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preliminary communication

Microbial Characterization of Table Olives Processed According to Spanish and Natural Styles**

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Summary

A study on the microflora of table olives »Bella di Cerignola«, produced according to Spanish style and natural processing, is presented. The samples (olives and brines) were taken at different fermentation phases; olives, before treatments, were analyzed too. pH was monitored and microbial populations were assessed by standard plate count. Determination of the following microbial groups was carried out: mesophilic bacteria, lactic acid bacteria, Enterobacteriaceae, Pseudomonadaceae, staphylococci, Micrococcaceae and yeasts. In the second phase, the identification of mesophilic bacteria, lactic acid bacteria and yeasts was performed. The amount of lactic acid bacteria and yeasts increased during the storage in all the samples, but no significant differences were observed between the two styles. At the end of fermentation an increase of Pseudomonadaceae cell load was observed, which was absent in the first phase of fermentation. The samples analyzed were extremely unsteady, therefore the addition of starter lactic acid bacteria could standardize olive processing. *Lactobacillus plantarum*, *Bacillus* spp. (mainly *B. subtilis*) and *Candida* spp. were the predominant species at the end of the processing.

Key words: Bella di Cerignola, table olives, Spanish style and natural processing, microorganisms

Introduction

The Unified qualitative standard applying to table olives in international trade defines table olives as »the sound fruit of specific varieties of cultivated olive tree (*Olea europea sativa*, Hoffm., Link), harvested at the proper stage of ripeness and […] processed as specified in this standard. Such processing may include the addition of various products or spices of good quality« (*1*). The main purpose of processing is, at least, the removal of bitterness of fruit, then the hydrolysis of the phenolic compounds and, particularly, the oleuropein.

Fernandez-Diez (*2*) reported that the table olive preparations of greatest importance in the world are: (*i*) Spanish (or Sevillian) style green olives in brine; (*ii*) Californian style black olives in brine; (*iii*) Greek style naturally black olives in brine. In the first two processings, bitterness is removed by adding lye; in the Greek style process, instead, fruits are placed directly in brine and oleuropein removal is slow and only partial (*3*). The fermentation of Spanish style treated olives is due to lactic acid bacteria, while in Greek processed black olives the organisms responsible for fermentation are yeasts, and lactic acid bacteria form only a small proportion of the total microflora (*3*).

In the Apulian area (Italy), table olives, processed according to Spanish and Greek (labeled also natural) styles, are fermented without the addition of lactic acid bacteria starter cultures; consequently, the control of the

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fermentation process is limited to the mantainance of the olive ecosystem (*4*). Olive ecosystem is influenced by many factors; the most important are: (*i*) indigenous microbial association; (*ii*) pH, water activity of olives and availability of nutrients, diffusing from tissue; (*iii*) levels of phenolic compounds and organic acids; and (*iv*) temperature of fermentation and the concentration of salt in brine (*5*).

Altough table olives are a popular product in Italy, to our knowledge, few studies have been conducted regarding the microbial characterization of Italian table olives (*6*). On that basis the aims of this work were: monitoring the microbial changes during the fermentation of table olives »Bella di Cerignola«, an indigenous cultivar of Apulian area; evaluating the differences in olives and brines, processed according to Spanish and natural (Greek) styles; and characterizing microbial populations.

Materials and Methods

Preparation of olives

Experiments were carried out with green olives of the »Bella di Cerignola« variety purchased from Santo Stefano, a local establishment of Apulian area. The samples were processed according to two different styles: Spanish style and natural processing, which can be distinguished on the basis of the treatment of the fruits with NaOH before brining.

On arrival, olives were subjected to grading: olives with blemishes, cuts and insect damage were discarded and the remainder was washed with tap water. In Spanish style processing, bitterness is removed by lye treatment, so the fruits were treated with NaOH (1.3–2.6 %) for 12–15 h before brining. If the lye treatment is correct, a quick rinse immediately after the treatment, followed by the first washing of 2–3 h and the second one of 10–12 h may be a suitable schedule. The following step was the fermentation in brine (this phase is common for Spanish style and natural processing). Fermentation took place in a container with 140 kg of olives and 80 L of brine (NaCl 10 %) at ambient temperature (20–25 °C). For each processing, two batches, labelled A and B, were analyzed.

Microbiological analyses

Microbiological analyses were performed both on olives and brine and for each fermentation phase (raw material and after 8, 17, 31 and 80 days). For each sample aliquots of 20 g of olives and 20 mL of their brines were diluted with 0.9 % NaCl solution (180 mL), homogenized with a Sterilmixer (PBI International, Milan, Italy) and the dilutions were plated in duplicate onto appropriate media. The media and the conditions were the following: plate count agar (PCA) incubated at 30 °C for 48 h for mesophilic bacteria; MRS agar with 0.17 g/L of cycloheximide (Sigma, Milan, Italy), incubated at 30 °C for 4 days in anaerobiosis for lactic acid bacteria; violet red bile glucose agar (VRBGA), incubated at 37 °C for 18–24 h for Enterobacteriaceae; Baird-Parker agar base, with egg yolk tellurite emulsion, incubated at 37 °C for 48 h for staphylococci and Micrococcaceae; Sabouraud dextrose agar, with 0.1 g/L of chloramphenicol (C. Erba, Milan, Italy), incubated at 25 °C for 3–4 days for yeasts and moulds; *Pseudomonas* agar base, with *Pseudomonas* CFC Supplement, incubated at 30 °C for 48 h for Pseudomonadaceae.

All the media and the supplements used were from Oxoid (Milan, Italy). Microbiological data are the average of two replicates. The cell load data, collected during the storage of the products, were analyzed by one-way ANOVA (Statsoft, Tulsa, USA) and modelled according to Gompertz equation modified by Zwietering *et al.* (*7*):

$$y=k+A^{-[(\mu_{max}\cdot e/A)\,\cdot\,\lambda_t+1]}$$

where k is the initial cell load (logCFU/g); A is the maximum bacteria growth obtained at the stationary phase (logCFU/g); μ_{max} is the maximal growth rate [ΔlogCFU/(g·h)] and λ the lag phase (days).

pH measurements

The pH measurements of brines and olives homogenate were obtained with a Crison pH meter model 2001 (Crison Instruments, Barcelona, Spain), calibrated with two standard solutions buffered at pH=4.00 and 7.02.

Identification of microorganisms

Bacteria

For identification, six colonies of each different bacterial morphological type were selected from primary cultures and kept on MRS agar (lactic acid bacteria) and on PCA (mesophilic bacteria) at 4 °C until they were identified. Mesophilic bacteria strains were grouped on the basis of staining reaction, catalase test, oxidative-fermentative metabolism of glucose, motility reaction, cell shape and spore formation by heating cultures at 80 °C for 10 min and successive plating on PCA, according to Collins *et al.* (*8*).

Lactic acid bacteria strains, instead, were grouped on the basis of staining reaction, catalase test, growth at 15 and 45 °C, esculin hydrolysis, and NH_3 and CO_2 production. The isolates were identified at the species level, using the appropriate API identification system (bioMérieux, France).

Yeasts

For identification, five colonies of each different bacterial morphological type were selected from primary cultures and kept on Sabouraud dextrose agar (Oxoid) at 4 °C until they were identified. The isolates were characterized according to the method of Van der Walt and Yarrow (*9*) and by using the API ATB ID32C system (bioMérieux). Identification was carried out by comparing the test results with the tables of Kurtzman and Fell in *The Yeasts: A Taxonomic Study* (*9*).

Results

Evaluation of cell load data

The fermentation of table olives involves a complex microflora of lactic acid bacteria, yeasts, Gram-positive and Gram-negative bacteria (*2*). The Gompertz parameters of lactic acid bacteria in olives and brines are reported in Tables 1 and 2. There were no significant dif-

Table 1. Gompertz parameters of lactic acid bacteria of table olives »Bella di Cerignola«, processed according to Spanish and natural styles

Samples	A/(logCFU/g)	μ_{max}/[ΔlogCFU/(g·h)]	λ/day	R
Spanish A	6.74±0.16	0.43±0.03	7.95±0.69	0.999
Spanish B	6.65±0.18	0.55±0.12	12.47±1.07	0.999
Natural A	6.81±0.36	0.39±0.06	7.55±1.49	0.999
Natural B	7.49±0.01	0.41±0.00	8.48±0.02	1.000

A – maximum cell load obtained at the stationary phase; μ_{max} – maximal growth rate; λ – lag phase; R – correlation coefficient

Table 2. Gompertz parameters of lactic acid bacteria of brines of table olives »Bella di Cerignola«, processed according to Spanish and natural styles

Samples	A/(logCFU/g)	μ_{max}/[ΔlogCFU/(g·h)]	λ/day	R
Spanish A	7.10±0.36	0.78±0.09	8.00±0.60	0.998
Spanish B	7.78±0.17	0.64±0.11	12.49±0.88	0.999
Natural A	6.18±0.35	0.42±0.11	12.73±1.58	0.999
Natural B	7.06±0.35	0.44±0.09	12.24±1.44	0.999

A – maximum cell load obtained at the stationary phase; μ_{max} – maximal growth rate; λ – lag phase; R – correlation coefficient

ferences between Spanish and natural style olives, as shown by Gompertz parameters; higher cell load in the batch B of natural processing and higher λ value of batch B of Spanish style could be attributed to an intrinsic variability of the sample.

In brines the value of parameter A of lactic acid bacteria (Table 2) in batches processed according to Spanish style was higher than that observed in the same batches but treated with natural processing.

Pseudomonadaceae (Fig. 1) were absent in the 1st phase and increased in the latter days of fermentation.

Microbiological analyses of yeasts in olives (Fig. 2) showed that their cell load was higher than 2 A/(log CFU/g) on the raw material and during the 1st fermentation phase and it increased during the storage of products. As for lactic acid bacteria, no significant differences

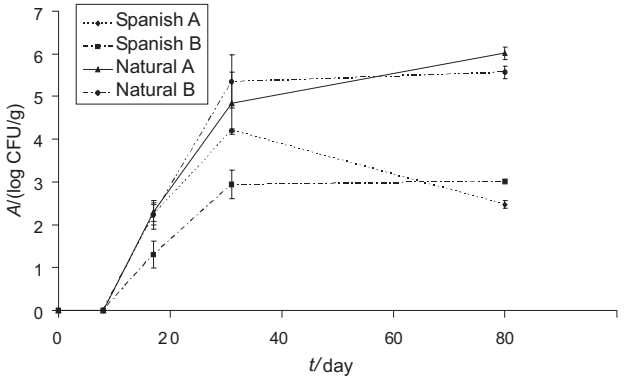

Fig. 1. Pseudomonadaceae cell load of table olives »Bella di Cerignola«, processed according to Spanish and natural styles

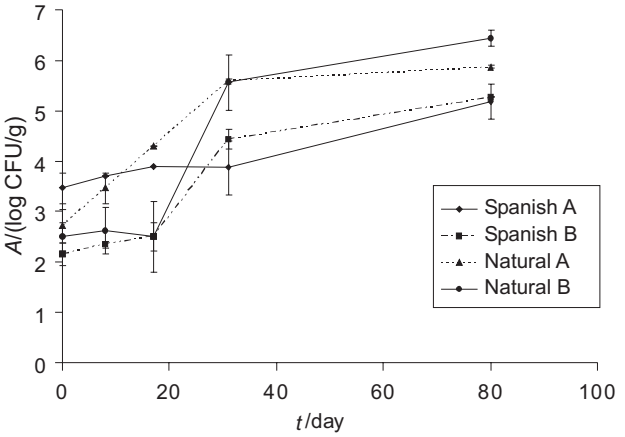

Fig. 2. Yeast cell load of table olives »Bella di Cerignola«, processed according to Spanish and natural styles

were observed between the two styles. Moulds were absent during all the fermentation phases (data not shown).

Staphylococci were at undetectable levels in samples processed according to Spanish style, both in olives and brines, at the beginning of the fermentation; they increased in the 1st phase of fermentation and reached the maximum cell load after 17 days of fermentation. In the latter phases, a decrease of cell load was observed (data not shown).

Enterobacteriaceae and Micrococcaceae remained constant at 2nd A/(logCFU/g) in all the samples of analyzed olives, during the 80 days of fermentation (data not shown).

As observed in Figs. 3 and 4, pH value increased during the 1st phase of fermentation, both in brines and olives treated according to Spanish style, as a consequence of the addition of lye. In the latter phases a decrease of pH value was observed.

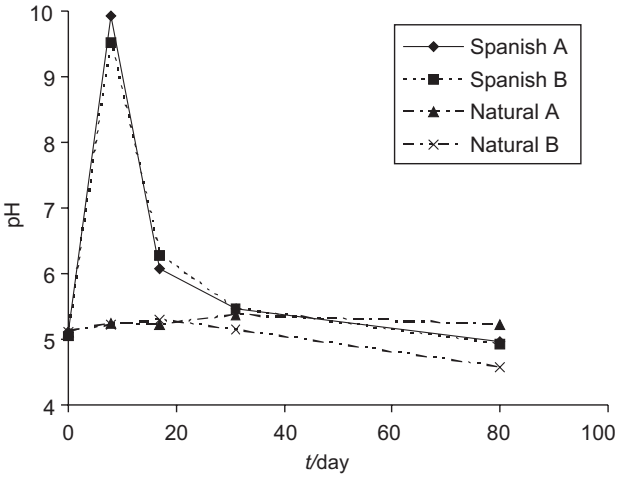

Fig. 3. Changes of pH in olives during fermentation

In samples processed according to natural style, the pH remained at ~5.0 during scientific observation. Only in batch B, both in olives and brine, a short decrease was observed.

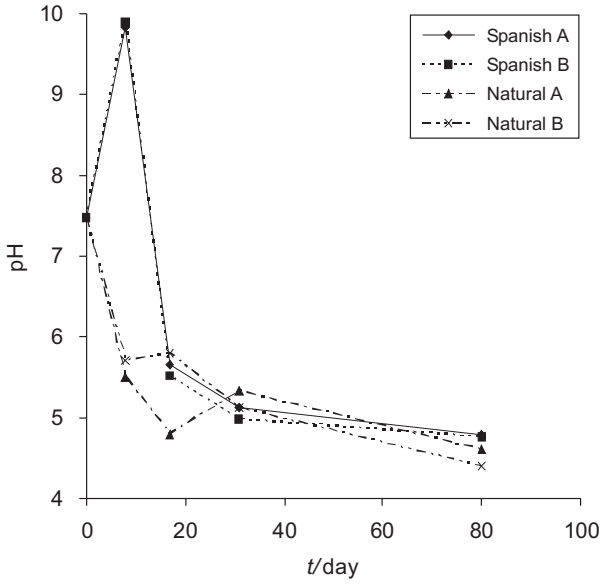

Fig. 4. Changes of pH in brines during fermentation

Characterization of microflora

The identification at the species level of lactic acid bacteria showed that *Lactobacillus plantarum* was the predominant species on the olives, although *Lactobacillus pentosus* and *Leuconostoc mesenteroides* were present too.

In Table 3 the frequency of the identified yeast species in table olives of Spanish style and natural processing is reported. Qualitative composition of yeast populations pointed out that *Candida* was the mainly isolated genus. *Rhodotorula mucilaginosa* and *Cryptococcus laurentii* were identified too.

Among the mesophilic population, *Bacillus* spp. was predominant on the raw material; in the 1st phase of fermentation *Enterobacter cloacae*, *Enterobacter amnigenus* and *Chryseobacterium* spp. were isolated. In the latter days of storage *Bacillus subtilis* was mainly isolated and identified on olives, among mesophilic bacteria population (data not shown).

Discussion

As reported in literature (*3*), the predominant microorganisms in Spanish style treated olives are lactic acid bacteria; yeasts, instead, are the organisms responsible for the fermentation of olives in natural processing.

Nychas *et al.* (*5*) reported that yeasts tended to predominate on the skin surfaces and in the stomal openings of olives, whereas bacteria predominated in the intercellular spaces of the sub-stomal cells.

Our observations confirmed the importance of yeasts and lactic acid bacteria in a correct development of fermentation, but there were no significant differences between Spanish style and natural processing. The fermentation, in fact, relied upon microflora naturally present on raw material or in the containers in which the olives were stored. This practice could lead to variation in the quality and flavour of the product and to the spoilage of olives (*6*).

During storage, the growth of cellulolytic yeasts and contaminant microorganisms of genus *Bacillus* can cause the softening of olives (*6*); on the contrary, the development of proteolytic microorganisms, like *Pseudomonas* spp., followed by decarboxylation and deamination of the resulting aminoacids by heterofermentative lactobacilli (*10*), could cause an unusual type of spoilage, characterized by a decrease in the acidity of brines and swelling.

The isolation of *Bacillus subtilis* and the increase of Pseudomonadaceae in the latter phases of fermentation, as well as the presence of *Candida* spp., could be the signs of the beginning of spoilage. Degradative species of yeasts, belonging to the genus of *Candida*, could cause organoleptic decay at cell load higher than 10^6 CFU/g (*11,12*), but this cell load was never reached in our experimental conditions.

Table 3. Qualitative composition of yeast population of table olives »Bella di Cerignola«, processed according to Spanish and natural styles

Time[b]/ day	Spanish style		Natural style	
	Batch A	Batch B	Batch A	Batch B
0	50 % *Candida pelliculosa*[a] 50 % *Candida ciferrii*	50 % *Candida pelliculosa* 50 % *Candida ciferrii*	50 % *Candida pelliculosa* 50 % *Candida ciferrii*	50 % *Candida pelliculosa* 50 % *Candida ciferrii*
8–17	60 % *Candida pelliculosa* 20 % *Rhodotorula mucilaginosa* 10 % *Candida ciferrii* 10 % *Candida glabrata*	80 % *Candida pelliculosa* 20 % *Candida ciferrii*	66.7 % *Candida ciferrii* 33.3 % *Candida pelliculosa*	42.8 % *Rhodotorula mucilaginosa* 28.6 % *Candida pelliculosa* 28.6 % *Candida glabrata*
31	100 % *Candida pelliculosa*	66.7 % *Candida pelliculosa* 11.1 % *Candida glabrata* 11.1 % *Candida ciferrii* 11.1 % *Cryptococcus laureentii*	50 % *Candida pelliculosa* 16.7 % *Candida glabrata* 16.7 % *Candida ciferrii* 16.7 % *Cryptococcus laurentii*	66.7 % *Candida pelliculosa* 33.3 % *Candida ciferrii*
80	100 % *Candida glabrata*	81.8 % *Candida pelliculosa* 9.1 % *Candida glabrata* 9.1 % *Cryptococcus laurentii*	60 % *Candida ciferrii* 40 % *Candida glabrata*	83.3 % *Candida pelliculosa* 16.7 % *Candida glabrata*

[a]calculated as [(number of isolated species/total number of isolated species) · 100]
[b]time of fermentation

pH values of the analyzed samples (about 5.0) were similar to those observed by other authors (*6*) in the olives of Apulian area and could explain the presence of *Bacillus subtilis* and Pseudomonadaceae in the latter phases of fermentation. However, both Spanish and natural processing (adopted by Santo Stefano establishment) include a thermal treatment which preserves the final product from microbiological spoilage.

Microflora of lactic acid bacteria was mainly composed of homofermentative strains; consequently, a good quality of the product was obtained thanks to the combination of the selection of homofermentative lactobacilli, which led the fermentation to a successful conclusion, and a thermal treatment, which preserved the product from further environmental contaminations.

The analyzed samples were extremely unsteady; the addition of starter lactic acid bacteria could standardize olive processing and it would be better to stop the fermentation in the 3rd phase when *Bacillus subtilis* and Pseudomonadaceae were at low levels.

Conclusions

The market of table olives is booming, although it is supported neither by an adequate productive facilities nor an appropriate training of personnel (*6*). In fact, table olives are the major fermented vegetables in the western countries (*13*). Their production in the Apulian area is based on the work of artisans, without the addition of microbial starter or acidified solution, which can standardize the processing and the quality of the product. These conditions and seasonable raw material caused a strong variability of samples in our experiments. Generally, in natural fermentation »good« strains of lactic acid bacteria prevailed on the natural microflora; they provided for the correct course of processing, but the absence of starter bacteria could favour the prevailing of spoilage microorganisms and this could be a critical point during the fermentation for the quality of product. In conclusion, it is necessary to control the fermentation process by adding lactic acid bacteria starter or technological adjuvants, in order to obtain high quality of products and good acidification of olives.

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Mikroflora stolnih maslina proizvedenih španjolskim i prirodnim postupkom

Sažetak

Istraživana je mikroflora maslina »Bella di Cerignola« proizvedenih španjolskim i prirodnim postupkom. Uzorci maslina i rasola uzimani su iz raznih fermentacijskih faza, a masline su analizirane i prije obrade. Praćena je promjena pH, a mikrobna je populacija utvrđena standardnom procjenom na pločama. Utvrđene su sljedeće mikrobne skupine: mezofilne bakterije, stafilokoki, bakterije mliječne kiseline, bakterije iz porodica Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae i kvasci. U drugoj fazi fermentacije identificirane su mezofilne bakterije, bakterije mliječne kiseline i kvasci. Tijekom skladištenja u svim je uzorcima porastao broj bakterija mliječne kiseline i kvasaca, ali ne postoji značajna razlika u uzorcima dobivenim španjolskim ili prirodnim postupkom. Na kraju fermentacije

povisio se broj stanica Pseudomonadaceae kojih nije bilo u prvoj fazi fermentacije. Ispitivani uzorci bili su vrlo nepostojani, pa se za standardizaciju prerade maslina dodala starter kultura bakterija mliječne kiseline. Na kraju prerade prevladavale su vrste bakterija *Lactobacillus plantarum*, *Bacillus* spp. (uglavnom *B. subtilis*) i kvasci roda *Candida*.