Abnormal fermentations in table-olive processing: microbial origin and sensory evaluation

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INTRODUCTION

The production of fermented foods is one of the oldest biotechnologies known to mankind. In particular, the fermentation of vegetables, which is a practice that originated in the Orient, has been used as a means of preserving food for more than 2,000 years. Table olives (Olea europaea L.) are fermented products that are widely diffuse (Garrido Fernandez et al., 1997). The most important production zone of table olives is the Mediterranean area, although olives are consumed on a large scale all over the world. Indeed, their consumption is also expanding, due to the increasing popularity of the Mediterranean diet. Olives are picked at different stages of maturity, and they are then processed to eliminate the characteristic bitterness caused by their oleuropein glucoside, and thus to make them suitable for human consumption. There are several ways to prepare table olives, but the most widespread methods are known as “treated green olives in brine in the Greek style” (Balatsouras, 1990). The process of transformation of table olives from tree to table is the result of complex biochemical reactions that are determined by the interactions between the indigenous microflora of the olives, together with a variety of contaminating microorganisms from different sources (fiberglass fermenters, polyvinyl chloride (PVC) tanks, pipelines, pumps, and water), with the compositional characteristics of the fruit. One of the most important aspects of improving the quality of table olives is the use of selected microorganisms to drive the fermentation. These can supplant the indigenous microflora and, in particular, the complementary microflora that are responsible for spoilage of canned olives. In this context, from a technological point of view, a well-characterized collection of microorganisms (lactic acid bacteria, yeast) that can be isolated from the matrix to be processed (the olive fruit) will provide the basis for the development of starter culture systems. These cultures can be fully compatible with the typical products and will guarantee high quality standards. Inoculation of the brine with such selected starter cultures will reduce the probability of spoilage, and help to achieve an improved and more predictable fermentation process. Control of the fermentation processes can thus occur through chemical, chemophysical and microbiological approaches, and since 2008, also through organoleptic evaluation (COI/CT/MO/Doc. No 1. Method for the sensory analysis of table olives). This last has established the necessary criteria and procedures for sensory analysis of the negative, gustatory and kinaesthetic sensations of table olives, which can also be attributed to abnormal proliferation of microorganisms. It also sets out the system for commercial classification, through assessment of the median of the defect predominantly perceived.

MAIN MICROBIAL ALTERATIONS DURING TABLE-OLIVE PROCESSING

Briefly, the Seville or Spanish system consists of alkaline treatment with NaOH or lye (1.5–4.5 ◦C) to remove the bitterness of the olive fruit, which is followed by water washing to eliminate the residual lye, and then fermentation in brine to enhance the nutritional and sensory characteristics of the fruit. After the alkaline treatment, the pH of the olive flesh reaches 11.0–13.0, which is reduced to 8.0–9.0 after repeated washing.

After the washing, the olive fruit are immersed in 6–10% NaCl solution (brine). Spontaneous fermentation starts as soon as the olives are in the brine. In the first phase of fermentation, when the Gram-negative bacteria prevail, the pH decreases from 8.0 to 9.0 to about 6.0. This phase lasts until the development of lactic acid bacteria (normally after 48–72 h). Most of the microorganisms that develop in this first phase are Gram-negative (Enterobacter, Citrobacter, Aeromonas, Escherichia, and Klebsiella). If the decrease in the pH during the first few days of fermentation is not fast enough, deterioration of the olives can quickly set in, due to Enterobacteraeaceae and other microbial groups that can reach high cell densities.
and form “gas pockets,” resulting in softening and breakage of the cuticle, and other defects.

During the first phase of the fermentation process, there are frequent cases where these gas-generating Gram-negative bacteria can take over, which consume the sugars and release CO₂. This CO₂ can accumulate as pockets of gas below the epidermis (hypocuticular gas pockets) or within the pulp itself (intramesocarpic gas pockets, Figure 1). The olives affected by these changes appear to show bubbles on their surface, which is known as “fish-eye” (Vaughn et al., 1972), or as a narrow belt, which is known as “olive gated” or “alambrado” (Gililland and Vaughn, 1946; Borbolla y Alcalá et al., 1959, Borbolla y Alcalá et al., 1960). High pH can also contribute to the development of Clostridium, which results in fermentation that is termed putrid (reminiscent of the smell of rancid butter). This malodorous fermentation caused by butyric anaerobes such as Clostridium butyricum produces olives that are completely fissured (Gililland and Vaughn, 1946). The gas evolved reduces the density of the fruit, which then float on the surface. This process can also lead to the appearance of olives without gas pockets, due to the development of the pulp cavity, which sometimes extends through the core, and which is due to the accumulation of CO₂ from the respiration of olive tissue and the activity of certain microorganisms that release CO₂ as a normal product of their metabolism (Table 1). To reduce the appearance of this defect, an aerobic fermentation system has been developed, in which air is blown into the container (the fermenter) where the processing takes place (Borbolla y Alcalá et al., 1968; Garrido Fernández et al., 1987; Marsilio et al., 2008a,b). This system removes the CO₂, which induces a change in the predominant microflora in the process, with particular reference to yeast. This treatment thus supports the aerobes with so-called oxidative metabolism (which transform nutrients in the presence of oxygen) at the expense of those of fermentation (metabolism that takes place in the absence of oxygen).

The development of pectinolytic (Saccharomyces oleaginosus, S. kluveri, Hansenula anomala, Pichia manshurica, Pichia kudriavzevii, Candida boidinii, Rhodotorula minuta, R. rubra, Rhodotorula glutinis, Aspergillus niger, Penicillium sp. and Fusarium sp.) and cellulolytic (Cellulomonas sp.) yeast and moulds is associated with “softening” of the fruit. This is due to the action of their degrading enzymes that, respectively, act on pectic substances that form the middle lamella, which leads to cell separation, and act on cellulose, hemicellulose and polysaccharides, which damages the cell walls (Vaughn et al., 1969; Vaughn et al., 1972; Arroyo-López et al., 2012; Golomb et al., 2013). The softening of the fruits is also associated with the presence of Bacillus and Gram-negative organisms that are normally present in this phase (Nortje and Vaughn, 1953). A second phase begins when the pH reaches about 6.0, and this stage lasts until the development of lactobacilli (normally 2 weeks). During this phase, the Gram-negative microorganisms progressively decrease, until they disappear altogether. Reducing sugars and glucosides, the basic sources of carbon needed for the development of lactobacilli and other microorganisms, pass from the olive flesh into the brine, where they are used by heterofermentative or homofermentative microorganisms that transform them into lactic acid.

Most of the microorganisms that grow in this second phase are lactococci, within the genus Pedococcus (homofermentative strain) and Leuconostoc (heterofermentative strain). These produce lactic acid, which contributes to the further lowering of the pH. This then favors the growth of lactobacilli (in the third phase) that are aciduric, with their optimal growth between pH 5.5 and 5.8. This phase is characterized by abundant growth of homofermentative lactobacilli, with a predominance of Lactobacillus plantarum. One of the most common abnormalities associated with olives treated with the Seville style is known in the industry as “yeast or white spots.” These are small white spots that can develop between the skin and the flesh of the olive. Microscopic and microbiological studies have shown that this defect is due to L. plantarum bacteria colonies, rather than to yeast (Vaughn et al., 1953; Samish and Dimant, 1963; Garrido Fernandez et al., 1987; Kallis and Harris, 2007).

A population of yeast with fermentative metabolism can then co-exist with the lactic acid bacteria. If these yeast do not become prevalent, they are not considered to be harmful to the process, and indeed, they can help to enhance the sensory properties of the

FIGURE 1 | Olive fruit showing hypocuticular (A) and intramesocarpic (B) gas-pockets.
Table 1 | The main microbial alterations during table-olive processing. Red, defects that are detectable by sensory analysis.

| Spoilage                     | Microorganism responsible | Reference                   |
|------------------------------|----------------------------|-----------------------------|
| Gas-pockets                  | Saccharomyces kluyveri     | Duran Quintana et al. (1979)|
| (fish-eye and alambriado)    | Saccharomyces cerevisiae   | Vaughn et al. (1972)        |
|                              | Pichia anomala             | Borbolla y Alcalá et al. (1960)|
|                              | Enterobacter sp.           | Gilliland and Vaughn (1946) |
|                              | Citrobacter sp.            |                             |
|                              | Aeromonas sp.              |                             |
|                              | Escherichia sp.            |                             |
|                              | Klebsiella sp.             |                             |
|                              | Clostridium butyricum      | Levin and Vaughn (1966)     |
| Putrid fermentation          |                             |                             |
|                              | Clostridium butyricum      | Gilliland and Vaughn (1946) |
| Butyric fermentation         |                             |                             |
|                              | Clostridium beijerininki   |                             |
|                              | Clostridium lemallocys     |                             |
|                              | Clostridium acetobutylicum |                             |
| Zapateria                    | Propionibacterium tetronae | Gonzalez Cancho et al. (1968)|
|                              | Propionibacterium zeae     | Gonzalez Canche et al. (1973)|
|                              | Propionibacterium acma     |                             |
|                              | Clostridium spongogas      | Kawamoto and Vaughn         |
|                              | Clostridium bifurcante     | (1956)                      |
| Musty                        | Penicillium crustosum      | Marsiko and Spotti (1967)   |
|                              | Penicillium diphtropium    |                             |
|                              | Penicillium roqueforti     |                             |
|                              | Penicillium simplicissimum |                             |
|                              | Penicillium aurantquisum   |                             |
|                              | Penicillium expansum       |                             |
|                              | Penicillium herquei        |                             |
|                              | Penicillium viridicatum    |                             |
|                              | Aspergillus niger          |                             |
|                              | Alternaria alternata       |                             |
| Winey-vinery                  | Yeast with alcoholic       | Borbolla y Alcalá et al. (1960)|
|                              | metabolism                 |                             |
|                              | Acetic bacteria            |                             |
| Yeast or white spots          | Lactobacillus plantarum    | Samilu and Dimont (1963)   |
|                              |                            |                             |
| Botulism                      | Clostridium botulinum      | Casioy b. et al. (2006)    |
| Softening                     | Saccharomyces cerevisiae   | Dolom et al. (2013)        |
|                              | Pichia anomala             | Arroyo-López et al. (2012) |
|                              | Saccharomyces kluyveri     | Vaughn et al. (1972)        |

(Continued)
lye has previously been used to remove the bitter glucoside. Thus, the importance of this type of starter is to reduce the lag phase and the risk of spoilage (Ruiz-Barba et al., 1994; Vega-Leal-Sánchez et al., 2003; Bevilacqua et al., 2010).

Secoiridoid glucosides (oleuropein, demethyl-oleuropein, ligstoside) and other β-glucosides are the principal fermentative substrates in the olive fruit. These are enzymatically hydrolyzed by β-glucosidase (E.C.3.2.1.21), which releases glucose and aglycones. The aglycones can then be completely degraded by esterases (E.C. 3.1.1.1., E.C. 3.1.1.2.) into simple and non-bitter phenolics, such as hydroxytyrosol, tyrosol, and elenolic acid. Some strains of L. plantarum can produce β-glucosidase and esterase and use the glucose from the β-glucosides as a source of carbon. Therefore, these strains can hydrolyse oleuropein and other bitter glucosides, which contributes to the debittering process. The degradation of oleuropein is evaluated by inoculation of the strains to be tested in MRS broth without glucose and with the addition of oleuropein, with analysis for the degradation products (hydroxytyrosol, aglycones) by gas chromatography (Ciafardini et al., 1994) or high performance liquid chromatography (HPLC; Lanzę, et al., 2010; Zago et al., 2013). The evolution of individual phenolic compounds upon processing of the Ascolana cv. olive fruit without and with lactobacilli as an inoculant has been described previously (Marsilio et al., 2006). In that study, the degradation rate of oleuropein was faster in the presence of the starter inoculants, with only trace levels after 15 days of fermentation, thereby defining a significant role of lactobacilli in olive debittering. Similarly, decreases in oleuropein and increases in hydroxytyrosol have been shown, which reduced the debittering phase to 8 days during controlled fermentation of Leccino cv. olives using L. pentosus 1MO as a starter culture (Servili et al., 2006). Six L. plantarum strains studied by Zago et al. (2013) showed a high degree of oleuropein degradation after 24 h, and this glucoside completely disappeared after a week. These results were confirmed by hydroxytyrosol accumulation. In particular, strains Lp793, B51, and Lp994 showed the highest degrees of oleuropein degradation and hydroxytyrosol accumulation. Other studies have isolated and selected oleuropeinolytic lactic acid bacteria from fermenting Moroccan green olives, including L. plantarum, L. brevis, and Pediococcus pentosaceus (Ghaibour et al., 2011).

**PHAGE ATTACK**

The presence of bacteriophages in the brine of table olives might be a cause for failure of the acidification process carried out by the lactic acid bacteria. This can have a more or less serious impact on the technological process and the final product characteristics (e.g., abnormal fermentation, inhibition of starter culture). A phage infection in the processing of table olives can be the real obstacle to the use of starter cultures as inoculum (Lanza et al., 2012). In addition to the safeguarding of traditional products, mixed starters or natural brine that are used as “mothers” are generally not very sensitive to phages as the complex microbial composition provides a kind of self-defense. This is because different strains can sustain an attack by a phage without serious repercussions on the fermentation (resistant strains prevail, while the sensitive strains are lysed). The bacteriophages are also a useful tool for the typing of strains. Phages can provide a sort of natural selection across strains with different phage sensitivities,
by helping to regulate the development of these microbial ecosys-
tems. Indeed, a protocol previously described for dairy starters was
adapted in our laboratory to search for the presence of viral parti-
cles in brine. This involves: (a) incubation for 24 h of strains used
as starters in MRS-Ca\textsuperscript{2}\textsuperscript{+} broth medium; (b) addition of an aliquot
of the brine to be tested (in this case the same brine as that used
for formation of the matrix of yeast) (COI/OT/MO No 1/Rev. 2). This
is applicable solely to the fruit of the cultivated olive tree (O.
 europaea L.) that has been suitably treated or pro-
cessed, and has been prepared for trade or for final consump-
tion as table olives, in accordance with the trade standards appling
to table olives (COI/OT/NC No 1, 2004). This method estab-
lished the necessary criteria and procedures for sensory analy-
sis of the quality of the processing of table olives. This includes:
(a) the presence of any visible defects, (b) the presence of any
inappropriate use of acids as correctives for acidity (e.g., citric
acid). High levels of acid sensation are also found in olives that
have been prepared with the addition of vinegar (e.g., Kalamata
olives). Finally, through this sensory evaluation, it is possible to
define the level of softening of the fruit, which corresponds to
the low levels of the kinaesthetic sensation of hardness. The main
microbial alterations in table olives that are detectable by sensory
analysis are shown in Table 1.

CONCLUSION
Transformation of table olives from the tree to the table is the
result of complex biochemical reactions that depend on interac-
tions between the indigenous microflora of the olives, together
with a variety of contaminating microorganisms from different
sources (fiber-glass fermenters, PVC tanks, pipelines, pumps, and
water), with the compositional characteristics of the fruit. One
of the most important aspects for the improvement of the qual-
ity of table olives would be the use of selected microorganisms
to drive the required fermentation. These would also supplant
the indigenous microflora and those that are responsible for
spoilage of canned olives. In this context, a well-characterized
collection of microorganisms (lactic acid bacteria, yeasts), possi-
bly isolated from the matrix to be processed (the olive fruit),
could provide the basis for the development of starter cul-
tures, while remaining fully compatible with the typical prod-
ucts, to guarantee the maintenance of high quality standards.
Finally, a phage-database is a natural and necessary comple-
ment of a collection of lactic acid bacteria of technological interest.

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