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Survival of yeasts inoculated in flavoured extra virgin olive oil

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Abstract - The survival of four strains of yeast belonging to the species *Saccharomyces cerevisiae, Candida wickerhamii, Candida boidinii* and *Williopsis californica* was studied in extra virgin olive oil flavoured with garlic, lemon, oregano and red chilli pepper. The ingredients used in the doses of 1%, 5% and 10% profoundly modified the habitat of the extra virgin olive oil, reducing drastically, in 90 days of storage, the survival of the yeasts by 20-50%, in the following decreasing order: lemon, garlic, oregano and red chilli pepper. Among the yeasts studied, *W. californica* strain 1639 was found to be one of the most sensitive, while *S. cerevisiae* strain 1525 was one of the most tolerant regarding the ingredients present in the flavoured olive oil. The observations carried out with a scanning electron microscope (SEM) highlighted the presence of frequent lesions on the cellular wall of *C. wickerhamii* 1532*, C. boidinii* 1638 and *S. cerevisiae* 1525 and only in a few rare cases in *W. californica* 1639. Nevertheless, since the survival of W*. californica* 1639 in the flavoured olive oil was compromised to a greater extent in respect to the other species, it is plausible to deduct that the damage to the cellular wall represents only one of the causes responsible for the death of the yeasts in the flavoured olive oil.

Key words: aromatic spices, flavoured olive oil, oil yeasts, survival of yeasts.

INTRODUCTION

The olive oil is obtained from the grinding and crushing of the olives picked at the beginning of the ripening period. The physico-chemical characteristics of the oil improve during the storage period due to the sedimentation of the suspended material and to the hydrolytic processes, which involve different forms of phenolic compounds. Some hydrolytic enzymes, deriving from the crushed fruits or from the activity of the microorganisms present in the oil, are able to improve oil quality during storage (Ciafardini and Zullo, 2002). In fact, some yeasts allowed the sweetening of an extra virgin olive oil through the production of a β-glucosidase active on the bitter glucoside of the oil know as oleuropein, hydrolysing it into simpler compounds which are no longer bitter in taste (Ciafardini and Zullo, 2002). The extra virgin olive oil represents a very selective habitat, where some types of microorganisms from the fruits grow according to the chemical composition of the oily mass and the storage conditions. Nevertheless, other than the extra virgin olive oil whose habitat is fairly defined, recently there has also been a rapid expansion of olive oils enriched with aromatic products commercialised as flavoured olive oil, whose habitat is modified differently according to the ingredient used. In a previous study carried out on some types of extra virgin olive oil enriched with lemon, oregano, garlic and red chilli pepper, it has been demonstrated that the aromatic ingredients are able to influence the composition of the natural micro-flora present in the oily mass. The microbiological analysis of the extra virgin olive oil flavoured with different ingredients showed the presence of the yeasts only in the oil enriched with oregano, whereas moulds were found also in the oil containing lemon, garlic or red chilli pepper (Ciafardini *et al.*, 2004a). However, since the discovery of the microorganisms in the oil was only made recently (Ciafardini and Zullo, 2002), at the moment it is not known whether the well-known antimicrobial activity of some compounds present in the ingredients (Hammer *et al.,* 1999), is able to condition the survival of the yeasts, which are normally present in extra virgin olive oil. The aim of the present research was to study the survival of four species of yeasts isolated from extra virgin olive oil and inoculated in different types of flavoured olive oils.

MATERIALS AND METHODS

Yeast preparation. The studies made on the survival of the yeasts in flavoured extra virgin olive oil were carried out using four strains of yeasts named *Williopsis californica* 1639, *Saccharomyces cerevisiae* 1525, *Candida boidinii* 1638 and *Candida wickerhamii* 1542. The strains of yeasts tested were isolated from samples of extra virgin olive oil and previously classed on the basis of the carbohydrate fermentation pattern and other physiological characteristics, according to conventional methods used in yeast taxonomy (Kurtzman and Fell, 1998; Ciafardini and Zullo, 2002; Ciafardini *et al*., 2004b). The cultures were prepared by growing the four strains separately in Petri dishes containing the MYGP Agar of the following composition: yeast extract (Biolife, Milan, Italy) 3 g, malt extract (BBL, Cockeysville, MD, USA) 3 g, beef

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extract powder (BBL) 5 g, D-glucose (Merck, Darmstadt, Germany) 10 g, agar agar (BBL) 20 g, distilled water 1000 mL, pH 5.5 (Kurtzman and Fell, 1998). After 3 days of incubation at 30 °C, the yeast colonies grown on the surface of the MYGP Agar medium were suspended in sterile distilled water. The stock suspensions were stored at 4 °C and used within 24 h for the tests described below.

Flavoured extra virgin olive oil preparation. In laboratory conditions four types of flavoured extra virgin olive oil were prepared using respectively lemon, oregano, garlic and red chilli pepper. After being washed under tap water and dried by air, the lemons were peeled by hand separating the peel from the rest of the fruit. Next, the peel was homogenized for 10 min with a Tefal Mixer (FAVS, Bologna, Italy) and finally added to the oil in the proportions reported below. The oregano flowers were air-dried for about a month, then finely triturated with a mortar and finally added to the oil. The garlic was first divided into cloves, then peeled by hand, homogenized for 10 min with the mixer and finally added to the oil. The red chilli peppers were air-dried for about a month, then finely triturated with a mortar and added to the oil. Each type of flavoured olive oil was prepared using an extra virgin olive oil of the "Leccino" variety enriched respectively with zero (control), 1, 5, and 10% (w/v) of lemon, oregano, garlic and red chilli pepper. The oil, enriched with the various ingredients in different proportions, was homogenized with a Turrax mod. T25 homogeniser (IKA-Milan, Italy) and sterilised by filtration through nitrocellulose filters with a porosity of 0.2 µm (Minisart NML-Sartorius, Göttingen, Germany), then transferred to 500 mL Pyrex flasks with screw-on caps which had been previously sterilised in autoclaves at 121 °C for 20 min. Each type of flavoured olive oil was kept in the dark at room temperature.

Yeast inoculation in flavoured olive oil. The survival of yeasts in flavoured extra virgin olive oil was studied using the four flavoured olive oil stocks reported above. A volume of 100 mL of sterilised flavoured extra virgin olive oil was inoculated respectively with 0.1 g of wet *W. californica* 1639, *S. cerevisiae* 1525, *C. boidinii* 1638 and *C. wickerhamii* 1542 yeast containing about 50% of dry weight suspended respectively in 1 mL of sterile extra virgin olive oil. The inoculated samples were mixed (Vortex, Mod. Maxi Mixer, FAVS, Bologna, Italy) and stored in a dark room for 90 days at 18-20 °C. The trials were accomplished using four repetitions. At zero time and after 45 and 90 days of incubation period, the samples were aseptically sampled after a brief mixing of the oily mass using a vortex.

Microbiological and chemical analysis of the flavoured olive oil. The evaluation of yeasts inoculated in four types of flavoured extra virgin olive oil was carried out after 45 and 90 days of incubation on a representative part of each sample that was first diluted with sterile distilled water and then used to inoculate the Petri dishes containing the MYGP Agar. The colony forms unit (CFU) of the yeasts was evaluated after 3 days of incubation at 30 °C. The routine chemical analyses of acidity, peroxides and other chemical parameters of the flavoured olive oil were made after 90 days of incubation according to the European Community's 2568/91 Regulation (EC, 1991).

Scanning electron microscopy (SEM) observation. Samples of 5 mL of flavoured extra virgin olive oil, inoculated with yeasts and stored for 90 days at 18-20 °C in a dark place, were transferred into sterile centrifuge plastic tubes. Centrifugation was carried out with a Hettich mod. Universal 32 (Tuttlingen, Germany), using 7000 *x g* for 20 min. The supernatant was discarded, whereas the pellet was suspended in 5 mL of sterile distilled water. After agitation for 1 min with a vortex, the samples were newly centrifuged at 4500 *x g* for 5 min. The pellet obtained was suspended in 1 mL of 0.1 M phosphate buffer (pH 7) and treated for SEM observation using the same procedure previously reported (Ciafardini and Marotta, 1988) with the follow minor variation. Samples were fixed overnight in phosphate buffer 0.1 M (pH 7) containing 3% (v/v) of glutaraldehyde. The samples were washed three times with the same buffer without the glutaraldehyde, than fixed 3 h at 20 °C in a solution of OsO_4 1% (w/v) and rinsed with the same buffer 3 times. Samples were deposed on poly-L-lysine-pretreated glass coverslips and dehydrated in an ethanol dilution series of 20, 40, 60, 80, 95 and 100% (v/v). The glass coverlips with specimens in 100% ethanol were critical point dried in CO_2 atmosphere using an Emitech K850 critical point drier (Emitech, Rome, Italy), mounted on aluminium stubs, gold-coated in a Emitech K550 sputter coater and examined with a SEM Zeiss DSM 940 (Zeiss, Rome, Italy).

RESULTS AND DISCUSSION

The investigation carried out using experimental flavoured olive oil demonstrated that the spices studied are able to condition the physico-chemical properties of olive oil and the survival of the yeasts present in extra virgin olive oil. In fact, the yeast inoculated in the flavoured olive oil during 90 days of storage time, survived in a different way according to the type and the concentrations of the ingredients. On average, the inhibition produced by each ingredient in a maximum concentration of 10%, took place in the following decreasing order: lemon, garlic, oregano and red chilli pepper while the chemical properties charactering the extra virgin olive oil, in 90 days of incubation worsened dramatically especially in olive oil flavoured with lemon and red chilli pepper (Table 1).

TABLE 1 – Physico-chemical properties of the flavoured olive oil evaluated after 90 days of storage

Parameter	Unflavoured olive oil (control)	Olive oil flavoured with garlic	Olive oil flavoured with oregano	Olive oil flavoured with lemon	Olive oil flavoured with red chilli pepper
FFA*	0.29 ± 0.09	0.58 ± 0.07	0.69 ± 0.03	1.0 ± 0.09	1.05 ± 0.07
PV	16 ± 1.01	10 ± 0.90	11.34 ± 1.02	8.8 ± 0.99	12.15 ± 1.2
K_{232}	0.78 ± 0.05	0.75 ± 0.05	0.76 ± 0.04	1.40 ± 0.08	0.76 ± 0.03
K_{270}	0.053 ± 0.001	0.051±0.02	0.052 ± 0.002	0.096 ± 0.005	0.051 ± 0.003
Δk	-0.002	-0.002	-0.002	-0.004	-0.002

* Mean ± standard deviation; *n* = 4; FFA (% oleic acid) = free acids; PV (meq O_2/Kg) = peroxide value.

Nevertheless, the results of the microbiological analyses indicated a different sensitivity of the yeasts according to the ingredients used. In fact, the lemon caused a reduction in the survival of *W. californica* 1639 of more than 50% even in the minimum concentration equal to 1%. The survival of *C. boidinii* 1638 and *C. wickerhamii* 1542 was gradually reduced by increasing the concentration of the ingredients while the survival of *S. cerevisiae* 1525 was not influenced by the different concentrations of lemon (Fig. 1).

Also, the studies carried out on the dynamics of the survival of the yeasts during the storage of the product confirmed the different sensitivity of the yeasts studied. In fact, unlike *C. boidinii* 1638 where the mortality of the cells increased gradually with time, in *C. wickerhamii* 1542 and *W. californica* 1639 the inhibitory action was found to be more marked respectively in the first 45 or after 45 days of storage of the product (Fig. 2). The observations carried out with the SEM allowed us to verify deterioration on the cellular wall of *C. wickerhamii* 1542 and morphological modifications to *C. boidinii* 1638 (Fig. 3). The inhibitory effect of the compounds originating from the lemon on the yeasts has been reported by other authors. In fact, Abe *et al.* (2003)

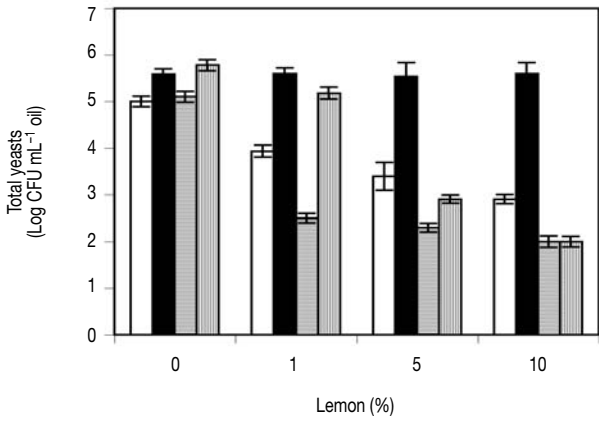

FIG. 1 – Survival of *Candida boidinii* 1638 (□), *Saccharomyces cerevisiae* 1525 (■), *Williopsis californica* 1639 (≡) and *Candida wickerhamii* 1542 (|||), evaluated in the experimental flavoured olive oil with lemon after 90 days of storage. Data are means ± standard deviation; *n* = 4.

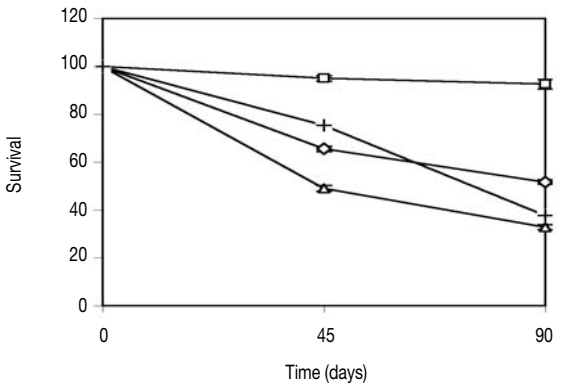

FIG. 2 – Survival of *Candida boidinii* 1638 (◊), *Saccharomyces cerevisiae* 1525 (□), *Williopsis californica* 1639 (Δ) and *Candida wickerhamii* 1542 (+) in experimental flavoured olive oil with 10% lemon during 90 days of storage. Data are means ± standard deviation; *n* = 4.

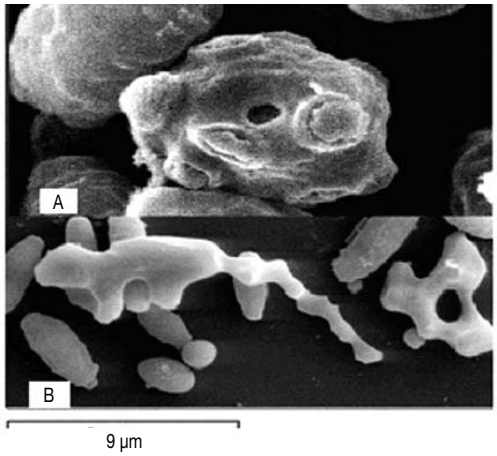

FIG. 3 – SEM observation of cells of *Candida wickerhamii* 1542 (A) and *Candida boidinii* 1638 (B) extracted from samples of olive oil flavoured with lemon, after 90 days of storage.

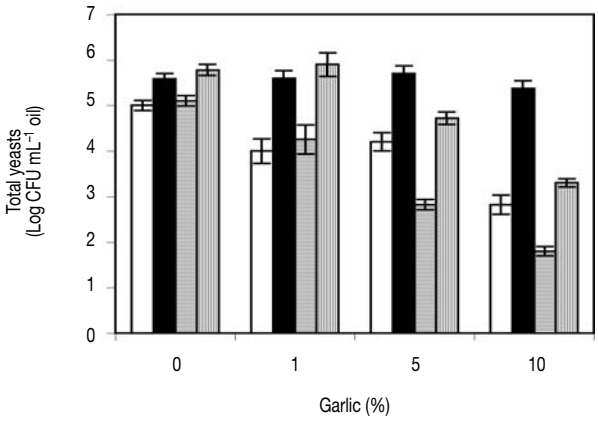

FIG. 4 – Survival of *Candida boidinii* 1638 (□), *Saccharomyces cerevisiae* 1525 (■), *Williopsis californica* 1639 (≡) and *Candida wickerhamii* 1542 (|||), evaluated in the experimental flavoured olive oil with garlic after 90 days of storage. Data are means ± standard deviation; *n* = 4.

report that the mycelial growth of *Candida albicans* was inhibited in the medium containing 100 µg/ml of essential lemon oil.

The survival of the yeasts inoculated in the oil flavoured with garlic varied in a different way according to the species used. In fact, with the exception of *S. cerevisiae* 1525 which did not undergo variations, the survival of *C. wickerhamii* 1542 was inhibited only when the concentration of garlic present in the oil exceeded 1%, while the survival of the other two species was inhibited proportionally to the concentration of the ingredient (Fig. 4). From the examination of the dynamics of the survival of the yeasts during the storage of the oil flavoured with 10% of garlic, it can be noted that, with the exception of the 1525 strain of *S. cerevisiae*, the other species of yeasts showed the same behaviour regarding the different concentrations of garlic (Fig. 5). Allicin is one the major antimicrobial components of garlic. It is a sulphur-containing compound able to inhibit the growth of both Gram-negative and Gram-positive bacteria where it interacts with the cellular wall (Johnson and Vanght, 1969; Dankert *et al.*, 1979). The components of garlic seem to manifest the same action also toward to the yeasts used in the present research.

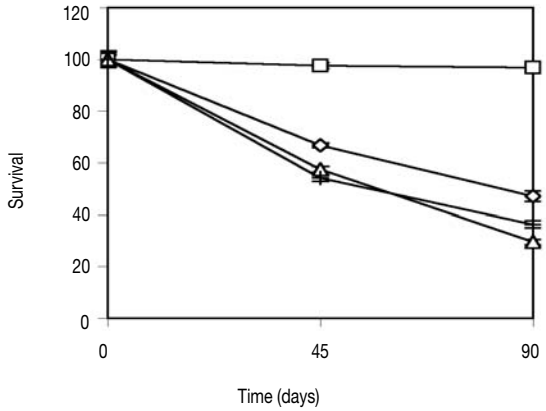

FIG. 5 – Survival of *Candida boidinii* 1638 (◊), *Saccharomyces cerevisiae* 1525 (□), *Williopsis californica* 1639 (Δ) and *Candida wickerhamii* 1542 (+) in experimental flavoured olive oil with 10% garlic during 90 days of storage. Data are means ± standard deviation; *n* = 4.

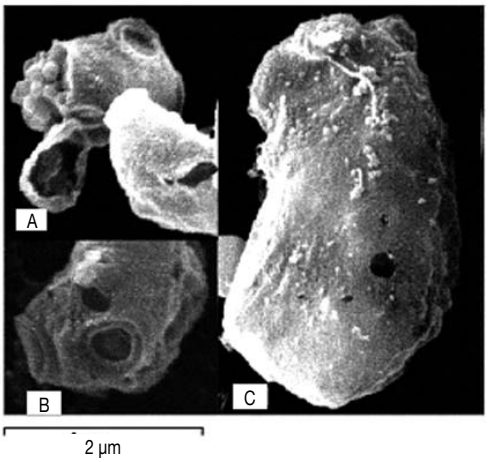

FIG. 6 – SEM observation of cells of *Candida wickerhamii* 1542 (A, B), *Candida boidinii* 1638 (C), extracted from samples of olive oil flavoured with garlic, after 90 days of storage.

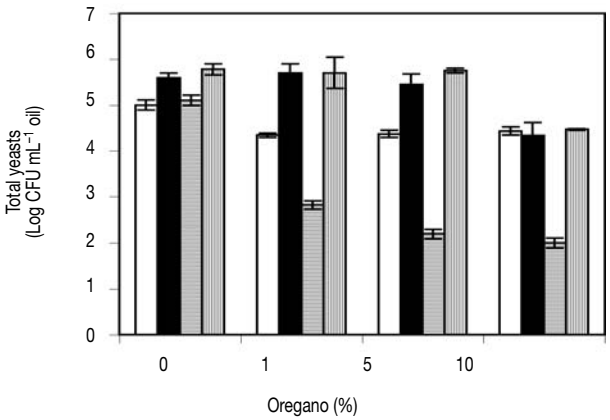

FIG. 7 – Survival of *Candida boidinii* 1638 (□), *Saccharomyces cerevisiae* 1525 (■), *Williopsis californica* 1639 (≡) and *Candida wickerhamii* 1542 (|||), evaluated in the experimental flavoured olive oil with oregano after 90 days of storage. Data are means ± standard deviation; *n* = 4.

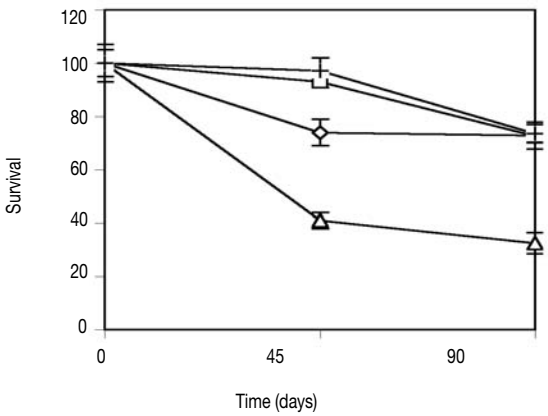

FIG. 8 – Survival of *Candida boidinii* 1638 (◊), *Saccharomyces cerevisiae* 1525 (□), *Williopsis californica* 1639 (Δ) and *Candida wickerhamii* 1542 (+) in experimental flavoured olive oil with 10% oregano during 90 days of storage. Data are means ± standard deviation; *n* = 4.

In fact, from the observations carried out with the SEM, it was possible to verify that a lot of the *C. wickerhamii* 1532 and *C. boidinii* 1638 cells extracted from samples of olive oil flavoured with garlic, after 90 days of storage, presented a seriously damaged wall (Fig. 6), showing much more devastating lesions than those reported for the lemon in Fig. 3. Nevertheless, such morphological deterioration was not found in *W. californica* 1639 which, among the yeasts tested, is without doubt one of the most sensitive species regarding the garlic (Figs. 4 and 5), thereby demonstrating how the lesions to the wall represent only one of the causes leading to the death of the yeast cells suspended in the oil flavoured with garlic. Also, in the olive oil flavoured with oregano the survival of the yeasts was influenced differently according to the species. In fact, the vitality of *W. californica* 1639 and *C. boidinii* 1638 was negatively influenced both in low and high concentrations of the ingredient. While in S*. cerevisiae* 1525 and *C. wickerhamii* 1542 the survival was reduced only when the concentration of the ingredient reached 10% (Fig. 7). Furthermore, upon examining the dynamics of the survival of the yeasts in the oil flavoured with 10% of oregano, it was noted that *W. californica* 1639 can be clearly distinguished from the other species, in fact the data reported in Fig. 8 demonstrates that, after only 45 days of incubation, the survival of this yeast was reduced by more than 50%. Among the compounds present in oregano, thymol and carvacrol are well-known for their antimicrobial activity regarding the various microorganisms, such as *Pseudomanas aeruginosa* and *Staphylococcus aureus,* to which membrane integrity damage is caused (Lambert *et al.,* 2001). Nevertheless, from Fig. 7 it is interesting to note that *S. cerevisiae* 1525, among all the ingredients studied, is only sensitive to oregano present in the oil in the dose of 10%. This result is confirmed by observations carried out with the SEM where serious lesions can be noted on the cellular wall of this specific species of yeast (Fig. 9). The wall alteration of *S. cerevisiae* 1525, due to oregano's essential oils, is reported by other authors (Bennis *et al.*, 2004; Chami *et al.,* 2005). Nevertheless, the observations carried out with the SEM also confirm in this case that the survival of the yeasts in the flavoured olive oil with oregano is not linked solely to damage of the cellular wall. In fact, no alterations to the cellular wall were observed on the *W. californica* 1639 cells (data

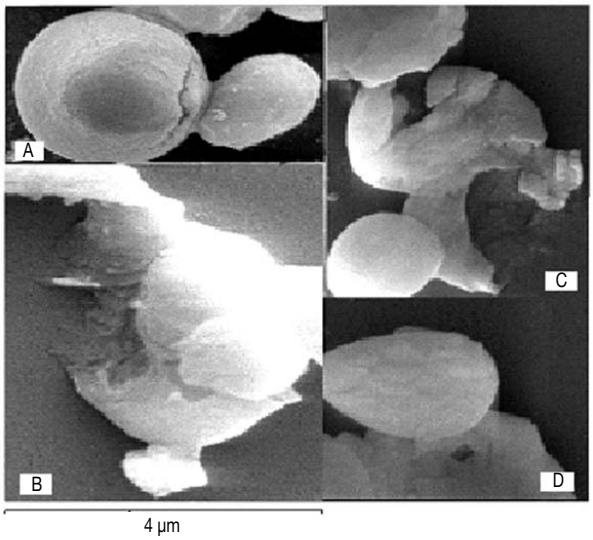

FIG. 9 – SEM observation of cells of *Saccharomyces cerevisiae* 1525 extracted from samples of olive oil flavoured with oregano, after 90 days of storage.

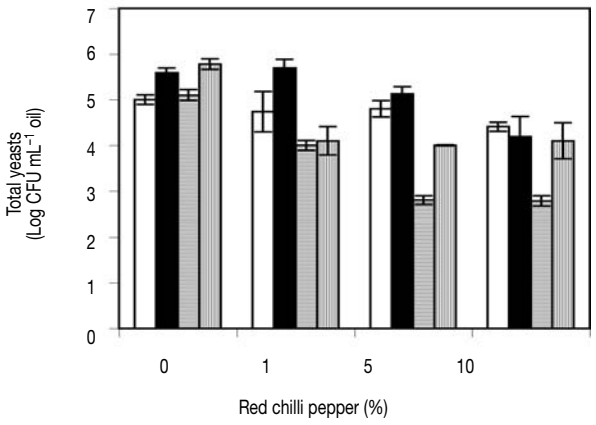

FIG. 10 – Survival of *Candida boidinii* 1638 (□), *Saccharomyces cerevisiae* 1525 (■), *Williopsis californica* 1639 (≡) and *Candida wickerhamii* 1542 (|||), evaluated in the experimental flavoured olive oil with red chilli pepper after 90 days of storage. Data are means ± standard deviation; $n = 4$.

not shown), which without doubt is one of the most sensitive yeasts. The olive oil flavoured with red chilli pepper inhibited the survival of *W. californica* 1639 variably from 20 to 40 % respectively for a concentration of 1 and 10% of the ingredient. The survival of other species of yeasts was negatively influenced only when the concentration of the ingredient exceeded 5% (Fig. 10). Such results are in agreement with those reported by Zaika (1988) that, unlike the other ingredients reported above, classify the red chilli pepper among the products with a weak inhibitory effect on the micro-flora. In conclusion, the above-reported ingredients are able to modify the habitat of extra virgin olive oil, reducing in different measures the survival of some yeasts which are normally a part of the natural micro-flora of this aliment, thereby demonstrating that the antimicrobial activity of the ingredients is manifested not only in the watery phase as reported by other authors, but also in the organic one represent in this case by the olive oil.

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