

The Impact of Production Technology on the Growth of Indigenous Microflora and Quality of Table Olives from Slovenian Istria

Vasilij Valenčič^{1*}, Dunja Bandelj Mavsar^{2,3}, Milena Bučar-Miklavčič^{1,2},
Bojan Butinar², Neža Čadež⁴, Terezija Golob⁴, Peter Raspor⁴ and Sonja Smole Možina⁴

¹LABS LLC, Institute for Ecology, Olive Oil and Control, Zelena ulica 8, SI-6310 Izola, Slovenia

²Science and Research Centre of Koper, University of Primorska, Garibaldijeva 1,
SI-6000 Koper, Slovenia

³Faculty of Mathematics, Sciences and Information Technologies, University of Primorska,
Glagoljaška 8, SI-6000 Koper, Slovenia

⁴Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana,
Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

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Summary

The objective of the research is to determine the leading microorganisms in spontaneous fermentations of table olives in Slovenian Istria. The influence of traditional regional and modified Spanish style technology on yeast and lactic acid bacteria population dynamics and on the quality of Istrska belica and Storta table olive varieties was studied during 180 days of fermentation. Apart from that, pH of the brine during fermentation and total biophenols in olive fruits before processing and after 60 and 180 days of fermentation were determined. The quality of the final product was determined with sensory analysis. Table olive fermentation was carried out by yeasts. *Aureobasidium pullulans*, *Cryptococcus adeliensis*, *Metschnikowia pulcherrima*, *Rhodotorula mucilaginosa*, *Pichia anomala* and *Candida oleophila* were isolated and identified using PCR-RFLP analysis of ITS regions and traditional phenotypic tests. High initial amount of total biophenols in olive fruits and their better preservation during traditional processing influenced microbial population dynamics and quality characteristics of table olives. The modified Spanish style technology was not confirmed as suitable for retaining positive characteristics of the product of traditional regional technology in Slovenian Istria.

Key words: table olives, Istrska belica, Storta, fermentation, yeasts, total biophenols, sensory characteristics

Introduction

The olive tree (*Olea europaea* L.) is a widespread and economically very important plant in the Mediterranean countries. Indeed in 2009, more than 92 % of the world olive production was concentrated in the Mediterranean basin (1). In Slovenian Istria olive growing has a long

tradition. It was mentioned for the first time in the 1st century BC. Nowadays, olive growing and olive oil production are important complementary economic activities and thus important for the local tradition, culture and lifestyle. In 2007, there were 1561 hectares cultivated with 416 070 olive trees (2), the annual olive produc-

*Corresponding author; Phone: ++386 5 640 2630; Fax: ++386 5 641 7228; E-mail: vasilij.valencic@gmail.com

tion was estimated at 2500 tonnes of fruits. The variety Istrska belica is the most widely spread in the region (approx. 63 % of the cultivated varieties), it can withstand low temperatures well. The autochthonous variety Storta is traditionally processed for table olives and characterized by a good texture and an ease of detachment of the flesh from the stone (3). However, in 2007, table olives represented less than 1 % of the annual Slovenian olive production.

Several table olive production technologies are known (4). Most frequently used are the Spanish style for green olives, the Californian style for ripe olives darkened by alkaline oxidation and the Greek style for naturally black olives. Many other traditional ways of processing differ according to the olive characteristics and fermentation conditions. However, scarce information is available about microorganisms in traditional fermentations. Microbial population of the olives before brine making is one of the factors that could affect the dynamics of the fermentation and the quality of the product (5,6). Lactic acid bacteria and yeasts are part of autochthonous microbiota of raw vegetables, adapted to their intrinsic characteristics. When lactic acid bacteria outgrow yeasts, lactic acid fermentation is favoured rendering a more acidic product with lower pH, which is greatly desirable in the fermentation of naturally black olives. The opposite happens when yeasts become the predominant group, resulting in a product with milder taste and fewer self-preservation characteristics. Yeast metabolites contribute to the formation of aroma compounds such as glycerol, higher alcohols, esters, volatile compounds, acetic, succinic and formic acid, ethanol, methanol and acetaldehyde, which affect the sensorial properties of table olives (7–10). Phenolic compounds, natural antioxidants and antimicrobials in vegetables and fruits, in the non-alkali treated green olive brine exhibit a pronounced bactericidal effect (6,11), alone and/or associated with NaCl (12).

The aim of this paper is to present microbial population dynamics during processing and quality characteristics of table olives produced by traditional regional technology compared to the modified Spanish style processing, which is also adapted by the producers of Slovenian Istria.

Materials and Methods

Plant material

The olive varieties Istrska belica and Storta (*Olea europaea* L.) were harvested in two olive orchards in Slovenian Istria in October 2006 and 2007. Before processing, the olive fruits were size-graded. The sizes of Istrska belica and Storta ranged from 321 to 350 fruits/kg and from 351 to 380 fruits/kg, respectively.

Processing of olive fruits

The olives of both varieties were processed by traditional regional and modified Spanish style production technologies. During traditional regional technology, one kilogram of olive fruits was placed in a 3-litre glass vessel and covered with 1.5 L of tap water for 10 days, which was replaced every other day. The debittering in water was followed by fermentation in brine solutions. At the

beginning the fermentations were set in 3.5 % brine solution for 2 days, then in 4.2 % for 5 days and continued in 6.2 % brine solution until the end of processing. Modified Spanish style technology involved alkaline treatment. One kilogram of olive fruits was placed in a 3-litre glass vessel and covered with 1.5 L of 2 % NaOH solution for 8 h. After lye treatment, the olives were washed 5 times with tap water for 24–36 h, using 1.5 L of water each time. Subsequently, the olives were covered with 6.2 % brine solution. All the phases (debittering in water or lye treatment, washing and fermentation) of the two processing technologies were monitored for 180 days. The fruits of Istrska belica were green, the fruits of Storta were turning colour. The olives fermented spontaneously without starter culture. Each cultivar was fermented in two parallel experiments in years 2006 and 2007.

Microbiological analysis

The fresh olive fruits were suspended in saline solution and decimal dilutions were obtained and spread on plates for quantification of yeasts and lactic acid bacteria. Then, the fermenting brine solutions were sampled after 3, 10, 20, 30, 60 and 180 days of fermentation, serially diluted and spread on culture media for population dynamics determination. From serial decimal dilutions with sterile physiological solution, yeasts were quantified by plate counting on oxytetracycline glucose yeast agar (OGY; Biolife, Milano, Italy) and lactic acid bacteria on De Man-Rogosa-Sharpe agar (MRS; Merck, Darmstadt, Germany) after incubation at 28 °C for 7 days and at 30 °C for 3 days, respectively. Two determinations for each of the two parallel experiments were made and the averaged results are presented.

Yeast isolation and identification

The yeast colonies were morphologically classified into groups on each countable plate, enumerated and the DNA of the purified representative colonies was isolated according to the method of Möller *et al.* (13). The ITS1, ITS2 and 5.8S rRNA gene regions were amplified with polymerase chain reaction (PCR) as described previously (14) and digested with *Hae*III, *Cfo*I, *Hinf*I (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The digests were separated on 2.5 % agarose gels. The molecular sizes of the ITS digests were determined by comparison with a DNA molecular marker using Quantity One v. 4.0.3 (15). The isolates sharing identical restriction patterns were classified into 6 groups and only representative colonies were characterized by traditional physiological testing in liquid media according to Yarrow (16). Phenotypic similarities were examined by the BioloMICS computer program (17) from the CBS database. Identified strains were deposited in the Collection of Industrial Microorganisms (ZIM, BF, Ljubljana, Slovenia).

Extraction and quantification of biophenols

The extraction was done with a modified method of Vinha *et al.* (18) with methanol, from 250 mg of freeze-dried and powdered sample with the addition of 1 mL of internal standard solution (syringic acid 0.15 mg/mL). Total biophenol content was determined by HPLC anal-

ysis. A Hewlett-Packard 1050 Series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with quaternary pump and automatic liquid sampler, C18 reversed-phase column (Phenomenex Luna C18(2), 250×4.6 mm, 5 µm; Phenomenex, Inc, Torrance, CA, USA), with UV detection at 280 nm was used. The separation was based on the method of Cortesi *et al.* (19). The mobile phase used was a gradient consisting of 0.2 % aqueous H₃PO₄ (by volume) (A) and methanol/acetonitrile 1:1 (by volume) (B). The initial gradient composition was 96 % A and 4 % B, which changed in 40 min to 50 % B, in the next 5 min to 60 % B and in the last 15 min to 100 % B. At 72 min from the start, the concentration of B was put at an initial value of 4 %. The column was then equilibrated for 10 min before the next injection. A volume of 10 µL of aliquot of methanolic extract was injected into the system. A calibration solution of tyrosol (0.030 mg/mL) and syringic acid (0.015 mg/mL) was prepared. All biophenol compounds were quantified using the response factor for tyrosol as reported in the original method. Biophenols were determined in two replicates for each of the two parallel fermentations.

pH measurement

The pH of the brine was measured with pH meter MA 5730 (Iskra, Kranj, Slovenia). The pH value of the brine solutions was determined after 3, 10, 20, 30, 60 and 180 days of fermentation.

Sensory analysis of table olives

The sensory attributes were determined by nine trained assessors according to the official method of the International Olive Council (20). The intensity of salti-

ness, bitterness, sourness, hardness and fibrousness, and the possible presence of defects as the flavour of anomalous fermentation, mustiness, rancidity, overheating and other negative attributes were determined and labelled on the specific profile sheet for table olive sensory assessment. The collected data were statistically analysed and the median for each attribute was determined.

Statistical analysis

Levene's test for equality of variances and *t*-test for equality of means were performed with SPSS Statistics v. 17.0 software (21).

Results and Discussion

The influence of processing on pH and biophenol content

The results of pH, total biophenol content and yeast population dynamics in traditional regional and modified Spanish style fermentations are presented in Figs. 1 and 2.

The pH of the brine solutions was measured for monitoring the course of fermentations. Significantly lower (*t*-test, $\alpha < 0.05$) pH values were determined in table olives produced with traditional technology. However, the results showed that the final pH values (Figs. 1 and 2) did not comply with the minimum requirements ($\text{pH} \leq 4.3$) of the Trade standard applying to table olives (22). Therefore, to assure the stability and safety of the product, table olives have to be acidified and/or pasteurized.

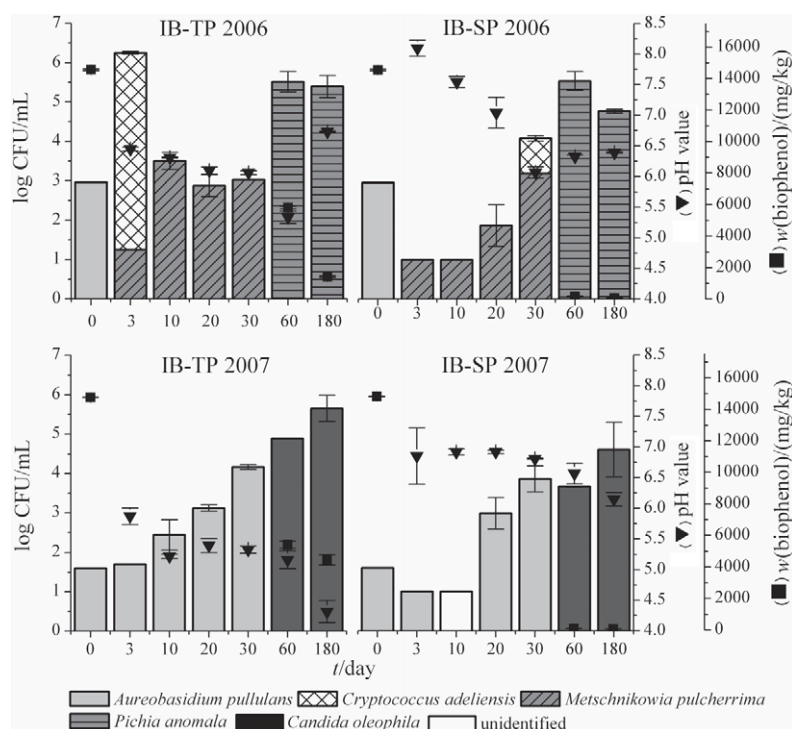


Fig. 1. Yeast population dynamics, pH values and total biophenol content during processing of Istrska belica (IB) olive fruits before debittering (0 days) and table olives, processed with traditional regional (TP) and modified Spanish style (SP) technology, crop years 2006 and 2007. The results are averaged from four replicates and are expressed as means±standard deviations (error bars)

Statistically significant differences (*t*-test, $\alpha < 0.05$) in total biophenol content between both olive varieties and between the two processing technologies were determined. Total biophenol content of Istrska belica olives before processing was 39 % (2006) and 34 % (2007) higher than in the olive fruits of Storta. In addition, much higher total biophenols were determined in table olives produced by traditional regional technology (544–5803 mg/kg) than in table olives produced by modified Spanish style technology (44–183 mg/kg). The total amount of biophenols was reduced during fermentation. After 60 days of traditional regional processing of Istrska belica and Storta table olives, 61.6 and 84.6 % reduction of total biophenols was determined, respectively. After 180 days of fermentation, the amount of biophenols was reduced for 79.9 and 90.5 % for Istrska belica and Storta, respectively. The reduction of 58 and 55 % in total biophenol content was also reported by Ben Othman *et al.* (23) after 67 days of spontaneously fermented green table olives (cv. Chétoui) and turning colour olives, respectively. The results of our research showed a drastic reduction of 99.1 and 98.7 % of total biophenols in table olives of Istrska belica and Storta, respectively, already after 60 days of fermentation using modified Spanish style technology (Figs. 1 and 2).

The influence of processing, biophenol content and crop year on microbial population

Yeasts and lactic acid bacteria were monitored during the course of fermentations. When the traditional regional technology was used, the yeast populations increased in the early stage of fermentations (e.g. from 3 to 6.25 log CFU/mL in Istrska belica table olives, crop year

2006, after 3 days; Fig. 1). However, this was not the case when using Spanish style technology due to alkaline treatment of olive fruits before fermentation in brine. Initial reduction of yeast population, e.g. from 3 to 1 log CFU/mL in 2006, was followed by gradual increase after 3 to 10 days, but never reached the concentration of the traditional regional technology (Figs. 1 and 2). The statistical analysis of the data showed statistically significant differences (*t*-test, $\alpha < 0.05$) in the quantity of yeasts between Istrska belica and Storta table olives regardless of the production technology. In the samples of Storta table olives, higher yeast counts and yeast species diversity were determined (Figs. 1 and 2). The observed differences can be related to the differences in total biophenol content of both olive varieties. This was higher in Istrska belica fruits and processed table olives than in Storta. Furthermore, the influence of crop year on the yeast count and species diversity was statistically evaluated. Significant differences (*t*-test, $\alpha < 0.05$) were determined in all samples of the crop year 2006 compared to the samples of the crop year 2007. We assume that in 2007 the higher total biophenol content in the fruits reduced the growth of yeasts, especially in the Istrska belica variety. In spite of the different geographical environments, the yeast population growth in table olives from Slovenian Istria is comparable to the already published data about yeast concentrations. Arroyo López *et al.* (24) stated that the concentrations of yeast in black Hojiblanca table olives and in green Aloreña table olives of Spanish origin increased up to 4 and 6 log CFU/mL, respectively, after five days of fermentation. In general, the populations of yeasts increase from 4 to 7 log CFU/mL during table olive fermentations (7).

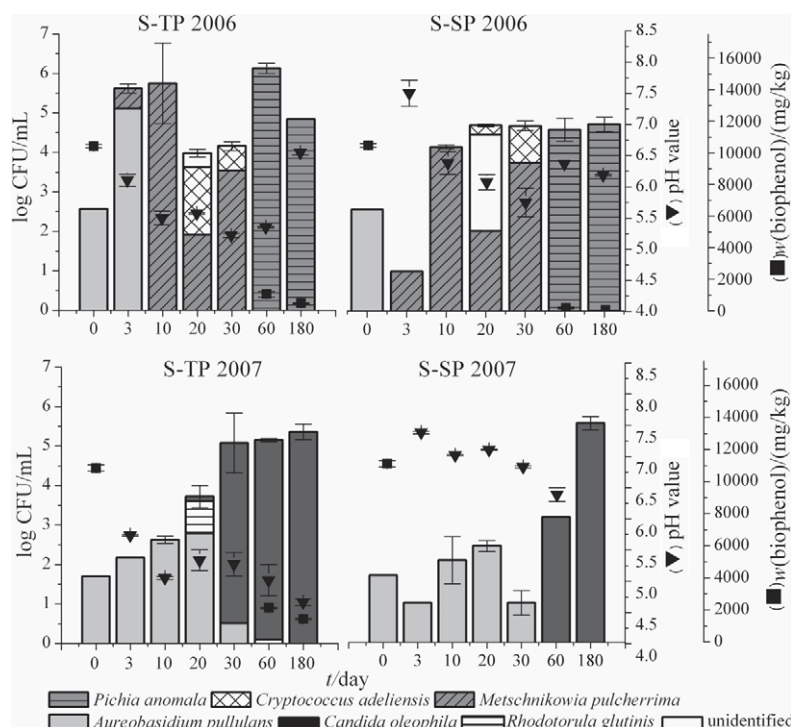


Fig. 2. Yeast population dynamics, pH values and total biophenol content during processing of Storta (S) olive fruits before debittering (0 days) and table olives, processed with traditional regional (TP) and modified Spanish style (SP) technology, crop years 2006 and 2007. The results are averaged from four replicates and are expressed as means±standard deviations (error bars)

Considering the results from the literature, we expected that besides yeasts, lactic acid bacteria would also evolve. The presence of lactic acid bacteria in the fermentations of table olives from Slovenian Istria could not be confirmed. The results from plates with MRS agar were negative. It was assumed that high biophenol content had a negative influence on lactic acid bacteria and, consequently, only yeasts were responsible for table olive fermentation. As it is reported in literature (6), products of biophenol degradation, formed during debittering processes, which have even stronger antimicrobial effect with respect to the original biophenol molecules in olive fruits, could as well contribute to the inhibition of lactic acid bacteria.

With this research, it was ascertained that yeasts are responsible for fermentation of Slovenian table olives with high biophenol content, which presumably inhibited the growth of lactic acid bacteria. The results of pH measurement and microbial growth showed that the use of starter culture can be recommended.

The influence of processing on yeast population dynamics

On the basis of restriction patterns of amplified internal transcribed spacers and 5.8S rRNA gene (PCR-RFLP of ITS), 48 yeast strains isolated in both crop years were grouped into six groups. The representatives of each group were identified by traditional yeast identification procedure and confirmed by *in silico* PCR-RFLP of ITS regions using the pDRAW v. 1.0 software (25) as suggested by Raspor *et al.* (26). The species of *Aureobasidium pullulans*, *Cryptococcus adeliensis*, *Metschnikowia pulcherrima*, *Rhodotorula mucilaginosa*, *Pichia anomala* and *Candida oleophila* represented the leading microflora of Slovenian table olive fermentations. Figs. 1 and 2 present yeast population dynamics during 180 days of fermentation of Istrska belica and Storta table olives, respectively. Because the species *Aureobasidium pullulans* was present in brine solution of all samples (1.5–3 log CFU/mL), it was assumed that it predominated on the surface of olive fruits, as it was reported for olives of Spanish (7,27) and Portuguese origin (28). In our samples of the crop year 2006, the species *Metschnikowia pulcherrima* was isolated after three days of fermentation of both olive varieties with both technologies used. It was confirmed that this species has the capacity to ferment D-glucose and it probably took an active role in the fermentations. Besides *Metschnikowia*, a non-fermentable *Cryptococcus adeliensis*, often isolated from the surface of grapes (14), was also present during fermentations of olives in the year 2006. In the same period of fermentation (days 3–30) of the year 2007, only *Aureobasidium pullulans* species was isolated. Nisiotou *et al.* (29) reported that *Metschnikowia pulcherrima* prevailed in the initial days of fermentation (36–56 %), while *Aureobasidium pullulans* (3–10 %), *Rhodotorula mucilaginosa* (4–9 %) and other species were encountered at rather low relative proportions (≤ 3.5 %).

After 30 days of fermentation of Storta table olives in the year 2006, *Pichia anomala* prevailed in both fermentations, while in the samples of the crop year 2007, the predominant species after 30 days of fermentations

was *Candida oleophila* (Fig. 2). Using the fermentation tests with Durham's tubes, it was confirmed that both prevailing species of late stages of fermentations were fermentative.

Similar yeast populations were also reported by Hurtado *et al.* (30), who isolated yeasts of genera *Cryptococcus* and *Candida* from table olives of Spanish origin, and also by Nisiotou *et al.* (29), who stated that the heterogeneity of population changed with the period of fermentation; they determined prevailing fermentative yeasts of *Pichia anomala*, *Pichia membranifaciens* and *Candida boidinii* between 7 and 35 days of fermentation. Besides *P. anomala* and *Candida boidinii*, Coton *et al.* (31) determined the presence of *Debaryomyces etchellsii* in black table olives of French origin. Nevertheless, the prevalent species during the production of green table olives of Portuguese and black olives of Spanish origin was *P. anomala* (27,32).

The importance of controlling microbial hazards during table olive productions was proven by Pereira *et al.* (28), who isolated fungal nosocomial pathogen *Candida krusei* from the brine of Portuguese table olives. They connected this fact with contamination during the production and packing of table olives, mainly due to the lack of hygiene. The authors stated that it is necessary to improve and optimize the table olive production, which are produced with traditional technology in Portugal, because of a good production practice and food safety.

As it can be seen in Figs. 1 and 2, it can be concluded that the crop year and the total biophenol content of the olives influenced the development of yeast species during the fermentation process more than the production technology of Slovenian table olives.

The influence of production technology on the quality of table olives

To evaluate the influence of production technology on the quality of table olives from Slovenian Istria, sensory analysis was performed after 60 and 180 days of fermentation. The bitterness, hardness, sourness and fibrousness of both table olives were more intense when traditional regional technology was used. Sensory characteristics of Istrska belica table olives are shown in Fig. 3. Statistically significant differences (*t*-test, $\alpha < 0.05$) in the perceived bitterness, sourness and saltiness were determined. It was also observed that the bitter and acid taste were more intense in both table olives produced in the year 2007, while saltiness, hardness and fibrousness were more intense in the samples of the year 2006 (data not shown). We found out that Storta table olives, regardless of the production technology, were more fibrous (data not shown) than Istrska belica table olives. The sensory characteristics of both table olives that were processed for 60 days were more intense compared to the table olives after 180 days of processing. The modified Spanish style technology with initial alkaline treatment was not suitable for retaining the characteristics of our local olive varieties. Istrska belica and Storta table olives after 180 days of processing using the modified Spanish style technology (Fig. 3b) were very mollified and the intensities of the sensory attributes were less expressed.

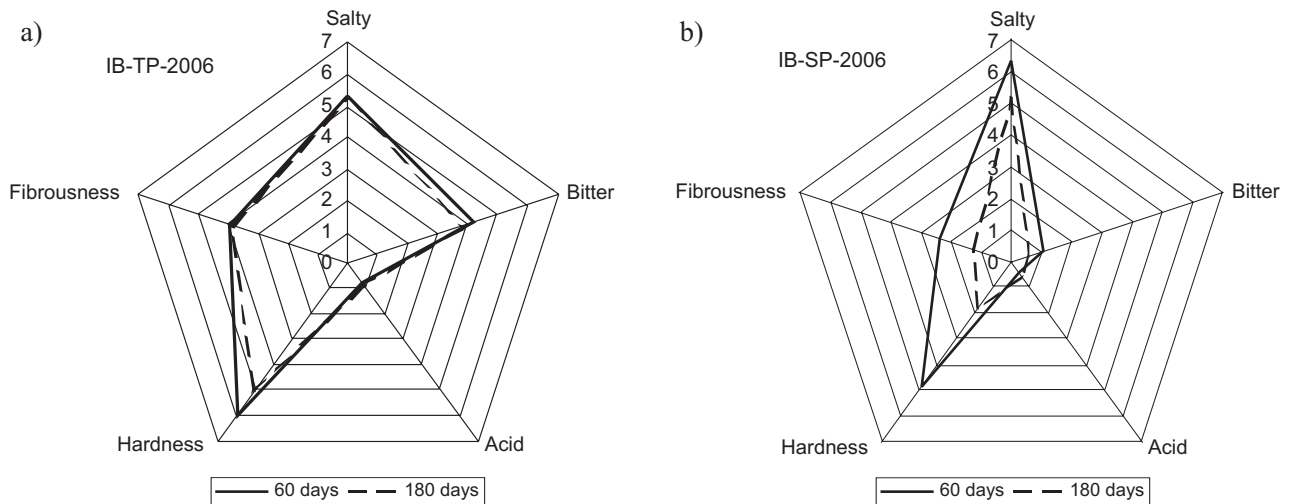


Fig. 3. Graphic representation of the sensory characteristics of Istrska belica (IB) table olives, produced by: a) traditional regional technology (TP), after 60 and 180 days of fermentation, crop year 2006, and b) modified Spanish style technology (SP), after 60 and 180 days of fermentation, crop year 2006. The results are the median intensities of the data obtained from nine assessors

Conclusions

According to the results of this research, yeasts play a dominant role in Slovenian table olive fermentation. Three fermentative and three non-fermentative yeast species were identified according to the restriction fragments of the ITS regions of rDNA and physiological tests. Traditional regional technology was found more suitable for retaining positive characteristics of the studied olive varieties. Using the traditional process, even after 180 days of fermentation, the olives preserved appropriate sensory characteristics. In order to overcome relatively high pH values of the final product, table olives have to be acidified and/or pasteurized. Table olives from Slovenian Istria, produced with traditional regional technology, are also a good source of biophenols as natural antioxidants. However, the high amount of total biophenols contributes to the bitter taste of the table olives, has a negative influence on lactic acid bacteria development and influences population dynamics of yeasts during fermentation.

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