**Lactobacillus pentosus DSM 16366 starter added to brine as freeze-dried and as culture in the nutritive media for Spanish style green olive production**

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1. **INTRODUCTION**

Modern large scale production of table olives is focused on the use of defined strain starter systems to ensure quality and consistency in the final product. Lactic acid bacteria have been exploited because of their ability to produce desirable changes in organoleptic attributes, improve shelflife and enhance food safety (Sánchez et al., 2001; de Castro et al., 2002; Chorianopoulos et al., 2005). In Spanish style green olive fermentation, as a consequence of the alkaline treatment, pH values during the first days after brining are not favorable for the growth of lactic acid bacteria, whereas potential spoilage microorganisms can proliferate and spoil the product.

*Lactobacillus plantarum* and *Lactobacillus pentosus* have been known to play an important role in many spontaneous processes of table olive fermentation. Therefore, these species have been used as starter cultures to improve the microbiological control of the process and to produce consistently high quality table olives (Sánchez et al., 2001; de Castro et al., 2002; Leal-Sánchez et al., 2003; Panagou et al., 2003; Chorianopoulos et al., 2005; Lamzira et al., 2005). The inhibitory action of lactic acid bacteria is due to the accumulation of main primary metabolites (lactic and acetic acids, ethanol and carbon dioxide) as well as to the production of other microbial compounds (formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins).

*Lactobacillus pentosus* DSM 16366, originally isolated from Spanish style olive fermentation brines was shown to produce the highest bacteriocin titers in environmental conditions that resembled green table olive fermentation (Delgado et al., 2005), suggesting its technological interest as a starter for table olives.

The objectives of the present study, concerning green table olives of the Portuguese Azeiteira cultivar, were to assess chemical and microbial fermentation profiles and to study the effect of *Lactobacillus pentosus* DSM 16366 as freeze-dried cells and as culture in nutritive media on the development of fermentation and final product quality.
2. MATERIALS AND METHODS

2.1. Preparation of olives

Green olives of the Portuguese cultivar Azeiteira (Olea europaea) were harvested from an olive grove in the Alentejo Northeast area. The steps of Spanish-style processing were as follows: Olives were submitted to an alkali treatment (1.5% w/v) for 6 hours until penetration of NaOH reached approximately 2/3 of the flesh (c.a. 6 hours) and then washed with tap water twice for 4 and 12 hours. Fruits were placed in brine (8.0% NaCl, w/v). It is very important that all the olives have been rehydrated or with an overnight culture in Man Rogosa and Sharpe agar (MRS) (Oxoid, Hampshire, England). Lactobacillus pentosus DSM 16366, originally isolated from green olive fermenting brines, was used as a starter culture. The inoculation was carried out suspending freeze-dried cells into brine from the corresponding fermentor and left for 4 h to rehydrate or with an overnight culture in Man Rogosa and Sharpe agar (MRS) (Oxoid, Hampshire, England). Lactobacillus pentosus DSM 16366 was added to brines giving a population in the vessels of approximately 10^2-10^3 CFU mL^{-1}. Inoculation took place at day 5 of fermentation, when brine pH was c.a. 6 (Delgado et al., 2005). Spontaneous fermentation by the environmental microbiota represented the control treatment. Trials were prepared in duplicate in 30 L plastic containers.

At the first and fifth days of fermentation and each week for a further 50 days, samples of brine were aseptically withdrawn from the centre of the fermentation containers to analyze microbial and chemical parameters in order to draw fermentation profiles.

2.2. Microbial growth in brines

Brine suspensions were serially diluted and plated on selective media for counting purposes. Enterobacteriaceae colonies were counted on crystal-violet neutral red bile glucose agar (Merck, Darmstadt, Germany) incubated at 30 °C for 24 h. Lactic acid bacteria were numbered on Man Rogosa and Sharpe agar (MRS) (Oxoid, Hampshire, England), supplemented with 0.01% (w/v) sodium azide (Sigma-Aldrich, Madrid, Spain) at 30 °C for 72 hours. Fungi were counted after 72 h of incubation at 28 °C on dichloran-glycerol (DG18) agar base (Oxoid, Hampshire, England) with chloramphenicol selective supplement (Oxoid, Hampshire, England).

2.3. Chemical profile of brines

Along with the microbiological analyses, brine samples from each fermentor were analyzed for pH, free acidity, and NaCl concentration (Díez et al., 1985). Free acidity was expressed as lactic acid percentage in brine (w/v).

2.4. Organoleptic evaluation

After 60 days of fermentation, representative samples were presented to a 5-member trained panel which was assigned to assess samples' overall appreciation on a 1 to 9 rating scale.

2.5. Statistical analysis

Mean and standard deviation values of the different parameters were plotted.

3. RESULTS AND DISCUSSION

This experiment was performed in three consecutive seasons and fermentation profiles were essentially the same. Results from one set of fermentations are shown below. Microbial and chemical spontaneous fermentation profiles of “Azeiteira” Spanish-type green olives, shown in Figures 1-5, can be characterized in the so-called three successive phases. Initially, Enterobacteriaceae predominated and brine pH decreased to a value approximately 6.0. During the second phase, lactobacilli and fungi developed quickly, and Enterobacteriaceae decreased until they disappeared completely at a pH around 4.2. In the third phase, lactic acid bacteria (10^9 CFU mL^{-1}) abounded and coexisted with fungi (10^6 CFU mL^{-1}).

Throughout fermentation there was no NaCl addition. In brines, by the diffusion phenomenon between brine and fruit, salt concentration declined until the equilibrium was achieved exhibiting values around 4% (data not shown). In fact, salt concentration at day 1 was between 4.7 and 5.1 % in all fermentors, which is a concentration easily tolerated by most strains of lactobacilli from olive fermentations including L. pentosus DSM 16366 (Delgado et al., 2005).

The results revealed an initial rapid drop in pH during the first 12 days of fermentation, followed by stabilization around 4 units and a negligible rate of drop until the end of the fermentation, achieving 3.8-3.9 pH units (Figure 1). The sharp decrease of pH is consistent with the remarkable increase in the lactic acid bacteria population and free acidity as described below (Figure 2 and Figure 3). Lactic acid bacteria produce mainly lactic acid; and to a lesser extent other acids, leading to a pH drop (Sánchez et al., 2000, 2001; de Castro et al., 2002; Panagou et al., 2003; Chorianopoulos et al., 2005).

In all fermentors, free acidity remained very low during the first five days (less than 0.10%) and afterwards raised rapidly (Sánchez et al., 2001; Leal-Sánchez et al., 2003). At 12 days of fermentation, 7 days post-inoculation, free acidity was 0.61% when...
The addition of growth factors to the ecosystem, notably vitamins, amino acids or trace elements could also play a role (Ruiz-Barba and Jiménez-Díaz, 1994).

A remarkable increase in lactic acid bacteria occurred during the week after inoculation, this increase being smaller in spontaneous fermentation (Figure 3). Throughout the second month of fermentation, a similar pattern and moderate fluctuation around $10^9$ CFU mL$^{-1}$ lactic acid bacteria were recorded for all fermentors (Sánchez et al., 2000, 2001). Nevertheless, it must be stressed that this delay at the onset of fermentation, when the risk of spoilage due to Enterobacteriaceae or butyric-acid-producing clostridia is highest, implies a higher likelihood of

The inoculum carrier was nutritive media, whereas freeze-dried starter application and spontaneous fermentation showed free acidity levels above 0.6% only after 26 and 33 days of fermentation, respectively (Figure 2). Therefore, induced lactic acid fermentation by Lactobacillus pentosus DSM 16366 culture accelerated acidification during the second stage of fermentation, thus reducing the risk of spoilage, as previously found in earlier studies on green olives using L. pentosus and L. plantarum starters (Sánchez et al., 2000, 2001; Leal-Sánchez et al., 2003; Panagou et al., 2003). The positive effect of MRS inoculum carrier could be explained by its protective effect on L. pentosus cells, as previously observed in L. plantarum in the presence of other stressing conditions (Durán Quintana et al., 1994).
spoilage if inoculation is not carried out. Furthermore, *Lactobacillus pentosus* DSM 16366 bacteriocin production could be a relevant factor in strain establishment and may contribute to the control of microbiological fermentation (Delgado et al., 2005). Moreover, the predominance of the lactic population in the olive brine is important to inhibit the fermentative metabolism of yeasts that produce bloaters (Lamzira et al., 2005).

*Enterobacteriaceae* showed an increase within the first 5 days of fermentation, which was followed by a noticeable decrease until they achieved undetectable levels (<10 CFU mL\(^{-1}\)) at 12 and 19 days using culture and freeze-dried cell starters, respectively, and 26 days in the control (Figure 4). The reduction of this microbiota coincided with the free acidity rising above 0.5% and the pH dropping below 4.2 (Figure 1 and Figure 2). *Enterobacteriaceae* were inactivated faster in started lactic fermentation than in the control (Sánchez et al., 2000; de Castro et al., 2002; Lamzira et al., 2005), especially when nutritive media was used as starter carrier, confirming the advantage of using *Lactobacillus pentosus* DSM 16366 to reduce the likelihood of spoilage.

With regard to fungi growth (Figure 5), after the 19\(^{th}\) day of brining, an early population increase seemed to occur in fermentors added by starter culture, but it soon disappeared, and the population, in both inoculated and uninoculated vessels, achieved similar values (Sánchez et al., 2000; 2001; Leal-Sánchez et al., 2003). At the end of the process, spontaneous and induced fermentation revealed a similar lactic acid bacteria (10\(^9\) CFU mL\(^{-1}\)) and fungi (10\(^4\) CFU mL\(^{-1}\)), as well as pH (3.8) and free acidity (1.0% lactic acid) (Figures 1-5). Suitability of using *L. pentosus* DSM 16366 as a starter culture may be emphasized by future studies focusing on the main fermentation substrates and end-products, which could reveal an
improved and more predictable fermentation process, as well as a greater safety and a reduced hygiene risk. No differences were found in the overall organoleptic evaluation of inoculated and naturally fermented olives (data not shown). Trials to confirm these results at industrial scale are underway.

4. CONCLUSIONS

At the beginning of fermentation, a beneficial effect could be seen on microbial growth and chemical characteristics of brines, namely Enterobacteriaceae, lactic acid bacteria and free acidity. Enterobacteriaceae were inactivated faster in inoculated fermentors than in controls, illustrating the advantage of using Lactobacillus pentosus DSM 16366 to reduce the likelihood of spoilage. The best results were attained using nutritive media as pure culture carrier rather than freeze-dried cells.

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REFERENCES


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