Food Microbiology 44 (2014) 278–287

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Production of potential probiotic Spanish-style green table olives at pilot plant scale using multifunctional starters

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form
4 March 2014
Accepted 10 March 2014
Available online 28 June 2014

Keywords:
Biofilm
Fermentation
Starter culture
Functional olives
Lactobacillus pentosus
Probiotic

ABSTRACT

This work evaluates the use of two multifunctional starters of *Lactobacillus pentosus* species (TOMC LAB2 and TOMC LAB4) during elaboration of Manzanilla olive fruits processed according to the Spanish-style. Data show that the use of inocula at the onset of fermentation led to a proper acidification and sugar consumption of brines compared to the spontaneous process, obtaining in a shorter period of time the maximum population for lactic acid bacteria. Both inoculated *L. pentosus* strains were recovered at high frequencies at the end of fermentation on the olive surface, which was corroborated by RAPD-PCR analysis. *In situ* observation of olive epidermis slices by scanning electron microscopy revealed a strong aggregation and adhesion between microorganisms, which reached population levels of approximately 6 and 7 $\log_{10}$ cfu/cm^2 for yeasts and lactic acid bacteria, respectively. *Enterobacteriaceae* on the olive surface were also found at the onset of fermentation (~9 $\log_{10}$ cfu/cm^2), but they declined during the process and were below the detection limit at the end of fermentation. Results obtained in this study show the advantage of using multifunctional starters with the ability to adhere to the olive epidermis because, ultimately, the fruits are the food ingested by consumers.

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1. Introduction

According to the International Olive Oil Council statistics, the last recognized production of table olives (2010/2011 season) was 2,563,000 tons (IOC, 2013). It is estimated that approximately 60% of this production was processed as Spanish-style green table olives, which implies a lye treatment followed by typical lactic acid fermentation after brining (Garrido Fernández et al., 1997). Although the main objective of the elaboration of this fermented vegetable is the preservation of the product by acidification and salting, the preservation of its texture and the development of pleasant flavor have allowed its worldwide implementation.

Spanish green table olive fermentation is a process typically dominated by lactic acid bacteria (LAB). These microorganisms may have potential benefits on human health, because, among others, the following individual or combined favorable effects have been described for some species: improvement of lactose assimilation, food digestibility, hypercholesterolemia, immune response, and the prevention of intestinal infections, cancer, food allergies and constipation (Champagne and Gardner, 2005). Moreover, table olives might also be considered as a functional food because of their high content in dietary fiber, antioxidant compounds, vitamins and anticancer compounds (Garrido Fernández et al., 2001).

Ranadhera et al. (2010) consider that the type of food carrier plays an essential role in buffering the probiotic throughout the gastrointestinal tract, regulating their colonization or interacting with the probiotic to alter functionality. Lavermicocca et al. (2005) used table olives as a vehicle to incorporate probiotic bacteria species into the human body. Particularly, one strain of *Lactobacillus rhamnosus* remained invariant and showed a good recovery (about 6 $\log_{10}$ cfu/g) after 30 days of its incorporation to fermented table olives. *Lactobacillus paracasei* IMPC2.1 successfully colonized the olive surface, dominating the natural LAB population until the end of the fermentation (De Bellis et al., 2010), making the product a suitable carrier for delivering probiotic bacteria to humans. According to these authors, the high survival rates observed for probiotic strains on olives implies that the consumption of about 80 g

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http://dx.doi.org/10.1016/j.fm.2014.03.023
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of olives allowed the ingestion of more than one billion *L. paracasei* or *Lactobacillus plantarum* live cells (Lavermicocca et al., 2010). Recently, it has been demonstrated that diverse *L. plantarum* and *Lactobacillus pentosus* strains establish polymicrobial communities on the surface of green Spanish-style Gordal (Domínguez Manzano et al., 2012) or Manzanilla (Arroyo López et al., 2012) cultivars. In both cases, *in situ* observation of the olive epidermis by scanning electron microscopy (SEM) showed strong aggregation and adhesion between bacteria and yeasts by the formation of a complex matrix which embedded the microorganisms.

Nowadays, a great diversity of bacterial populations are found during Spanish-style green table olive processing (Doulgeraki et al., 2013; Hurtado et al., 2012). Such diversity may be caused by the empirical production process (Botta and Cocolin, 2012). To improve the fermentation profile, the use of starter cultures for the initiation of the process and to control the microbiological population in the brines has been proposed (Sánchez et al., 2001; De Castro et al., 2002; Peres et al., 2008). A recent study showed that the inoculation with a starter culture led to higher LAB and yeast populations, to decrease in the *Enterobacteriaceae* populations and to faster acidification of the brines, but did not assure *per se* the presence of the added strains in the brine solutions at the end of fermentation (Rodríguez-Gómez et al., 2013). Most of the above mentioned works did not control the survival and imposition of the specific inoculated strain and the favorable effects on fermentation could not be attributed with total certainty to the inoculum activity. On the contrary, Argyri et al. (2014) and Blana et al. (2014) have evaluated the use of potential probiotic LAB strains originally isolated from olive fermentation as starters with promising results, paying particular attention to their imposition and presence at the end of fermentation. Therefore, a proper selection of the starter strain and manipulation of the fermentation process is essential for succeeding in the production of functional olives and the imposition of selected strains.

With the present study we aim to determine the performance, at pilot plant scale, of two preselected LAB strains (*L. pentosus* TOMC LAB2 and TOMC LAB4) for the fermentation and production of functional table olives. The research was based on a multidisciplinary approach using molecular biology, analytical chemistry, modeling, scanning electron microscopy and food microbiology techniques to determine the microbial growth, acidification kinetics, imposition of the inoculated strains in the brines and on the olive surface as well as their ability to form biofilm, which is an essential characteristic to turn table olives into an appropriate bacteria food carrier.

2. Material and methods

2.1. Olive processing

The fruits used in the present study were of the Manzanilla variety (*Olea europaea pomiformis*), picked by hand at the green maturation stage during the 2010/2011 season and supplied by JOLCA S.A. (Huevar del Aljarafe, Seville, Spain).

Six cylindrical PVC fermentation vessels with a total volume of 100 L (Ø 0.4 m × 0.8 m high), provided with a reduction in the top (Ø 0.15 m × 0.15 m), were filled with 64 kg of olives. For debittering, fruits were lye-treated with a 2.2% NaOH (40 L) solution for 5 h (until the lye penetrated 2/3 of the flesh), followed by immersion in tap water for 20 h to remove excess alkali. Then, a brine solution with 11% (w/v) NaCl and 35 mL of HCl 37% was added to partial neutralize of the remaining NaOH. After 2 days, CO_2 was bubbled through the fermentation vessels (olives and brine) to reach a pH of nearly 7.5 units. Anaerobic conditions were achieved by using a floating closing device placed on the top of the vessels.

2.2. Inoculation and fermentation

After pH adjustment, the fermentation vessels were inoculated with overnight cultures (MRS at 37° for 18 h) of strains *L. pentosus* TOMC-LAB2 and TOMC-LAB4, selected from previous experiments because of their potential probiotic characteristics (Bautista Gallego et al., 2013), ability of adhesion to olive epidermis (Arroyo-López et al., 2012) and good performance in previous trials carried out at laboratory scale (Rodríguez-Gómez et al., 2013). These cultures were grown until early stationary phase and then an aliquot of the suspension was added to the fermentation vessels in a proportion of about 0.03% to reach an initial inoculum level of approximately 6 $\log_{10}$ cfu/mL in the brines. The experimental design consisted of: F1, spontaneous and un-inoculated treatment; F2, treatment inoculated with LAB2 strain; and F3, treatment inoculated with LAB4 strain. Each treatment was carried out in duplicate and monitored for 135 days.

The fermentation vessels were kept during the entire process at the Instituto de la Grasa pilot plant (CSIC, Seville, Spain), where the room temperature decreased progressively from 28 °C (October) to 14 °C (January), which was maintained until the end of the experiments (February). After 18 days of fermentations, 2 L of brine from the bottom of the vessels were removed and substituted with the same volume of fresh brine containing 5% NaCl and 15% glucose (to reach a final concentration in the brines of 7.5 g/L of glucose). On the 54th day of fermentation, the brine was again supplemented with a 2.8 g/L glucose solution. This practice is common during Spanish-style olive processing to achieve adequate final pH values (<4.2) and ensure the safe storage of the fermented olives (Garrido-Fernández et al., 1997; Chorianopoulos et al., 2005).

2.3. Physicochemical analyses of the brines and modeling

Analysis of pH and titratable acidity of the fermentation brines was carried out using the methodology described by Garrido-Fernández et al. (1997). Sugars (glucose, fructose, sucrose and mannitol), organic acids (lactic and acetic) and ethanol were determined by HPLC according to the protocols described by Rodríguez Gómez et al. (2012). The evolution of these parameters through fermentation was modeled using the following equations:

i) Exponential decay function (for pH and total sugar concentration):

$$Y = D + S{*}e^{-(K{*}t)}$$

where Y is the dependent variable, t is the time (days), D is the minimum asymptotic value when $t \to \infty$, S is the estimated value of change, and K is the kinetic constant of change (days^{-1}).

ii) Reparameterized Gompertz function (Zwietering et al., 1990) (for lactic acid, acetic acid and titratable acidity):

$$Y = A{*}\exp\{-\exp[((\mu_{max}{*}e)/A){*}(\lambda - t)) + 1]\}$$

where Y is the dependent variable, A is the maximum asymptotic value reached when $t \to \infty$, μ is the maximum rate of production (days^{-1}), and λ is the period of time without production (days).

Model parameters were obtained by a non-linear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e., loss function (observed-predicted)^2. This task was accomplished using the non-linear module of the Statistica 7.1 software package (StatSoft Inc,

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