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Use of Starter Cultures in Olives: A Not-Correct Use Could Cause a Delay of Performances

Marianne Perricone, Maria Rosaria Corbo, Milena Sinigaglia, Antonio Bevilacqua*

Department of Agriculture, Food and Environmental Science, University of Foggia, Foggia, Italy.
Email: *a.bevilacqua@unifg.it, *abevi@libero.it

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ABSTRACT

The role of lactic acid bacteria for a correct course of fermentation of table olives has been extensively reviewed and it is a common idea that the *inoculum* of selected strains could exert a strong benefit for product quality; however, the basic assumption of this research is that a not-correct preparation of starter could affect its performances in brines and delay the benefit of a starter *inoculum*. Thus, some selected strains of LAB (lactic acid bacteria) and yeasts were inoculated into brines of Bella di Cerignola olives (Spanish style), as a single starter (LAB or yeasts) or a combined preparation (LAB + yeasts) and compared to a natural fermentation, focusing on the performances of the process (acidification, production of lactic acid) and trying to verify the basic assumption. LAB and yeasts were grown in the opportune lab media and then directly inoculated in brine after the lye treatment. LAB exerts a kind of benefit (increased content of lactic acid) only for 2 - 3 weeks; then, the inoculated samples behave in the same way as not-inoculated ones. Thus, this research underlined that a starter culture, not prepared in the correct way, could not be useful to guide the fermentation.

Keywords: Olives; Lactic Acid Bacteria; Yeasts; Delay in Fermentation; Starter

1. Introduction

Fermentation of table olives starts spontaneously and is strongly influenced by olive cultivar, indigenous microbiota and methodological factors, such as temperature and salt concentration in the brine [1].

Moreover, olive fermentation relies on a complex microbiota, including *Enterobacteriaceae*, lactic acid bacteria and other *genera* of Gram positive microorganisms and yeasts; the traditional fermentations are usually based on the maintenance of this association [2].

In a natural olive fermentation after an initial stage, characterised by the growth of Gram negative bacteria, lactic acid bacteria and yeasts coexist until the end of fermentation; in particular, in Bella di Cerignola olives, yeasts were found at concentrations of approximately 5 - 6 log·cfu/g on olives and 6 log·cfu/ml in the brines [3,4]. On the other hand, Hernandèz, Martin, Aranda, Pèrez-Nevado and Còrdoba [5] recovered a yeast number of about 3.0 log·cfu·g/l and 4.9 log·cfu/ml in Spanish green olives and their brines, respectively. The role of lactic acid bacteria for a correct course of fermentation of table olives has been extensively reviewed and it is a common idea that the *inoculum* of selected strains could exert a strong benefit for product quality [2].

For a better course of the process and to control naturally occurring microflora, many authors recommended the inoculation of brine with commercial strains; the use of suitable *Lactobacillus plantarum* starter cultures has the potential to improve the microbiological control of the process, increase the lactic acid yield and, accordingly, provide the production of Spanish-style fermented green olives of consistently high quality [6]. Recently, some authors proposed a mixed starter, containing a combination of LAB and yeasts [4].

During the fermentation, weak acidification rates are often registered, because most of the sugars and nutrients are lost by the effect of lye treatment and strong washing, thus pH is often unsuitable for a safe storage of product [7].

However, many times a delay or a lack of fermentation of olives, especially those of the Manzanilla variety, occurs and there is no explanation for this phenomenon; many variables can affect the progress of lactic acid fermentation such as salt concentration, temperature, nutria-

*Corresponding author.

nutrient content, inhibitors and others. Earlier investigations related the difficulties of LAB growth in olive brines with the presence of polyphenols [8].

However, many times the incomplete acidification cannot be related to phenols (Bevilacqua, unpublished results); the basic assumption of this research is that a not-correct preparation of starter could affect its performances in brines and delay the benefit of a starter *inoculum*.

Thus, some selected strains of LAB and yeasts were inoculated into brines of *Bella di Cerignola* olives, as a single starter (LAB or yeasts) or a combined preparation (LAB + yeasts) and compared to a natural fermentation, focusing on the performances of the process (acidification, production of lactic acid) and trying to verify the basic assumption.

2. Materials and Methods

2.1. Strains

4 strains of *Lactobacillus plantarum* were used in this study: the strains c1, c16 and c19 isolated from a natural fermentation of *Bella di Cerignola* olives [9], whereas the isolate DSMZ 2601 was purchased from a Public Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Moreover, 3 strains of *Candida* spp. (labelled as 22-51-66 and isolated from a natural fermentation of *Bella di Cerignola* olives) were used as selected yeasts; in particular the strains 22 and 51 were identified as *C. famata*, while the strain 66 as *C. guilliermondii* [2].

The strains of *Lb. plantarum*, stored at −20˚C in MRS broth (Oxoid, Milan, Italy) added with 33% of sterile glycerol (J.T. Baker, Milan, Italy), were grown over 2 passages in MRS broth, incubated at 30˚C for 48 h. The strains of *Candida* spp. were maintained on Sabouraud Dextrose Agar slants (Oxoid), stored at 4˚C and transferred monthly; before each assay, the strains were grown in Sabouraud broth (Oxoid), incubated at 25˚C for 48 h.

2.2. *Inocula* Preparation

LAB cocktail was prepared as follows: 50 ml of the culture of each strain were centrifuged at 4000 rpm for 10 min. then, the supernatant was discarded and 50 ml of saline solution (9% of NaCl) was added to the pellet (cell suspension).

The cocktail was prepared by mixing 50 ml of cell suspension for each LAB strain to obtain a final volume of 200 ml; this volume was used for the inoculation of 10 l of brine (the level of each strain was about 8 log·cfu/ml).

Concerning yeast cocktail, 70 ml of the culture of each strain were centrifuged at 4000 rpm for 10 min; then, the pellet was suspended in 70 ml of brine. Yeast cocktail was prepared by mixing cell suspension of each strain (ca. 6 - 7 log·cfu/ml), thus attaining a final volume of 210 ml. This suspension was used for the inoculation of 10 l of brine (high *inoculum*); for the batches inoculated with a low level of yeasts (low *inoculum*), the suspension was diluted at 5 log·cfu/ml and then used for brine inoculation.

2.3. Olive Preparation and Fermentation

The fermentation was performed on *Bella di Cerignola* olives, size GGG (*i.e.* 70 to 80 olives/kg). After harvesting, olives were subjected to grading, to remove fruits with visible damages, washed with tap water and treated with lye (1.3%) for 12 to 15 h. After 2 washings (2 to 3 h and 12 to 13 h), olives were brined (9% of NaCl) and inoculated with LAB and/or yeasts.

The fermentation took place in vessels of 25 l, containing 10 kg of olives and 10 l of brine; olives were maintained dipped in the brines through a grid. The vessels were stored at room temperature for the entire fermentation and mixed periodically to avoid the formation of a mould layer on the top of the brine. NaCl was checked periodically and maintained for the entire fermentation at 8% - 9%.

Table 1 shows the different batches prepared for the analysis.

Although performed at laboratory level, the conditions used for the fermentation (ratio olive/brine 1:1, use of a grid, mixing periodically the brines, fermentation at room temperature) mimic the conditions encountered throughout olive production in Apulian region.

2.4. Microbiological Analyses

Microbiological analyses were performed both on olives and brines throughout the entire fermentation; 20 g of olives were diluted with sterile saline solution (0.9% NaCl) and homogenized through a Sterilmixer (Pbi International, Milan, Italy) at 16,500 rpm for 2 min. Serial dilutions of olive homogenates and brines were performed and plated onto selective media. All the media and the supplements were purchased from Oxoid.

Table 1. Batches prepared for the fermentation assays (T, olives; S, brines).

T1/S1	Control
T2/S2	Batch inoculated with LAB (6 - 7 log·cfu/ml)
T3/S3	Batch inoculated with yeasts (high inoculum, 5 log·cfu/ml)
T4/S4	Batch inoculated with LAB and yeasts (high inoculum)
T5/S5	Batch inoculated with LAB and yeasts (low inoculum, ca. 3 log·cfu/ml)

The media and the conditions were the following:

1) Mesophilic bacteria: Plate Count Agar (PCA), incubated at 30˚C for 24 - 48 h.

2) Spore-former bacteria: PCA, incubated at 30˚C for 24 - 48 h. The evaluation of spore-former bacteria was carried out after heat-shocking olive homogenates and brines at 80˚C for 10 min.

3) LAB: MRS Agar, added with 0.17 g/l of cycloheximide (Sigma-Aldrich, Milan, Italy), incubated at 30˚C under anaerobic conditions for 48 - 72 h.

4) Yeasts: Sabouraud Dextrose Agar, added of 0.1 g/l of chloramphenicol (C. Erba, Milan, Italy), incubated at 25˚C for 2 - 4 days.

5) *Pseudomonadaceae*: Pseudomonas Agar Base, added with CFC selective supplement (containing cetrimide, a selective antibiotic for pseudomonas), incubated at 25˚C for 48 - 72 h.

6) Staphylococci: Baird Parker Agar Base, added with egg yolk tellurite emulsion and incubated at 37˚C for 24 - 48 h.

7) *Enterobacteriaceae*: Violet Red Bile Glucose Agar (VRBGA), incubated at 37˚C for 18 - 24 h.

2.5. pH Evaluation

pH measurements were performed on olive homogenates and brines through a pH-meter Crison 2001 (Crison Instruments, Barcelona, Spain).

2.6. Determination of Organic Acids

The content of D- and L-lactic acids, as well as the concentration of acetic, citric, D- and L-malic acids, D-glucose and D-fructose in the brines was determined through enzymatic kits (Megazyme, Bray, Ireland) just after the brining (beginning of the fermentation) and after 7, 21, 28, 35 and 42 days of fermentation. The analyses were performed as suggested by the producer.

2.7. Statistical Analyses

All the analyses were performed in duplicate over 2 independent batches, labelled A and B. Data were submitted to one-way analysis of variance (one-way ANOVA) and Tukey's test ($P < 0.05$) through the software Statistica for Windows version 10.0 (Statsoft, Tulsa, Okla., USA).

3. Results

3.1. Microbiology

Figure 1(a) reports the growth of lactic acid bacteria (LAB) in olives. In particular, in the batches inoculated with the starter (T2-T4 and T5), cell counts were 4 log·cfu/g higher than in the control after 7 days, whereas the difference was not significant after 21 days, as the population attained 5 to 5.75 log·cfu/g in all the samples, except for the batch inoculated with yeasts (T3) showing a LAB count 1 log·cfu/ml lower.

Concerning brines (**Figure 1(b)**), LAB were ca. 6 log·cfu/ml just after *inoculum* in the batches T2, T4 and T5. Moreover, after 7 days inoculated samples showed a LAB count higher than un-inoculated ones, while the differences were not significant after 14 days.

Figure 2 shows yeast evolution in brine. As expected, after 7 days the highest cell number was observed in the inoculated batches (T3, T4 and T5), with a cell load ranging from 5.6 to 6.6 log·cfu/ml; otherwise, in the un-inoculated samples (T1 and T2) yeast number was approximately 4.3 to 5.3 log·cfu/ml. As reported for LAB, the difference between inoculated and inoculated batches was not significant after 14 days. A similar trend was recovered in olives (data not shown).

Enterobacteriaceae were at 3 log·cfu/g in olives and 4 log·cfu/ml in brines, whereas pseudomonads, staphylococci and spore-former bacteria were at low levels (1 - 2 log·cfu/ml) or below the detection limit for the entire running time (data not shown).

3.2. pH

Figure 3 shows pH values of olives. After 7 days, olives showed a pH of about 7 in the batches T2, T5 as well as in the control, whereas the pH was ca. 8 in the samples T3 and T4. After 2 week pH was ca 6 - 6.5 and decreased until 5 at the end of fermentation, without significant differences amongst the different samples. A similar trend was recovered in brines (data not shown).

3.3. Organic Acid Content

Figures 4(a) and **(b)** show the concentration of D- and L-lactic acids in the brine; both L- and D-lactic acids increased significantly after 21 days of fermentation, then they decreased by 2 - 3 g/l; namely, in the sample T2 (sample inoculated with LAB) the concentration of L and D-lactic acids was the highest one (9.15 and 4.70 g/l), whereas the acids were at the lowest levels in the sample inoculated with a high level of yeasts.

Regarding acetic acid, its concentration increased throughout the entire fermentation, without any difference amongst the various batches (**Figure 5**). Finally, D-glucose and D-fructose decreased during the fermentation until to about 0.5 g/l or below the detection limit (data not shown).

4. Discussion

4.1. Why This Research?

The use of a starter culture could shorten the processing

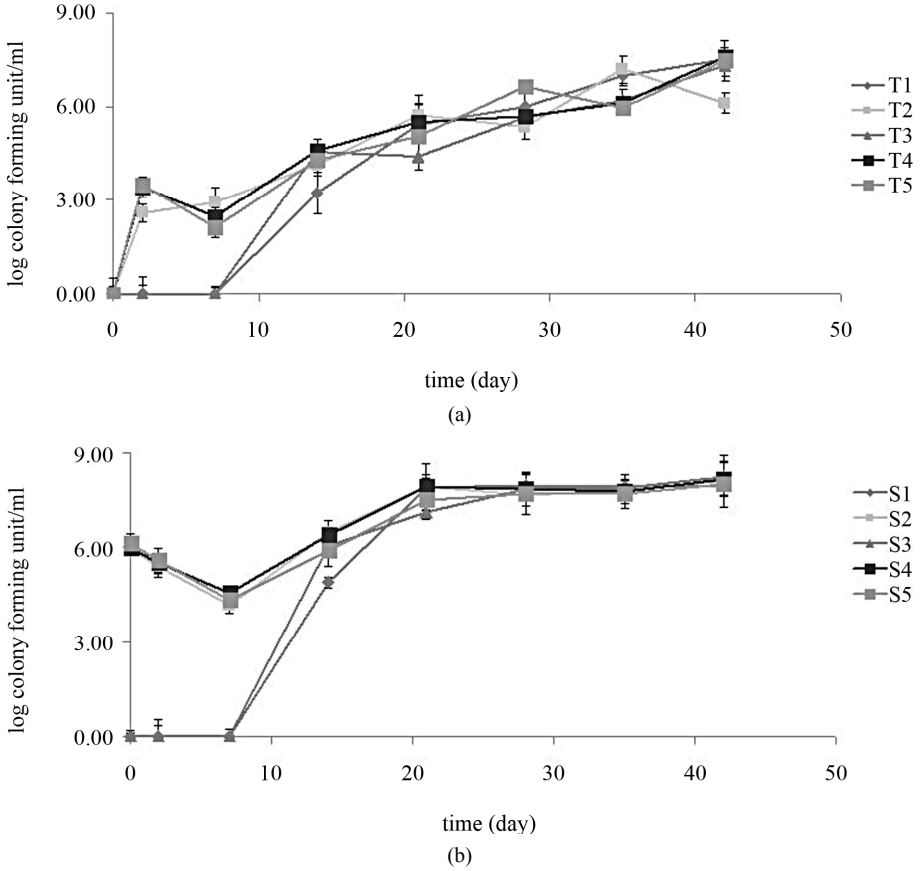

Figure 1. LAB microflora in olives (a) and brines (b). Mean values ± standard deviation.

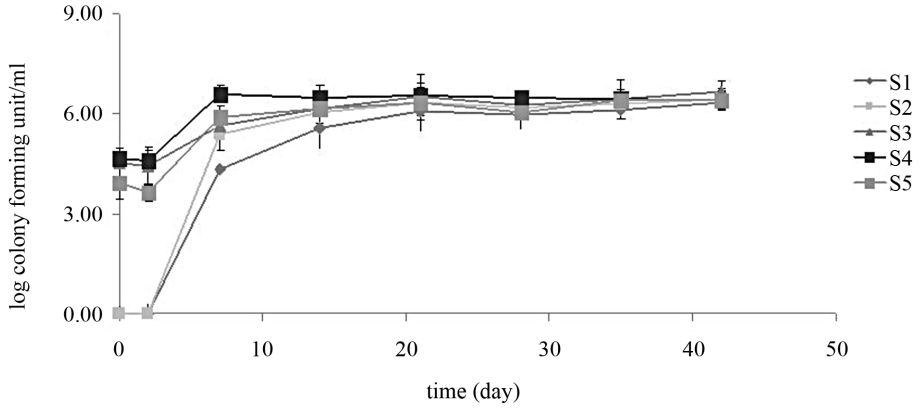

Figure 2. Yeast microflora in brines. Mean values ± standard deviation.

time for the preparation of naturally green olives and affects positively fermentation, acidification rate and flavour of the product [7].

Many authors recommended the inoculation of brine with starter colture; in particular, the use of suitable strains of *Lb*. *plantarum* could assure a correct course of fermentation [6-9]. Some benefits could be obtained also by a controlled *inoculum* of yeasts [10]. However, the focus of this study is different, as this research presents a new perspective on the use of starter cultures.

Many food producers select promising strains jointly with Universities and Research Centres; the main result of these collaboration is the selection of "the best strains". In the past a joint venture of the section of Applied Microbiology (University of Foggia) and some food producers of table olives of Apulian region resulted in the selection of some promising strains of lactic acid bacteria with interesting functional traits [2] and the evaluation of the technological and spoiling impact of yeast strains of Bella di Cerignola table olives [2]. Following these results,

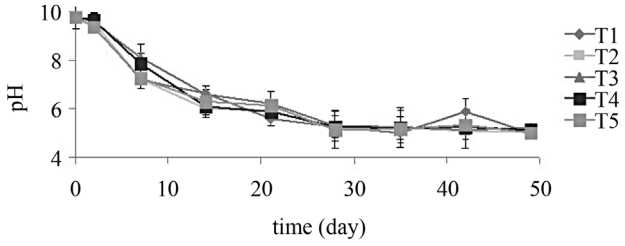

Figure 3. pH of olives. Mean values ± standard deviation.

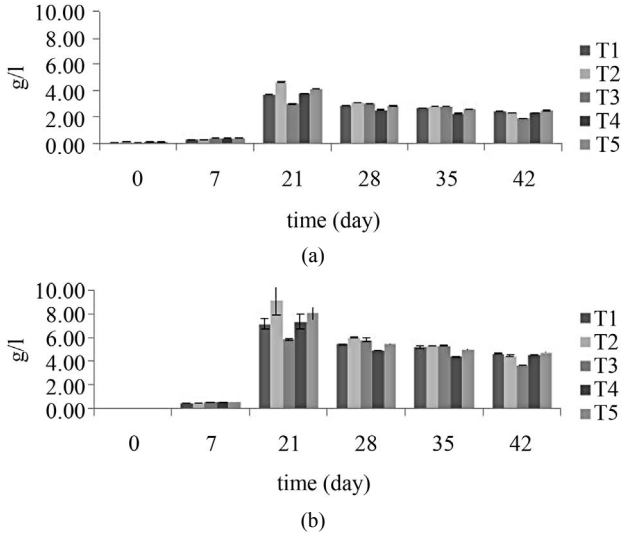

Figure 4. Content of D- and L-lactic acids (g/l) in the brine. (a) D-lactic acid; (b) L-lactic acid. Mean values ± standard deviation.

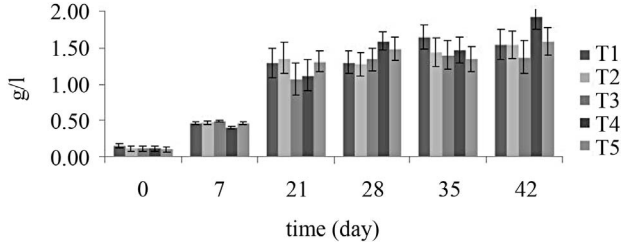

Figure 5. Content of acetic acid (g/l) in the brine. Mean values ± standard deviation.

lactic acid bacteria and yeasts were suggested to producers for a controlled fermentation; however, what happened was that producers reported a decrease of the technological impact of these strains under *in vivo* conditions. After a brief evaluation of the problem in our lab, we supposed that the same strains, showing optimal performances in a lab fermentation, could be affected in a real fermentation by the way of the preparation of *inoculum*; thus, we prepared the starters in the same way reported by producers (strain cultivation in lab media, followed by harvesting and cell suspension in brine).

The initial planning included also some batches inoculated with starters, grown in media containing increasing concentration of salt; then, we decided to delete these samples, as the main aim of the research was to verify if also under lab conditions the strains behave in the same way than in factories.

What we found was that, as suggested by many producers, the differences between inoculated and inoculated samples were significant only for 2 - 3 weeks of fermentation (cell number, pH, content of lactic acid); after this time, the samples inoculated with the starter showed the same trend than the uninoculated ones. A practical implication of this result is that a preliminary adaptation could be useful to assure the success of the starter for the entire process. A particular kind of adaptation is the back-slopping, *i.e*. inoculation often carried out with brines from previous fermentations [11]. However, such lactic cultures exhibit a diversity of metabolic activities, even among strains of the same species, including differences in growth rate, adaptation to a particular substrate, antimicrobial properties, flavour and quality attributes as well as competitive growth behaviour in mixed cultures, resulting in non-consistent and low quality final products. This kind of protocol was used in the past by Italian producer of table olives; although the process run faster, the quality was not constant.

Another element confirming the strong implications of a not-correct use of the starter was the pH; the pH in a safe table olive must be about 4.5. In our fermentation, pH decreased until to about 5 after 28 days, then it remained constant during the entire fermentation.

5. Conclusion

In conclusion, why this research? A possible answer: underline the risk associated with a not-correct use of the starter and the importance of a good step of starter preparation. Using the starter only after a growth phase in a laboratory medium could result a delay in this performances in brines.

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