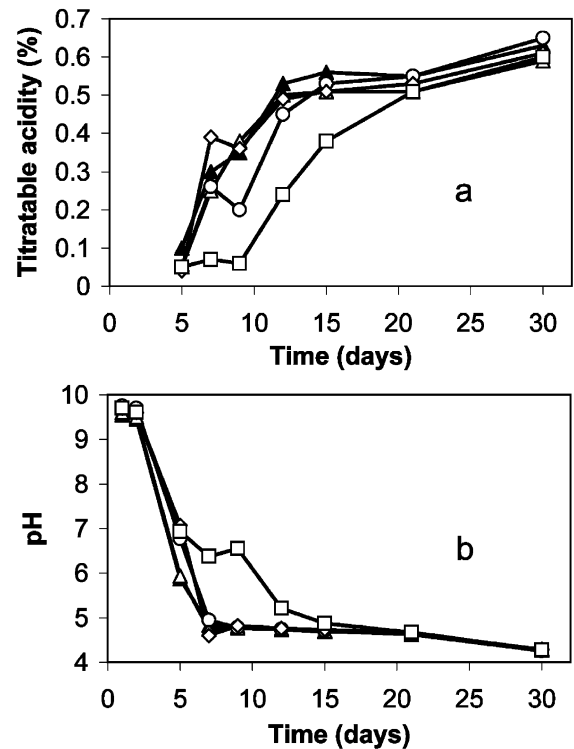


Fig. 2. Changes in (a) titratable acidity (% as lactic acid) and (b) pH in brine during fermentation of green olives by an *L. pentosus* CECT 5138 starter culture added at day 0.5 ( $\blacktriangle$ ), day 2 ( $\triangle$ ), day 3.5 ( $\circ$ ), and day 5 ( $\diamond$ ), and in uninoculated control ( $\square$ ). Points are means of duplicate fermentations. Coefficients of variation (%) were: day 0.5—14.6 and 2.2, day 2—11.6 and 1.7, day 3.5—6.4 and 0.8, day 5—3.6 and 0.5, and control—6.5 and 1.1, for acidity and pH, respectively.

tectable for the first days, since pH values are above 7, but from days 5–9, a sharp increase of acidity was noticed in all inoculated vessels, whereas acidity in the controls remained steady until lactobacilli increased in numbers. Differences were diminishing and were not detectable by day 21. This means that the more noticeable and recordable effects of the inoculation are present only during the so-called first and second stage of fermentation. The fact that differences in acidity or pH between inoculated and control brines could hardly be detected once the lactic acid fermentation was established might explain earlier results (Borbolla y Alcalá et al., 1964) from the use of pure cultures of lactobacilli for the fermentation of Spanish-style green olives. In that work, pH and acidity values through the whole

fermentation process led the authors to conclude that inoculation did not provide any significant improvement. On the other hand, the suitability of using starter cultures is emphasized by studying Table 2 in our work, which presents mean concentration values for the most important substrates and products after 12 days of fermentation. The most notable results were, firstly, the accelerated acidification when inoculation is carried out. Interestingly, this higher rate of acid production was not only lactic acid, but there was also a tendency to produce acetic acid, most likely as a result of metabolism of citric acid, which had disappeared by day 12 from inoculated brines (but not from controls). Acetic acid might be the sole major final metabolite of citric acid metabolism since other possible products, succinic acid, acetoin and 2,3-butanediol, were not detected (Hugenholtz, 1993). Secondly, the concentration of the main fermentation substrate, glucose, was nearly negligible in inoculated brines, whereas ca. 19 mM glucose was still available at day 12 in controls. That means that more glucose is accessible to potential spoilage microorganisms during the first stages, when growth of putrefactive and Gram-negative bacteria can take place. On the other hand, rapid consumption of glucose by an efficiently established culture implies an improved and more predictable fermentation process, as well as greater safety and a reduced hygiene risk. These are desirable properties in a starter culture to be used both at a household scale and in

industrialized, but not fully developed, fermentation processes (Holzapfel, 1997).

Data collected throughout three seasons are presented in Fig. 3. Results, expressed as difference between  $\log \text{CFU ml}^{-1}$  at the time of inoculation ( $N_0$ ), and  $\log \text{CFU ml}^{-1}$  24 h later ( $N_{24 \text{ h}}$ ), compare inoculum survival as a function of brine pH (Fig. 3a) or day (Fig. 3b) at the time of application. No decrease in population was observed when inoculation was performed at pH values below 7.5 or after day 4, whereas lethality tended to increase when the pH was higher or the inoculation earlier. However, in many instances, only a slight lethality was observed even when inoculation was performed at pH values above 9. Major factors that may limit the growth of lactobacilli during the first days of brining Spanish-style green olives are (in summary): (i) salt concentration, (ii) lack of nutrients, (iii) natural inhibitors, and (iv) high pH. Salt concentration should not be inhibitory against common strains of *L. pentosus* or *L. plantarum* when cultures are applied after day 1 of fermentation. The balance between surrounding brine and flesh is almost total within 24 h (Borbolla y Alcalá, 1979). In fact, salt concentration at day 1 was between 4.3 and 4.7% in all fermenters—nearly the same concentration used for preparing the inocula—and a concentration easily tolerated by most strains of lactobacilli from olive fermentations (Balatsouras, 1985; Rozés and Peres, 1996; Marsilio and Lanza, 1998). With regard to

Table 2

Metabolic products and substrates remaining in brine after 12 days of fermentation<sup>a</sup>

	<i>L. pentosus</i> CECT 5138 inoculum added at				Uninoculated control
	0.5 Day <sup>b</sup>	2 Days	3.5 Days	5 Days	
<i>Products (mM)</i>					
Lactic acid	96.3	106.9	79.2	114.6	58.8
Acetic acid	16.9	17.3	14.8	23.4	13.2
Succinic acid	ND <sup>c</sup>	ND	ND	ND	2.5
Ethanol	11.2	13.4	11.3	10.2	13.2
<i>Substrates (mM)</i>					
Glucose	1.6	1.2	1.8	1.1	18.5
Sucrose	0.3	ND	0.1	0.1	ND
Mannitol	ND	ND	ND	ND	3.3
Citric acid	ND	ND	ND	ND	4.0

<sup>a</sup> Values are means of duplicate fermentations.<sup>b</sup> Days after brining.<sup>c</sup> ND, not detected.

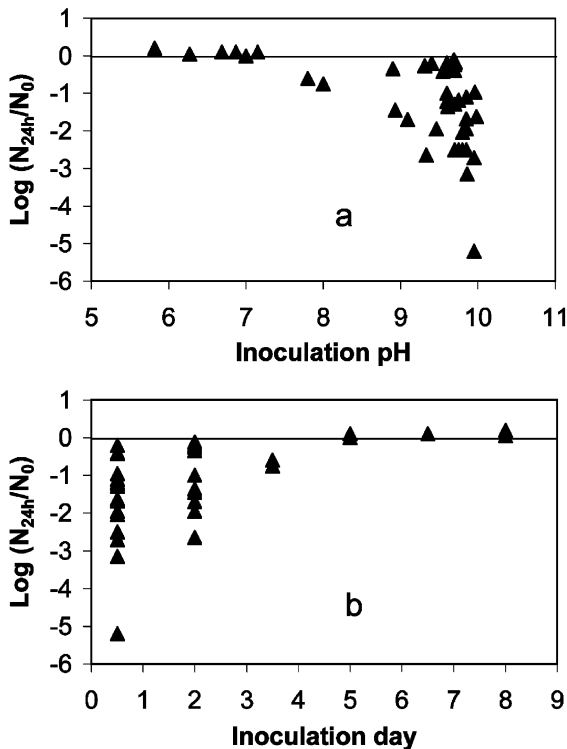


Fig. 3. Starter culture survival expressed as difference between log CFU ml<sup>-1</sup> at the time of inoculation ( $N_0$ ), and log CFU ml<sup>-1</sup> 24 h afterwards ( $N_{24\text{ h}}$ ). Each point corresponds to one fermentation batch inoculated at the given (a) pH and (b) day.

nutrients, there is insufficient data to know the composition in essential requirements for lactobacilli during the first 48 h in brine, although reducing sugars are rapidly available (Fernández-Díez et al., 1985). Ruiz-Barba and Jiménez-Díaz (1994) determined the vitamin and amino acid requirements of *L. plantarum* strains isolated from olive fermentations, and the same authors verified later that at least B-group vitamins were available by day 7 and throughout the fermentation process (Ruiz-Barba and Jiménez-Díaz, 1995). Most amino acids are present in brines (Montaño et al., unpublished data), but it is likely that enrichment of brine with nutritive supplements at the time of inoculation will increase viability and recovery of starter cultures and improve their efficiency (Roig and Hernández, 1991). There is a large quantity of information dealing with natural inhibitors from olives and their effects on microorganisms (Juven and Henis, 1970; Fleming et al., 1973; Nychas et al., 1990; Ruiz-Barba et al., 1993). Oleu-

ropein and polyphenols from its hydrolysis have been related to this inhibitory effect, but the alkaline treatment applied to Spanish-style green olives destroys most of these compounds, in fact making this product easily fermentable in comparison with untreated olives (Ruiz-Barba et al., 1993). Apart from interactions between factors, in our experiments high pH was most likely the main factor explaining loss of inoculum viability. Information on high pH resistance of lactobacilli is scarce. Growth of *L. plantarum* and *L. pentosus* at pH values above 9.0 has been recorded (Tanasupawat et al., 1992), and an alkali-tolerant strain of lactobacilli has been used previously (Liepe and Pfeil, 1976). It has been suggested that the ability of *L. plantarum* to shift between the production of neutral (acetoin) and acidic (acetic acid) compounds may help to maintain pH homeostasis in alkaline environments (McFall and Montville, 1989), as may the presence of a Na<sup>+</sup>/H<sup>+</sup> antiport (Krulwich, 1995), whose requirement for Na<sup>+</sup> reentry into the cell would be solved by the external high Na<sup>+</sup> concentration due to NaCl and residual NaOH. In summary, when starter cultures of *L. plantarum* or *L. pentosus* were applied at day 0 or day 2 in brines with pH above 9, a marked decrease of viability was noticed as a result of the unfavourable conditions. Nevertheless, survivors can withstand this hostile environment, and eventually grow and initiate the lactic acid fermentation of alkali-treated green olives. The lethality may be calculated as a function of day of inoculation (Fig. 3b), and is, on average, two log cycles when applied at day 0 and 1 log cycle at day 2. This indicates the inoculum size necessary (depending on the day of application) to achieve an initial population of lactobacilli close to 10<sup>6</sup> CFU ml<sup>-1</sup>, which is considered the minimum for significant results. Our results can be applied industrially for the inoculation of Spanish-style green olives without the requirement of previous pH correction or waiting for the pH to be reduced by other microorganisms—a practice not recommended.

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