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Selezione e caratterizzazione di lieviti isolati da olive fermentate al naturale ad azione bioprottetiva

Isolation and characterization of yeasts isolated from naturally fermented olive brine with bioprotective function

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PhD thesis

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Introduction

1. TABLE OLIVE

The olive tree (*Olea europaea* L.) is one of the most ancient domestic, cultivated plants characteristic of the Mediterranean Basin. Clear signs of olive domestication (olive oil presses) come from the Near East during the Early Bronze Age (second half of the fifth millennium BP). The crop (*O. europaea* subsp (ssp) *europaea* (*O. europaea* var. *sativa* Lehr) has been of immense importance. It is currently assumed that individual oleaster trees showing superior performance for size and/or oil content of fruit were selected empirically and propagated vegetatively as clones, using cuttings that were planted directly or, more recently, grafted onto indigenous oleasters. From these propagated individuals, olive cultivars were developed and distributed by various successive human migrations throughout the Mediterranean Basin, especially from East to West. Deforestation probably reduced native oleaster populations and the geographic dissemination of cultivars favoured multiple contact areas and hybridisation between cultivated olive and indigenous oleasters that are fully interfertile. This may have led to the progressive disappearance of the original genetic characteristics of the wild oleasters. In South Australia, where it has been introduced 200 years ago, the olive has become a major woody weed that displaces native vegetation. (Lumaret *et al.,* 2004)

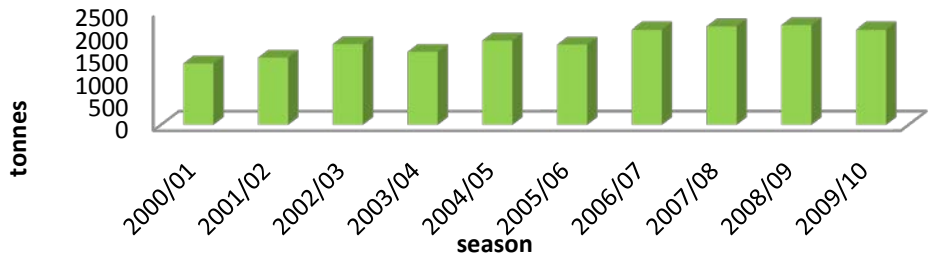
The International Olive Council (IOC), an international intergovernmental body that has a long-standing cooperation with Codex in the development and revision of Codex texts concerning olive-related products, has actively participated in the development and updating of the Codex Standard for Table Olives. Moreover, it

has recently adopted a revised *Trade Standard applying to Table Olives* (IOC/OT/NC no.1) to take account of changes in science, technology and marketing practices within their membership. This proposal relates to the revision of the Codex Standard for Table Olives .

Table olives production

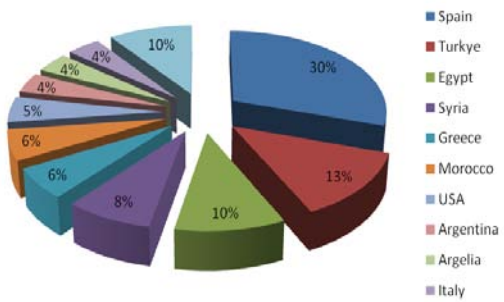
Table olives production, in 2010/11 crop year, is expected to be similar to the previous season. EU production is estimated at 700 000 t, showing a year-on-year increase of 4.3% (Fig. 1).

Figure 1: World's table olives production



Spain is by far the world's top producer and exporter of table olives (Fig. 2). Official sources at the Spanish Agriculture Ministry report that table olives production for 2010/11 estimates for assess production at above 470 000 t, in which case the IOC estimates would only have to be adjusted by 15 000 t.

Figure 2: World's table olives production



According to Association of Producers and Exporters of Table Olives (www.asemesa.org, 2009) data, Spanish exports, including to

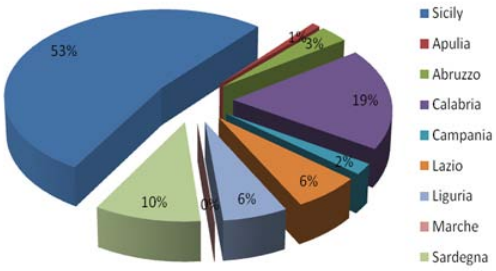
the countries of the EU, total 252,322 metric tons, worth 583.213 million euros. That is more than 30% of total world exports of this product. Spain is followed at a great distance by Turkey, Argentina, Morocco and Greece (data from ASEMESA).

Outside the EU, the biggest production increases are expected to occur in Israel (18 500 t = +95%) and Iran (47 000 t = +77%), followed by Syria (155 000 t = +15%) and Albania (22 000 t = +22%). In the other direction, estimates put production at lower levels in Egypt (290 000 t, down by -3%), Tunisia (20 000 t, down by -9%) and above all Turkey (300 000 t, down by -23%).

The data received by IOC for Argentina do not yet provide a clear snapshot, although table olive production in this country may rise quite significantly as it appears to be a move towards switching from oil-olive production to table olive production owing to the low oil content of some varieties.

In Europe, Greek and Italy follow Spain with a production respectively of 115 and 68 tons for the 2009-2010 season. In Italy table olives production mainly regards the south regions; in particular Sicily is the major producer; with most processing of table olives concentrated in the Trapani area, in particular in Castelvetrano (Fig. 3).

Figure 3: Olives producing Regions of Italy



IOC data show that world consumption of table olives in the 2008-2009 season was 2.159,5 and for the 2009-2010 is provisory estimated for 2.150,5 tons in net, drained weight. Of that amount, by regions, the European Union accounted for 32.96%, Arab countries 28.77%, other Nations 15.27%, US/Puerto Rico/Canada 13.45%, Central/South America 5.82% and Eastern Europe 3.72%.

By country, the world's largest consumers of table olives are Spain, the United States, Egypt, Turkey, Italy, Syria, Russia, Algeria, Brazil, Germany, Iran, Morocco, Jordan, Britain and Canada.

Market research on China, Russia and USA/Canada

As requested by the IOC, marketing research have been carried out on olive oil and table olive consumption in China, Russia and the USA/Canada prior to launching promotional campaigns for the implementation of actions and measures to highlight the biological value of olive oil and table olives; in particular antioxidant properties against cancer have been mainly promoted from Spain and Italy.

Table olive value chain is becoming increasingly more complex, in particular in the current context of economic crisis, in which product price is more important than the attributes of table olives. Promotion campaigns are therefore needed to highlight the worth of table olives.

Moreover, impact of the crisis on the olive oil and table olive sector is due to different causes, such as:

- Generalised losses in recent years, especially in the EU producer countries;
- Farms have very serious structural problems (fragmentation, ageing, lack of training), which make it hard for them to cope with the crisis, especially in traditional countries;
- There is the threat of relocation to emerging countries;
- The dichotomy has increased between larger, modern, high yielding farms which account for a growing volume of product and the rest of the farms.

OLIVES COMPOSITION

- The olive fruit is a drupe. It is characterized by a bitter component (oleuropein), a low sugar content (2.6-6%) compared with other drupes (12% or more) and an high oil content (12-30%) depending on the period of year and variety. These characteristics make it a fruit that cannot be consumed directly from the tree and that has to undergo a series of processes that differ considerably in relation to region and olive variety. Some olives are, however, an exception to this rule

because as they ripe and become sweeter on the tree; in most cases this is due to fermentation. One case is the Thrubolea variety in Greece. Oleuropein, which is distinctive to the olive, has to be removed as it has a strong bitter taste: it is not, however, pernicious to health. Depending on local methods and customs, the fruit is generally treated in sodium or potassium hydroxide, brined or successively rinsed in water.

TYPES OF OLIVES

According to IOC the most important table olive production can be distinguish in three type of preparations according to the degree of ripeness of the fresh fruits:

1. Green olives: fruits harvested during the ripening period, prior to colouring and when they have reached normal size. They may vary in colour from green to straw yellow.
2. Olives turning colour: fruits harvested before the stage of complete ripeness is attained, at colour change. They may vary in colour from rose to wine rose or brown.
3. Black olives: fruits harvested when fully ripe or slightly before full ripeness is reached. They may vary in colour from reddish black to violet black, deep violet, greenish black or deep chestnut**.**

The main olive processing can be explained as follows:

<u>Green Table Olives</u>

Green Table Olives are obtained from olives harvested during the ripening cycle when they have reached normal size, but prior to colour change. They are usually hand-picked when there is a slight change in hue from leaf-green to a slightly yellowish green and when the flesh begins to change consistency but before it turns soft. Colour change should not have begun. Trials have been run to machine

harvest table olives, but owing to the high %age of bruised fruit they had to be immersed in a diluted alkaline solution while still in the orchard. After harves the olives are processed on the same day if possible. Green olives are processed in two principal ways: with fermentation (Spanish type) and without fermentation (Picholine or American type).

a) Spanish or Sevillian style

The olives are treated in a diluted lye solution (sodium hydroxide) to eliminate and transform the oleuropein and to release sugars, by increasing the permeability of the fruit. The lye concentrations vary from 2% to 3.5%, depending on the ripeness of the olives, the temperature, the variety and the quality of the water. The treatment is performed in containers of varying sizes in which the solution completely covers the fruit. The olives remain in this solution until the lye has penetrated two thirds of the flesh. The lye is then replaced by water, which removes any remaining residue and the process is repeated. Lengthy washing properly eliminates soda but also washes away soluble sugars which are necessary for subsequent fermentation.

Fermentation is carried out in suitable containers in which the olives are covered with NaCl brine. Traditionally, this was done in wooden casks. More recently, larger containers made with inert materials have come into use. The brine causes the release of the fruit cell juices, forming a culture medium suitable for fermentation. Brine concentrations are 9-10% to begin with, but rapidly drop to 5% owing to the olive's higher content of interchangeable water.

At first Gram-negative bacteria multiply, but after a week and a half they disappear. They are a consequence of contamination produced in the plant installations, atmosphere and brine and can be avoided by stepping up hygiene measures. At a pH level of 6 and upwards, lactobacilli develop massively until the Gram-negatives disappear; the brine attains a pH of 4.5 and there is a predominance of *Lactobacillus plantarum* which produces lactic acid from glucose. When the

fermentable matter is spent, acid formation ceases. Yeasts appear together with the lactobacilli. Fermentative yeasts do not cause deterioration but oxidant yeasts consume lactic acid and raise the pH level and may therefore jeopardise the process.

Under certain conditions normal fermentation processes can be altered by the presence of undesirable microorganisms which can transmit undesired sensory properties to the olives or impair their keeping properties. Gas pocket fermentation is caused by the Gram-negative bacilli in the first stage of fermentation, but can be controlled by intensifying hygiene precautions when the olives are delivered to the plant, as well as during lye treatment and washing. If gas pockets still appear in spite of these measures, the pH level can be lowered to 4 by adding an acid. Butyric fermentation is well controlled by ensuring the proper pH level. Putrid fermentation is caused by poorly-kept containers and bad water. Lastly, there is a type of deterioration known by its Spanish name of "zapatería" (cobbler's) which produces an unpleasant taste and odour at the end of the fermentation process, often coinciding with rising temperatures in the spring or early summer. It is produced by bacteria belonging to the *Clostridium* and *Propionibacterium* genera. The right combination of brine concentration and pH level (5% salt and 4.5 pH) helps to control fermentation process.

When properly fermented, olives are characterized by a long shelf-life. If they are in casks, the brine level must be topped up. At the time of shipment, the olives have to be classified for the first or second time as the case may be. The original brine is replaced and the olives are packed in barrels and tin or glass containers. Sometimes they are stoned(pitted) or stuffed with anchovies, pimento, etc. The most commonly used varieties are Manzanillo, Gordal and Moroccan Picholine.

b) Picholine style

Olives belonging to the Picholine variety from Languedoc and Lucques in southern France are prepared in this manner, as are other varieties from Morocco and Algeria.

The bitterness of the olives is removed by treating them in a 3-3.% lye solution in which they are left for 8 to 72 hours until the lye has penetrated three-quarters of the flesh. They are rinsed several times over the next day or two days, and then placed in a 5-6% brine solution for two days. A second 7% brine solution is prepared, and acidity is corrected with citric acid (pH 4.5). After 8-10 days they are ready to be eaten and retain their intense green colour. Sometimes the consignment has to be postponed, and it is necessary to store the olives. This is easy, as long as temperatures do not rise. The olives can stay in an 8% brine solution until spring, but then it has to be raised to 10%. In larger installations they can be kept in cold storage, at a temperature of between 5-7° C, in a 3% brine solution.

Before shipment, the olives are washed repeatedly, sorted and packed in suitable containers in brine at 5-6°C.

Californian style (Semi-ripe olives)

These are obtained from olives that are picked when their colour is starting to change. They are harvested before full maturity, when the flesh is quite firm and oil formation has not concluded. The process of darkening the fruit by oxidation is typical of California. Olives suitable for processing as green olives are selected as they enter into the factory, then placed in brine at concentrations between 2.5 and 10 % in inverse relationship to fruit size, and they are protected from air.

The olives are placed in large concrete tanks in an initial solution of 2 % lye until they are ready for subsequent preparation. When they are to be prepared for the market, they are placed in low-concentration lye and then washed in water into which compressed air is injected. Further treatments in dilute lye, each followed by

aeration, facilitate penetration through to the stone. Next, the olives are washed to eliminate lye residue and to lower the pH up to the neutrality. Solutions of 0.1 % ferrous gluconate or lactate are often applied to enhance fruit darkening. After placement in brine for a few days, the olives are ready for canning. Heat processing, as temperature and pressure-controlled sterilisation , is fundamental to ensure the product shelf-life.

Ripe olives

There are olives that are harvested when the fruit is close to full ripeness, once it has attained the colour and oil content corresponding to each particular variety. The are many types of preparations depending on local tastes. Those in greatest commercial use are now outlined.

a) Black olives in brine

These are typical of the eastern Mediterranean countries; in Greece they are from the Conservolea variety, which grades at around 200 fruits per kilogram, and in Turkey they are made with the Gemlik variety. The fruit is picked by hand when black ripe, but before the olives overripen or are shrivelled by frost. They have to be transported as quickly as possible to the processing plant where they are sorted, washed and immersed in 8-10% brine. Large-scale plants use big 10-20 tons tanks while small-scale processors continue to use wooden vats. At the start of fermentation the tanks are tightly sealed because the olives must not be exposed to air. The brine stimulates the microbial activity for fermentation and reduces the bitterness of the oleuropein. It drops to a concentration of 6 %, which is then increased to 8 -10 %, while homogenising it by operating a pump to activate circulation.

When the bitterness has been sufficiently weakened -how long this takes can vary greatly - the fruit can be sold. The colour fades during the process, but is corrected by aerating the olives for two or three days, although sometimes they are treated

with 0.1 % ferrous gluconate or lactate to make them a deeper black. Lastly, the olives are selected and packed in barrels or internally varnished cans, which are filled with 8 % fresh brine. They are popular on the market because of their slightly bitter taste and aroma. They are also packed in vinegar (25 % of brine volume) and may even be heat processed; a few grams of oil are then added to each can to form a surface layer. The Kalamata variety is prepared in this way; the elongated, medium-sized olives are slit to absorb the flavour of the marinade and then canned.

b) Black olives in dry salt

Also of Greek origin, they are prepared using overripe olives of the Megaritiki variety. They are vigorously washed and placed in baskets with alternating layers of dry salt equivalent to 15 % of the weight of the olives. The end product is not bitter, but salty, and it looks like a raisin; it is for local consumption.

Finally, a mention should also be given to the numerous styles of table olive preparations in the different olive-growing regions. Some examples are olives treated solely with water to sweeten them prior to crushing or splitting, which facilitates washing. In many cases, the olives are eaten after being seasoned with herbs, pieces of orange, lemon, garlic, paprika, oregano, etc. Until the turn of the twentieth century, the table olive market was local, but since then it has expanded to non-producing areas where table olives have become popular. This is particularly true of the Spanish, Greek and California types.

TRADE PREPARATIONS

According to the PROPOSED DRAFT CODEX STANDARD FOR TABLE OLIVES (Revision of CODEX STAN 66-1981) (25th Session Bali, Indonesia, 25 – 29 October 2010) different trade preparations are allowed depending on the processes, alkali or biological treatments. The product so obtained may be preserved in brine

according to its specific characteristics, in dry salt, in a modified atmosphere, by heat treatment, by preservatives, or by acidifying agents. The colour of green olives may vary from green to straw yellow, that of olives turning colour may vary from rose to wine rose or brown, and the colour of black olives may range from reddish black to violet black, deep violet, greenish black and deep chestnut.

Olives shall undergo the following preparations:

a) **Treated olives:** Green olives, olives turning colour or black olives that have undergone alkaline treatment, then packed in brine in which they undergo complete or partial fermentation, and preserved or not by the addition of acidifying agents:

1) Treated green olives in brine

2) Treated olives turning colour in brine

3) Treated black olives.

b) **Natural olives:** Green olives, olives turning colour or black olives placed directly in brine, in which complete or partial fermentation undergo, preserved or not by the addition of acidifying agents:

1) Natural green olives

2) Natural olives turning colour

3) Natural black olives.

c) **Dehydrated and/or shrivelled olives:** Green olives, olives turning colour or black olives that have undergone or not mild alkaline treatment, preserved in brine or partially dehydrated in dry salt and/or by heating or by any other technological process:

1) Dehydrated and/or shrivelled green olives

2) Dehydrated and/or shrivelled olives turning colour

3) Dehydrated and/or shrivelled black olives.

d) **Olives darkened by oxidation:** Green olives or olives turning colour preserved in brine, fermented or not, and darkened by oxidation with or without alkaline medium. They shall be a uniform brown to black colour. Alkaline-darkened olives shall be preserved in hermetically sealed containers and subjected to heat sterilisation. Olives darkened without alkaline treatment shall fulfil the requirements of **Packing Media (packing brines)**

1) Black olives.

2) Green Ripe Olives: Green olives or olives turning colour, not fermented, not preserved in brine and not oxidized, but undergo alkaline treatment, preserved in hermetically sealed containers subject to heat sterilization

e) **Specialities:** Olives may be prepared by means distinct from, or additional to, those set forth above. Such specialities retain the name "olive" as long as the fruit used complies with the general definitions laid down in this Standard. The names used for these specialities shall be sufficiently explicit to prevent any confusion, in purchasers' or consumers' minds, as to the origin and nature of the products and, in particular, with respect to the designations laid down in this Standard.

VARIETAL TYPES

For table olive production any commercially cultivated variety (cultivar) suitable for canning may be used.

STYLES

Olives may be offered in one of the following styles:

1 **Whole olives**

a) Whole olives**:** Olives, with or without their stem, which have their natural shape and from which the stone (pit) has not been removed.

b) Cracked olives: Whole olives subjected to a process whereby the flesh is opened without breaking the stone (pit) which remains whole and intact inside the fruit.

c) Split olives: Whole olives that are split lengthwise by cutting into the skin and part of the flesh.

2 **Stoned (pitted) olives**

a) Stoned (pitted) olives: Olives from which the stone (pit) has been removed and which basically retain their natural shape.

b) Halved olives: Stoned (pitted) or stuffed olives sliced into two approximately equal parts,

perpendicularly to the longitudinal axis of the fruit.

c) Quartered olives: Stoned (pitted) olives split into four approximately equal parts along and perpendicularly to the major axis of the fruit.

d) Divided olives: Stoned (pitted) olives cut lengthwise into more than four approximately equal parts.

e) Sliced olives: Stoned (pitted) or stuffed olives sliced into segments of fairly uniform thickness.

f) Chopped or minced olives: Small pieces of stoned (pitted) olives of no definite shape and

practically devoid (no more than 5 per 100 of such units by weight) of identifiable stem-insertion units as well as of slice fragments.

g) Broken olives: Olives broken while being stoned (pitted) or stuffed. They may contain pieces of the stuffing material.

3 **Stuffed olives:** Stoned (pitted) olives stuffed either with one or more suitable products (pimiento, onion, almond, celery, anchovy, olive, orange or lemon peel, hazelnut, capers, etc.) or with natural pastes.

4 **Salad olives:** Whole broken or broken-and-stoned (pitted) olives with or without capers, plus stuffing material, where the olives are the most numerous compared with the entire product marketed in this style.

5 **Olives with capers:** Whole or stoned (pitted) olives, usually small in size, with capers and with or without stuffing, where the olives are the most numerous compared with the entire product marketed in this style.

6 **Olive paste:** Exclusively olive flesh, finely crushed.

2. *BACTROCERA OLEAE* PEST

The olive fruit fly *Bactrocera oleae*

Taxonomic classification

Class: Insect

Order: *Diptera*

Family: *Tephritidae*

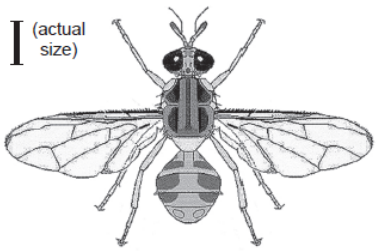
Genus: *Bactrocera* (Dacus)

Species: *oleae*

Common name: olive fly

Common host: olives

Figure 4: Olives fruit fly adult.



The olive fly, *Bactrocera oleae* (Rossi) (Fig. 4), formerly *Dacus oleae* is a serious pest of olives in most of the countries around the Mediterranean Sea. Larvae are monophagous and feed exclusively on olive fruits. Adults feed on nectar, honeydew and other opportunistic sources of liquid or semi-liquid food. Until 1998, the fly had not been detected in the United States, and its range coincided with the range of the olive tree in the Eastern Hemisphere: northern, eastern and southern Africa, Canary Islands, India, western Asia.

The olive fruit fly was first detected in North America infesting olive fruits on landscape trees in 1998. In the Western Hemisphere, it is currently restricted to California and Baja California, Mexico (Rice *et al.,* 2003).

The olive fruit fly development can be continuous throughout the year. The cause of olive fly damage is larvae. Egg laying females deep their ovipositor in the fruit surface. The larvae (maggots) of the fly feed inside the fruit, destroying the pulp and allowing the entry of secondary bacteria and fungi that rot the fruit and lowering/decreasing the oil quality. Feeding damage can cause premature fruit drop and reduce fruit quality for both table olive and olive oil production.

Insects have been reported to establish symbiotic associations with a variety of microorganisms which affect many aspects of host biology and physiology. Insect symbionts are divided into two main groups: “primary” and “secondary” symbionts. Primary symbionts (for example is *Buchnera*, the primary symbiont of aphids) live with bacteriomes,specific host organs, and have the ability to make available nutrients such as essential amino acids, vitamins and other cofactors for their host. Secondary symbionts, also called “guest” microbes, typically establish optional symbiotic associations that can be deleterious or beneficial (Capuzzo *et al.,* 2005).

Since the beginning of the last century, some authors (Petri 1909) reported the presence of symbiotic bacteria in flies belonging to the subfamily *Tephritinae*. The olive fly population affected the number of epiphytic bacteria,with the main role of protein source, present on the olive surface.

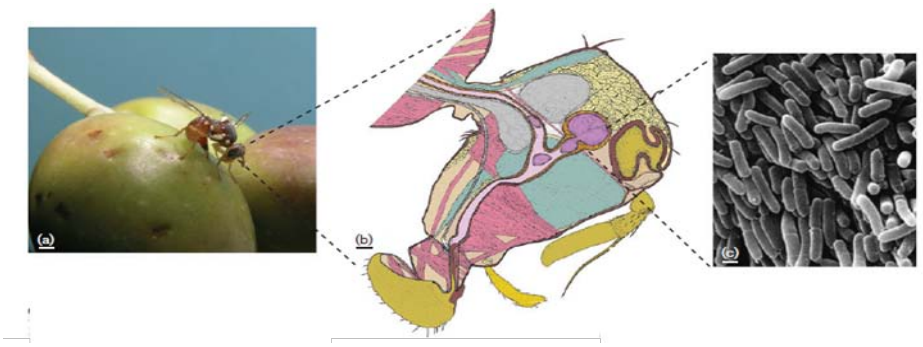
Adults of *B. oleae* harbour microorganisms inside a cephalic organ in which the symbionts multiply rapidly, forming conglomerate that arrive at the mid-gut. The mother diffuses symbionts to the eggs during oviposition. ‘Bacterium’ *Pseudomonas savastanoi*, is supposed to be the symbiont and the causal agent of the olive knot disease, as it has been isolated from larvae. Recently another the olive fly symbiont has been described and designated as ‘Candidatus *Erwinia dacicola*’ (Capuzzo *et al.*, 2005) (Fig.5).

Control

The losses, caused in this crop by insect pests, fungi and weeds, have been estimated by some authors to be as high as 30% of production in Greece, Italy and Tunisia. Hence It is reasonable to calculate that the damage caused to harvested fruits by insect pests to be at least 15% of production, (Saour and Makee, 2004), damage threshold level for oil production is 10% and for commercial table fruit is 1%, but California table fruit processors have zero tolerance for olive fruit fly damage. For back yard olive producers, wanting to make a few table olives, the damaged fruit can be sorted out by hand, therefore the damage threshold level (tolerance of some olive fruit fly damage) can be greater.

The control of *Bactrocera oleae* has been based mostly on bait sprays with conventional pesticides, (organophosphate insecticides ,usually dimethoate and fenthion) for about 40 years (Panagiotis *et al.,* 2006).

Figure 5: Outline of the bacterial location within adult *Bactrocera oleae*: (a) Female fly laying its egg in an olive; (b) Anatomical depiction of the fly head in longitudinal section, with indication of the pharyngeal bulb; (c) Scanning electron micrograph of the bacterial content present in the pharingeal bulb.



Even if there is a need to use more environmentally compatible control methods, i.e. mass-trapping, attract and kill, sterile insect technique, alternative natural phytosanity products (e.g. kaolin-based participle films) and biological control, the control of *B. oleae* remains almost totally based on insecticides, particularly organophosphates (Roessler, 1989). Among them dimethoate is extensively used because of its low residual persistence in olive oil. The extensive and long use of

insecticides for the control of *B. oleae* might lead to an increasing resistance to insecticide, especially when only one kind of insecticides with a particular mode of action is applied. The expansion of resistance to insecticides is the central problem in pest control since resistant populations have been reported in almost all economically important insect pests.

Organic agriculture

In the last years there has been an explosion in the use of common foodstuffs and other "organic" products that are associated with an image of naturalness and hygienic safety by the public.

In reality, it can't be considered so different from the most modern "conventional" strategies, as it is largely based on the use of active ingredients, which are natural but anyway toxic, and that have on a different level all the problems of "pesticides" (residues on foodstuffs, side effects on non-target organisms, etcetera). Botanical insecticides and natural foods fit well together. Increasing information helps in choosing the best materials and in insuring their effectiveness and safety. The natural chemical defenses of plants, used as botanical insecticides, offer continue benefits in organic agriculture with confidence that the consumer and environment will be adequately protected (Lozzia and Rigamonti, 2002). This new trend is going to be more studied even because residues of pesticides have been detected in olive oil and in the environment where olives are grown.

Barrier film spry: kaolin

Substitute to synthetic chemical pesticides are necessary to reduce the impact of pesticides in the environment and to insure a safer food supply for consumers. Kaolin-based particle film, a novel approach to contain insects in agricultural crops, is now commercially available under the trade name Surround WP Crop Protectant (Engelhard Corporation, Iselin, NJ, USA). The chemical component film is a highly

refined kaolinic mineral [$Al_4Si_4O_{10}(OH)_8$], white in appearance and hydrophilic (Perri *et al*., 2005; Liang and Liu, 2002). Even if it is not directly lethal to insect pests, its insecticidal properties are thought to be a result of its repellent nature, anti-ovipositional qualities and/or because of its highly reflective white coating. In practice, when the product is sprayed onto tree foliage as a liquid suspension, water evaporates leaving kaolin as a white porous protective powdery film on the surface of the leaves and fruits (Saour *et al.,* 2004).

Study on Castelvetrano table olive orchards, using different combinations of clays products, Surround WP, compared to copper hydroxide, oxichloride and bentonite products during 2003, 2004 and 2005 have shown good results and new opportunities in organic farm for table olive production (Caleca and Rizzo, 2006).

Copper salts

Copper fungicides can be highly effective if applied prophylactically (before infection) and with complete exposure of all plant foliar surfaces, including the undersides of leaves where the pathogen typically sporulates. After the absorption into the fungus or bacterium, the copper ions will link to various chemical groups (imidazoles, phosphates, sulfhydryls, hydroxyls) present in many proteins and disrupt the function of these proteins. Therefore, the mode of action of copper hydroxide (or any other copper fungicide) is the nonspecific denaturation (disruption) of cellular proteins (http://extoxnet.orst.edu/pips/coppersu.htm, 1996). In yeast cells, although beneficial at subtoxic levels, is demonstrated that, at moderately toxic levels, copper induces extensive apoptosis. At even higher concentrations, necrosis takes over. The toxic copper ion is absorbed by the germinating fungal spore and thus for best results copper must be reapplied as plants grow to maintain coverage and prevent disease establishment. Many similarities occur between apoptotic programs in yeast and mammalian cells. Copper sulfate and hydroxide has been shown to cause toxic effects to human health. Copper does not degrade in soil and there are serious concerns about the

cumulative effect (Baker, 1990). In humans, Cu toxicosis can be the result of inherited abnormalities (such as in Wilson's disease and likely some cases of Indian childhood cirrhosis) or environmental poisoning, and it often leads to hepatic cirrhosis and degeneration of the basal ganglia (Liang and Zhou, 2007).In the European Union, copper fungicides have been banned completely in the Netherlands and Denmark, and use has been restricted to 6kg/ha/year (5.4 lbs/A) elemental copper in other EU countries since 2006.

Bait Sprays

GF-120 NF Naturalyte Fruit Fly Bait, is an organically acceptable product containing the biologically produced insecticide spinosad, and is the only insecticide registered in California.

Spinosad is a fermentation by-product from the actinomycete bacteria called *Saccharopolyspora spinosa*. The bait is a formulation of hydrolyzed protein.

GF-120 attracts olive fruit fly adults, which feed on the bait, and causes adult mortality.

Trapping for detection

Traps baited with food lures can be used to reduce adult fly densities by mass trapping (Broumas *et al.,* 2002). Yellow sticky traps baited with a male sex lure (spiroketal pheromone capsule) and a feeding attractant capsule (ammonium bicarbonate) are used to capture both male and female adult flies. The attraction packet is inserted between the panels of the yellow sticky trap and the pheromone capsule hangs on the top outside edge.

Another kind of trap is the so called McPhail trap, that is used extensively in Europe, mainly for monitoring, but in some cases for mass trapping (control) as well. They are constitute of glass or plastic with a tank for liquid bait containing bait attractants, usually 4% solution of ammonium salts (ammonium bicarbonate or ammonium phosphate). Flies enter from below and sink in the solution. These

traps also work in a non-breeding host such as citrus, cherry, plum and nectarine orchards (Vossen *et al.,* 2004).

OLIPE Trap

OLIPE trap was developed in Spain and It is a homemade trap used to suppress olive fruit fly populations in organic olive groves and in sensitive areas near homes and natural parks. The OLIPE trap is made from a 1- to 2-liter plastic bottle with 13⁄64 inch (5 mm) holes. Traps are hung in the shade, and flies are attracted to the trap, crawling inside and dying. Current research in California suggests that *Torula* yeast tablets dissolved in water are very effective as bait.

"Attract and Kill" Trap

Olive fly species uses a sex pheromone as part of its mating behaviour. A sex pheromone released by virgin females attracts male *B.oleae*. The principal component of this sex pheromone was identified as 1,7-dioxaspiro undecane (Rice, 2000).

Flies attracted to the trap are killed when they contact the insecticide. No chemical residue is left on the fruit or in the orchard environment. The "Attract and Kill" method integrating pheromone and ammonium bicarbonate as lures has been tested with good results in Spain and in Greece and is going to be tested all over the Mediterranean basin.

Biological Control

In sub-Sahara Africa, where it is believed to have originated, and in the Mediterranean area, the olive fruit fly is attacked by a number of parasitic wasps. On the other hand, the parasites common in the Mediterranean area do not provide acceptable control in commercial situations. Some naturally occurring parasites are recognized to attack olive fruit fly in California, but they do not provide sufficient control (Kapatos *et al*., 1977; Navrozidis *et al.,* 2000).

IPM (Integrated Pest Management)

IPM are all the Technologies and techniques which have found a useful role in fight against olive fly, which include: the use of resistant plants varieties, cultural practices, the use of predators and parasites, microbial pesticides (entomopathogenic bacteria, viruses and fungi), botanical insecticides, insect growth regulators and semiochemicals. Many of these have been researched as constituents of an IPMstrategy for olive pests. (Montiel Bueno, 2002). Moreover is necessary a research on the relationships between macro- and microclimates, fly behaviour, fruit maturity and susceptibility of table and oil cultivars to infestation, and a better understanding of when and how to apply various integrated pest management (IPM) strategies such as bait spray applications, trapping options, parasite releases and harvest timing. For example an olive fly phenology model based on trapping and temperature, could be used to determine optimum harvest timing, or the release of parasites in urban trees or organic olive production

In the future the best strategy for a high recovery from harvesting of olive drupe, managing olive fruit fly, will depend on a combination of tactics including bait sprays, attract-and-kill trapping of adult flies, harvest timing, fruit sanitation after harvest, and biological control.

3. MICROBIAL POPULATION OF ROW OLIVES

Olives surface harbour large and diverse populations of microorganisms. Gram-negative bacteria are mainly belonging to *Pseudomonas*, *Enterobacter* or *Erwinia* species. Lactic acid bacteria, involved in developing the spontaneous or started lactic fermentation of table olives (Garrido Fernandez *et al.,* 1997), have been also detected in olives (Lavermicocca *et al.,* 1998; 2005; Nychas et al., 2002); the most isolated are (Tab. 1): *Lactobacillus plantarum, Lb. paracasei, Lb. pentosus, Lb.*

brevis, Steptococcus salivarus, Pediococcus pentiocaseus, P. acidilactici, Tetragenococcus alophilus. Some probiotic strain have been tested on table olive and showed good probiotic and technological attitude.

The probiotic strain *Lb. paracasei* IMPC2.1 can be considered an example of a strain used in the dual role of starter and probiotic culture which allowed the control of fermentation processes and the realization of a final probiotic product with functional properties (De Bellis *et al.,* 2010). The most isolated genera of moulds were *Pennicillum*, *Botrytis* and *Monilia* (Kacem and Karam, 2006).

Table 1: Lactic Acid Bacteria isolated from table olives.

Authors	**Products**	**Lactic Acid Bacteria**
Floriano *et al*., 1998	Spanish-style green olives	*L. plantarum* *Enterococcus sp.*
Maldonado *et al.,* 2003	Fermented green olive of South Spain	*L. plantarum*
Ruiz-Barba *et al*., 1991; Ruiz-Barba and Jiménez-Diaz,1994; 1995	Spanish fermented olives	*L. plantarum*
Fernàndez-Dìez, 1983; Van den Berg *et al*., 1993; Oliveira *et al.,* 1993	Portuguese fermented olives	*L. plantarum* *L. paracasei* *L. pentosus* *Ln. pentosaceus*
Asehraou *et al*., 2002	Moroccan fermented olives	*L. plantarum*
Borcakli *et al*., 1993	Algerian fermented olives	*L. plantarum*
Kacem *et al*., 2004a	Algerian fermented olives	*L. plantarum* *L. lactis* *E. faecalis*
Korukluoglu *et al*., 2002	Turkish fresh olives	*L. plantarum* *L. brevis* *L. lactis* *Ln. mesenteroides* *P. damnosus*
Randazzo *et al.,* 2004	Sicilian (Italy) fermented green olives	*L. casei* *L. plantarum* *L. brevis* *E. faecium*

Yeast species

Yeast species associated with table olive are species selected from the condition of the brine environmental conditions.

High salt concentration, low pH, polyphenols content are some example of factors that can influence the microbial population. Anyway due to the capability of yeast to adapt to different environments yeasts play an important role in different food fermentation.

The count and isolation of yeast species has been done on both olive drupe and brine (Arroyo-Lopez *et al.,* 2008). Considering the olive surface low values (1 log 10 cfu g^{-1}) have been detected, while olive brine allows to get an higher value, if the sampling is made correctly on different fractions.

Yeast species isolated and identified vary from different treatment and process of table olive.

According to different techniques of identification, traditional morphological and biochemical using the taxonomic keys of Barnett et al. (1990) and Kurtzman and Fell (1998) or new recent methods as molecular tools that allow to get an higher degree of accuracy, independently from variability of phenotypical characteristics.

According to different olive process the main species identified are:

Spanish style olive

Yeast species are usually detected during the latest fermentation process at low level, as in this process LAB starter culture are inoculate subsequently to lye treatments. *Candida krusei, C. parapsilosis, C. tropicalis, Pichia anomala, Rodothorula glutinis and Saccharomyces cerevisiae* were isolated and identified in different production years in Spain and USA (Mrak *et al.,* 1956; González Cancho, 1965, 1966). Moreover yeast oxidative species were identifies and considered cause of film-forming as for example *C. rugosa, P. membranifaciens.*

Directly brined

Several species were isolated from directly brined green and turning colour olives. Pelagatti (1978) and Marquina et al. (1992) detected *Candida, Debaryomyces, Kluyveromyces, Pichia, Rhodotorula* and *Saccharomyces* species , while Marquina et al. (1997) in studies carried out with olive brines from seven locations in Morocco mainly isolated *C. boidinii, P. membranifaciens* and *Torulaspora delbrueckii.*

Moroccan olive were studied under different aspect; altered green olive fruits harvested by the pole slender method were studied for the microorganisms involved in post-harvest alterations of the fruits before the fermentation process. Samples showed an high presence of the species *Debaryomyces hansenii, R. glutinis, P. membranefaciens, P. anomala and C. bacarum* (Faid et al., 1994).

Hernández et al. (2007) found *P. anomala, Kluyveromyces marxianus, D. hansenii and S. cerevisiae* to be the main species present during the processing of directly green table olives from Portugal. In another study carried out on natural turning colour olives from Portugal *C. boidini* with *C. glabrata* where the most isolated species from both olive brine and pulp and *C. krusei* only from olive brine (Pereira *et al.,* 2008).

Arroyo López et al. (2006) identified the species *S. cerevisiae, Issatchenkia occidentalis* and *Geotrichum candidum* from Spanish green seasoned table olives. Hurtado et al. (2008) monitored *C. boidinii, C. diddensiae, C. membranifaciens, Kluyveromyces lactis, P. membranifaciens, P. kluyveri,* and *R. glutinis* species during processing of Arbequina table olives in Spain, produced according to a traditional process involving a spontaneous fermentation in brine.

In Tunisian olive fermentation brine the dominant eukaryotic phylotype was identified to be *P. membranifaciens* (Chamkha *et al.,* 2008). The microbial diversity was analysed using a culture-independent approach based on the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). SSCP patterns showed a remarkably simple microbial community but higher for bacterial community than for the eukaryotic community.

Attacked fruits were collected to study the microbial population in bloaters. Only yeast specie were isolated and the high presence was of *S. cerevisiae, P. anomala, C. etchellsii, C. versatilis and R. glutinis* species (Asehraou *et al.,* 2000).

C. parapsilosis, P. guilliermondii and *P. kluyveri* were the three most isolated species from four green Sicilian table olive cultivars (Brandofino, Castriciana, Nocellara del Belice and Passalunara) and the Spanish Manzanilla (Aponte *et al.,* 2010).

Black olive

Balatsouras (1967) reported the presence of yeasts of the genera *Trichosporon, Candida, Pichia, Kloeckera, Torulopsis* and *Debaryomyces* during the fermentation of directly brined natural black olives from Greek cultivars, and Borcakli et al. (1993) isolated species of *Debaryomyces* from Turkish cultivars.

Spanish natural black olive fermentations were characterized by an high presence of *S. cerevisiae* and *P. anomala* (González Cancho *et al.,* 1975). Kotzekidou (Kotzekidou 1997) identified, based on 65 morphological and physiological characteristics, *T. delbrueckii, D. hansenii* and *Cryptococcus laurentii* as the predominant species in Greek-style black olives; the species *Cryptococcus albidus*, *Dekkera bruxellensis*, *Sporobolomyces roseus*, *Bullera variabilis*, *P. farinosa*, *P. membranaefaciens*, and *Zygosaccharomyces bailii* were sporadically identified.

Arroyo López et al. (2006) isolated *C. boidinii* and *Hanseniaspora guilliermondii* from processed black olives; Coton et al. (2006) identified *P. anomala, C. boidinii* and *D. etchelsii* as the predominant species in French black olive natural fermentations using the same methodology (molecular). *C. boidinii* , mostly detected on olive pulp, and *C. glabrata* were also isolated from different black ripe olive from different Portuguese markets (Pereira *et al.,*2008).

Greek-style processing of natural black Conservolea olives in different brine solutions were analysed and *Metschnikowia pulcherrima* was the dominant yeast

species at the onset of fermentation, followed by *D. hansenii* and *Aureobasidium pullulans*. Species heterogeneity changed as fermentations proceeded and *P. membranifaciens* along with *P. anomala* evolved as the main yeasts of olive elaboration, prevailing at 17 and 35 days of the process. Molecular techniques allowed for the identification of five yeast species, namely *A. pullulans, Candida* sp., *C. silvae, Cystofilobasidium capitatum* and *M. pulcherrima*, which have not been reported previously in black olive fermentation (Nisiotou *et al.,* 2010).

4. IDENTIFICATION TECHINIQUES / MOLECULAR TOOLS

Yeast taxonomy and species identification (Fleet , 2007) (Obera-Ratòn, 2004)

Yeasts are agents responsible for the damaging of fresh and elaborated food for human consumption. For these reasons a fast and accurate identification of industrially, environmentally and clinically yeasts is necessary. Yeast taxonomy has been supported by conventional techniques , based on morphological and physiological descriptions of species and genera requiring the laborious completion of 80 to 100 morphological biochemical and physiological analyses. Therefore, strain culture conditions have introduced errors in yeast taxonomy and originated the duality of their nomenclature. These difficulties have been solved with the application of molecular techniques, based on the sequence analysis of nucleic acids.

The DNA sequences of the genes encoding the D1/D2 domain of the large (26S) subunit of ribosomal RNA are known for all yeast species, and the sequence of the ITS1-ITS2 region of rRNA, as well as other genes, is known for many. These sequence–phylogenetic data have led to a complete revision

of yeast taxonomy, and the description of many new genera and species. Although sequencing of ribosomal genes is now the accepted method for yeast identification,

restriction fragment length polymorphism(RFLP) analysis of the ITS1-ITS2 region is a less expensive, faster alternative, and databases containing the results of such analyses have been established for food yeasts and easily to be found in literature.
Nucleic acid probes and real-time PCR detection methods have been described for some species, such as *S. cerevisiae, Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* and a novel probe-flow cytometric assay has been reported for various *Candida* species (Page and Kurtzman, 2005; Rawsthorne and Phister, 2006).

Culture dependent analysis / Strain differentiation

The distinctive character of many fermented foods can be linked to the particular yeast strains used for the fermentation. Therefore, differentiation of yeasts at a subspecies level is an important requirement. For example, it is now known that the fermentation of breads, beers, cheese, salami and wines and other products not only involves the successive contributions from many different species of yeast, but consecutive growth of numerous strains within each species also occurs (Fleet, 2003).
Molecular methods developed for this purpose especially include karyotiping electrophoresis (pulsed-field gel electrophoresis, PFGE, of chromosomal DNA), macrorestriction analysis (REA-PFGE) and PCR-based methods such as random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), RFLP, δ-PCR, PCR Melting Profile (PCR MP) and profiling of microsatellite DNA. A simpler, faster method is based on RFLP analysis of mitochondrial DNA, where no PCR amplification of DNA is required (de Llanos *et al.,* 2006; Krawczyk *et al.,* 2009; Muller and McCusker 2009). These methods are not only useful for quality assurance typing of yeast starter cultures and spoilage species, but they have been used to reveal the ecological complexity of the yeast

flora associated, at both species and strain level, with many food and beverage fermentations.

Culture-independent analysis

Most branches of microbial ecology now accept that viable but non-culturable species occur in many habitats, including foods and beverages. Detection of these organisms requires extraction and analysis of the matrix DNA and different techniques are available nowadays.

PCR amplification of the yeast community ITS rDNA, targeting the fungal 5.8S-26S rDNA spacer region, was performed using DNA directly extracted from brine samples of different Sicilian table olives (Aponte *et al.,* 2010); its results were comparable with culture dependent methods.

Study of ITS region was also performed on PCR of DNA extracted directly from wine-related samples using primers ITS3 and ITS4, the latter being partially labelled by FAM (f-ITS PCR). The non-cultivation analysis of the fungal microbial community was carried out using total DNA from grape and wine must samples. The PCR results varied slightly between different PCR repetitions, however dominant fragments were the same and gave a good reproducibility of the technique (Brežná *et al.,* 2010) .

One approach that is finding increasing application is PCR in conjunction with Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE). Total DNA is extracted from the food and yeast DNA is specifically amplified using PCR and primers targeting regions of rDNA. The yeast DNA is then resolved into amplicons for individual species by DGGE or TGGE. These amplicons are extracted from the gel and their species identity determined through sequence analysis. PCR-DGGE/TGGE has been applied to analyse the yeast communities associated with grapes, wine, sourdough, cocoa bean, coffee bean and meat sausage fermentations (Nielsen *et al.,* 2005).

There is good agreement in the results obtained by cultural and PCR-DGGE/TGGE methods, although in some cases species that were not identified by agar culture were recovered by PCR-DGGE, suggesting the presence of non-culturable flora. However, the reverse also occurs, where PCR-DGGE has not detected yeasts that were isolated by culture. Many factors affect the performance of PCR-DGGE/TGGE analyses and further research is required to understand and optimize the assay conditions (Prakitchaiwattana, 2004).

Analysis using a culture-independent approach based on PCR-SSCP (Polymerase Chain Reaction-Single Strand Conformation Polymorphism) has been used to study the evolution of microbial species and to established two small subunit (SSU) rRNA clone libraries of Bacteria and Eucarya populations. The diversity and succession of yeast populations in three traditional Registered Designation of Origin (R.D.O.) Sellers cheeses has been determined by using phenotypic diagnoses SSCP analysis to amplify the V4 region of the 18S rRNA gene. Isolates were identified by phenotypic tests and the sequencing of the D1-D2 domains of the 26S rRNA gene. Ninety-two % of the isolates were identified as the same species in both approaches (Callon *et al.,* 2006). The technique was suitable also to study the microbial diversity of a Tunisian olive fermentation brine and Olive mill waste water sludge (Abid *et al.,* 2007; Chamkha *et al.,* 2008).

A novel multiplex method Polymerase Chain Reaction coupled to Electrospray Ionization Mass Spectrometry (PCR/ESI-MS), was used to identify the genus and species of microorganisms found to cause human bloodstream infections, with DNA directly extracted from blood cultures, without the need for cultivation. The methods has a high analytical accuracy in comparison to routine subculture of blood cultures bottles and phenotypic identification of microbes and a fast results efficiency in 5-6 hours. Anyway the method is still very expensive for routine analysis and a widespread use (Kaleta *et al.,*2010).

5. ROLE OF YEASTS IN TABLE OLIVE FERMENTATION

The complex role of yeasts in table olive processing is summarized in Table 2

Table 2. Role of yeast in table olive process

Fermentation				**Packing**	
Positive role	**Yeast species**	**Negative role**	**Yeast species**	**Negative role**	**Yeast species**
Production of desirable volatile compounds and metabolites	Not related clearly with yeast species	**Gas pocked spoilage/ excessive production of CO2**	*S. cerevisiae* *P. anomala*	**Production of CO2**	*P. anomala* *S. cerevisiae*
Antioxidant activity	*P. anomala*	**Polygalactunorase activity**	*R. glutinis* *R. minuta* *R. rubra*	**Increase of the cell number (clouding of the brines)**	*I. occidentalis* *S. cerevisiae*
Improvement of the lactic acid bacteria growth	*D. hansenii* *S. cerevisiae*	**Product with a milder taste a less self-preservation**	Not related clearly with yeast species	**Production of off-flavours and off odours**	Not related clearly with yeast species
Killer activity	*D. hansenii* *K. marxianus* *P.membranifaciens*			**Softening of fruits**	*P. anomala* *S. cerevisiae*
Biodegradation of polyphenols	*C. tropicalis*			**Resistance to high preservative concentrations**	*I. occidentalis*

Table from (Arroyo-Lopez *et al.,* 2008)

Improvement of the lactic acid bacteria growth

During table olive fermentation LAB and yeast coexist as in many other food matrix as for example in sourdough, cucumbers fermentation, wine.

In table olive the correct equilibrium between the two taxa depends on different factor:

1. Type of olive production (use of NaOH). The role of yeasts is especially important in directly brined green and natural black olives (Fernández Díez *et al*., 1985) because olives are not debittered with NaOH, and LAB are partially inhibited by the presence of phenolic compounds, in particular oleuropein, in the flesh. Due to β- glucodisicase activity yeast can hydrolyze oleuropein into fermentable sugars and aglicon.

2. Initial condition of fermentation (NaCl concentration, pH). If the levels of NaCl are high (≥8% NaCl in the equilibrium) yeasts can be the dominant microorganisms during fermentation. In natural olive fermentation, olives are placed into brine with a salt concentration between 8 and 10 % (wt/vol), although in colder areas lower concentrations (about 6 %) are used.

Gram-positive lactic cocci, *Pediococcus* and *Leuconostoc* genera, are detected during the first days; also, *Lactobacillus* growth during the whole fermentation period, if the salt concentration is not raised above 8.0 % (Garrido *et al.,* 1987).

Salt concentration influences the diffusion of water soluble components from olive flesh into brine. The sugars diffused in brine are used by microorganisms and converted to organic acids. Changes in chemical components are associated to simultaneous microbiological populations development during spontaneous fermentation (Romeo *et al.,* 2010). For example in Greek black dry-salted olives (cv. Thassos), yeasts became the dominant microbial group, with a population which ranged from 4.7 to 6.0 log10 CFU g−1 in olives with ≈7.5% NaCl (Panagou, 2006) and LAB are rarely detected. Arroyo López et al. (2007) found populations of yeasts around 6.5 log10 CFU ml−1 in directly brined green cracked olives of the Manzanilla–Aloreña variety with 11% initial NaCl.

In Sicilian green table olive Yeasts and moulds were countable from the day 42 (2 log CFU/ml) till the end of fermentation with 8% NaCl brine and acidified with 1 ml/l lactic acid (95%) (6 log CFU/ml) while *Pseudomonaceae*, *Staphylococcaceae*, *Enterobacteriaceae* and spore-forming bacteria remained undetectable up to the end of fermentation (Aponte *et al.,*2010). Tassou et al. (2002) reported that only

yeasts were able to grow in naturally black olives (Conservolea cv.) when the NaCl concentration was 8%. During the process of black table olives of Hojiblanca cv. by means of both anaerobic and aerobic processes with 4% NaCl and 0.3% acetic acid, yeasts reached their maximum populations 10 days after brining, with 7 log10 CFU ml−1 for the aerobic process and 4 log10 CFU ml−1 for the anaerobic process (Arroyo López *et al.,* 2006). Özay and Borcakli (1996) as well found a population of yeast of≈6 log10 CFU ml−1 as predominant microorganisms in naturally black olives of the Gemlik cv.

Studies on the effect of of sorbic and benzoic acids on the yeast population during fermentation of naturally black olives have been performed. The use of sorbic and benzoic acids can reduce yeast population without affecting the LAB growth, although a marked concentration of both preservatives in olive flesh was observed (Turantas *et al.,* 1999). The single and combined effects on a yeast cocktail (*S. cerevisiae, P. anomala, Issatchenkia occidentalis, and C. diddensiae,* isolated from table olives) have been studied with MIC (minimum inhibitory concentration), FIC (fractional inhibitory concentrations) and ELPM (efficacy of combined inhibitorsequation); this last one was the most suitable, showing that *I. occidentalis* was the most resistant yeast to the combined effect of sorbic and benzoic acids (Arroyo-Lòpez *et al.,* 2008). Anyway the use of benzoic acids, widely employed in USA, is not allowed in EU, opening new scenarios for implementation of fermentation process and a higher safety of the final product.

Vitamins. The olive cultivars showed different fermentation performance when directly brined, due to chemical, physical and biological cultivar characteristics. If a safe pH is achieved and kept during the fermentation process in brines, a proper environment could be created for LAB growth. If an initial high contamination of resistant microorganisms could be held, as for example mesophilic bacteria or yeasts and moulds, their importance throughout the brining process would be

leading during the whole process. The sequence of appearance of microbial populations in olive brines might be related to certain characteristics of the cultivar, such as high concentration of vitamins and nutrients and high sugar content, which could facilitate the growth of microorganisms.

LAB are not able to grow in a basal medium and need thiamine (vitamin B1), nicotinic acid, pyridoxine (vitamin B6) and pathogenic acid and other amino acids and purines (Abbas, 2006). Yeast accumulated and/or synthesized these vitamins and other enzyme cofactors that are essential for the growth of *Lactobacillus* (Viljoen, 2006). On the other side, LAB excrete lactic acid which leads to a lowering of the pH, which either inhibits the growth on undesired pathogens (Gram negative pathogens, enterobacteria and clostridia) or promotes yeast growth (Viljoen, 2006).

Antioxidant activity. Yeasts can synthesize a number of bioactive compounds which can contribute as antioxidants. Numerous *Candida* and *Saccharomyces* species produce compounds, for example, carotenoids, citric acid, glutathione and tocopherol with attractive antioxidant properties (Abbas, 2006). The secretion of these substances can be induced in yeasts grown under stress conditions or as a response to fermentation medium composition as phenolic or additives that are known to be toxic to aerobic cells (Cruz et al., 1999). The screening of yeasts for free-radical scavenging activity is an active area of research. As a result, yeasts from table olives could produce bioactive antioxidants, retarding oxidative degeneration of fatty substances and improving human health, for example *P. anomala,* broadly isolated from different table olive fermentations, is studied due to *in vitro* high antioxidant activity (Garrido Fernández *et al*., 1997; Gazi *et al.,* 2001).

Biodegradation of polyphenols. As previously said, olives have oleuropein which is responsible for their bitter taste. The green Spanish process degrades oleuropein

using NaOH. This treatment leads to large quantities of olive wastewater containing high levels of phenolic compounds, which is a serious environmental problem in almost all Mediterranean countries. Hence many studies have been carried out on yeasts with a capacity to reduce the amount of polyphenols in olives, olive brines and their wastewater. Strains of *C. tropicalis* and *Yarrowia lipolytica* have shown capacity to decrease the chemical oxygen demand, monophenols and polyphenols in olive mill wastewater (OMW) (Ettayebi *et al*., 2003; Lanciotti *et al.,* 2005). *C. tropicalis* has also been isolated from processing of black olives by Durán Quintana et al. (1986).

Studies of OMW consumption showed an high lipolytic activity of *C. cylindracea* CBS 7869; the phenols degradation was quite difficult, mostly when more easily degradable carbon source was still present in the medium (Gonçalves *et al*.,2009).

Production of desirable volatile compounds and metabolites. Esters produced by yeasts can contribute to both aroma and flavour, in the case of alcoholic and non-alcoholic fermentations. Several factors can contribute to aroma production by yeasts. These include changes in fermentation conditions such as temperature, pH, aeration, and the nature and concentration of the substrates used (Suomalainen and Lehtonen, 1979). In table olives, Sánchez et al. (2000) reported the formation of acetic acid, succinic acid, formic acid and ethanol during green olive fermentations. Montaño et al. (2003) also found the production of high amounts of ethanol, methanol and acetic acids in industrially fermented green olives, with acetaldehyde in small amounts. However, the formation of these compounds in table olives has not yet been linked with yeast growth, and far from it, with the yeast species. Hernández et al. (2007) studied the esterase and lipase activity of several yeasts isolated from green table olives.

Most of the yeast strains investigated showed esterase activity, but fewer cases had lipase activity. So the yeast population may therefore contribute to increasing

the free fatty acid content in olives during fermentation. This case has been reported in olive oil by Ciafardini et al. (2006) who found only two strains of *Williopsis californica* and *S. cerevisiae* with lipase activity.

ENZYMATIC ACTIVITY ASSOCIATED WITH POSITIVE ASPECT OF TABLE OLIVE

a. **Lypolytic Activity:** Olives are fruits with high fat concentrations, so the presence of lipolytic yeasts in table olives could modify the nutritional composition of the fruits and their sensory characteristics. More studies on olive yeast enzymatic activities can help to fix the role of the non-*Saccharomyces* versus *Saccharomyces* species in increasing the free fatty acid content during the fermentation stage of green olives. Only in recent times it has been shown that triglycerides, fatty acids, non-polar compounds (oxidized triglycerides, diglycerides and free fattyacids),sterols, aliphatic alcohols and triterpenic alcohols can be modified through ripe olive processing and that previous storage is the key step to such a modification. Volatile compounds, such as propanol and 2-butanol found in Spanish-style green olives (Montaño *et al*., 2003), can be generated by the catabolism of free fatty acids (Collins *et al*., 2003). As said, Hernández et al. (2007) studied the esterase (able to degrade tributyrin) and lipase activity of several yeasts isolated from green table olives Most of the yeast strains investigated showed esterase activity, but fewer cases had lipase activity*. D hansenii* and *T. delbrueckii* strains, characterized for their use as potential starter cultures in Greek-style black olives, showed an high lipolytic activity, hydrolyzing both tributyrin and olive oil (Psani and Kotzekidou 2006), as reported in olive oil by Ciafardini et al. (2006) for *Williopsis californica* and *S. cerevisiae* species. Studies by different methods, Rhodamine olive-oil agar method and spectrophotometric assay, on yeasts isolated from Hojiblanca and Manzanilla fruits evidenced an higher qualitative sensibility of the ROA method while the spectrophotometric measure

was quantitative better. *C boidinii* was the species with the highest enzymatic activity. In this case, *S. cerevisiae* strains with *P. galeiformis* didn't shown any activity suggesting an high variability among this species that can be due to table olive production. (Rodríguez-Gómez *et al*., 2010).

Pichia caribbica, *Zygosaccharomyces fermentati* (*Lachancea fermentati*) and *Pichia holstii* (*Nakazawaea holstii*), *Pichia mississippiensis*, *Lachancea* sp., *Kluyveromyces thermotolerans* and *Saccharomyces rosinii*. strains isolated from oleic ecosystem haven't shown any lipase activity (Romo-Sánchez *et al*., 2010).

b. **Oleuropein detoxification:** The main phenolic glycosides found in olive fruit are oleuropein, ligustroside, and demethyloleuropein. Other as verbascoside, an elenolic acid glucoside, luteolin- 7-glucoside, apigenin-7-glucoside, rutin, and quercetin-3-rutinoside have also been identified in fruits from different cultivars and maturation stages.

The aglycon derivatives resulting from the enzymatic hydrolysis of oleuropein, ligstroside, and demethyloleuropein, identified as dialdehydic forms of decarboxymethyl oleuropein and ligstroside aglycones (DGO and DGL, respectively) and aldehydic forms of oleuropein and ligstroside aglycones (AGO and AGL, respectively), are commonly known as secoiridoid compounds and are frequently found in table olive and olive oil.

Previous studies have proved that the concentration of phenolic compounds in the olive is greatly affected by the cultivar, maturation stage of olive fruit and processing parameters such as temperature, time, or exposure to air (Bouaziz *et al*., 2010), while the dependence of biophenols concentration on the irrigation conditions is still controversial (Visioli *et al.,* 2002). These factors probably modulate the activity of key enzymes within the metabolism of phenolic compounds and could be used for controlling the production processes and correlating the sensorial characteristics to the polyphenolic pattern.

Recent studies have been focused the antimicrobial and antiradical activity of these compounds.

In vitro antibacterial effects of different classes of important and common dietary phytochemicals has been proofed against pathogens such *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus.* The application of dual combinations of phenolic compounds with antibiotics, in particular streptomycin with isothiocyanates, were effective inhibitors of the *in vitro* on Gram-negative and Gram-positive pathogenic bacteria (Saavedra *et al*., 2010). Interest in phenolic compounds is related also to their antioxidant activity. They have shown an important biological activity in vivo and may contribute to prevent diseases related to oxygen radical formation when this exceeds the antioxidant defense capacity of the human body (Morello *et al*., 2004). The importance of β-glucosidases to food quality is due to their ability to hydrolyze flavor precursors, liberating aglycone moieties that provide desirable organoleptic properties to plant-derived foods. Microbial β-glucosidases have been investigated from both bacteria and yeast species (Romero-Segura *et al*., 2009).

The predominant secoiridoids of olive fruit pulp are oleuropein and ligustroside. Some oleuropein derivatives are demethyloleuropein, oleuropein aglycone, and elenolic acid (Amiot *et al.,* 1986; Esti *et al.,* 1998; Romani *et al.,* 1999; Soler-Rivas *et al.,* 2000). Others secoiridoids are verbascoside and nuzhenide.

They are typical of olive pulp and are present at a lower concentration in skin and seed. Apart from their antimicrobial and healthy characteristics, they are the main cause of sensorial profile of table olives due to their role in the bitter flavor.

Oleuropein can be enzymatically hydrolyzed and Marsilio and Lanza (1998) reported a two-step pathway for oleuropein metabolism. The first step is the hydrolysis of glycoside linkage by β-glucosidase with formation of oleuropein-aglicone. In the second step, the aglicone is hydrolyzed to elenolic acid and hydroxytyrosol. This last compound is unstable and diffuses in brine, depending on to the permeability of the olive skin (Poiana and Romeo, 2006).

From oleuropein hydrolyses the free glucose can have inhibitory action on β-glucosidase but no influence on esterase activity. the complete hydrolysis of hydroxytyrosol is fundamental because these compounds are toxic for LAB , responsible of stability of table olive, and can lead to a protein degradation , bringing to a final product with less nutritional characteristics. The role of LAB, in particular *Lb. plantarum*, has been highlighted in these last years because they are able to use hydroxytyrosol, when glucose level decrease (Ciafardini and Peca, 2003), and many studies are focused in these species as starter culture.

The cultivar characteristic is important in fermentation for amount and type of phenols and their ability to diffuse outside the fruit. The higher the content of water in the drupe the faster is the speed of phenol diffusion in the first brining.

In the last years there has been the tendency to prefer the natural fermentation of table olive vs the chemical Spanish style, due to not only to wastewater purification, low value of BOD and COD and lower use of water for washing steps , but to possibility of maintaining polyphenol in the final product because NaOH washing leads to polyphenol and nutritional losses for the final table olive product.

Moreover enzymatic debittering of oleuropein in natural fermented olives not only preserves polyphenol but also tocopherol and carotenoid of olive drupe which antioxidant and antiradical action is nowadays indubitable (Ciafardini and Peca, 2003).

The role of yeast in the detoxification from oleuropein has been highlighted; β-glucosidase activity (ßG) has been reported to be particularly marked in *Pichia* strains (Romo-Sánchez et al., 2010) anyway also *C. boidinii, C. wickerhamii* and *S. cerevisiae* strains isolated from olive oil displayed ßG althought in *S. cerevisiae* species this activity is not common (Ciafardini and Zullo 2002; Spagna et al., 2002).

ENZYMATIC ACTIVITY RELATED WITH NEGATIVE ASPECT OF TABLE OLIVE

a. **Polysaccharolytic activity**

Polysaccharolytic activity comprehends different enzymatic as pectolytic and xylanolytic activities usually associated with softening and gas-pocket formation in olives (Vaughn *et al.,* 1972; Duràn Quintana *et al.,* 1986).

Pectolytic activity vary from different species and vary in the same species, depending on tested isolated from different table olive production. In green seasoned olive. *P. anomala* and *K. marxianus*, isolated from brine, were highly variable, ranging from absence to strong activity. Strains of other minority species in olive brine, such as *D. hansenii, C. rugosa*, and *R. minuta*, were highly pectolytic.

Most of the strains isolated from fresh fruit (*Cryptococcus ssp* and *Candida* spp, *R. glutinis*) showed a high pectolytic activity, except for one of *C. humicola* and another of *C. laurentii*.

Cryptococcus spp, didn't show high enzymatic activity in strains isolated from brine disaccording to data in literature (Hernández *et al*., 2007). No *S. cerevisiae* showed relevant pectolytic activity.

b. **Xylanol acitivity**

R. minuta exhibited an intense xylanolytic activity, unlike most of the studied strains that were isolated from olive brine. Species of *Rhodotorula* genus have been often associated with softening of black olives (Vaughn *et al*., 1969).

c. **Polygalactunorase activity**

One of the most studied and widely used commercial pectinases is polygalacturonase (PG). Therefore pectinase enzymes are commonly used in processes involving the degradation of plant materials, such as speeding up the extraction of fruit juice from fruit, including apples and sapota. PG hydrolyzes of the α-1,4-glycosidic bonds of the galacturonic acid units forming the pectins. This reaction leads to the softening of the olive pulp and a separation of the drupe cuticle.

Pink yeasts identified *as Rhodotorula glutinis* var. *glutinis, R. minuta var. minuta,* and *R. rubra* are the first yeast species to produce polygalacturonases which cause a slow softening of olive tissue, probably proceeding from the olives' surface. *S. kluyveri* and *S. oleaginosus*, isolated from olive brine, caused softening of olives under in vitro conditions. Both pectin methyl esterase and polygalacturonase are produced when cultures are grown in appropriate media while the cultures of *H. anomala* var. *anomala (P. anomala*) didn't cause softening of olives under different conditions (Vaughn RH *et al.,* 1969, 1972).

To avoid softening in olive drupe, during fermentation or storage, is recommended to use a concentration of NaCl higher than 6% otherwise more than 0.1% actual acetic acid as part of the total acidity (in normal storage of Spanish-type fermentation brines, the quantity of acetic acid may amount to as much as 50% of the total acidity).

Pasteurization, low pH of olive brine (about 4) and potassium sorbate in the product and packed product can play an inhibitory effect on this kind of alteration and on the microbial population responsible of it (Asehraou *et al.,* 2002).

6. ANTAGONISTIC ACTIVITY

Starter culture, both bacteria and yeast, have been extensively investigated with the goal of optimizing microbial metabolism for industrial purposes especially in wine sector because alcoholic fermentation and malolactic fermentation, lead to lactic acid and CO2 production, are processes that yield compounds that influence and condition the organoleptic characteristics of wines and thus determine their quality (Magliani *et al.,* 1997).

Killer activity it's a so called antagonistic activity of yeasts due to different mechanisms.

S. cerevisiae was the first yeast species to be studied for the capability of inhibiting the growth of other species without affecting itself (immune to the toxic effects of the protein due to an intrinsic immunity).

The killer phenotype has been studied also in species as for example *Pichia, Debaryomyces, Kluyveromyces, Ustilago maydis* .

The mechanisms of recognizing and killing sensitive cells differ for each toxin. The most studied species and killer activity are:

- *S. cerevisiae* K1, K2, and K28 toxins, encoded by different cytoplasmically inherited satellite dsRNAs (M1, M2, and M28), encapsidated in virus-like particles (VLPs) and dependent on another group of helper yeast viruses (L-A) for their replication and encapsidation. *S. cerevisiae* are toxins encoded by a double-stranded RNA virus, transcribed as a precursor, cleaved and secreted outside of the cells, where they may affect susceptible yeast;
- *S. cerevisiae* KHR and KHS, which are encoded on chromosomal DNA;
- *Ustilago maydis* dsRNA viruses with segmented genomes. Killer strains can secrete one of the three different toxins that have been identified so far. These toxins, designated KP1, KP4, and KP6, have killer activity against susceptible cells of the same and closely related species;
- *K. lactis* killer strains secrete a heterotrimeric toxin. This finding was unexpected because the phenomenon was associated with DNA linear plasmids. Killer strains always contain 50 to 100 copies per cell of each of two cytoplasmically inherited linear plasmids designated pGKL1 (k1) and pGKL2 (k2). this toxin inhibits the growth of a wide range of susceptible yeasts in the genera *Candida, Kluyveromyces, Saccharomyces, Torulopsis*, and *Zygosaccharomyces*, as well as nonkiller strains of *K. lactis*;
- *Tetrapisispora phaffii* (formerly known as *Kluyveromyces phaffii*) secretes a killer toxin known as Kpkt, that is lethal to spoilage yeasts, *Hanseniaspora uvarum*, under winemaking conditions. The mode of action of the glycoprotein Kpkt, and the specific damage produced by this toxin on sensitive yeasts has been

investigated using castanospermine, a beta-glucanase inhibitor, demonstrating that beta-glucanase activity is essential for the Kpkt killer activity in vivo (Comitini *et al.,* 2009);

- *P. acaciae* killer strains have been shown to possess two linear plasmids, designated p*Pac*1-1 (13.6 kbp) and p*Pac*1-2 (6.8 kbp). These plasmids are quite similar in both function and structural organization to those found in *K. lactis* (Meinhardt and Klassen, 2009)*;*
- *P. kluyveri* killer toxin, a 19-kDa acidic glycoprotein, induces the formation of ion-permeable channels, in the same way of *S. cerevisiae* K1, which causes leakage of potassium ions and ATP, decrease of the cellular pH, and inhibition of amino acid uptake (Middelbeek *et al.,* 1979);
- *P. farinosa* , termed SMK (salt-mediated killer toxi*n),* has been recently described. This toxin shows its maximum killer activity in the presence of 2 M NaCl and is a heterodimer (14.214 kDa), whose subunits (a, 6.6 kDa; b, 7.9 kDa) are tightly linked under acidic conditions;
- *Williopsis saturnus* var*. mrakii* toxin designated HM-1 or HMK, first described in 1983; and a second one, described more recently and designated K-500;
- *Mrakia frigida*, strain 2E00797 isolated from sea sediment in Antarctica, was found to be able to produce killer toxin against *Candida tropicalis*, *C. albicans* and the pathogenic yeast in crab, *Metschnikowia bicuspidata* WCY. The highest killer activity is reached in medium with pH 4.5 and 3.0% (wt/vol) NaCl and at 15°C (Hua *et al.,* 2010);
- *P. anomala*. The attention results from its activity against a wide range of unrelated microorganisms, such as yeasts, hyphomycetes, and bacteria, including important opportunistic pathogens such as *C. albicans*. Antibodies (Abs) with antibiotic activity, internal image of a *P. anomala* killer toxin (PaKT) characterized by microbicidal activity against microorganisms expressing ß-glucans cell-wall receptors (PaKTRs), are nowadays synthesize and test in vivo against HIV-1 and Influenza A virus (Polonelli *et al.,* 2010);

- *P. ohmeri* 158 was tested against *Penicillium expansum* at 25°C by measuring hyphal length and %age conidia germination. showing high inhibition of mycelial growth (66.17%), indicating a probable mechanism associated with killer activity. This killer toxin (molecular mass <3 kDa) was partially purified and tested against *P. expansum*: causing % inhibition from 39.32 to 91.12% (*P. ohmeri*) ($p < 0.05$). The one-step purification process was adequate in isolating killer toxin from culture supernatant and also increased anti-*Penicillium* activity (Coelho *et al.,* 2009).

Killer yeasts and their toxins have many potential applications in environmental, medical and industrial biotechnology. They are also suitable to study the mechanisms of protein processing and secretion, and toxin interaction with sensitive cells.

Application of killer toxin in food industry has been investigated since the beginning of its discover due to the optimal pH of action of some yeast toxin, similar to those of some fermented food such as wine and beer.

Killer phenotype is considered an important characteristic for starter culture depending on:

- Microbial spectra: killer yeast are first of all antagonistic of strains of the same species but some killer toxins are found to be decrease or inhibit the growth of other yeast species ,bacteria and molds. It has been postulated that the ideal candidate for an inoculum starter would be an engineered yeast resistant to microbial toxins and producing a toxin lethal to indigenous yeasts, molds, and bacteria. To avoid the failure of the fermentation process the yeast starter should be a killer or at least a neutral strain but not a susceptible strain as killer phenotypes are widespread.
- Inoculum ratio: initial ratio between killer and susceptible yeast cells that is needed to predominate. Experimental studies have shown that the killer yeast predominates when it is prevalent or at least equivalent in number to

susceptible yeasts at the time of inoculation. In the case of *S. cerevisiae* inoculum in grape juice the correct ration was higher than 1:2 and less than 2:1 because in this last option complete elimination of susceptible cells was not achieved. However, it has been found that during the fermentation process, the killer effect is detectable at lower concentrations of the killer cells up to 1:500 (Heard *et al.,* 1987; Tredoux *et al.,* 1986).

Killer toxin have been tested for their antagonistic activity in several food, untill today the main application were on:

- **Wine** In axenic cultures on grape juice, without toxin addition, specific growth rates of *P. membranifaciens* CYC 1086, *S. cerevisiae* SC1 and *B. bruxellensis* was monitored. *B. bruxellensis* is a constant wine inhabitant but it develops mainly at the end of fermentation. The presence of the killer strain or its purified killer toxin did not change the growth parameters of the fermentative *S. cerevisiae* strain, indicating that these two yeasts are compatible in winemaking conditions, whereas *B. bruxellensis* 1D007 showed significant inhibition in mixed cultures. Furthermore, no killer activity remaining in solution was detected in mixed cultures during the first 3h, indicating that the produced toxin was attached to the sensitive cells. Probably, when all the binding sites of the toxin in the sensitive cells were saturated the presence of the toxin would be detected in the medium (Shimizu, 1993).

- **Bread** *C. tropicalis*, possible cause of contamination in the starter cultures of *S. cerevisiae* fermentation used in the Turkish baking industry, was tested against *S. cerevisiae* K3 and *Hansenula anamola* K8, showing killer phenotype. This led to a conclusion that K8 and/or K3 killer traits could be used for the construction of industrial strains resistant to *C. Tropicalis* contamination (Izgü et al., 1997).

- **Fruit** *P.anomala* and *M. pulcherrima* strains have been proposed in antagonistic activity against *Botrytis cinerea* on apples surface (Janisiewicz, 2001; Jijakli and Lepoivre, 1998).

Panomycocin, a novel exo-beta 1,3 glucanase, was tested as an antifungal agent against green and blue mold diseases, the most important causes of post-harvest decay in citrus fruits. All tested isolates of *Penicillium digitatum* and *P. italicum* were susceptible to panomycocin in vitro. In tests on fruit, panomycocin at concentrations equal to in vitro MIC-0 value protected lemon fruit from decay (Izgu *et al.,* 2010).

- **Salted fermented food** Killer phenotype is studied for its application not only in the wine fermentation but also in other sector, also of potential industrial interest is the finding of osmophilic killer yeasts, whose toxic activity was demonstrated only in the presence of high concentrations of salts such as NaCl or KCl.

Kluyveromyces strains with killer activity against *Z. rouxii* in the presence of salts were screened to detect isolates useful in developing natural preservatives to prevent refermentation of salted fermented foods. In high salt concentration food *D.hansenii, C. farinose, S. cerevisiae* and *P.anomala* killer strains have been frequently isolated (Llorente *et al.,* 1997).

In table olive in particular not only NaCl concentration plays an important role in killer activity but also pH (Hernández *et al.,* 2008).

- **Silage** Killer spectra were also investigated to select killer yeasts, such as *K.lactis*, that might inhibit the growth of wild yeasts, potentially causing aerobic deterioration of silage.

Killer yeasts such as *P. anomala*, *P. guilliermondii*, and *S. cerevisiae* have been used in the biocontrol of mold growth in high-moisture wheat stored under airtight conditions due to mycotoxigenic contamination of species such as *Aspergillus* and *Penicillium*.

A genetically modified killer yeast, *K. lactis* killer strain PCK27, defective in growth on lactic acid due to disruption of the gene coding for phosphoenolpyruvate carboxykinase, a key enzyme for gluconeogenesis, inhibited the growth of *P. anomala* inoculated as an aerobic spoilage yeast and prevented a rise in pH in a model of silage fermentation. To improve the aerobic stability of silage can be used as an additive to prolong the aerobic stability of maize silage. In the laboratory-scale experiment of maize silage, the addition of a killer yeast changed the yeast flora and significantly reduced aerobic spoilage (Kitamoto *et al.,* 1999).

The yeast *W. mrakii* produces a mycocin or yeast killer toxin designated HMK. Two food, yoghurt ,and feed production, mature maize silage, systems prone to yeast spoilage were used as models to assess the ability of mycocin HMK to act as a biocontrol agent. Thus, mycocin HMK has potential applications in controlling both silage spoilage and yoghurt spoilage caused by yeasts (Lowes *et al.,* 2000).

- **Crabs** *W. saturnus* WC91-2, *P. guilliermondii* GZ1, *P. anomala* YF07b, *D. hansenii* hcx-1 and *Aureobasidium pullulans* HN2.3 produced killer toxin against the pathogenic yeast *Metschnikowia bicuspidate*, which could cause milky disease in *Portunus trituberculatus* (Wang *et al.,* 2007; 2008).

7. *Pichia anomala* species

The genus *Pichia* is one of the largest yeast genera in view of the number of species. Since the genus was described in 1904, the number of species included in this taxon has changed considerably. *Pichia* currently contains 91 species with 30 being related to food production and processing. However, the majority of them can be considered as food contaminants (spoilage organisms). *Pichia* appears to be extremely heterogeneous. The genus nearly doubled in size with the transfer of nitrate-positive *Hansenula* species to *Pichia*. *H. anomala* is now called *P. anomala*

but taxonomic studies are proposd for a new reclassification of the genus according to molecular profile obtained with the sequences D1/ D2 (26S) and PCR and restriction of 5.8S-ITS. *P.anomala* is a widespread species, isolated in several different environments, as shown in table 3. *Pichia* is consider able to make a positive contribution in the producing of fermented foods (tab. 4). As one of the most frequently encountered and important species in foods, it is considered a safe microorganisms as it is used by the WHO for the development of vaccines, as a producer organism (*e.g.* phytases and other enzymes) and for other enzymatic production of biotechnological interest (Tab. 5, 6**)**.

Table 3. Some diverse *Pichia anomala* habitats

Isolation from	**Reference**
Homo sapiens (human skin, faeces etc.)	Murphy *et al* (1986); Mo*et al* (2004)
Palm sugar	Nagatsuka *et al* (2005)
Bread/fermenting dough	Lanciotti *et al* (1998); Daniel *et al* (2010)
Insects (eg. *Drosophila* and malaria mosquito *Anopheles)*	Kurtzman (2001); Ricci *et al* (2010)
Sea urchins	Kajikazawa *et al* (2007)
Pharmaceutical wastewater	Recek, Cadez and Raspor (2002)
Cereal silos and silage	Olstorpe (2008)
Oil-contaminated soil	El-Latif *et al* (2006)
The sea	Wang *et al* (2007)
Hospitals	Thuler *et al* (1997); Chakrabarti *et al* (2001); Reyes *et al* (2004)
Table Olive	Hernández *et al* (2007)
Yogurt	Caggia *et al* (2001)
Wine	Spagna *et al* (2002)
Fruit	Jijakli and Lepoivre (1998)
Coffee	Vilela *et al* (2010)
Sugarcane Silage	Avila *et al* (2010)
Ethnic Meat	Rai *et al* (2010)
Banh Men	Thanh *et al* (2008)
Rutab	Hamad (2008)
Hamei	Jeyaram *et al* (2008)
Gowé	Vieira-Dalodé *et al* (2007)
Leaf Surfaces Of Trees	Sláviková *et al* (2007)
Soft Drinks	Ancasi *et al* (2006)

The main species *P. anomala* is described as a safe producer organism, since this yeast does not contain pyrogens or “viral inclusions” and is not a pathogen.

Nevertheless, there are sporadically report of *P. anomala* as responsible for cases of fungaemia in a Brazilian paediatric intensive care unit (Pasqualotto *et al*. 2005). The source of the infection was never found. Patients with *P. anomala* fungaemia seem to have risk factors in common with those who have candidaemia. Is common that fungemias are detected to have causes in yeast species frequently used, other example are referred to *S. cerevisiae* strains associated with bakery industry.

Table 4. Important roles of *Picha anomala* in foods, feeds and beverages

Food/beverage	**Applications**	**References**
Flavour enhancement	Volatile (eg esters) and savoury(eg nucleotides) flavours	Eun-Kyoung *et al* (2003); Lee *et al* (2004)
Food/feed bio-preservation	Biological control of fungi in fruits and cereals	Petersson and Schnürer (1995); Jijakli(2010); Olstorpe *et al* (2009b); Izgu *et al* (2010)
Dairy fermentations	Probiotic effects	Mo *et al* (2004)
Baking	Sourdough fermentations (not necessarily beneficial)	Daniel *et al* (2010)
Winemaking	Volatile aromas, low alcohol wines, malic acid reduction	Ertin and Campbell (2001); Swangkeaw et al (2009)
Enzymatic food/feed processing	Phytase, amylase, peptidase	Ray and Nanda (1996);Satyanarayana (2010)
Brewing	Anti-gushing potential in malting barley	Olstorpe *et al* (2009); Laitila *et al* (2007;2010)

Because of positive roles of this specie in several industrial process and its safety is has reached the QPS status (Qualified Presumption of Safety) referred to EFSA standards, (www.efsa.europa.eu/en/scdocs/doc/s587.pdf) together with *P. angusta* , *P. jadinii* and *P. pastori,* and can be compared for its widely potential and new research interest to one of the most studied species as *S.cerevisiae* (tab. 7).

Table 5. Summary of antimicrobial properties of *P. anomala*

Antimicrobial Activity	**Examples of microbes suppressed**	**References (examples)**
Antifungal	*Aspergillus*, *Botrytis, Penicillium, Fusarium*	Jijakli and Lepoivre (1998); Masih *et al* (2000); Jijakli (2010); Laitila *et al* (2007)
Antizymal	Various yeasts, incl. *C. albicans*	Sawant *et al* (1988)
Antibacterial	*Erwinia* spp.; *Enterobacteriaceae*; *Streptococci*	Polonelli and Morace (1986); Conti *et al* (2002)
Antiviral	Influenza virus	Conti *et al* (2008)

(Walker 2010)

Table 6. *P. anomala* products of biotechnological potential

Product	**Potential application**	**Reference**
Sophorolipids	Biosurfactants	Thaniyavarn *et al* (2008)
γ-aminobutyric acid, GABA	Pharmaceuticals (GABA acts as a neurotransmitter, improves cerebral blood flow)	Kaku and Hagiwara (2008)
Volatile organic compounds	Fragrances	Buzzini *et al* (2003)
Isobutanol	Biofuels	US Patent (2009)
Beverage starter culture	Low-alcohol wines; aromas	Ertin and Campbell (2001)
Panomycocin	Novel zymocidial agents	Izgü, Altinbay and Türeli (2006)
Antiviral agent	Influenza virus therapy	Conti *et al* (2008)
Amoebicidal agent	Therapy of *Acanthamoeba* infections	Fiori *et al* (2006)
Anti-*Pneumocystis* agent	Therapy of *Pneumocystis carnii*	Seguy *et al* (1996)
Antibacterial agent	Therapy of Streptococcal infections	Conti *et al* (2002)
Biocontrol/biopreservative	Stored grain, vines, fruit	Jijakli (2010); Olstorpe (2010)
Enzymes	Phytase, esterase, peptidase, β- glucosidase, amylase	Ray and Nanda (1996); Satyanarayana, T (2010)
Bioethanol (indirectly)	Maintenance of airtight stored grain (biofuels)	Passoth *et al* (2009)

From (Walker 2010)

Table 7. Cell physiological and other characteristic differences between *P. anomala* and *S. cerevisiae*

P. anomala	***S. cerevisiae***
Budding/pseudomycelia	Mainly budding
Crabtree negative	Crabtree positive
Predominantly respiratory	Predominantly fermentative
Oxygen sensitive	Glucose sensitive
Glucose uptake by H^+ symport	Facilitated glucose diffusion
Malic acid utilisation	Malate only utilised with glucose
Several enzymes secreted	Few enzymes secreted
Antifungal action	Rarely antifungal (some strains)
High ethyl acetate	Low ethyl acetate
Widespread in Nature	Not widespread in Nature
Halotolerant	Not very halotolerant
Moderate ethanol tolerance	Ethanol tolerant
QPS (EFSA)	GRAS (FDA)
Opportunistic pathogen (some strains)	Doubtful opportunistic pathogenicity

(Walker 2010)

Objectives

The aim of this work was to evaluate the potential use of yeasts as starter and bioprotective cultures for fermented food as table olives.

Therefore, the work has been developed with the following aims:

1. Isolation, identification and characterization of yeast population from Sicilian naturally fermented olive brine differently treated in fields against the olive fly *Bactrocera oleae*.

2. Characterization of β-glucosidase enzyme isolated from an olive brine strain of *Pichia anomala* considering the potential as key enzymes in the biological debittering of olives and in the release of aromatic compounds from glycosidic precursors present in raw materials, which lead to a mostly unused flavour potential during the processing of foods and beverages

3. *Pichia anomala* as potential bioprotective microorganism in food processing and molecular characterization of killer phenotype.

Results and Conclusion

The first stage of the study was the isolation of yeasts from naturally fermented olive brine with culture dependent and independent method. Ecological studies of microbiota are necessary for the understanding of different species trends during fermentation process and the possible causes of spoilage. More over a technological characterization of the *S. cerevisiae* strains was carried out on enzymatic activities, in order to improve the overall quality of final product.

The second stage was the enzymatic characterization of an alkaline β-glucosidase from *P. anomala* strain. In fact, in the production of naturally fermented table olives and biological debittering can be considered as an alternative strategy to Spanish style production, with use of lye treatment with sodium hydroxide solution, and subsequent washing process and wastewater discarding problem.

The study of bioprotective activity of *P. anomala* strains was the third stage of this work. The collection of yeast isolates was tested *in vitro* against yeast sensitive reference strains and Gram positive and Gram negative clinical isolates . Among them three strains of *P. anomala* were selected for a molecular study of the genetic codification process of the antagonist activity for a deeper understanding of the mechanism of action of the killer toxin. The use of P. *anomala* killer toxin, as food bioprotective, is nowadays proposed in the food industry to control spoilage yeasts, molds and bacteria as the toxin is not considered of potential risk for human health.

In conclusion among different yeast species isolated from naturally fermented olives it was possible not only to select *S. cerevisiae* strains as possible *starter cultures*, as already applied in oenological and baking sectors, but also to highlight the prospective of using *P. anomala* which recently has reached the QPS status referred to EFSA standards.

Strains isolates of this last mentioned species showed both unusual β-glucosidase activity, useful for example in olive wastewater detoxification, and antagonistic activity against other yeast species and bacteria.

The use of *P. anomala* killer strains as potential bioprotective agent against different spoilage yeasts, bacteria and molds can be proposed, considering the wide action spectrum of this species and the safety for consumers.
Gene identification, sequencing and study of the molecular target of killer toxin was, finally, realized.

The methodology, results, discussion and conclusions obtained in this work are presented in the following section as scientific articles and proceedings :

1. **Isolation and characterization of yeast and LAB from olive brine**
   - Yeast dynamics during fermentation of brined green olives differently treated in field (submitted to International Journal of Food Microbiology)
   - Caratterizzazione tecnologica di lieviti isolati da olive verdi al naturale (OLIVE DA TAVOLA proceedings)
   - Caratterizzazione della popolazione di lieviti in olive verdi al naturale diversamente trattate in campo (QUALICIBI proceedings)
   - Identificazione e caratterizzazione di batteri lattici isolati da olive in salamoia (QUALICIBI proceedings)

- Impiego di colture starter selezionate per la produzione di olive da tavola (QUALICIBI proceedings)

2. Enzymatic Activity of *Pichia anomala*

- An alkaline β-glucosidase isolated from an olive brine strain of *Pichia anomala* (submitted to FEMS Yeast Research)
- Preliminary characterization of a beta-glucosidase from a *Pichia anomala* yeast strain of olive brine (ECB, European Congress on Biotechnology proceedings)

3. *Pichia anomala* strains with potential bioprotective activity

- Molecular Genetics of *Pichia anomala* Killer Strains Isolated From Naturally Fermented Olive Brine
- Killer toxin of *Pichia anomala* strains isolated from olive brine and active against human pathogens (BioMicroWorld2009 proceedings)
- Molecular Genetics of *Pichia anomala* Killer Strains Isolated From Naturally Fermented Olive Brine (IBS 2010 proceedings)

4. Other works. In the present thesis are also reported works regarding:

- Valutazione della sopravvivenza di *Lactobacillus rhamnosus* in confetture di pesche (QUALICIBI proceedings)
- Effetto di *Lactobacillus rhamnosus* sulle caratteristiche chimico-fisiche in confettura di pesche probiotica (CISETA proceedings)
- Employment of *Saccharomyces* Hybrids as a tool for Improving Quality of Moscato di Siracusa Doc Wine (In press, Italian Journal of Food Science)

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Isolation and characterization of yeast and Lactic Acid Bacteria from olive brine

Yeast dynamics during fermentation of brined green olives differently treated in field

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ABSTRACT

The yeast microbiota associated with naturally fermented and inoculated table green olives, differently treated in field with non-conventional repellent and antiovipositional products in the control of *Bactrocera oleae*, was analyzed using a combination of culture dependent and independent molecular fingerprinting. Routine yeast isolation gave rise to 118 strains, whose identification was carried out by PCR-RFLP of ITS region. Total DNA was extracted directly from brine throughout fermentation, by an experimental protocol set right to remove Taq polymerase inhibitors, and yeast community was highlighted by Denaturing Gradient Gel Electrophoresis (DGGE) of 26S rRNA gene PCR amplicons. Comparison of both culture-dependent and independent methods indicated that the yeast species *Saccharomyces cerevisiae*, *Pichia anomala*, *Candida diddensiae* and *Issatchenkia orientalis* were dominant during fermentation despite *Lactobacillus plantarum* starter used in brining. Isolated species resulted as unaffected by treatments in field except for *C. diddensiae* which can be proposed as an index of fly infestation.

Keywords: Table olives, Copper, Kaolin, Natural fermentation, Yeast population, Molecular identification, DGGE

1. Introduction

Olives are the major fermented vegetables in western countries (Garrido Fernandez et al., 1997). Homemade production of naturally fermented table olives is very common in Mediterranean countries; the production methods vary according to local tradition and most of the times the process is guided by the indigenous microbial population. Particularly, the microbial composition 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. It can be influenced also by treatment used on olives in fields to control *Bactrocera oleae*. Kaolin form a protective barrier that control or suppress the pest by repelling or irritating the ovipositing females and copper salts make fruits less attractive to ovipositing females because of the lack of some attractive microbial compounds on the surface of fruits (Belcari et al., 2003; Saour and Makee, 2004). However, the effect of copper-containing pesticides on yeast population has been investigated only on grapes, since copper residues in musts may cause lagging fermentation and affect wine quality detrimentally (Tromp and De Klerk, 1988; Brandolini et al., 1995), but not on other fermented products such as table olives.

Yeast play a critical role in all olive fermentations, especially in directly brined green and natural black olives, since lactic acid bacteria are partially inhibited by the presence of toxic phenolic compounds. The presence of yeast during the fermentation of green olives was reported in the earliest studies of this product. Thus, González-Cancho (1965) isolated yeast of the genera *Candida*, *Hansenula*, *Pichia*, *Torulopsis* and *Saccharomyces* from these fermentations. Balatsouras (1967) reported the presence of yeasts of the genera *Trichosporon*, *Candida*, *Pichia*, *Kloeckera*, *Torulopsis* and *Debaryomyces* during the fermentation of directly brined natural black olives from Greek cultivars. Pelagatti (1978) and Marquina et al. (1992) isolated several species of the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula* and *Saccharomyces* from directly brined green and turning colour olives. Borcakli et al. (1993) isolated species of *Debaryomyces*

from Turkish cultivars. Kotzekidou (1997) identified *Torulaspora delbrueckii*, *Debaryomyces hansenii* and *Cryptococcus laurentii* as the predominant species in Greek-style black olives, while Marquina et al. (1997), in studies carried out with olive brines from seven locations in Morocco, isolated *Candida boidinii*, *Pichia membranifaciens* and *Torulaspora delbrueckii*. Hernández et al. (2007) found *Pichia anomala*, *Kluyveromyces marxianus*, *D. hansenii* and *Saccharomyces cerevisiae* to be the main species present during the processing of directly green table olives from Portugal.

Until present, the characterization of yeast associated with table olives has mainly been made through biochemical and morphological methods, using the taxonomic keys of Looder (1970), Barnett et al. (1990), and Kurztman and Fell (1998) and more recently, by molecular methods as PCR-RFLP of 5.8S rRNA-ITSs. In the last way, Arroyo López et al. (2006) identified the species *S. cerevisiae*, *Issatchenkia occidentalis* and *Geotrichum candidum* from green seasoned table olives, and *C. boidinii* and *Hanseniaspora guilliermondii* from processed black olives; Coton et al. (2006) identified *P. anomala*, *C. boidinii* and *D. etchelsii* as the predominant species in black olive natural fermentations from France using the same methodology. Finally, Hurtado et al. (2008) found the species *C. boidinii*, *C. diddensiae*, *C. membranifaciens*, *Kluyveromyces lactis*, *P. membranifaciens*, *P. kluyveri* and *R. glutinis* during processing of Arbequina table olives in Spain.

The culture independent approach is a recent trend in the study of the microbial ecology of foods (Ercolini, 2004). A very recent study carried out on Sicilian olives for microbial detection by different approaches, including a culture-independent methodology, revealed the presence of three yeast species during the entire fermentation period: *C. parapsilosis*, *P. guilliermondii* and *P. kluyveri* (Aponte et al., 2010). However polyphasic approach studies, including dependent and independent culture methods, such as DGGE still need to be carried out to broaden our current knowledge of such fermentation.

90 The aim of this study was to characterize and monitor yeast community of Sicilian naturally
91 fermented green olives using a combination of culture-dependent methods (PCR-RFLP of ITS
92 regions and D1-D2 sequencing) and culture-independent method Denaturing Gradient Gel
93 Electrophoresis (DGGE). Samples of olives differently treated on plant with copper or kaolin and
94 one sample of untreated olives were analyzed for yeast population from the first day of direct
95 brining to 180 days of fermentation.
96
97
98 **2. Materials and methods**
99 *2.1. Olive processing*
100 Green Sicilian olives, cultivar Nocellara dell'Etna, were treated in field for the control of the olive
101 fruit fly *Bactrocera oleae* (Rossi), the key pest of olive groves. The olives were differently treated
102 on trees with kaolin and Bordeaux mixture. A lot of untreated olives was used as control.
103 Olives were harvested at a maturity stage suitable for processing (mid-October) and selected.
104 Screw-capped PVC vessels (10 kg fruits plus 10 l brine capacity) were filled with olives, and the
105 steps of naturally fermented green olive preparation were carried out: (a) washing with water at
106 room temperature; (b) brining, at 8% NaCl; storage at environmental temperature (20°C). For each
107 experimental trial a lot of olives was inoculated with 1×10^6 cells ml^{-1} of *Lactobacillus plantarum*.
108 Experimental design is reported in Table 1.
109 The pH of the brine solution, throughout the fermentation process, was determined electrometrically
110 with a pH meter (pH 510, XS Instruments, Giorgio Bormac s.r.l., Carpi (MO), Italy).
111

112 *2.1 Yeast reference strains*

113 The following strains were used as reference, together with sequenced yeast isolates: *Rhodotorula*
114 *mucilaginosa* CBS 316, *Rhodotorula glutinis* CBS 322, *Pichia triangularis* 4094, *Issatchentia*
115 *orientalis* DSM 3433, *Candida diddensiae* CBS 2214, *Saccharomyces cerevisiae* DSM 70449, *P.*
116 *anomala* CBS 5759, *P. membranifaciens* DSM 70169, *Hanseniaspora guiliermondii* CBS 465.
117

118 *2.2 Yeast isolation*

119 Brine samples were taken immediately after brining and at 7, 15, 30, 60, 90, 120, 150 and 180 days
120 of fermentation from each olive lot. Brine samples (1 ml) and appropriate decimal dilutions in
121 sterile physiologic solution (90 g l^{-1} NaCl) were plated on Sabouraud Dextrose Agar supplemented
122 with chloramphenicol (Oxoid, Basingstoke, UK). Plates were incubated at 25 °C for 48-72 h.
123 Approximately 20% of the yeast colonies were randomly selected from plates that showed counts
124 between 30 and 300 colonies, and then purified three times on the same medium. A representative
125 number of 118 yeast isolates were microscopically observed and submitted to molecular
126 identification.
127

128 *2.3. Yeast identification*

129 DNA extraction from pure cultures: DNA was isolated using the Hoffman and Winston protocol
130 (1987). Pure cultures were grown in YPD medium (g $l^{-1}$of distilled water: Yeast extract 10, Peptone
131 10, Dextrose 20; Oxoid, Basingtoke, UK) at 25 °C for 18 h at 250 rpm on an orbital shaker. Two
132 milliliters of cell culture were then centrifuged at 9,000×g for 2 min at room temperature. The cells
133 were resuspended with 400 µl of equilibrated phenol (pH 6), and 400 µl of lyses buffer (10 mM

Tris–HCl, pH 8, 10 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS) and 0.6 g of sterile glass beads (∅ 0.50 mm) (Biospec Products, Bartlesville, OK, USA) were added. Tubes were vortexed for 4 min, centrifuged at 9,000×g for 1 s and, after adding 200 µl of TE (10 mM Tris–HCl, 1 mM EDTA), centrifuged at 14,000×g for 5 min. Supernatant was transferred in new tubes, 500 µl of chloroform/isoamyl alcohol (24:1) were added, and the tubes were centrifuged at 14,000×g for 2 min. Supernatant was transferred in new tubes with an equal volume of cold isopropanol and put at −20 °C for 30 min at least. Eppendorf tubes were then centrifuged at 14,000×g for 10 min at 4 °C. The pellet was then washed with EtOH 70%, and resuspended in 60 µl of TE buffer. To verify the amount of extracted DNA, Qubit® Fluorometer was used (Invitrogen-Life Technologies, Carlsbad, CA, USA).

Amplification reactions: For the PCR amplification of the 5.8S rRNA gene and the intergenic spacers ITS1 and ITS2, the protocol described by Esteve-Zarzoso et al. (1999) was used. The amplification reactions of the 5.8S-ITS regions were carried out under the following conditions: each 50 µl reaction mixture contained 20–30 ng template DNA; 10 mM Tris–HCl, pH 9.0; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.5 µM of each primer and 1 U Taq DNA polymerase (Invitrogen-Life Technologies, Carlsbad, CA, USA). The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) described by White et al. (1990) were used. Amplification was performed in a Tpersonal Thermocycler (Whatman Biometra, Göttingen, Germany) programmed as follows: initial denaturation at 94 °C for 5 min; 30 s 94 °C for denaturing, 30 s at 57 °C for annealing, 1 min at 72 °C for extension, repeated for 35 cycles; and a final extension step of 5 min at 72 °C. Aliquots of 5 µl of amplified product were separated electrophoretically in 1.4% (w/v) Certified Molecular Biology Agarose (Bio-Rad Hercules, CA, USA) gel.

157 Restriction analysis: Aliquots of the PCR-amplified products were separately digested with *Hha*I,
158 *Hae*III, *Hinf*I and *Msp*I restriction enzymes (New England Biolabs, Ipswich, MA, USA). Reaction
159 mixtures contained 2.5 μl 10× digestion buffer (supplied by the manufacturer), 6.5 μl deionized
160 H_2O, 1 μl restriction enzyme and 15 μl PCR product. The mixtures were incubated for 2 h at 37 °C.
161 Resulting DNA fragments were analysed by electrophoresis through 2% (w/v) UltraPure™ Agarose
162 1000 (Invitrogen-Life Technologies, Carlsbad, CA, USA) gels in 1× TAE buffer. DNA molecular
163 weight marker 100 bp DNA ladder (Invitrogen-Life Technologies, Carlsbad, CA, USA) was used as
164 standard. Restriction profiles generated were recorded and compared with those available in
165 literature.

166 Sequence analysis of the 26S rRNA gene: Yeast strains belonging to each cluster were analysed by
167 sequencing of domains D1 and D2 of the 26S rRNA gene. For this purpose, PCR amplification of
168 the 26S rRNA gene with the universal primers NL1 (50-GCATATCAATAAGCGGAGGAAAAG-
169 30) and NL4 (50-GGTCCGTGTTTCAAGACGG-30) (Kurtzman and Robnett, 1998) was carried
170 out and the resulting products commercially sequenced. The corresponding sequences were finally
171 compared with the sequences present in public data libraries (GenBank) using the Blast search
172 program in order to determine their closest known relatives (Altschul et al. 1997;
173 http://www.ncbi.nlm.nih.gov/BLAST/).

174

175 *2.4 Genomic DNA extraction from olive brine*

176 For the extraction of genomic DNA, the protocols of Chamkha et al. (2007) and Godon et al. (1997)
177 were used with a few modifications. Ten millilitres of the olive fermentation brine sample were
178 collected and centrifuged at 4,000×g for 10 min. The pellet was suspended in 2 ml of 4 mol l^{-1}

guanidine thiocyanate–0.1 mol l^{-1} Tris pH 7.5 and 600 µl of *N*-lauroyl sarcosine 10% (Sigma, Taufkirchen, Germany). Two hundred fifty µl of treated samples were transferred in 2 ml screw-cap polypropylene tubes and stored frozen at –20°C. After the addition of 500 µl of 5% *N*-lauroyl sarcosine–0.1 M phosphate buffer (pH 8.0), the 2-ml tube was incubated at 70°C for 1 h. One volume (750 µl) of 0.5-mm-diameter silica beads (BioSpec products Inc., Bartlesville, OK, USA) previously sterilized by autoclaving was added, and the tube was shaken at maximum speed for 10 min in a BeadBeater (BioSpec products Inc., Bartlesville, OK, USA). Polyvinylpolypyrrolidone (30 mg) was added to the tube, which was vortexed and centrifuged for 3 min at 12,000×g. After recovery of the supernatant, the pellet was washed with 500 µl of TENP (50 mM Tris [pH 8], 20 mM EDTA [pH 8], 100 mM NaCl, 1% polyvinylpolypyrrolidone) and centrifuged for 3 min at 12,000×g, and the new supernatant was added to the first supernatant. The washing step was repeated three times. The pooled supernatants (about 2 ml) were briefly centrifuged to remove particles and then split into two 2-ml tubes. Nucleic acids were precipitated by the addition of 1 volume of isopropanol for 10 min at room temperature and centrifuged for 15 min at 12,000×g. Pellets were resuspended and pooled in 450 µl of 100 mM phosphate buffer, pH 8, and 50 µl of 5 M potassium acetate. The tube was placed on ice for 90 min and centrifuged at 12,000×g for 15 min. The supernatant was transferred to a new tube containing 20 µl of RNase (1 mg ml^{-1}) and incubated at 37°C for 30 min. Nucleic acids were precipitated by the addition of 50 µl of 3 M sodium acetate and 1 ml of absolute ethanol. The tube was incubated for 10 min at room temperature, and nucleic acids were recovered by centrifugation at 12,000×g for 15 min. The DNA pellet was finally washed with 70% ethanol, dried, and resuspended in 150 µl of TE buffer. The extracted DNA was quantified as previously described.

202 *2.5 PCR-DGGE analysis*

203 To detect the dominant yeast populations, the D1–D2 loop of the 26S rRNA gene was amplified by
204 PCR using the primers NL1GC (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG
205 GCG GCG GGC CAT ATC AAT AAG AGG AGG AAA AG-3') and LS2 (5'-ATT CCC AAA
206 CAA CTC GAC TC-3') (Cocolin et al., 2000).
207 PCR amplifications were performed in a final volume of 50 μl using TPersonal Thermocycler
208 system (Biometra, Göttingen, Germany) and the reaction mix consisted of 10 mmol l^{-1} Tris–HCl
209 (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} MgCl2, 0.2 mmol l^{-1} dNTPs, 1.25 U of Taq DNA
210 polymerase recombinant (Invitrogen, Carlsbad, CA, USA), 0.2 μmol l^{-1} concentration of each
211 primer and 2 μl of the extracted DNA standardized to 100 ng μl^{-1}.
212 Thermal cycling with primers NL1GC and LS2 was as follows: an initial denaturation of 95°C for 5
213 min, 30 cycle of 2 min at 95°C, 1 min at 55°C, 2 min at 72°C, and 10 min of final extension.
214 PCR products were analysed by electrophoresis in a 1.5% agarose gel using Tris-acetate-EDTA as
215 running buffer and a 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as size marker.
216 The Dcode universal mutation detection system (BioRad, Hercules, CA, USA) was used for DGGE
217 analysis. PCR products were loaded onto a 8% (wt/vol) polyacrylamide gel (acrylamide-
218 bisacrylamide, 37.5:1) with a denaturant gradient from 30% to 60%, in 0.5× TAE buffer (2 M Tris
219 base, 1 M glacial acetic acid, 50 M EDTA [pH 8.0]). A 100% denaturant corresponds to 7 M urea
220 and 40% (vol/vol) formamide. Electrophoresis was performed at a constant voltage of 85 V and a
221 temperature of 60°C for 16 h. The DNA bands were visualized by silver staining and developed as
222 previously described (Sanguinetti et al., 1994).
223

224 **3. Results**

225 *3.1 Yeast counts*

226 Samples of brine for yeast analysis were collected immediately after brining and after 7, 15, 30, 60,
227 90, 120, 150 and 180 days of fermentation from each olive lot. Inoculum with starter culture didn't
228 lead to a faster pH decrease with respect to the spontaneous one. Values of pH rapidly decreased
229 within the first 10 days of fermentation for olives treated in field with copper and kaolin, while in
230 untreated samples the decrease was slower. After the tenth day, pH values of untreated olive brine
231 continued to decrease while in treated samples slightly increased; at the end of fermentation pH of
232 brines stabilized at values ranging from 4.2-4.3 for treated samples to 4.5-4.7 in untreated ones (Fig.
233 1). Immediately after brining, yeast counts on treated samples remained at undetectable levels (1 log
234 cfu ml^{-1}). Untreated samples were 1 log unit higher than the treated samples. After 7 days, the yeast
235 population of each sample sharply increased up to 5 log cfu ml^{-1}, to reach maximum values after 30
236 days of fermentation, for all thesis, of 6 log cfu ml^{-1} (Fig. 2). Yeast population continued to
237 maintain high counts up to the end of considered period (5 log cfu ml^{-1}).

238

239 *3.2 Yeast identification*

240 A total of 118 yeast strains was isolated during the fermentation of table olives differently treated in
241 field. Yeasts were grouped according to the PCR-RFLP method developed by Esteve-Zarzoso et al.
242 (1999). PCR amplification of the ITS region allowed strains to be referred to four amplicon size,
243 ranging from 480 to 850 bp, while digestion, by using three restriction endonucleases (*Cfo* I, *Hinf* I
244 and *Hae* III), presumptively evidenced the existence of 8 species (Tab. 2). The species
245 *Saccharomyces cerevisiae* (Cluster 1), *Pichia anomala* (Cluster 2), *P. membranifaciens* (Cluster 3),
246 *Candida aaseri* (Cluster 5), *C. diddensiae* (Cluster 6), *Issatchenkia orientalis* (Cluster 7) and
247 *Rhodotorula mucilaginosa* (Cluster 8) were recognized following a comparison of the molecular

248 mass of restriction products with those previously described (Esteve-Zarzoso et al. 1999; Villa-
249 Carvajal et al., 2006; Nisiotou et al., 2010). Isolates belonging to Cluster 4 showed a specific RFLP
250 pattern that did not match any data reported to date in the literature. Species identity of the
251 representative strains of the above genotypic clusters was confirmed and the uncertain isolates were
252 identified by 26S rRNA gene sequencing (Tab. 2). Previous identification based on restriction
253 analysis of the 5.8 ITS region was confirmed, except for cluster 3. The D1⁄D2 domain assigned 66
254 isolates to *S. cerevisiae*, 26 to *P. anomala*, 3 to *C. aaseri*, 10 to *C. diddensiae*, 1 to *I. orientalis* and
255 2 to *R. mucilaginosa*. The 8 strains of cluster 3, identified as *P. membranifaciens*, were ascribed to
256 *P. manshurica*, confirming that these species are closely related (Naumov and Naumova, 2009). In
257 the case of Cluster 4, sequence alignments clearly placed isolates within the genus *Pichia*, being
258 most closely related to *P. manshurica* species but with a low identity percent (97%).
259

260 *3.3 Population dynamics by PCR-DGGE*

261 Dynamics of yeast species during spontaneous fermentation of brined green olives were also
262 investigated by extracting DNA directly from brine and then submitting the amplicons, obtained
263 with primers NL1GC and LS2, to PCR-DGGE analysis (Cocolin et al. 2000). The amplified
264 fragments generated from the reference strains of yeast species identified in this study by culture-
265 dependent methods and those usually found in olives, also included in the work, were differentiated
266 according to migration distance in a DGGE gel and used to recognize and distinguish yeast species
267 from brine total DNA (Fig. 3). Yeast population profiles at 7, 15, 90, 120 and 180 days of
268 fermentation are reported, since at 30, 60 and 150 days no remarkable difference was observed.
269 Generally, DGGE profiles from different vessels did not present relevant variability in yeast species
270 composition, assessed by number of bands and migration position in DGGE gels; a few changes
271 were noticed, particularly in terms of band intensity. In all cases, *P. anomala* and, at a less extent, *S.*

cerevisiae were the dominant yeast species at early fermentation (7 days); also the species *C. diddensiae* was encountered in all samples, but with rather low intensity. Species heterogeneity slightly changed as the fermentation proceeded, since *P. anomala* and *S. cerevisiae*, along with *C. diddensiae*, demonstrated to be dominant in olive fermentation, prevailing at 15 days of the fermentation. *C. aaseri* also counted for a significant proportion of the total yeast population at this stage, but only in the copper-treated olives. After 90 days of fermentation, the intensity of *P. anomala* band seemed to decrease, while *S. cerevisiae* was the prevailing species in all considered samples. The band corresponding to *C. diddensiae* was detectable in untreated and, weakly, in copper treated samples; on the other hand, *C. aaseri* was detected in all samples. Yeast population after 120 and 180 days indicated a stabilization of the olive fermentation process, thus achieving a sort of ‘‘steady-state’’ condition, where the selective environment results in the dominance of *S. cerevisiae* species, both in naturally fermented and inoculated olives, followed by *P. anomala* and *C. diddensiae*, the latter reaching the maximum intensity at the end of fermentation.

It’s interesting to evidence the appearance of a weak *I. orientalis* band, in all samples, after 120 days of fermentation, intensity of which gradually increased up to the end of the considered period.

4. Discussion

The natural fermentation of olives relies upon indigenous microbial populations from raw material or containers used for storage. This practice may lead to fluctuations in the final characteristics of the product (Lanciotti et al., 1999; Panagou et al., 2003). The microbial ecosystem in brine is influenced by (a) the indigenous microbiota present in olives, (b) intrinsic factors of the fruit such as pH, water activity, diffusion of nutrients from the drupe (depending on the structure of the olive skin), levels of antimicrobial compounds such as oleuropein, and (c) extrinsic factors such as temperature, oxygen availability and salt concentration in brine (Nychas et al., 2002). The

296 occurrence and role of yeasts in different types of olive brine fermentations is well documented
297 (Arroyo-López et al., 2008), but no information on relationship between olive treatments in field
298 and yeast population present during olive fermentation is available.
299 We studied samples of naturally fermented and inoculated table green olives, differently treated in
300 field with unconventional repellent and antiovipositional products in the control of *Bactrocera*
301 *oleae*, with the aim of studying yeast ecology.
302 The yeast species identified throughout olive fermentations were *S. cerevisiae, P. anomala, P.*
303 *manshurica, C. aaseri, C. diddensiae, I. orientalis* and *R. mucilaginosa,* and, according to Garrido
304 Fernández et al. (1997), this population reached about 10^4-10^6 CFU/ml, despite the addiction of
305 LAB starters.
306 As it is shown in this paper, the combined use of culture-dependent and independent molecular
307 methods has allowed the identification of *P. anomala* and *S. cerevisiae* as dominant yeast species
308 throughout the fermentative process and in all experimental trials, both in treated and untreated
309 samples. *C. diddensiae* and *C. aaseri* also accounted for a significant proportion of the total yeast
310 population, but starting from the 15th day of fermentation. The other species, *P. manshurica* and *R.*
311 *mucilaginosa* are rather common members of olive fermenting flora However, as far as we know,
312 this is the first time that *I. orientalis* is related to table olive fermentation.
313 *P. anomala* has found to be the most abundant yeast throughout the fermentation process in all
314 kinds of olives (Hurtado et al., 2008). Moreover, the relevance of the species *P. anomala* in table
315 olive fermentation has been pointed out by other authors, proposing its use as starter culture
316 (Hernández et al., 2007). Gazi et al. (2001) reported that *P. anomala* produced the highest
317 freeradical-scavenging activity in a laboratory medium; therefore, strains could be selected also on
318 the basis of the production of bioactive antioxidants, retarding oxidative degeneration of fatty
319 substances and improving human health. Moreover, *P. anomala* isolates from olive brine were able
320 to produce killer toxins endowed with a broad spectrum of activity against human pathogens, thus

321 focusing on the possibility to use such yeast strains as bioprotective agents in table olives (Muccilli
322 et al., 2010) reducing, at the same time, the requirement for salt and preservatives.
323 *S. cerevisiae* is associated with olive fermentation since the initial scientific studies of this product
324 (González Cancho, 1965). The high occurrence of this species during storage is in agreement with
325 the results obtained by Rodríguez-Gómez et al. (2010) and Hernández et al. (2007) for directly
326 brined green olives, although other authors reported lower proportions in diverse olive brine
327 solutions (Marquina et al., 1992; Nisiotou et al., 2010).
328 *C. diddensiae* are widespread on the carposphere of the olive fruits as they were found in the waters
329 of seasoned table olive fermentation (Hernández et al., 2006; 2008). This species has been
330 described as a strong fermentative yeast, well adapted to the first stage of the fermentation (Ruiz-
331 Barba and Jiménez-Díaz, 1995). Moreover, the same authors demonstrated the ability of this species
332 to produce nicotinic acid, pantothenic acid and vitamin B6, essential for the growth of *Lactobacillus*
333 *plantarum.* The source of C. *diddensiae* species was postulated to be olive flies *Bactrocera oleae*
334 larvae (), so we could assume that *C. diddensiae* presence is directly correlated with fly infestation.
335 Based on this hypothesis and on our results, the kaolin clay has demonstrated to be more effective
336 against *B. oleae* than Bordeaux mixture (copper), as DGGE profile of kaolin treated olives showed
337 absence or very weak band corresponding to *C. diddensiae* species, especially at the beginning of
338 fermentation. The band appeared only by the 120th day of fermentation and this could be explained
339 by the fact that this species is able to degrade olive polyphenols and to grow also on olive mill
340 wastewater (Chakri et al., 2007). More effective action of kaolin was also demonstrated by Pennino
341 et al. (2006), due to the fact that rainfalls wash copper away more than kaolin film-particle.
342 Moreover, the kaolin clay has some advantages: unlike copper it has no environmental toxicity.
343 As reported by Nisiotou et al. (2010), *Candida aaseri* was encountered at relatively low percentages
344 (<10%) in different brine solutions both at the middle and at the final stages of fermentation. This
345 species constitutes part of the olive associated yeast community, as it has been characterized so far.

346 The former yeast has been isolated previously from the mid-fermentation stage of directly brined
347 green olives of Arbequina variety (Hurtado et al., 2008) and has been associated with olive bloater
348 spoilage (Faid et al., 1994).
349 Two clusters of yeast isolated were assigned to *P. manshurica* species by D1-D2 sequencing,
350 although one of these with a low sequence homology. As far as we know, few strains belonging to
351 this species have been isolated from fermented foods and molecularly identified; only in the study
352 of Rodríguez-Gómez et al. (2010) the existing genetic variability between yeast isolates of *P.*
353 *manshurica*, from ripe table olives, was recently demonstrated by RFLP analysis of the 5.8S-ITS
354 rDNA region. Specific phylogenetic studies should be carried since we can suppose that the two
355 strains might belong to an unknown species, closely related to *P. manshurica*.
356 *Issatchenkia orientalis* has been found in foods such as cheese (Prillinger et al. 1999), sourdough
357 (Pulvirenti et al., 2001; Vogelmann et al., 2009) grape wine pomace (Seo et al. 2007), cocoa and
358 coffee beans (Daniel et al. 2009; Masoud et al., 2004), and rice bran (Koh and Suh. 2009), but not
359 in olive brine. However, in a recent study of Kitagawa et al. (2010) *I. orientalis* strain MF-121 was
360 described as a multiple stress-tolerant yeast, being ethanol tolerant, salt tolerant, acid tolerant, and
361 thermotolerant. This specie was evidenced as a dominant species in the late stage of brine
362 fermentation only by PCR-DGGE probably because culture medium used for the isolation favoured
363 growth of the other species, thereby biasing the results.
364 The yeast biodiversity highlighted by PCR-DGGE was composed of 5 distinct species belonging to
365 4 genera. Some species, such as *P. anomala*, *S. cerevisiae*, *C. diddensiae* and *C. aaseri* were
366 detected both by culture dependent and independent methods. *Issatchenkia orientalis* was more
367 abundantly detected by PCR-DGGE but only after 120 days of fermentation. *P. manshurica* and *R.*
368 *mucilaginosa* were only detected by culture dependent methods at a very low number, reinforcing
369 the evidence that a polyphasic approach should be used to evaluate biodiversity.

In this study, we used for the first time DGGE method for elucidating the yeast population of green table olives during fermentation; the species evolution evidenced by a polyphasic approach allowed us to assert that yeast diversity is not affected either by LAB starter or by copper treatment. Only kaolin application delayed the growth of *C. diddensiae*, presuming an higher efficacy of this treatment to prevent fly infestation.

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522

Table 1

Experimental scheme of olive fermentations.

Vessel	Treatment in field	Starter	%NaCl
N1	Untreated	-	6%
N2	Untreated	*Lb. plantarum*	6%
N3	Untreated	-	8%
N4	Untreated	*Lb. plantarum*	8%
CU1	Copper	-	8%
CU2	Copper	*Lb. plantarum*	8%
CU3	Copper	*Lb. plantarum*	8%
CA1	Kaolin	-	8%
CA2	Kaolin	*Lb. plantarum*	8%
CA3	Kaolin	*Lb. plantarum*	8%

523

524

Table 2

Molecular identification of yeast isolates.

Species	Ampl. (bp)	*Hha*I	*Hae*III	*Hinf*I	*Msp*I	Isolates	Cluster	D1+D2 clostest match
S. cerevisiae	850	380+330+140	320+230+180+ 140	350+120	725+135	BCA11, 32, 41, 43, 44, 51, 52, 61, 64, 65, 71, 72, 81, 82, 83, 84, 91, 92, 102, 103, 111, 112; BCU23, 26, 33, 34, 41, 42, 44, 51, 61, 65, 71, 73, 74, 81, 82, 91, 92, 101, 102; BS61, 62, 63, 64, 65, 66, 71, 72, 73, 74, 75, 76, 82, 86, 101, 102, 103, 104, 111, 112, 113, 114, 122, 131, 141	1	*S. cerevisiae* 99%
P. anomala	650	580+60	620	310		BCA12, 13, 14, 15, 21, 42, 45, 46, 53, 62, 63, 101; BCU13, 21, 22, 24, 25, 32, 43; BS81, 84, 91, 92, 93, 94, 121	2	*P. anomala* 99%
P. membranifaciens	480	240+100	320+80	270+210		BS14, 15, 21, 22, 23, 25, 26, 31	3	*P. manshurica* 99%
Unknown	480	250+100	320+80	290+230		BS51,52	4	*P. manshurica 97%*
C.aaseri	650	280+280+70	420+130+80	300+190+170		BS32, 33, 35	5	*C. aaseri* 99%
C. diddensiae	650	290+190+140	420+130+80	320		BCA85, 93; BCU12, 14, 15, 45, 75; BS11, 34, 132	6	*C. diddensiae* 99%
I. orientalis	550	220+190	400+120	230+170		BS36	7	*I. orientalis* 99%
R. mucillaginosa	650	370+270	420+230	320+220+70		BS13, 16	8	*R. mucillaginosa* 99%

Figures

Fig. 1. Mean pH values of untreated, copper and kaolin treated olives throughout fermentation process.

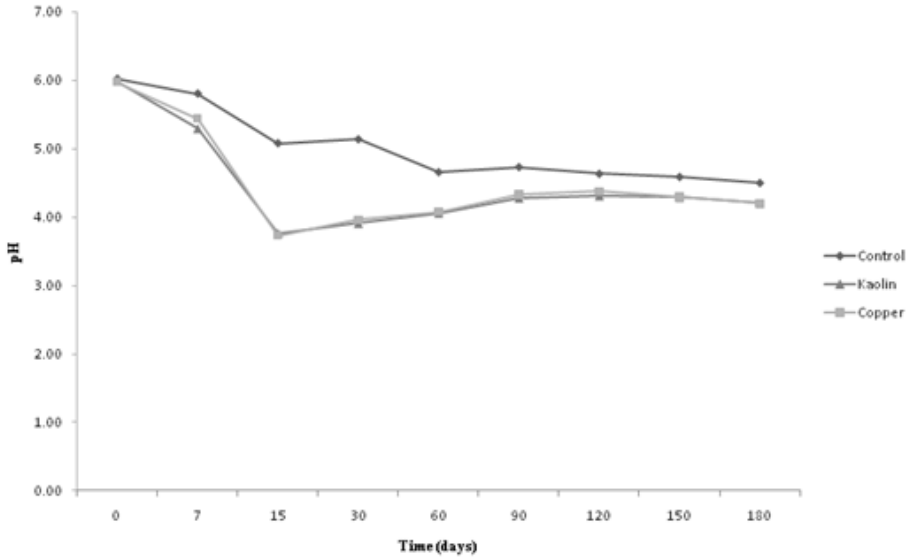


Fig. 2. Mean yeast count values in untreated, copper and kaolin treated olives throughout fermentation process.

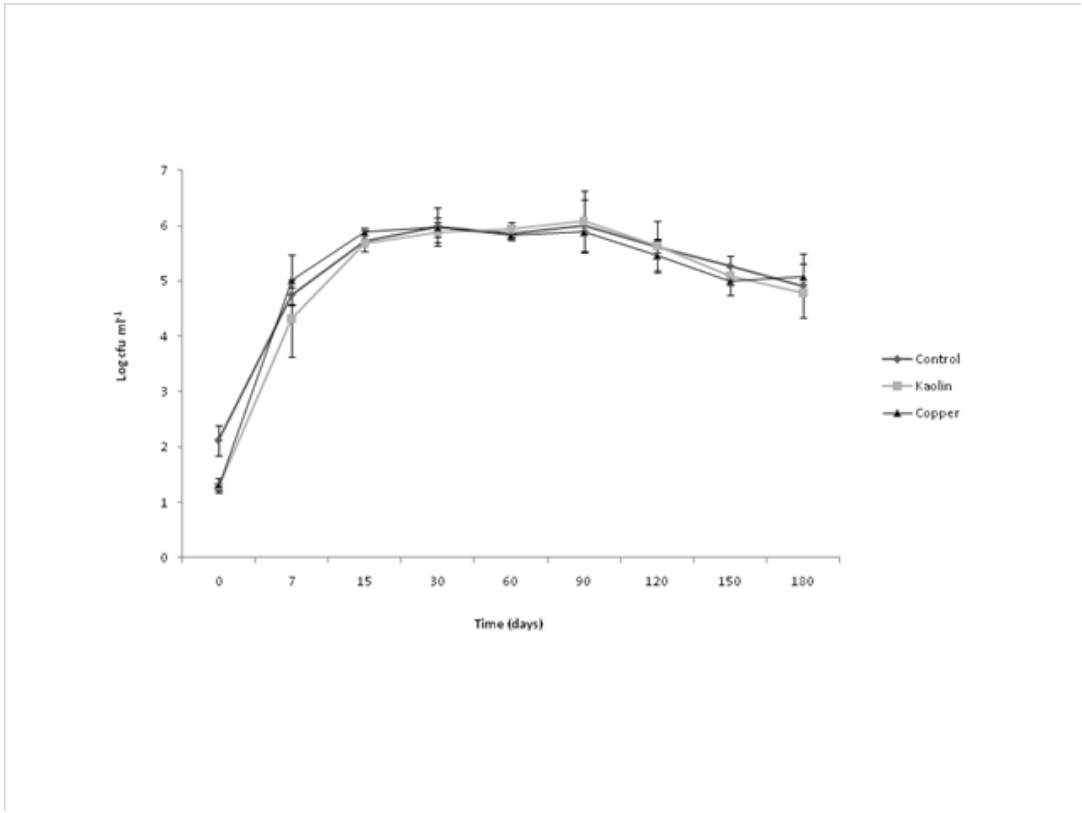
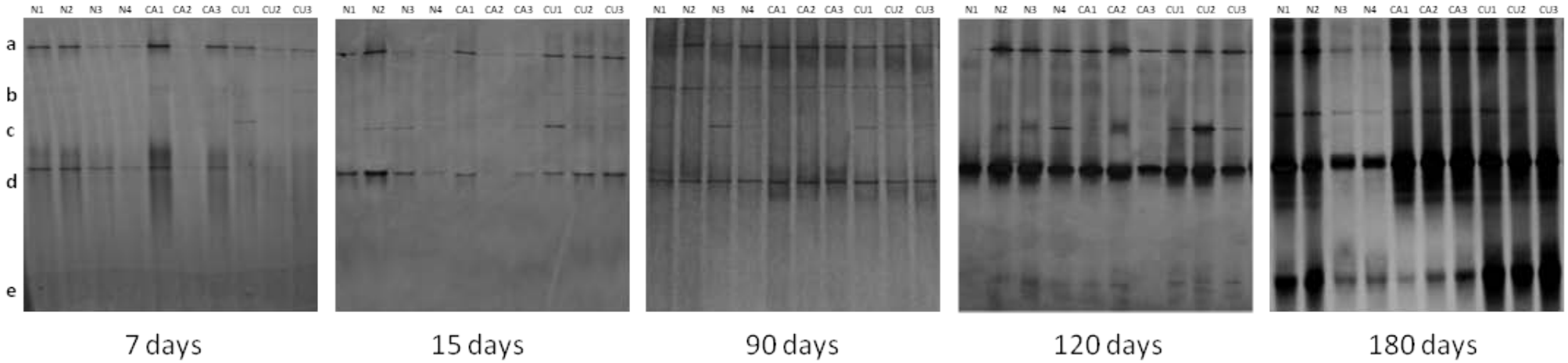


Fig. 3. PCR-DGGE profiles of yeast population during fermentation; a: *Pichia anomala*; b: *Candida aaseri*; c: *Candida diddensiae*; d: *Saccharomyces cerevisiae*; e: *Issatchenkia orientalis*.

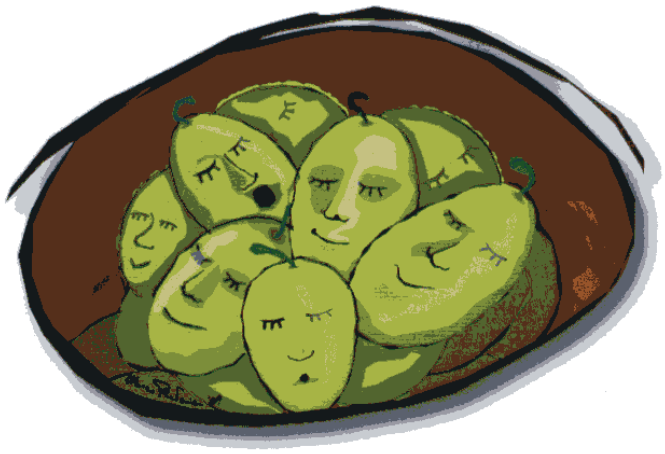


OLIVE DA TAVOLA

LA FERMENTAZIONE AL NATURALE FRA TRADIZIONE E INNOVAZIONE

ATTI DEL CONVEGNO
CATANIA, 25 GIUGNO 2009

a cura di
Cinzia Lucia Randazzo



Caratterizzazione tecnologica di lieviti isolati da olive verdi al naturale

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Riassunto

Durante il processo di fermentazione delle olive da tavola, i lieviti giocano un importantissimo ruolo in quanto essi, essendo presenti durante tutto il processo fermentativo, sono in grado di influenzare la qualità del prodotto finito. Tra le specie di lievito, *Saccharomyces cerevisiae* è stata quella più frequentemente isolata in campioni di olive fermentate al naturale prodotte in Sicilia. Una più ampia conoscenza delle proprietà tecnologiche dei ceppi potrebbe contribuire a chiarirne il ruolo durante il processo fermentativo e a proporne l'utilizzo come colture starter, vista la capacità di produrre composti aromatici, antiossidanti, enzimi, tossine killer e di promuovere lo sviluppo dei batteri lattici.

Introduzione

La preparazione delle olive da tavola è tradizionalmente basata sulla fermentazione lattica che si avvia spontaneamente non appena le olive sono poste in salamoia. Non si deve tralasciare comunque il ruolo che i lieviti rivestono all'interno delle salamoie, per la qualità finale del prodotto e per la loro azione di supporto ai batteri lattici. Sebbene alcuni ceppi di lievito siano stati spesso associati a fenomeni

di rammollimento o di formazione di gas all'interno delle confezioni (Vaughn *et al.*, 1972; Durán Quintana *et al.*, 1986), successivi studi hanno dimostrato il loro contributo alle caratteristiche sensoriali delle olive da tavola (Sanchez *et al.*, 2000) e il potenziale effetto benefico che essi apportano durante la fermentazione, in quanto produttori di vitamine, aminoacidi, composti aromatici, enzimi e tossine killer la cui potenziale azione di controllo varia dai lieviti alteranti ai batteri patogeni (Hernández *et al.*, 2008).

Scopo del presente lavoro di ricerca è stata la caratterizzazione tecnologica di lieviti appartenenti alla specie *S. cerevisiae* isolati da salamoie di olive, allo scopo di selezionare ceppi in grado di garantire un prodotto di elevata qualità, e allo stesso tempo svolgere la loro attività metabolica in equilibrio con i batteri lattici.

Materiali e metodi

Ceppi

I 72 ceppi di *S. cerevisiae* sottoposti a caratterizzazione erano stati precedentemente isolati da olive in salamoia provenienti da diverse aziende siciliane; i lieviti sono stati codificati in base al trattamento che le olive avevano subito in campo, con le sigle BCA (trattate con caolino), BCU (trattate con il rame), BS (non trattate), seguite da un numero progressivo.

Capacità di crescita a differenti pH, concentrazioni saline e 15°C

Sono state utilizzate provette contenenti di YM–broth inoculate con 0.1 mL di colture di 24 ore (McFarland 1), così come riportato da Psani e Kotzekidou (2006).

Resistenza al solfato di rame

È stato utilizzato un terreno liquido con aggiunta di solfato di rame (Yeast Nitrogen Base 0.67%; glucosio 2%; $CuSO_4$ 1M). Dopo l'inoculo le provette sono state incubate a 25°C e la verifica della capacità di crescita è stata effettuata mediante letture spettrofotometriche a 530 nm all'inoculo (t0) e dopo 24 ore (t24).

Attività β–glucosidasica, lipolitica, pectinasica e poligalatturonasica

La presenza dell'enzima β–glucosidasi è stata rilevata su terreno solido contenente arbutina come unica fonte di carbonio, incubato a 25°C fino a 14 giorni.

La capacità di produrre lipasi e pectinasi è stata valutata utilizzando il metodo riportato da Hernandez et al. (2007).

Lo screening qualitativo della poligalatturonasi è stato condotto su mezzo solido (YNB 0.7%; glucosio 0.5%; acido poligalatturonico 0.5%; agar 2%) inoculato tramite spot ed incubato per 48 ore a 25 °C. La presenza dell'enzima è stata evidenziata versando HCl 6M sulla superficie delle piastre e verificando, dopo qualche minuto, la formazione attorno ai ceppi positivi di un alone più chiaro rispetto al resto del terreno.

Carattere killer

L'analisi è stata condotta su un mezzo solido YEPD–MB medium (Hernandez *et al.*, 2008) impiegando come ceppi di riferimento il ceppo sensibile K6 e i ceppi produttori di tossine K1, K2 e K28, tutti appartenenti alla specie *S. cerevisiae.*

Produzione di vitamine del gruppo B

È stato effettuato uno screening qualitativo mediante prove di crescita in terreni privi della vitamina di cui verificare la sintesi: biotina, acido nicotinico, piridossina, acido pantotenico (Ruiz–Barba e Jimenez–Diaz, 1995).

Risultati e discussione

Prove di crescita alle diverse condizioni del mezzo

Per le prove relative alla capacità di crescita alle basse temperature (15°C), a diverse concentrazioni di NaCl e a diversi valori di pH, i lieviti isolati dai diversi campioni di salamoia hanno mostrato buona capacita di crescita in tutte le condizioni considerate.

I risultati relativi alla resistenza al rame sono stati ottenuti mediante il calcolo del Δ24, differenza di assorbanza della brodocoltura dopo 24 ore. Su un totale di 72 isolati soltanto 7 sono risultati incapaci di

sviluppare in presenza di rame: 2 isolati da olive trattate con caolino e 5 isolati da olive non trattate. Tutti i ceppi isolati da olive trattate con rame hanno evidenziato buona resistenza alla concentrazione testata. I risultati confermano i dati riportati in letteratura secondo cui il rame, a seconda della concentrazione e del ceppo, può portare all'apoptosi (Liang e Zhou, 2007).

Prove enzimatiche

Il saggio per la verifica della presenza di attività β–glucosidasica non ha evidenziato alcun risultato positivo, in accordo con i dati disponibili in letteratura sulla frequenza estremamente bassa di tale attività enzimatica all'interno della specie *S. cerevisiae* (Restuccia *et al.*, 2002).

Dai risultati delle prove di lipolisi effettuate in piastra è stato possibile evidenziare che la maggioranza degli isolati di *S. cerevisiae* possiede tale capacità, visto che solo 6 su 72 ceppi sono risultati negativi.

I saggi qualitativi per rivelare la presenza di pectinasi negli isolati hanno evidenziato risultati positivi soltanto per pochi ceppi, in particolare 3 isolati da olive trattate con caolino e 2 da olive non trattate.

Simile andamento è stato evidenziato per le prove della poligalatturonasi, sebbene il numero di isolati positivi sia stato più alto. I 14 ceppi che possedevano tale attività enzimatica erano tutti isolati da olive trattate con caolino. Questo è un dato confortante visto che nelle olive gli enzimi pectici possono essere causa di alterazione come il rammollimento e devono, pertanto, non essere prodotti dalle colture starter.

Carattere killer

Le prove effettuate hanno permesso di evidenziare che fra i ceppi isolati di *S. cerevisiae* 11 ceppi sono stati in grado di produrre tossine killer contro il ceppo sensibile K6. Per quanto riguarda le prove di resistenza alle varie tossine killer, i risultati sono stati alquanto promettenti, visto che tutti hanno mostrato resistenza alle tossine K7, K8 e K9, 58 ceppi alla tossina K2, 54 alla K19, 67 ceppi alla K30 e 61 ceppi alla K37.

Produzione di vitamine del gruppo B

La disponibilità di vitamine appartenenti al gruppo B durante la fermentazione delle olive è indispensabile per la crescita di *Lb. plantarum,* in quanto se la concentrazione non è ottimale la crescita dei batteri lattici potrebbe essere stentata (Ruiz–Barba e Jimenez-Diaz, 1995).

Hanno mostrato capacità di sintesi di tutte e quattro le vitamine i ceppi BCA 32,41, 44, 51, 52, 71, 92, 111; BCU 33, 41, 42 e BS 71. I ceppi BCA 72, BCU 81, BS 113 erano in grado di sintetizzare ac. nicotinico, ac. pantotenico e piridossina; i ceppi BCA 61,83,84 e BCU 24 e 44 biotina, ac. nicotinico e piridossina; i ceppi BCA 82 e il BS 43 biotina, piridossina e ac. pantotenico.

Conclusioni

Il presente lavoro di ricerca, condotto sui ceppi di *S. cerevisiae* isolati da salamoie di olive fermentate al naturale, si è proposto di analizzarne le proprietà tecnologiche. Tutti i ceppi hanno mostrato buona attitudine a crescere nelle condizioni caratteristiche della salamoia.

Importante si è dimostrata non solo la capacità fra i ceppi isolati di produrre lipasi, fondamentale per l'aumento dell'acidità della salamoia, ma anche la scarsa presenza di pectinasi e poligalatturonasi, portandoci a riconsiderare il ruolo alterante dei lieviti, come molti autori hanno evidenziato in studi precedenti.

L'attività delle tossine killer ad azione antimicrobica ha permesso infine di asserire che un'eventuale selezione sulla base di tale carattere può essere utile per l'applicazione di colture starter come colture protettive sia contro microrganismi alteranti che patogeni.

Sulla base dei risultati ottenuti è possibile, pertanto, affermare che la caratterizzazione tecnologica sugli isolati di *S. cerevisiae* si è rivelata un valido contributo alla comprensione del ruolo svolto dai lieviti nella realizzazione di un prodotto che, oltre a essere sicuro sotto il profilo igienico, abbia elevati standard qualitativi.

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Cibi di ieri e di domani: qualità e sicurezza tra tradizione e innovazione

Programma e Riassunti

Le Agavi Hotel a Positano
28 – 30 maggio 2008

Caratterizzazione della popolazione di lieviti in olive verdi al naturale diversamente trattate in campo

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Le olive da tavola rappresentano uno dei vegetali fermentati più diffusi in Italia, la cui preparazione è tradizionalmente basata sulla fermentazione lattica che spontaneamente si avvia non appena le olive sono poste in salamoia.
I lieviti sono presenti in numero elevato a partire dalla fine della prima settimana dalla messa in salamoia e sino all'esaurimento del materiale fermentescibile.
Sebbene alcune specie di lievito siano state storicamente associate a fenomeni di alterazione, recenti studi hanno dimostrato il loro positivo effetto sia sul processo fermentativo che sulle caratteristiche sensoriali delle olive da tavola. Essi producono, infatti, vitamine, aminoacidi, composti aromatici e tossine killer, il cui effetto si esplica sia sui lieviti alteranti che sui batteri patogeni.
La presente ricerca ha avuto lo scopo di selezionare ceppi di lievito da impiegare come starter nella produzione di olive verdi naturali in salamoia.
La cultivar di olive in studio è stata la Nocellara dell'Etna, sottoposta a trattamenti in campo con poltiglia bordolese (rame) e caolino; un campione non trattato è stato utilizzato come riferimento. Sui diversi campioni è stato condotto l'isolamento di un elevato numero di ceppi durante tutto il corso della fermentazione e la successiva identificazione tramite PCR/RFLP delle regioni ITS.
I risultati della conta dei lieviti hanno evidenziato in tutte e tre le tesi un aumento della popolazione di *S. cerevisiae* a partire già dalla prima settimana dalla messa in salamoia con un picco intorno ai 90 giorni. Gli altri isolati sono risultati appartenenti alle specie *Pichia anomala* e *Candida diddensiae.*
Le caratterizzazione tecnologica sugli isolati di *S. cerevisiae* ha confermato la capacità di questi ceppi di crescere a intervalli di pH tra 2.5 e 6.5, a concentrazioni di NaCl tra l'8 e il 12% e alla temperatura di 18°C, di resistere a elevate concentrazioni di rame e di produrre tossine killer.

Identificazione e caratterizzazione di batteri lattici isolati da olive in salamoia

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Il processo produttivo delle olive da tavola nei paesi del bacino del mediterraneo risulta ancora oggi spesso affidato a fermentazioni spontanee, che possono portare allo sviluppo di microrganismi alteranti e patogeni. La messa a punto di adeguate colture *starter* isolate da prodotti in fermentazione naturale rappresenta la migliore strategia per ottenere, nel rispetto della tradizione, prodotti sicuri. Lo scopo del presenta lavoro è stato quello di costituire una ceppoteca di batteri lattici (LAB) isolati da salamoie di olive delle varietà Nocellara dell'Etna e Nocellara del Belice, provenienti da differenti aziende siciliane, a differenti periodi dalla messa in salamoia. Gli isolati sono stati sottoposti all'identificazione a livello di specie e alla caratterizzazione tecnologica. In dettaglio 100 ceppi sono stati sottoposti ai seguenti test preliminari: colorazione di Gram, prova della catalasi, crescita a diverse temperature e produzione di CO_2. I presunti LAB sono stati identificati attraverso l'analisi della lunghezza dei frammenti di restrizione (RFLP) del 16S rDNA, utilizzando appropriati enzimi di restrizione. I ceppi sono quindi stati sottoposti alle seguenti prove: crescita a diverse concentrazioni di NaCl; crescita a diversi valori di pH; valutazione dell'attività β-glucosidasica; produzione di amine biogene (istamina e tirosina) e valutazione dell'attività antimicrobica. I risultati ottenuti hanno evidenziato che il 57% degli isolati appartengono alla specie *Lactobacillus plantarum,* riportata in letteratura come specie dominante nelle fermentazioni spontanee delle olive da tavola al naturale. Sulla base dei risultati, è stato possibile individuare ceppi di *Lb. plantarum* che presentano le migliori combinazioni dei caratteri tecnologici testati. Attualmente si sta procedendo alla differenziazione dei ceppi isolati mediante comparazione dei profili PFGE (Pulsed-Field Gel Electrophoresis) così da ottenere l'impronta digitale dei ceppi.

Impiego di colture *starter* selezionate per la produzione di olive da tavola

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Ceppi selezionati di batteri lattici (LAB) sono da tempo impiegati per la fermentazione delle olive in salamoia. Lo scopo del presente lavoro è stato quello di testare in vivo una coltura starter mista costituita da un ceppo di *Lactobacillus plantarum,* in associazione con un ceppo di *Lactobacillus casei,* precedentemente isolati da matrici vegetali in fermentazione e appartenenti alla collezione del DOFATA. I ceppi sono stati inoculati in rapporto 1:1 su olive della varietà Nocellara dell'Etna sottoposte in campo a trattamenti antiparassitari di tipo biologico nei confronti della *Bactrocera oleae* (mosca dell'olivo). In particolare le olive sono state sottoposte al trattamento con sali rameici (poltiglia bordolese) e con composti argillosi (caolino). La prova è stata effettuata in due anni consecutivi sia in laboratorio sia presso un'azienda locale che produce in biologico. L'andamento della popolazione microbica è stato monitorato sino a 210 giorni dalla messa in salamoia. I risultati hanno mostrato la capacità dello *starter* di sviluppare prontamente sin dalle prime fasi della fermentazione, raggiungendo livelli di 10^{12} unità formanti colonia (UFC)/mL già a 30 giorni dalla messa in salamoia.

Per quanto riguarda le olive trattate in campo, differenze sono state riscontrate tra l'effetto del caolino e quello della poltiglia bordolese. La coltura *starter,* pur presentando livelli di UFC/mL più bassi, ha mostrato la capacità di condurre con successo la fermentazione.

Enzymatic Activity of *Pichia anomala*

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An alkaline β-glucosidase isolated from an olive brine strain of Pichia anomala

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Keywords:	naturally fermented olives, biological debittering, Pichia anomala, β-glucosidase, esterase, biochemical characterization

An alkaline *β*-glucosidase isolated from an olive brine strain of *Pichia anomala*

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Abstract

An efficient β-glucosidase (βG) producing strain, *Pichia anomala* BS81, was isolated from naturally fermented olive brine and identified based on PCR/RFLP of internal transcribed spacer rDNA and sequence analysis of D1-D2 region of 26S rDNA.

The relative molecular weight of *P. anomala* βG was determined to be 135 kDa by sodium dodecylsulfatepolyacrylamide gel electrophoresis. The hydrolytic activity of the βG had an optimum pH of 8.5 and an optimum temperature of 35°C. The enzyme showed high substrate specificity and high catalytic efficiency (Km 0.99 mM, Vmax 14 U g^{-1} of cells) for p-nitrophenyl-β-D-glucopyranoside. The enzyme was activated at increasingly concentrations of NaCl, with maximum activity at concentrations of 15% (w/vol).

Although βGs have been purified and characterized from several other sources, *P. anomala* βG is distinguished from other βGs by its relative maximum activity values reached at alkaline pH and temperature of 35°C, never evinced in other yeast isolates. Moreover, the yeast strain was endowed with esterase activity, acting in synergism with β-glucosidase in degradation of oleuropein for debittering of table olives and olive oil.

Keywords

naturally fermented olives; biological debittering; *Pichia anomala*; β-glucosidase; esterase; biochemical characterization

Introduction

β-Glucosidase (EC 3.2.1.21) catalyses the hydrolysis of alkyl and aryl β-glycosides as well as disaccharide glucosides and gluco-oligosaccharides. This enzyme has been classified into glycohydrolase families 1 and 3 (Henrissat & Bairoch 1993, 1996; Li *et al*., 2002). The hydrolytic activity of this enzyme has been exploited in various industrial applications (Bhatia *et al*., 2002a).

During the processing of foods and beverages, and especially in winemaking, β-glucosidases are key enzymes in the enzymatic release of aromatic compounds from glycosidic precursors present in raw materials, which lead to a mostly unused flavour potential (Winterhalter & Skouroumounis, 1997; Gueguen *et al*., 1998; Palmeri & Spagna, 2007). Most research on β-glucosidases in yeasts has focused on species isolated during wine-making and high activities were demonstrated, especially in non-*Saccharomyces* yeasts (Rosi *et al*., 1994; Ferreira *et al*., 2001; Belancic *et al*., 2003; Rodriguez *et al*., 2004; Villena *et al*., 2005) while the majority of *Saccharomyces* isolates does not show β-glucosidase activity on a natural substrate like arbutin (Rosi *et al*., 1994; Spagna *et al*., 2002b; Rodriguez *et al*., 2004).

Another interesting field of β-glucosidase application in food processing is production of naturally fermented table olives and olive oil. Olives have a bitter compound, named oleuropein, which must be eliminated during the olive elaboration process. Nowadays, the most usual industrial debittering methods consist of treating the fruits with a sodium hydroxide solution that produces the hydrolysis of this compound. The lye treatment and subsequent washing process comprises a very complex mechanism of removal of some water-soluble compounds, like reducing sugar and organic acids, from the olive fruit as well as oleuropein (Garrido-Fernandez *et*

al., 1997). In addition, the lye could dissolve the epicuticular waxy coating and enhance the diffusion from fruit flesh and result in the softening (Marsilio *et al*., 1996a; Sanchez- Romero *et al*., 1998).

Furthermore, these technologies present huge drawbacks such as the highly contaminant discharges generated and the large quantity of water used for rinsing. The wastewaters originating from table olives processing industries pose an important environmental threat, as they are characterized by a very high organic load and high concentration of phenolic compounds, which are toxic to living organisms, together depriving this food of precious biological functions. After the debittering and washing steps, in fact, the olive fruit loose the major part of its phenolic content, and in particular of hydroxytyrosol, which is present in larger amounts in the waste of the washing stage (Parinos *et al*., 2007).

The alternative strategy is biological debittering that take place during naturally fermented table olives (Ciafardini & Zullo, 2000) and in newly produced olive oil (Ciafardini & Zullo, 2002b) production, in which microbial β-glucosidase and esterase enzymes hydrolize oleuropein (Esti *et al*., 1998). This procedure, typical of Greek style table olives, both eliminates wastewater discarding problem and avoids the reduction of such a precious sources of phenolic antioxidants as untreated olives are. In fact, many molecules isolated from *O. europaea* fruits and leaves are thought to have originated from oleuropein, via aglycon, by the opening of the elenolic acid ring, many forms of elenolic acid and simple phenolic compounds, such as hydroxytyrosol (Gariboldi *et al*., 1986; Montedoro *et al*., 1993). These molecules are known for their free radical scavenging activity (Visioli *et al*., 1998a; Manna *et al*., 1999) and demonstrated to prevent several pathological processes, such as certain tumours (prostate and colon cancers) (Lipworth *et al*., 1997) and coronary heart disease (Keys, 1995) since exhibited a pronounced hypolipidemic

effect, reduced the lipid peroxidation process and enhanced the antioxidant defence system in experimental atherogenic model (Jemai *et al*., 2008). The advantages of Greek-style olive processing is confirmed by the study of Boskou *et al*. (2006) in which they demonstrated that table olives available in the Greek market are a good source of phenolic compounds since the consumption of 50 g of table olive provides about 56 mg polyphenols from flesh.

Characterization of microbial degrading enzymes has important practical implementations, to obtain new enzymes to be used in commercial applications and new information that allows more effective use or improvement of microbial cultures.

The present study was designed to characterize β-glucosidase enzyme of a strain of *Pichia anomala* isolated from naturally fermented olives. Isolating source, the brine, is usually characterized by different parameters and therefore the isolation, purification and deeper knowledge of enzymatic characteristics could represent a useful tool applicable under different culture conditions and on different substrates.

Materials and methods

Yeast isolation

Brine samples of naturally fermented biological green olives were collected during all fermentation time (120 days), suitably diluted and inoculated into plates containing Sabouraud Dextrose Agar supplemented with chloranphenicol (Oxoid, Basingstoke, UK). The plates were then incubated at 25 °C for 48 h. One hundred twenty yeast colonies were isolated and purified

on the same medium, then transferred to YPDA (g l^{-1} distilled water: yeast extract, 10; peptone, 10; dextrose, 20; agar, 20) and refrigerated (4.0◦C) prior to testing.

β-glucosidase and esterase assays

A qualitative detection of glucoside hydrolase activity was carried out on 120 yeast isolates onto agar plates containing arbutin as substrate. Solid medium, adjusted to pH 5, consisted of: 6.7 g l^{-1} Yeast Nitrogen base (YNB), 5 g l^{-1} arbutin, 0.2 g l^{-1} ferric ammonium citrate, bacteriological agar 20 g l^{-1}, as described by Rosi *et al*. (1994). Yeast strains with β-glucosidase activity hydrolyse arbutin producing a brown colour of variable intensity around the colony. One litre of each yeast culture, grown at 25 ◦C for 48 h was centrifuged at 4100×*g* for 20 min at 4 °C. The supernatant was ultrafiltered using a membrane Pellicon XL (Millipore, Billerica, MA, USA) with a cut-off of 10,000 kDa. The enzyme was quantitatively recovered from the membrane with citrate-citrate (C-C) buffer 0.1M at pH 5.0 to a total volume of 25 ml. In order to carry out the cell count, 9.0 ml of distilled water were added to 1.0 ml of the homogenised pellet suspension, diluting as required in order to obtain a maximum absorbance value against distilled water of 1.0 at a wavelength of 600 nm.

β-Glucosidase (βG) was determined in the yeast pellet suspension and solution separately. One hundred microlitre of citrate–phosphate buffer (C-P) 0.1M at pH 5.0 were added to 100 µl of enzyme solution followed by 100 µl of substrate (0.55×10^{-2} M) dissolved in the same buffer and stirred at 30 °C for a reaction time that varied according to each sample. The synthetic substrate *p*-nitrophenyl β-d-glucopyranoside (*p*NPG) was supplied by Sigma Aldrich (St. Louis, MO, USA).

The reaction was stopped by adding 600 µl of 1M sodium carbonate (Merck, Darmstadt, Germany), allowing the yellow colour of the *p*-nitrophenolate ion to develop. In order to eliminate the turbidity, caused by precipitation of the proteins present in the pellets, 2.0 ml of ethanol were added and centrifugation was carried out at 4100 × *g* for 15 min at 4 °C.

The absorbance of the samples was read spectrophotometrically against the blank at λ of 400 nm, using an ε of 18,300 M^{-1} cm^{-1}.

One enzyme unit was defined as the quantity of enzyme required for hydrolysis of 1 µmol min^{-1} substrate under the previous experimental conditions. Activity data were expressed as specific productivity, the ratio between the enzyme units and the weight of the yeast cells in grams, using the following equation: Abs (600 nm) = 2.2256 × (g l^{-1} of yeast cells). All analyses were done in triplicate. The variability was ±1% (with a 95% of confidence interval).

The esterase activity was determined by the method of Blanco *et al*. (2004) modified as follows. Hydrolysis of *p*-NPA (0.4mM in 50mM sodium phosphate buffer pH 7.0) was assayed on 50 µL of yeast suspension diluted in phosphate buffer (50mM, pH 7.0) at 25 °C for 30 min. The absorbance of solution was read spectrophotometrically against blank at 348 nm. One esterase unit corresponds to consumption of 1 mol *p*-NPA/min (ε*p*-NPA = 5150M−1 cm−1). Activity data are expressed as specific productivity, the ratio between the enzyme units and the weight of the yeast cells in grams.

Yeast identification.

Strain BS 81, which exhibited the highest enzymatic activity, was identified by PCR/FRLP of ITS regions, since the amplification and subsequent restriction of ITS regions is

considered a valid method for identifying yeast at species level (Esteve-Zarzoso *et al*., 1999). Yeast strain was grown overnight in liquid YPD at 28 °C whilst stirring. DNA was extracted from 3 ml of this culture, according to the method described by Hoffman & Winston (1987) with a few modifications (Pulvirenti *et al*., 2001). The amplification procedure of rDNA ITS regions (Molina *et al*., 1992) was carried out using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers and purified DNA. The DNA was suspended in a solution containing recombinant Taq polymerase (Invitrogen, Carlsbad, CA, USA).

Amplification conditions were: a cycle at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 s. Digestion was done directly on 5–10 µl of amplified DNA, to a final volume of 20 µl with *Hae* III, *Hinf* I, *Hha* I and *Msp* I (New England BioLabs Beverly, MA, USA). Restriction fragments were separated for 1.5 h in a 2% Nusieve 3:1 agarose gel containing ethidium bromide in 0.5× TBE buffer. Strain CBS 5759 of *Pichia anomala* was used as reference.

To confirm species attribution of the investigated strain, PCR amplification of D1/D2 region of the 26S rDNA was achieved by using primers NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTTCAAGACGG-3′ (Kurtzman & Robnett 1998). 26S rDNA PCR product, obtained from the isolate, was commercially sequenced and the sequence data were compared with the sequences present in public data libraries (GenBank) using the Blast search program in order to determine their closest known relatives (Altschul *et al*., 1997; http://www.ncbi.nlm.nih.gov/BLAST/).

Properties of β-glucosidase from identified strain of Pichia anomala

βG of BS 81 yeast strain has been characterized. The following parameters were established: optimum pH (between 2.0 and 10.0 in 0.1 M C-P buffer) at 30 °C, optimum temperature (between 10 and 60 °C), inhibition by NaCl (between 5.0 and 30%); kinetic parameters *K*m and *V*max (at pH 5.0 and 30 °C) were determined from Lineweaver–Burk plots using standard linear regression techniques (Linewaver & Burk, 1934).

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.0% as described by Laemmli (1970), with a Mini Protean III electrophoresis unit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The determination of subunit molecular mass was performed both on pellet and supernatant in denaturant solution at two different concentrations. βG from almond was used as standard (Sigma, Saint Louis, MO, USA). The gels were stained for two hours with Coomassie brilliant blue R-250 (Bio-Rad Laboratories Inc., Hercules, CA, USA), washed with water and subsequently incubated in acetic acid (7% vol/vol) over night.

Results

β-glucosidase screening

Among the 120 yeast isolates, 40 strains were able to hydrolyze arbutin producing a brown colour of variable intensity developing around the colony on the agar plates.

Both the liquid fraction and pellets of the culture medium of the β-glucosidase positive strains were analysed by means of the quantitative assay. The β-glucosidase activity of all strains was found exclusively in the pellets, confirming that such activity is not exogenous. Strain BS 81 exhibited the highest enzymatic activity, with 13 U g^{-1} cells.

Strain identification

The yeast strain was identified by molecular methods at species level. In fact, the correct attribution of a strain as belonging to a species is an essential condition for being able to evaluate and characterize a strain at enzymatic level.

BS81 strain was analyzed by the restriction pattern of the rDNA ITS regions and the results were compared with those of the *P. anomala* type strain and those reported in literature. The strain had an amplification procedure of 620 bp and a restriction profile identical to the *P. anomala* type strain CBS 5759 (Fig. 1). To be more specific, the restriction pattern with *Hae* III did not have any cut site either of the isolated strain or the *P. anomala* comparison strain. Restriction enzymes *Hin*f I and *Hha* I had a single cut site in the defined region. In the first case, the enzyme produced two fragments with the same number of base pairs (310 bp); in the second case the two fragments had different molecular weights (580 and 60 bp). The *Hinf* I fragments were the same as reported by Esteve-Zarzoso *et al*. (1999), whereas slight differences were found in the size of the amplicon (620 and 650 bp reported); moreover, in the above-mentioned publication, *Hae* III produced two fragments, one of them only 50 bp long and hardly detectable by conventional gel electrophoresis. For the same reason the 60 bp produced by *Hha* I was not detectable by electrophoresis.

The results of PCR-RFLP was confirmed by sequencing of D1/D2 region of the 26S rDNA since PCR product, obtained from the isolate, was compared with the sequences present in GenBank using the Blast search program and the closest match resulted *P. anomala* (accession number FJ865436.1) at 98%.

Properties of β-glucosidase and esterase assay

By ascertaining β-glucosidase activity in *P. anomala* strain BS81 under saturation conditions, it emerged that the enzyme shows typical Michaelis–Menten-type kinetics for βG activity with increasing pNPG concentrations (Table 1). The Lineaweaver-Burk plot (Lineaweaver & Burk, 1934) obtained for the conversion of pNPG showed a Km of 0.99 mM. The high activity (Vmax 14 U g^{-1} of cells) and the value of Km revealed that the enzyme had an high affinity for the pNPG substrate (Segel, 1976).

As regards the behavior of the enzyme at increasingly concentrations of NaCl, the βG of BS81 strain was activated by its presence, with maximum activity at concentrations of 15%, while a 50% reduction of enzymatic activity was noticed at 30% of NaCl (Fig. 2).

The pH profile of the enzyme (Fig. 3) indicated the activity between pH 2.0 and 11.5, with an optimum value at pH 8.5, in citrate–phosphate buffer, with pNPG as substrate. The activity of the enzyme increased with increase in pH from 5 to 8.5, with a drastic loss of activity at pH values higher than 9.0.

As shown in Fig. 4, activity of the enzyme increased with the increase in temperature from 30 to 35 °C, in correspondence of which the highest activity was observed. The activity decreased considerably with further increase in the temperature up to 45°C, therefore, enzyme maintained a constant activity up to 60 °C.

The strain BS81 was assayed for esterase activity in presence of *p*-NPA, as described in "Materials and Methods", and the specific esterase activity was of 0.33 U/g of cells. The esterase from *Pichia* species is an intracellular activity, and it contributes positively to biological debittering of olives by promoting transformation of oleuropein into hydroxytyrosol (Capozzi *et al*., 2000; Segovia-Bravo *et al*., 2009).

SDS-PAGE

SDS-polyacrylamide gel electrophoresis of partially purified enzyme was performed as shown in Fig. 5. Analysis of the enzyme, in the presence of SDS, revealed one band with a molecular weight of 135 kDa, in both lanes of the pellets (lanes 4, 5), while there wasn't any band in the lane of supernatant. This analysis confirm that the enzyme is intracellular.

Discussion

The main phenolic compounds in the olive flesh are oleuropein (an heterosidic ester of elenolic acid and hydroxytyrosol), hydroxytyrosol 4-β-D-glucoside (4-β-D-glucosyl-3-hydroxyphenylethanol), hydroxytyrosol (3,4-dihydroxyphenylethanol) and other minor compounds, such as tyrosol, tyrosol glucoside, rutin and verbascoside (Romero *et al*., 2002; Ucella, 2001), which can be degraded through the concerted activity of different enzymes, such as β-glucosidases and esterases (Marsilio *et al*., 1996b). Esterases hydrolyze the ester bonds of oleuropein, producing hydroxytyrosol and glucosyl derivate (Capozzi *et al*., 2000); β-glucosidases break the liaison of glucose with the aglycon which, in turn, may also react with esterases, releasing hydroxytyrosol and elenolic acid (Briante *et al*., 2004; Mazzei *et al*., 2006).

Especially at the beginning of the fermentation process of the olives not treated with alkali, the hydrolysis of oleuropein can be attributed to the β-glucosidase produced by oleuropeinolytic microrganisms. Since it has been proven that oleuropein and its hydrolysis products induce leakage of glutamate and inorganic phosphate from the bacterial cell as well as degradation of the cell wall itself (Ruiz-Barba *et al*., 1990), biodegradation of polyphenols by yeast is a particularly interesting biotechnological application which promotes lactic acid bacteria growth.

A more recent attempt to improve olive oil quality, by increasing bioconversion of healthy phenolic compounds and their solubility in olive oil and by preventing the oxidization of the triglycerides, was carried out by Kachouri & Hamdi (2004). They demonstrated that incubation of olive oil samples with fermented OMW by *Lactobacillus plantarum* caused polyphenols decrease in OMW and increase in oil with multiple biological effects. However, only yeasts are able to survive in micro-drops of vegetation water present in newly produced olive oil, since it contains various simple and complex phenolic compounds characterized by high antimicrobial activity (Ciafardini & Zullo, 2002a). So the use of a yeast strain, such as BS81, which possesses both β-glucosidase and esterase activity, could also highlight the opportunity to inoculate olive oil for enforcing its functional properties.

An additional important aspect of the natural fermented brined olives or olive oil technology is related to the wastewaters management that, due to the high organic and phenolic content, is a serious environmental problem in almost all Mediterranean countries. Since polyphenolic compounds are toxic also for microorganisms (i.e. metanogenic bacteria) this could be considered as a limiting factor for conventional methods of microbial degradation usually applied to olive mill wastewaters. Some yeast strains of *Candida tropicalis* and *Yarrowia lipolytica* have shown capacity to reduce the chemical oxygen demand, monophenols and polyphenols in olive mill wastewater (Lanciotti *et al*., 2005; Martinez-Garcia *et al*., 2009).

Candida rugosa, *C. cylindracea* and *Y. lipolytica* have shown the ability to grow on olive mill wastewater (OMW)-based medium and to produce high-value compounds while degrading this waste (Brozzoli *et al*., 2009; Gonçalves *et al*., 2009). So there is a potential demand for yeasts with a capacity to reduce the amount of polyphenols in olive wastewater, that cannot be released into the environment, but previously and suitably treated (Amaral *et al*., 2008). A rapid polyphenol degradation may have a positive impact on the environmental problems of OMW management, since it can act also as a first step of effluent treatment.

In the present study β-glucosidase of a *P. anomala* strain was characterized by mean of biochemical parameters. Kinetic parameters were similar to those of the intracellular βG from the methylotrophic yeast *P. pastoris* (Turan & Zheng, 2005).

The βG isolated from BS81 resulted as actived at low NaCl concentrations and inhibited at high NaCl concentration with a similar behaviour of the βG isolated from *Aspergillus niger* (Rashid & Siddiqui, 1997).

So far, an alkaline pH optimum of the βG was not been evidenced in yeast strains, whose optimum pH range is between 3.5 and 7.0. In particular, for *P. anomala* strains, βG optimal pH was found to be 5.5 (Jijakli & Lepoivre, 1998; Spagna *et al*., 2002a). A maximal activity at about pH 8.0 have been found for βG isolated from *Agrobacterium tumefaciens* (Singh *et al*., 1995) and *Mucor miehei*. Moreover, Ciafardini & Zullo (2001) isolated a strain of *Leuconostoc mesenteroides* associated with fermentation of Coratina cultivar olives endowed with β-glucosidase activity that showed maximum catalytic activity at pH 8, probably demonstrating that isolating source could influence enzyme pH optimum.

The high value of optimum temperature (35°C) and temperature range between 40 and 60 °C was similar to βG isolated from *P. pastoris* (Turan & Zheng, 2005) and other microrganisms such as the cellobiose-fermenting yeast *C. wickerhamii* and the yeast *Metschnikowia pulcherrima,*

endowed with potential applications in wine-making (Singh & Hayashi, 1995; González-Pombo *et al*., 2008). Reports based on experimental data and theoretical considerations pointed to increased number of hydrogen bonds and salt bridges as the two main factors contributing to thermal stability (Macedo-Ribeiro *et al*., 1996; Vogt *et al*., 1997). On the contrary, the βG of a strain of *P. anomala* isolated from wine fermentation had an optimum temperature of 20 °C, above which there was a reduction in enzyme activity tending towards zero at a temperature of 35–40 °C; also this finding could support the thesis that microbial environment strongly effect enzyme structure-function relationship.

The molecular weight of the enzyme is in accordance with the values of literature; in fact, for the microbial βG, the Mw is between 43 and 330 kDa. From the SDS-polyacrylamide gel electrophoresis it was possible to observe one band in the pellet, with a molecular size of 135 kDa.

In addition, this is the first report of contemporary presence of esterase together with β-glucosidase activity in a *P. anomala* strain isolated from olive brine; this could be of biotechnological interest since, as well as it has been demonstrated that both activities are involved in degradation of oleuropein for debittering of table olives and olive oil (Marsilio e Lanza, 1998; Capozzi *et al*., 2000) and in flavour of plant derived foods and beverages (Lomolino *et al*., 2005; 2006).

Relative maximum values activity reached at pH 8.5 and temperature of 35°C, 165 and 64 (U mg^{-1}) respectively, were not previously highlighted in other studies and could represent the condition to lead important practical applications of the new enzyme in food processing.

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Table 1. Chemical and physical characteristics of βG from BS81 yeast strain

Vmax	Km	Optimum pH	Optimum Temperature (°C)	Relative Activity at 15% NaCl (%)	Molecular Weight (kDa)
14	0.99	8.5	35	100	135

Figure legends

Fig. 1. Restriction analysis of amplified ITS regions. Lane 1: 100 bp DNA ladder (New England BioLabs Inc., Ipswich, MA, USA); lane 2: CBS 5759 + *Hha* I; lane 3: BS 81 + *Hha* I; lane 4: CBS 5759 + *Hinf* I; lane 5: BS 81 + *Hinf* I, lane 6: CBS 5759 + *Hae* III; lane 7: BS 81 + *Hae* III; lane 8: CBS 5759 PCR product; lane 9: BS 81 PCR product; lane 10: 100 bp DNA ladder.

Fig. 2. Effect of NaCl concentration on β-glucosidase relative activity.

Fig. 3. Effect of pH on β-glucosidase relative activity.

Fig. 4. Effect of temperature on β-glucosidase relative activity.

Fig. 5. SDS-polyacrylamide gel electrophoresis of β-glucosidase. Lane 1: 10µl of protein molecular weight standard; Lane 2: 10 µl of crude enzyme; Lane 3: 20 µl supernatant; Lane 4: 20 µl of enzyme extract; Lane 5: 10 µl of enzyme extract; Lane 6: 10µl of protein molecular weight standard.

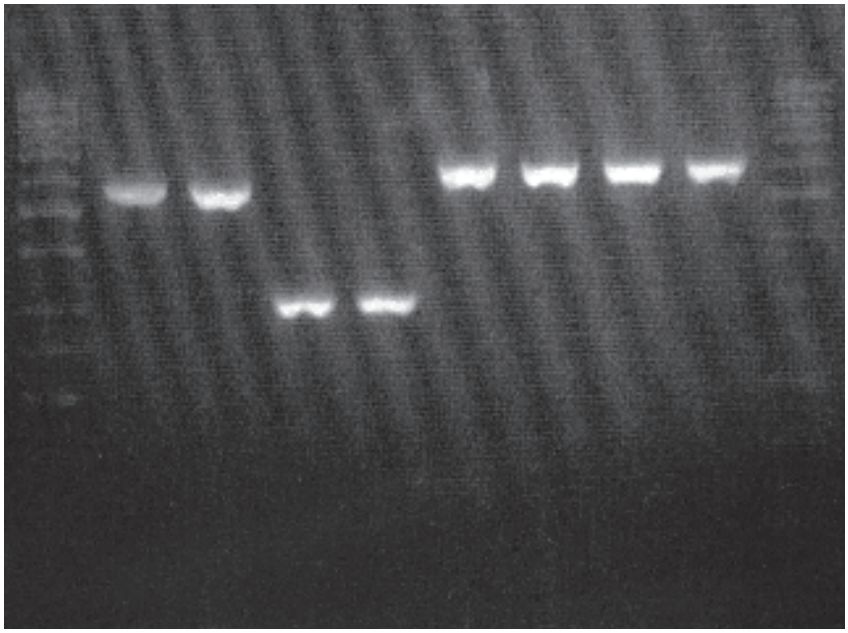

Fig. 1. Restriction analysis of amplified ITS regions. Lane 1: 100 bp DNA ladder (New England BioLabs Inc., Ipswich, MA, USA); lane 2: CBS 5759 + Hha I; lane 3: BS 81 + Hha I; lane 4: CBS 5759 + Hinf I; lane 5: BS 81 + Hinf I, lane 6: CBS 5759 + Hae III; lane 7: BS 81 + Hae III; lane 8: CBS 5759 PCR product; lane 9: BS 81 PCR product; lane 10: 100 bp DNA ladder.
136x101mm (300 x 300 DPI)

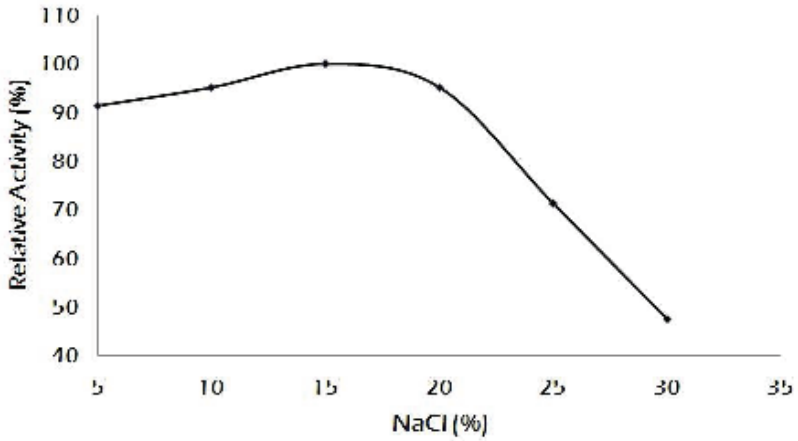

Fig. 2. Effect of NaCl concentration on β-glucosidase relative activity.
325x188mm (72 x 72 DPI)

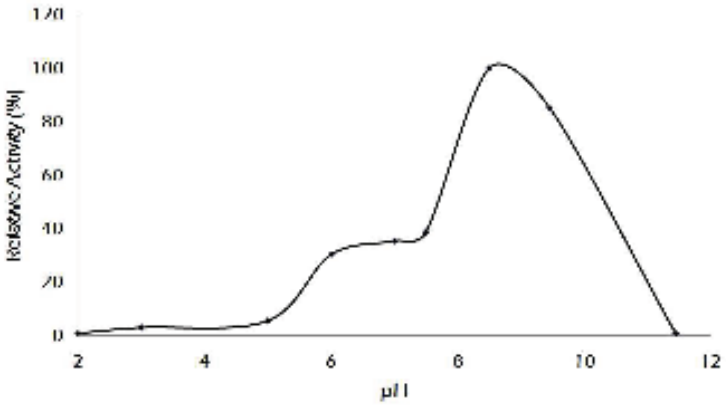

Fig. 3. Effect of pH on β-glucosidase relative activity.
400x230mm (72 x 72 DPI)

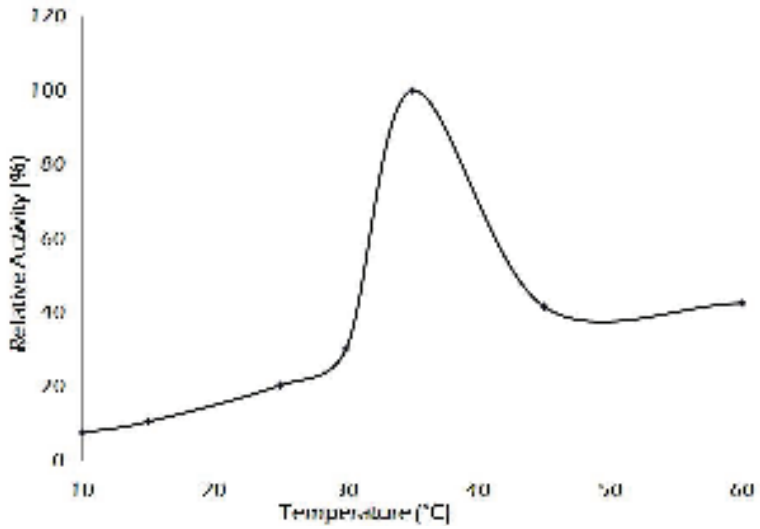

Fig. 4. Effect of temperature on β-glucosidase relative activity.
352x225mm (72 x 72 DPI)

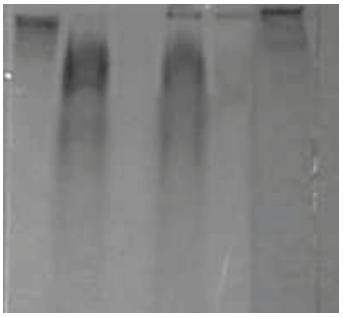

Fig. 5. SDS-polyacrylamide gel electrophoresis of β-glucosidase. Lane 1: 10µl of protein molecular weight standard; Lane 2: 10 µl of crude enzyme; Lane 3: 20 µl supernatant; Lane 4: 20 µl of enzyme extract; Lane 5: 10 µl of enzyme extract; Lane 6: 10µl of protein molecular weight standard.
70x64mm (72 x 72 DPI)

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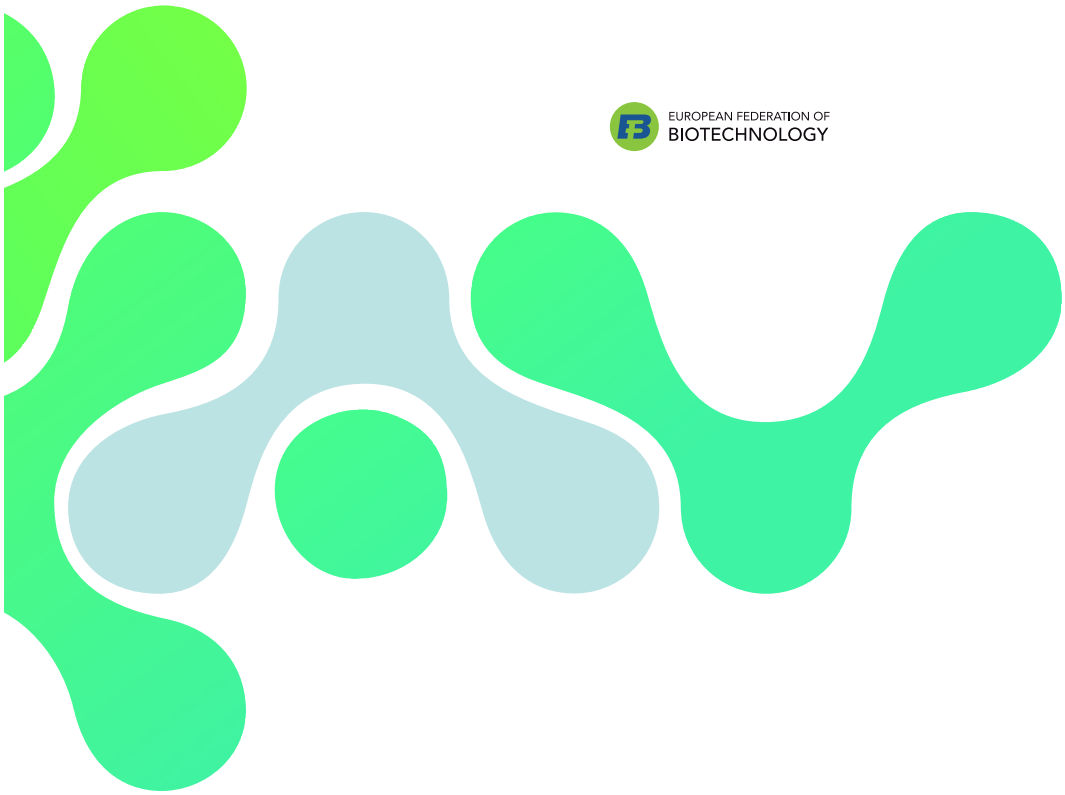

In the studies involving other microorganisms it was shown that addition of carboxylic acids with carbon chains containing five and more carbons as substrate can induce incorporation of the respective β-hydroxy acids into the PHA. In our experiments luminous bacteria were grown on media supplemented with valeric acid. PHAs synthesized by the most of studied strains of *P. leiognathi* contained significantly larger amounts of β-hydroxyvaleric acid in such conditions. It was shown that *V. fisheri*, strain 2089, included up to 1.5% β-hydroxyvalerate in PHAs. For *V. harveyi* influence of valeric acid on polymer composition was not detected. Also, no minor hydroxy acids were found in the PHAs produced by *P. phosphoreum* regardless of the addition of valerate.

Thus, *P. leiognathi* and *V. fisheri* cells grown in the medium supplemented with valerate, which is hydroxyvalerate precursor substrate, can produce polymers containing increased amounts of this hydroxy acid. By contrast, *P. phosphoreum* strains were found unable to synthesize multi-component PHAs.

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2.1.039

Recombinant protein production by Mut$^+$ strain of *Pichia pastoris* using dual carbon sources: methanol and sorbitol

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[2] Cambridge, United Kingdom

The methylotrophic yeast *Pichia pastoris* has become a popular host for the expression of recombinant proteins, due to its ability to produce foreign proteins at high levels using one of the strongest and most tightly regulated eukaryotic promoters, alcohol oxidase I (*AOX*1). In fermentations by *P. pastoris*, methanol is used not only as the carbon and energy source, but also as an inducer of the expression of recombinant proteins; and because, at high concentrations it inhibits growth, fed-batch operations have been preferred. Moreover, fed-batch strategy for Mut$^+$ (methanol utilization positive) strain of *P. pastoris* using mixed substrates of glycerol and methanol has been widely used in the past decade to increase productivity, cell density, and reduce the induction time. However, the optimal level of protein expression is not achievable with mixtures of glycerol and methanol, due to a partial repression of the *AOX*1 promoter by glycerol, which may result in lower specific productivities of r-protein. By contrast, sorbitol is a non-repressing carbon source for *AOX*1 promoter, thus sorbitol accumulation during the induction phase does not affect the expression level of r-protein.

In this study, batch-wise sorbitol addition as a co-substrate at the induction phase of methanol fed-batch fermentation by *P. pastoris* (Mut$^+$) was proposed as a beneficial recombinant protein production strategy and the metabolic responses to methanol feeding rate in the presence of sorbitol was systematically investigated. In the presence of sorbitol, 130 mg L^{-1} of recombinant human erythropoietin (rHuEPO) was produced at $t = 24$ hours, compared to 80 mg L^{-1} of rHuEPO ($t = 24$ hours) on methanol alone. Considering many other advantages to be presented, this work demonstrates the ease and efficiency of incorporating sorbitol to the fermentations by the popular methylotrophic yeast *P. pastoris*, for the production of any bio-product.

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2.1.040

Artificial pathway design: from enzyme discovery to designer bugs

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Industrial 'white' biotechnology is regarded as a central feature of the sustainable economic future of modern industrialized societies. Highly effective enzymes, heavily engineered microorganisms and 'designer bugs' promise improvement for existing processes or could enable novel product ideas. For any industrial application, enzymes and biosynthetic pathways need to function sufficiently well according to several application-specific performance parameters. Instead of designing a process to fit a mediocre enzyme, it is conceivable that a comprehensive access to the microbial diversity might be used to find a suitable natural enzyme(s) that optimally fits process requirements.

In view of multi-step bioconversions and the production of specialty- and bulk chemicals from renewable resources current technologies and screening strategies for the development of optimised biocatalysts from microbial biodiversity as well as from 'metagenome' libraries will be presented. To tap into the next generation biocatalysis using engineered 'designer' microorganisms for multi-step bioconversions, it is necessary to move into the construction of artificial operons and the heterologous expression of modified biosynthetic pathways. As a case study the construction of designer microorganisms for the production of 1,2-propanediol will be discussed.

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2.1.041

Preliminary characterization of a beta-glucosidase from a *Pichia anomala* yeast strain of olive brine

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An interesting field of β-glucosidase (βG, EC 3.2.1.21) application in food processing is production of naturally fermented table olives and olive oil. Olives have a bitter compound named oleuropein, which must be eliminated during the olive elaboration process. Nowadays, the most usual industrial debittering method consists of treating the fruits with a NaOH solution. However, these technology presents huge drawbacks due to the highly contaminant discharges generated; moreover, wastewaters originating from table olives processing pose an important environmental threat, as they are characterized by a very high organic load and

high concentration of phenolic compounds, which are toxic to living organisms, together depriving this food of precious biological functions. The alternative strategy is biological debittering that take place during natural fermentation of table olives, by the endogenous and microbial βG which specifically hydrolyses oleuropein to produce highly reactive molecules. This procedure, typical of Greek style table olives, both eliminates wastewater discarding problem and avoids the reduction of such precious sources of phenolic antioxidants as untreated olives are. In fact, many molecules isolated from *O. europaea* fruits and leaves, such as hydroxytyrosol, are thought to have originated from oleuropein, via aglycon, and are known for their free radical scavenging activity. The advantages of Greek-style olive processing are confirmed by the study of Boskou, which demonstrated that table olives available in the Greek market are a good source of phenolic compounds. The present study was designed to characterize βG of a strain of *Pichia anomala* isolated from naturally fermented olives. Identification of such strain, which exhibited the highest enzymatic activity, was carried out by PCR/FRLP of ITS regions. βG of the *P. anomala* strain has been characterized. The enzyme showed an high activity (13 U/g cells), and the established kinetic parameters, V_{max} (14 U mg^{-1}) and K_m (0.09 mM), according to Lineweaver—Burk, were in contrast to the results reported in the literature where βG is always inhibited by its product of hydrolysis. The inhibition by NaCl and physicochemical parameters optimum pH (8.5) and temperature (35°C), with high values of activity 165 and 64 U mg^{-1}, respectively, were also established representing the condition to lead important practical applications of the new enzyme.

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2.1.042

Influence of different origins of replication on transformation efficiency, plasmid copy number and plasmid stability in different industrial lactic acid bacterial strains

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Lactic acid bacteria (LAB) play an important role in industrial feed and food fermentation, where they are used in a variety of food and feed stuff such as meat and dairy products, fermented vegetables and ensiled forage. Because of the economic importance of LAB, there is a growing interest in the development of strains with improved properties and controlled processes. One advantage of LAB is a very efficient secretory pathway in contrast to *Escherichia coli* expression systems. In this work extrachromosomally maintained vectors for expression in different industrial LAB strains were constructed. Our main objective was the testing of influence of different origins of replication (oris) on transformation efficiency, plasmid copy number (PCN) and plasmid stability.

Expression vectors were constructed based on the pUC19 vector containing a pMB1 ori for replication in *Escherichia coli*, an ampicillin resistance gene for selection in *E. coli* and a chloramphenicol resistance gene from pC194 for selection in LAB. Four oris from different plasmids of LAB (pWV01, pWV02, minimal ori from p256) and the plasmid from pE194 from *Bacillus subtilis* were tested in respect to their replication in six different industrial LAB strains. Transformation experiments showed the individual potential of the four oris in the appropriate LAB strain. Plasmids containing the minimal origin from p256 and plasmids containing the ori pWV01 from *Lactococcus lactis*, lacking the ColEI ori from pMB1, showed the highest transformation efficiency.

Transformation experiments revealed that the same plasmids showed very different behavior in different industrial LAB strains. One reason for the strain specificity of some origins of replication may depend on the individual mechanisms of replication. It also could be shown that the presence of the ColE1 ori has a negative influence on the transformation efficiency in LAB. Further investigations regarding to PCN and plasmid stability are currently being performed.

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2.1.043

Identification of a fumonisin B1 degrading gene cluster in *Sphingomonas* spp. MTA144

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Fumonisins are a group of mycotoxins, produced by phytopathogenic filamentous fungi. They frequently contaminate corn and corn-based products and cause, when ingested, several severe diseases in humans and animals. Fumonisin B_1 (FB_1) is the most prevalent fumonisin and hence, holds the highest risk for human and animal nutrition. Most physical and chemical methods to remove FB_1 from contaminated food or feed are not very effective or are difficult to implement into production processes. Some efforts to find a biotechnological solution to this problem have been made. *Sphingomonas* spp. MTA144 was previously shown to decompose FB_1 effectively. In this work the nucleotide sequence of a gene cluster connected with FB_1 degradation in *Sphingomonas* spp. MTA144 was elucidated and functionality of two of the identified genes was tested by FB_1 degradation assays.

A nucleotide sequence of a size of 15,420 bp was elucidated by different PCR-based methods and submitted to the NCBI database. Using appropriate software, 11 ORFs, showing similarity to sequence records of the NCBI database, could be identified. The two most promising genes, encoding a carboxylesterase, type B and an aminotransferase, class III, were expressed heterologously in *P. pastoris* and in *E. coli*, respectively. Functionality of the obtained recombinant enzymes was tested by fumonisin degradation assays and subsequent LC—MS analysis. The carboxylesterase was shown to catalyze effectively the hydrolyzation of FB_1 to hydrolyzed FB_1 (HFB_1). The aminotransferase was demonstrated to eliminate the C2-amino group of HFB_1 in the presence of a cosubstrate.

A gene cluster associated with fumonisin degradation by the bacterium *Sphingomonas* sp. MTA144 was identified and its nucleotide sequence was elucidated. The consecutive action of two enzymes, encoded by this gene cluster, is sufficient for complete detoxification of the mycotoxin FB_1. In contrast to former works,

Pichia anomala strains with potential bioprotective activity

Molecular Genetics of *Pichia anomala* Killer Strains Isolated From Naturally Fermented Olive Brine

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Abstract: Due to its killer toxin production, the biosafety class 1 organism *Pichia anomala* is a useful tool in several respects as it displays a wide spectrum of microbial inhibition comprising not only members of the same species but also other yeasts and even moulds and bacteria. *P. anomala* killer strains are not only of clinical importance for fighting pathogenic yeasts but are successfully applied in the food industry to control harmful yeasts and bacteria. Here we focus on the genetic characterization of three *P. anomala* killer strains (BS91, BCA15 and BCU24) isolated from naturally fermented olive brine. The disruption of genes instrumental in β-glucan synthesis of a toxin sensitive *Saccharomyces cerevisae* strain conferred resistance to the toxins of the above three isolates as for other known *P. anomala* killer toxins. Consentaneously, extracellular exoglucanases, the genes of which (*PaEXG1* and *PaEXG2*) were sequenced from the three strains, display high overall similarities to those of known *P. anomala* killer strains.

Keywords: *Pichia anomala*, killer toxin, exo-β-glucanase

Introduction

Pichia anomala, a rather widespread, ubiquitiously occurring yeast species, is a non-pathogenic, biosafety class 1 organism. Because of positive roles of this specie in several industrial process and its safety is has reached the QPS status (Qualified Presumption of Safety) referred to EFSA standards. Nevertheless, there are sporadically report of *P. anomala* as responsible for cases of fungaemia in a Brazilian paediatric intensive care unit (Pasqualotto et al., 2005).

As for other yeasts several strains of the species secrete killer toxins capable of killing sensitive strains belonging either to the same or another species (for detailed information on yeast killer toxins see a recent review, Meinhardt and Klassen 2009). Killer toxin producers of *P. anomala* have been isolated from clinical and industrial environments (Baeza et al, 2008; Buzzini & Martini 2001; Hernández et al., 2008; Mathews et al. 1998; Rosini 1983) they are supposed to damage the integrity of the vital β-glucan scaffold in target yeast cell walls thereby inducing rapid cell death by osmotic lysis. With the cell wall being the target it is not surprising that killer toxin activity and production is sensitive to temperature, pH changes, NaCl and sugar concentrations (Sawant et al. 1989, Suzuki & Nikkuni 1989). Indeed, most of the killer strains isolated and studied until today have been shown to produce a toxic glycoprotein with β-1,3 or β-1,6 glucanase activity such as strain K which was used as a biocontrol agent on apples; strain K5 (NCYC 434) active against dermatophytes; strain YF07b for controlling pathogenic yeasts in crab; strain WC65, UP25F and ATCC 96603 useful for bio-typing of medically important pathogenic yeasts and fungi (Jijakli and Lepoivre 1998; Izgü et al., 2007; Sawant et al., 1988; Wang et al., 2008). Though its toxic mechanism is still not completely understood, the lethal action is apparently mediated by direct interaction of the above glycoprotein with β-glucan (β-1,3-glucan and β-1,6-glucan), the latter functioning as the specific cell wall receptors.

Only quite recently purification of the rather small (8 kDa) and determination of the amino acid sequence of the *P. anomala* DBVPG 3003 toxin suggested a diverse toxic mechanism as the N-terminal sequence was shown to be identical to ubiquitin of *Saccharomyces cerevisiae* (De Ingeniis et al., 2009).

Since *P. anomala* has rather simple nutritional requirements and is able to colonize dry surfaces for long periods of time it turned out to be a useful biotechnological tool in particular in the food and feed industry. Furthermore besides their capability to rapidly utilize available nutrients strains of *P. anomala* have the capacity to sustain a number of pesticides used in post-harvest treatment. As the suggested modes of action of *P. anomala* killer yeasts are not likely to constitute any hazard for the consumer´s health a main safety aspect for application in the food matrix is successfully addressed.

The growing interest in the development of safer and effective alternative compounds with modest environmental impact, mammalian toxicity and a low tendency to elicit resistance are pointing the attention to *P. anomala* toxin which have shown to have Antifungal, Antizymal, Antibacterial, Antiviral activity as reported from several authors Masih et al (2000); Izgu (2010); Laitila et al (2007); Sawant et al (1988); Polonelli and Morace (1986); Conti et al (2002); Conti et al (2008). Moreover *P. anomala* species is consider a safe producer organism, since this yeast does not contain pyrogens and is not a pathogen.

Here we focus on the characterization and genetic analysis of the killer phenotype of three *P. anomala* strains isolated from naturally fermented olive brine in Sicily (Catania).

MATERIAL AND METHODS

Strains and growth conditions

Strains used in this study are listed in Table 1. Cultivation was performed in complete YPD (1 %, w/v, yeast extract, 2%, w/v, peptone, 2.2 %, w/v, glucose), TSB (Tryptone Soya Broth, Oxoid Ltd., Cambridge, UK) or YNB (Yeast Nitrogen Base, Difco, Detroit, USA) media supplemented with (L-leucine 10 mg ml^{-1}, uracil 200 mg ml^{-1}) when required. Strains were cultivated at 30 °C in liquid or on agar medium. *Escherichia coli* were grown in LB (1 %, w/v, peptone, 0.5 %, w/v, yeast extract, 0.5 %, w/v, NaCl, pH 7.3) at 37 °C (Sambrook et al., 2001) with a working concentration of ampicillin of 100 mg ml^{-1} when required.

Killer toxin production

P. anomala strains BS91, BCA15 and BCU24 were isolated from differently treated olive brine. Olive drupe, cultivar Nocellara dell'Etna, were fermented during the season 2007/08. Strains were coded according to different field treatment against the olive fly, *Bactrocera oleae*. BCA strains were isolated from olive treated with kaolin; BCU strains from Olive treated with copper (poltiglia bordolese) and BS strains from olive with no field treatment (do you have a first paper about the isolation of your three strains? citation). Strains were tested for their killer toxin production against different bacteria, yeasts and clinical isolates (Table 1) with the eclipse assay (Kishida et al., 1996). Yeast strains were spotted on YPD agar buffered at pH of 4, 5, 6, and 7 with citrate-phosphate buffer and incubated at 30°C. Bacteria were tested on TSB agar (Tryptone Soya Agar, Oxoid) at pH 7 and incubated at 32°C. Colony material of the killer strains BCA15, BCU24 and BS90 were transferred to the rim of the strain's drop zone and growth was compared after 1 day of incubation.

Killer toxin assays

Killer toxins were partially purified from supernatants of *P. anomala* BS91, BCA15 and BCU24 by ultrafiltration using Vivaspin 20 ultrafiltration units (Vivascience AG, Hannover, Germany), equipped with 50 kDa cut-off membrane. Toxin-mediated growth inhibition of strains was determined using the microtiter plate assay. 1 µL of an overnight preculture of the yeast sensitive strain was used to inoculate 200 µl YPD, buffered at pH 4.3, containing various amounts of killer toxin; relative toxin concentration factor (RCF) was determined based on the concentration factor obtained by ultrafiltration and growth was determined photometrically at 600 nm following incubation for 16 h at 30°C (Klassen and Meinhardt, 2002).

Generation of mutants

Gene disruption experiments were performed by the PEG/lithiumacetate method (Gietz and Schiest, 1995). Deletion constructs were generated by PCR using the template pUG72 (*URA3*) (Gueldener et al., 2002) and transformed into sensitive *S. cerevisiae* CEN.PK2-1c cells (van Dijken et al., 2000); transformants were selected on YNB medium (Difco, Detroit, USA) supplemented with L-leucine (10 mg ml^{-1}). Deletions were verified by PCR using primers located outside of target genes (KRE1 and KRE6 outF and outR primers as well as marker specific ones (URAup and URAdown; see Table 2 for sequences).

*kre*1 and *kre*6 mutants were analysed for killer resistance with the eclipse assay as described above, using YPD agar buffered at pH of 4.3.

Cloning and Sequencing

PaEXG1 and *PaEXG2* genes were isolated, cloned, and sequenced by making use of the corresponding *P. anomala* strain K sequences for the primer design (Accession number AJ002195 and AJ222862). Primers deduced and applied for amplification and sequencing are listed in Table 2. Molecular cloning procedures were carried out essentially as described in Sambrook and Russell (2001).

E. coli strain DH5αF' was used as the cloning host. Bacterial cultures were maintained in LB media. Incubation was done overnight at 37 °C and 180 rpm in a shake incubator (innova42, New Brunswick Scientific, New Jersey, USA).

Plasmid pUCBM20 were used as cloning vectors for *PaEXG1* and *PaEXG2*, respectively. Both genes were isolated by PCR using primers PaEXG1-NdeI-for/-PstI-rev or PaEXG2-NdeI-for/-PstI-rev, respectively (Table 2). Inserts and plasmids were cut with *Pst*I and *Nde*I. Plasmid carrying *E.*

coli strains were selected on LB agar supplemented with ampicillin/IPTG/X-Gal (100 mg ml^{-1}/ 0,5 Mm/ 40 mg ml^{-1} respectively). Recombinant plasmid-carrying colonies were identified visually (white color).

Plasmids were routinely isolated using the "peqGOLD Plasmid Miniprep Kit II" by peqLab Biotechnologie GmbH (Erlangen, Germany). The correct integration of *PaEXG1* or *PaEXG2* into pUCBM20 was verified by restriction analysis with *Pst*I and *Nde*I in single and double digestions.

Southern analysis

For Southern analysis, 8 µg of chromosomal DNA was cut with different restriction enzymes, *Aat*I, *Acc*I, *Eag*I, *Fsp*I, *Hpa*I, *Spe*I for analysis of *PaEXG1* and *Bam*HI, *Eco*RV, *Hinc*II, *Kpn*I, *Pst*I, *Sac*I for *PaEXG2*. Hybridizations were carried out with digoxygenin (DIG) labeled probes at 68 °C (Southern, 1975). Signals were detected on X-ray films using the chemiluminescent substrate CDP-Star (Roche Diagnostics GmbH, Mannheim, Germany). Primers PaEXG1 f1-r1 and PaEXG2 f1-r1 (Table 2) were used for generating the probes; *PaEXG*1 *and PaEXG*2 probes encompassed almost the entire coding regions, i.e. nucleotides 1 to 1497 of 1497 (*PaEXG1*) and 42 to 1275 of 1283 (*PaEXG2*), respectively.

RESULTS

Killer toxin production and assay

Among 118 yeast isolates from olive brine, 26 strains of *P.anomala* were tested for their antagonistic property. *P. anomala* strains BS91, BCA15 and BCU24 were selected for the more intense antagonistic activity. The three strain displayed higher killing activities at pH 4.3 which is - by the way - frequently observed for strains isolated from fermented food due to the adaption to low pH values. Such activities were seen in a temperature range from 22°C to 30°C antimicrobial action included Gram positive (*Staphylococcus aureus* and *Staphylococcus hominis*) and Gram negative bacteria (*Enterobacter aerogenes*). With respect to yeast clinical isolates no killer activity was seen against *Candida albicans* and *Cryptococcus spp.*, however, all of the three strains were active against the clinical *S. cerevisiae* and the *S. cerevisiae* CEN.PK2-1c reference strain (see Table 1) (clinical isolated were provided from Dr A. Sciacca, Laboratory of microbiology of Azienda Poliniclinico Vitt. Em., Catania). Partially purified toxins (see Material and methods) of all of the three strains were tested against *S. cerevisiae* CEN.PK2-1 by making use of the microtiter

assay revealing the highest inhibition activity with a RCF of 50 (Fig.1) with only 20% relative growth left.

The target of the killer toxins is ß-glucan

In order to check the primary target site of the toxins and for possibly proving that the cell wall is the prime target we constructed two mutants carrying disrupted, *kre1* and *kre6* genes. Kre1, acting as the plasma membrane receptor for the *S. cerevisiae* K1 viral toxin (Killer toxin REsistant), is involved in cell wall 1,6-β-glucan assembly presumably by adding linear side chains of 1,6-linked glucose units to the a highly branched 1,6- and 1,3-linked glucan backbone. In a *kre1* null mutant, the total amount of cell wall 1,6- β -glucan is approximately 40% lower than in the wild-type and the resulting glucan polymer shows a lower degree of polymerization and possesses significantly fewer β 1,3-glycosidic branches (Boone et al., 1990).

The Kre6 protein is crucial for β-1,6 glucan biosynthesis, it is considered to be a β-glucan synthase; (Lesage and Bussey, 2006). The disruption cassettes *Δkre1::KlURA3* and *Δkre6::KlURA3* (Fig. 2 A) were constructed and transformed into *S. cerevisiae* CEN.PK2-1c; the correct integration was finally proven by PCR. (Fig. 2 B).

Eclipse assays were performed to test the killer sensitivity of the mutants against the three *P.anomala* killer strains: BS91, BCA15, BCU24. Inhibition halos visible with the wild type of the sensitive *S.cerevisiae* CEN.PK2-1c were not formed with the mutants *Δkre1* and *Δkre6* (Fig. 3).Other *P.anomala* reference killer strains, ATCC 96603 and CBS 5759 (NCYC 432) reported to have an high β 1,3-glucanase activity(Izgü et al, 2006; Magliani et al, 2008), and kindly provided by Prof L. Polonelli (Universita' degli Studi di Parma) likewise failed to inhibit the *kre1* and *kre6* deletion mutants in eclipse assays (not shown).

Isolated killer strains encode exoglucanases highly similar to other *P. anomala* killers

Sequencing alignment of the three sequences of the *PaEXG1* gene showed an high similarity among the thee strain with the one of the Strain K. Strain BS91 and BCU24 have the same sequences; BCA15 showed the same sequence, 100% with strain K. The protein aligment showed a 99% of matches between Strain K and BS91, BCU24. *PaEXG1* of Strain K and BS91, BCA15 and BCU24 code for a protein of with molecular weight of 58 KDa; all the four translated proteins have the identical Isoelectric Point (pI) of 4.6.

PaEXG2 sequences of strains BS91, BCA15 and BCU24 showed an homology of 99 and 100% with strain K and YF07b (AC number EF029071).

The translated products of strains BS91, BCA15 and BCU24 are proteins with a 49 KDa molecular mass and a pI of 4.5 showing similarity with already sequenced exo-β-1,3-glucanase of *P.anomala* strains NCYC432, NCYC434 and YF07b.

Southern blot

Southern blot analysis with genomic DNA revealed that there was a single copy of the *PaEXG1* and *PaEXG2* genes in the genome. The three strains, BS91, BCA15 and BCU24 , showed the same band profile indicating an high homology of the sequences of the genes and chromosomal similarity (Fig. 4).

Strain BCA15 gave two hybridization bands with *EcoR*V as restriction enzyme in the case of *PaEXG2* probe. This is due to the diploidy of the strain assuming that the two fragments hybridizing with the *PaEXG2* probe correspond at two allels of each gene.

Discussion

The purpose of this study was to provide deeper insight into the mechanism of *P. anomala* BS91, BCA15 and BCU24 killer toxins. The mechanism of action of these toxins is assumed to be due to a exo β-1,3 glucanase, similar to strain K toxin.

Testing *S. cerevisiae* sensitive strain deletants in two cell wall genes enabled us to show that the mode of action of the three toxins arise at the cell wall level. The resistance of the two different mutants, *kre*1 and *kre*6, led us assume that the enzymatic activity of the toxin is not only β-1,3 glucanase but also β-1,6 as confirmed from resistance of *kre*6 mutants, lacking on β-1,6 glycosidic bonds on cell wall.

Deletant mutants strategy is often used as method for the identification of yeast killer toxin target and pathway through the cell wall of sensitive strain as reported for *S. cerevisiae* killer toxin HM-1 (Miyamoto et al., 2010).

The antagonistic activity was effective against different pathogens as Gram positive bacteria (*Staphylococcus aureus*), Gram negative (*Enterobacter aerogenes*) and yeasts (*S.cerevisiae*) which are reported to have an low laminarase resistance due to cell wall composition. Several studies confirmed a broad range of activity of P. anomala toxin, Guyard et al. (1999) identified a 85 kDa toxic glycoprotein of *P. anomala* ATCC 96603 (PaKT) that is used as internal image for the production of Abs (antibodies with antibiotic activity) active against *Candida* spp, *Cryptococcus* spp, *Aspergillus*, HIV-1 and Influenza A virus (Polonelli et al., 2010). NYNC 434 killer toxin, a 49KDa glycoprotein ,(Izgu et al., 2010) reported a 100% growth inhibition of *Penicillum italicum*

and *P. digitatum* on citrus fruit due to killer activity of strain. The same strain previously tested against several *Candida* spp (dermatophytes) (Izgu 2007).

Comparison of sequences and translated products showed an high similarity among *P. anomala* killer strains (strain K, YF07b, NCYC 432 and NCYC 434), in particular considering the *PaEXG2*.

The deduced amino acid sequence of our three strains from *PaEXG2* shared a 100% or 99% homology with the N-terminal sequence of strains NCYC432, NCYC434 and YF07b toxins, starting after the predicted KEX2-type endoproteinase processing site of the exo-β-1,3-glucanase precursor of *P. anomala* strain K (Fig. 5) (Izgü and Altinbay, 2004; Izgü et al, 2006; Wang et al, 2007). The high degree of homology to *PaEXG2* of *P. anomala* strains K and YF07b is in accordance with the observed resistance of the *S. cerevisiae kre1* and *kre6* mutants.

The molecular weights of *P. anomala* killer toxins vary from 49 kDa to 85 kDa, however, their mode of action (glucanase activity) appears to be similar among the different toxins studied.

In line with the assumption that β-glucan represent the primary binding/target sites are reports concerning the toxins of *P. anomala* NYNC 432 and NYNC 434 (Izgü and Altinbay 2004, Izgü et al., 2006) and the determination of the internal amino acid sequence of a glycoprotein that shaped up as being identical with the corresponding sequence of the strain K toxin (Grevesse et al., 2003).

Studies on the Strain K activity demonstrate that the killer activity is influenced from different factors and the *in vivo* antagonist mechanism are due to both *PaEXG1* and *PaEXG2* gene as confirmed from deletants mutants tested on apple surfaces against *Botrytis cinerea* (Friel et al, 2007). Anyway the complete mechanism of action is not yet known and other genes are possibly involved in the antagonist pathway.

The comparison of the *Pst*I genomic DNA, probed with PaEXG2, of the three strain with the one on the strain K, KH6 and KU1(descendant of Strain K and uracil auxotrophic derivate of KH6) showed the same band at about 4.5 kb, suggesting an high conservation among this species also if the probe used has different length (Jijakli and Lepoivre 1998).

This work highlighted different tools for *P.anomala* killer toxin identification and characterization. Further studies are needed to deeper understand the mechanism and investigate genes involved *in vitro* and *in vivo*, considering that the application on large scale food processes of killer yeast have the consumer safety and satisfaction as first aim.

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Table 1. Bacteria and yeast tests strains used in this study. Strains *P. anomala* BS91, BCA15 and BCU24 were tested for their killing activities against different bacteria, yeasts and clinical isolates with the eclipse assay.

TEST STRAINS	**REFERENCES**	**BS91**	**BCA15**	**BCU24**
Bacillus megaterium DSM319	Vary et al. (2007)	-	-	-
Bacillus licheniformis MW3	Waschkau et al. (2008)	-	-	-
S. cerevisiae CEN.PK2-1c	van Dijken et al. (2000)	+	+	+
S. cerevisiae BY4741	Mehlgarten and Schaffrath (2004)	-	-	-
S. cerevisiae GS1731	Hayman and Bolen (1991)	-	-	-
Kluyveromyces lactis AWJ137	Kämper et al. (1991)	-	-	-
Pichia acaciae NRRL Y-18665	Worsham and Bolen (1990)	-	-	-
CLINICAL ISOLATES	**SOURCE***			
Candida albicans	Vagina, Skin, Sputum, Surgical wound	-	-	-
Saccharomyces cerevisiae	Vagina	+	+	+
Cryptococcus spp	Vagina, Sputum	-	-	-
Enterococcus faecalis	Wound	-	-	-
Escherichia coli	Peritoneum, Wound, Urine, Faeces	-	-	-
Pseudomonas aeruginosa	Neonatal pharyngeal tampon	-	-	-
Enterobacter faecium	Neonatal gastric aspirate	-	-	-
Enterobacter aerogenes	Gastrointestinal, Exudate	+	+	+
Enterobacter aerogenes	Surgical wound exudate	-	-	-
Enterobacter aerogenes	Neonatal pharyngeal tampon	+	+	+
Staphylococcus aureus	Blood, Peritoneal drainage	+	+	+
Staphylococcus aureus	Ulcer	+	+	+
Staphylococcus hominis	Skin	+	+	+
Staphylococcus epidermidis	Skin	-	-	-

*Strains were identified with vitek 2 (bioMerieux), in the Laboratory of Microbiology of Azienda Poliniclinico Vitt. Em, Catania.

Table 2. Oligonucleotides used in this study.

Oligonucleotides	5′ → 3′ sequence
PaEXG1 for 1	ATGTTGTTCAATATCCTGATCCTTTC
PaEXG1 rev 1	TTTCCAGAGGCAGCACTTTC
PaEXG1 for2	GCTTTGGAATGGGCCAAAC
PaEXG1 rev2	ATAGCACCTGACCACTCACCAAC
PaEXG1 for3	TGATGAATCACAATTGGTTAG
PaEXG1 rev3	CACCACCATATTTACCAAAG
PaEXG1 NdeI for	GCCATATGATGTTGTTCAATATCC
PaEXG1 PstI rev	GCCGCTGCAGTCTAAAGTAATGTTGATAATG
PaEXG2 for1	AGCACTAGCAAACCCTATCC
PaEXG2 rev1	TTGACCAGGATATTGTCTTG
PaEXG2 for2	TCAAGGCCAAGATGTTCCTG
PaEXG2 rev2	ACGAGAACCACGACCAACAC
PaEXG2 for3	TTACATGGTGCTCCAGGTTC
PaEXG2 rev3	CATTGCAGCTGACCATTCAC
PaEXG2 for4	TATTTCCACTTGGGATGATG
PaEXG2 rev4	AACCACCCACCTAAATTAAC
PaEXG2 for5	CCAAACTTGGGACTCTTTC
PaEXG2 rev5	CATCATCCCAAGTGGAAATATC
PaEXG2 NdeI for	GCTACATATGCTTATTTCAACTTTTATC
PaEXG2 PstI rev	GGACCAGTTACACTAATTTGACGTCGGTC
kre1ko for	AAATGATGCGTCGCACGCTATTACATTCATTCGCTACGCTGCTACCAGCTG AAGCTTCGTACGC
kre1ko rev	AACCGTTAATAGCCCACCCAAAATTGAAGTAAACGAACTCATACCGCATAG GCCACTAGTGGATCTG
KRE1outF	CAGCGTGTATGGTGATATCG
KRE1outR	CGGCGGTAGAAAGATTGCTG
kre6ko for	CTAATGCCTTTGAGAAATCTAACTGAAACGCACAACTTTAGTAGCCAGCTGA AGCTTCGTACGC
kre6ko rev	TAAGAGGATAACTTGAACTTCGAGCTAGTACATTTACCAGTTAGGGCATAG GCCACTAGTGGATCTG
KRE6outF	TTCGTCCTCTCGTACTAGTG
KRE6outR	TTAAAGCCGAGGGCTTGTAG
URAup	GACGCTGGCGTACTGGC
URAdown	GCCAGTACGCCAGCGTC

Figure 1. Microtitre plate assay to monitor the response of *S. cerevisiae* CEN.PK2-1c sensitive strains to BS91(♦), BCA15 (■) and BCU24 (▲) partially purified toxin. Relative growth was determined photometrically at 600 nm and refers to the OD_{600nm} value in toxin-free medium. Relative concentration factor (RCF) was calculated relating to the toxin concentration of the supernatants.

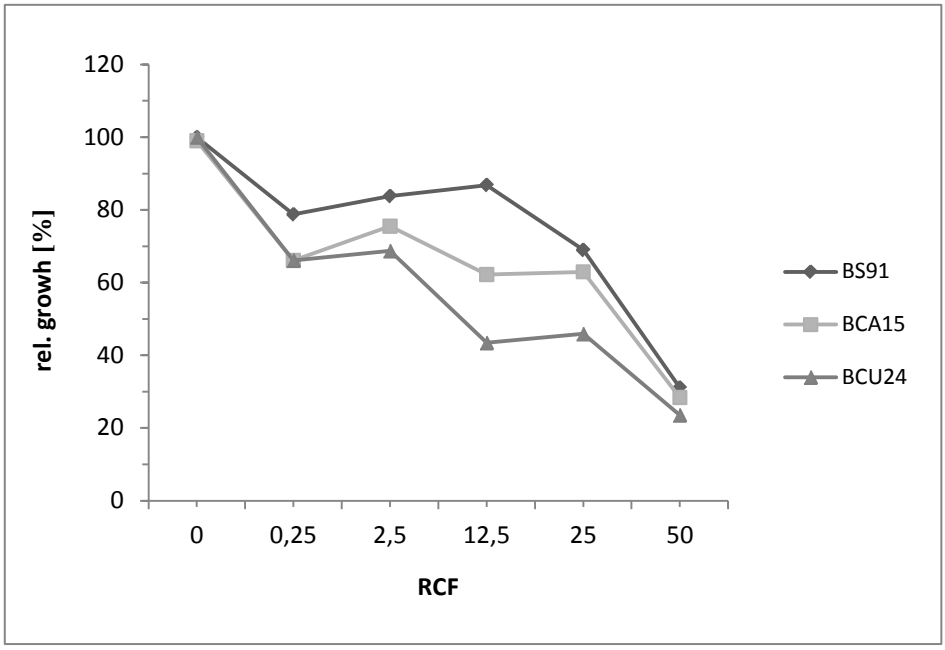

Figure 2. Genotypic analysis of *S. cerevisiae* CEN.PK2-1c *kre1* and *kre6* mutants. (left) The genetic organization at the *KRE1* and *KRE6* loci before and after integration of a disruption cassette harbouring the *Kluyveromyces lactis URA3* gene is represented. (right) To verify the correct integration at the corresponding loci, PCR was carried out on transformants (*kre1* and *kre6*) and wild type cells using combinations of primers complementary to sequences within the disruption cassette (URAup/URAdown) and to sequences flanking the target genes (KRE1outF/outR or KRE6outF/outR). The products obtained were subjected to agarose gel electrophoresis. The expected length of the products are indicated on the left. M: GeneRuler™ 1kb DNA ladder (Fermentas, St. Leon-Rot, Germany).

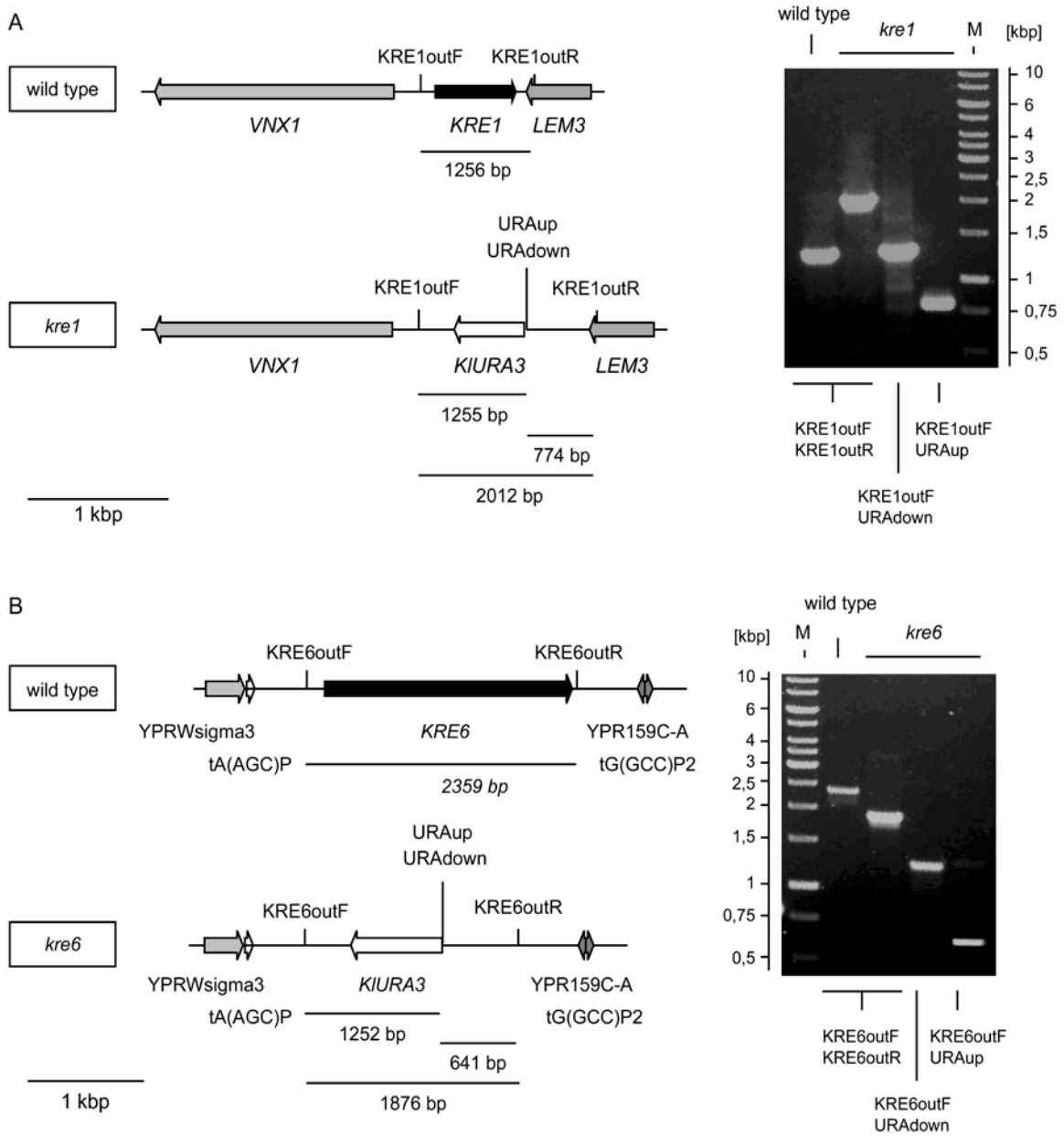

Figure 3. Eclipse assay of *P. anomala* BS91, BCA15 and BCU24 killer strains on *S.cerevisiae* sensitive wild type CEN.PK2-1c, *kre1* and *kre6* mutants.

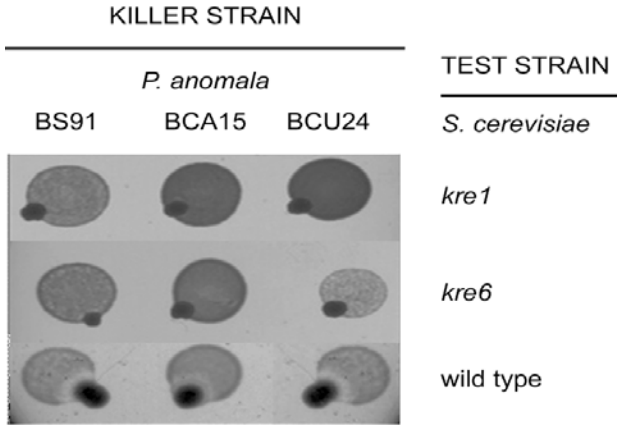

Figure 4. Southern analysis of total genomic DNA from strain BS91 cutted with different restriction enzymes and hybridized with *PaEXG1* (A) and *PaEXG2* (B) probes. (A) M: Gene Ruler^{TM} 1kb DNA ladder (Fermentas, St. Leon-Rot, Germany)

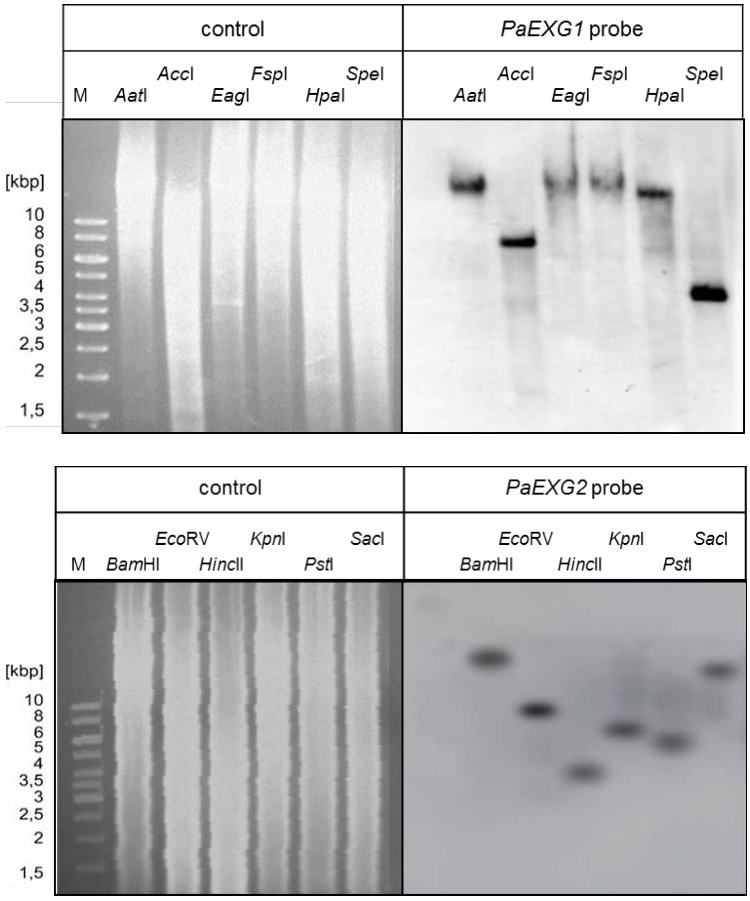

Figure 5. CulstalW alignment of *PaEXG2* from BS91, BCA15, BCU24, YFO7b and strain K translated protein with already known purified killer toxin from *P.anomala* (NYCY432).

```
BS91            MLISTFIISSLLSIALANPIPSRGGTQFYKRGDYWDYQNDKIRGVNLGGWFVLEPFITPS 60
BCA15           MLISTFIISSLLSIALANPIPSRGGTQFYKRGDYWDYQNDKIRGVNLGGWFVLEPFITPS 60
BCU24           MLISTFIISSLLSIALANPIPSRGGTQFYKRGDYWDYQNDKIRGVNLGGWFVLEPFITPS 60
YF07b           MLISTFIISSLLSIALANPIPSRGGTQFYKRGDYWDYQNDKIRGVNLGGWFVLEPFITPS 60
StrainK         MLISTFIISSLLSIALANPIPSRGGTQFYKRGDYWDYQNDKIRGVNLGGWFVLEPFITPS 60
NYCY432         ------------------------------------------------------------

BS91            LFEAFENQGQDVPVDEYHYTKALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPI 120
BCA15           LFEAFENQGQDVPVDEYHYTKALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPI 120
BCU24           LFEAFENQGQDVPVDEYHYTKALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPI 120
YF07b           LFEAFENQGQDVPVDEYHYTKALGKDLAIERLDQHWSSWIVEADFQSIAGAGLNFVRIPI 120
StrainK         LFEAFENQGQDVPVDEYHYTKALGKDLAKERLDQHWSSWIVEADFQSIAGAGLNFVRIPI 120
NYCY432         ------------------------------------------------------------IPI 3
                                                                             ***

BS91            GYWAFQLLDNDPYVQGQESYLDQALEWAKKYDIKVWIDLHGAPGSQNGFDNSGLRDSYEF 180
BCA15           GYWAFQLLDNDPYVQGQESYLDQALEWAKKYDIKVWIDLHGAPGSQNGFDNSGLRDSYEF 180
BCU24           GYWAFQLLDNDPYVQGQESYLDQALEWAKKYDIKVWIDLHGAPGSQNGFDNSGLRDSYEF 180
YF07b           GYWAFQLLDNDPYVQGQESYLDQALEWAKKYDIKVWIDLHGAPGSQNGFDNSGLRDSYEF 180
StrainK         GYWAFQLLDNDPYVQGQESYLDQALEWAKKYDIKVWIDLHGAPGSQNGFDNSGLRDSYEF 180
NYCY432         GYWAFQLLDNDPY---------------------------------------------- 15
                ************

BS91            QNGDNTQVALDVLQYISKKYGGSDYGDVVIGIELLNEPLGSVLDMGKLNDFWQQGYHNLR 240
BCA15           QNGDNTQVALDVLQYIS-KYGGSDYGDVVIGIELLNEPLGSVLDMGKLNDFWQQGYHNLR 239
BCU24           QNGDNTQVALDVLQYIS-KYGGSDYGDVVIGIELLNEPLGSVLDMGKLNDFWQQGYHNLR 239
YF07b           QNGDNTQVALDVLQYISNKYGGSDYGDVVIGIELLNEPLGSVLDMGKLNDFWQQGYHNLR 240
StrainK         QNGDNTQVALDVLQYISNKYGGSDYGDVVIGIELLNEPLGSVLDMGKLNDFWQQGYHNLR 240
NYCY432         -------------------YGGSDYGDVVIGIELL------------LNDFWQQGYHNL- 43
                                    ****************            ************

BS91            NTGSSQNVIIHDAFQTWDYFNDKFHTPDYWNVVIDHHHYQVFSPGELSRSVDEHVKVACE 300
BCA15           NTGSSQNVIIHDAFQTWDYFNDKFHTPDYWNVVIDHHHYQVFSPGELSRSVDEHVKVACE 299
BCU24           NTGSSQNVIIHDAFQTWDYFNDKFHTPDYWNVVIDHHHYQVFSPGELSRSVDEHVKVACE 299
YF07b           NTGSSQNVIIHDAFQTWDYFNDKFHTPDYWNVVIDHHHYQVFSPGELSRSVDEHVKVACE 300
StrainK         NTGSSQNVIIHDAFQTWDSFNDKFHTPDYWNVVIDHHHYQVFSPGELSRSVDEHVKVACE 300
NYCY432         ------------------------------------------------------------

BS91            WGANSTKENHWNLCGEWSAAMTDCTKWLNGVGRGSRYDQTFDYDPSQNQNYIGSCQGSQD 360
BCA15           WGANSTKENHWNLCGEWSAAMTDCTKWLNGVGRGSRYDQTFDYDPSQNQNYIGSCQGSQD 359
BCU24           WGANSTKENHWNLCGEWSAAMTDCTKWLNGVGRGSRYDQTFDYDPSQNQNYIGSCQGSQD 359
YF07b           WGANSTKENHWNLCGEWSAAMTDCTKWLNGVGRGSRYDQTFDYDPSQNQNYIGSCQGSQD 360
StrainK         WGANSTKENHWNLCGEWSAAMTDCTKWLNGVGRGSRYDQTFDYDPSQNQNYIGSCQGSQD 360
NYCY432         ------------------------------------------------------------

BS91            ISTWDDNKKSNYRRYIEAQLDAFEKRSGWIFWTWKTETTLEWDFQKLSYYGIFPSPLNSR 420
BCA15           ISTWDD-KKSNYRRYIEAQLDAFEKRSGWIFWTWKTETTLEWDFQKLSYYGIFPSPLNSR 418
BCU24           ISTWDDDKKSNYRRYIEAQLDAFEKRSGWIFWTWKTETTLEWDFQKLSYYGIFPSPLNSR 419
YF07b           ISTWDDDKKSNYRRYIEAQLDAFEKRSGWIFWTWKTETTLEWDFQKLSYYGIFPSPLNSR 420
StrainK         ISTWDDDKKSNYRRYIEAQLDAFEKRSGWIFWTWKTETTLEWDFQKLSYYGIFPSPLTSR 420
NYCY432         ------------------------------------------------------------

BS91            QYPGQCD 427
BCA15           QYPGQCD 425
BCU24           QYPGQCD 426
YF07b           QYPGQCD 427
StrainK         QYPGQCD 427
NYCY432         -------
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BOOK OF ABSTRACTS

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Book of Abstracts III International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009)
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Killer toxin of *Pichia anomala* strains isolated from olive brine and active against human pathogens

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Killer activity has been shown in many *P. anomala* isolates; the killer system described in this species shows toxic activity against a wide variety of nonrelated microorganisms, such as hyphomycetes and bacteria, including important opportunist pathogens, such as *Candida albicans*. Because the killer phenotype could not be cured by the application of cycloheximide, high temperature, ethidium bromide or acridine orange, it was suggested that the killer genes are chromosomally located.

In this study, 22 *Pichia anomala* strains, isolated from naturally fermented olive brine, showing high killer capacities against sensitive strain (strain 5×47) of *Saccharomyces cerevisiae*, were characterized according to the interactions among the isolates and prokaryotic and eukaryotic human pathogens (*Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli , Enterobacter aerogenes, Enterococcus faecalis*) and examined for detection of genetic elements, which code for the killer character (dsRNA, ds linear DNA or a chromosome).

Killing ability and resistance among *P. anomala* isolates and pathogenic *C. albicans* and *S. cerevisiae* were determined on YEPD-MB agar (1.0% yeast extract, 2.0% peptone, 2.0% glucose, 0.1M citrate-phosphate buffer pH 4.5, 0.01% methylene blue and 2.0% agar). The strains with killer activity appeared surrounded by a clear zone, surrounded by a blue precipitated halo indicative of cellular death.. Killing assay against bacterial clinical isolates was carried out in Trypticase Soy Agar; in this case antimicrobial activity was recorded as growth free inhibition zones.

Nucleic acids were extracted from *P. anomala* isolates; RNA/DNA samples were treated with Nuclease SI, DNaseI, RNaseH and RNAseA buffered at different ionic strength.

The killer yeast strains of *P. anomala* showed considerable activity against bacterial pathogens, both gram positive and gram negative. In fact, 12 strains of *P. anomala* have shown antimicrobial activity against *Staph. aureus* and *Staph.hominis* and 14 strains were active against *Enterobacter aerogenes*. These result confirmed previous studies, which suggested a widespread antimicrobial activity of yeast killer toxin against gram positive but also demonstrate action against gram negative bacteria, probably due to the cell wall composition and permeability among different species.

Regarding yeast clinical isolates, no killer activity was detected against *Candida albicans* and *Cryptococcus* sp., while it was effective against *S. cerevisiae*.

At a molecular level, all K+ isolates of *P. anomala* do not display any EGEs, suggesting that the phenotype is encoded in the genome of the cells.

Keywords *Pichia anomala*, killer toxin; clinical yeast and bacterial isolates; killer phenotype encoding

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Molecular Genetics of *Pichia anomala* Killer Strains Isolated From Naturally Fermented Olive Brine

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Introduction: Due to its killer toxin production, *Pichia anomala* is a useful tool in the food industry with a wide spectrum of microbial inhibition comprising not only members of the same species but also other yeasts and even mould and bacteria. Hence, *P. anomala* killer strains are being applied for the biocontrol of pre and postharvest infection by mould and yeast in grape and apple, and for controlling pathogens in caught crab (1,2).
Here we focus on the genetic characterization of three *P. anomala* killer strains (BS 91, BCA 15 and BCU 24) isolated from naturally fermented olive brine.

Methods: Strains were tested against different bacteria, yeast killer sensitive reference strains and killer sensitive *S. cerevisiae* CENPK.1c mutants (*kre1* and *kre6*), deficient in β-glucanase assembly. Cloning, sequencing and RFLP analysis of PaEXG2 gene, β-glucanase encoding, were performed.

Results: Mutants of the *S. cerevisiae* reference strain CENPK.1c (*kre1* and *kre6*) turned out to be resistant against the three *P. anomala* strains. The toxin was genetically identified as the β-glucanase as for other *P. anomala* strains (3). Hence, the corresponding genetic loci of the three strains were cloned, sequenced and compared to the known *PaEXG2*. The high degree of homology to *PaEXG2* of *P. anomala* strains K and YF07b is in accordance with the observed resistance of the *S. cerevisiae kre1* and *kre6* mutants.
As there is only one band in Southern analysis with a *PaEXG2* probe, there is presumably only one copy of the gene in all of the three strains.

Discussion: The identification and molecular characterization of the β-glucanase as the toxic principle of the *P. anomala* strains from olive brine favours the use of such microbes for controlling the fermentation process and to minimize possible and undesired effects of spoilage yeast or other potentially pathogenic microrganisms.

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Valutazione della sopravvivenza di *Lactobacillus rhamnosus* in confetture di pesche

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Nel presente lavoro 10 ceppi di *Lactobacillus rhamnosus* isolati da formaggio pecorino tradizionale sono stati studiati per le caratteristiche probiotiche e la loro sopravvivenza è stata valutata *in vivo* in confetture di pesche biologiche di Leonforte.

I ceppi sono stati sottoposti ad uno *screening* preliminare per valutarne la sopravvivenza *in vitro* a bassi valori di pH ed in presenza di sali biliari e per la capacità di produrre esopolisaccaridi. I risultati ottenuti hanno evidenziato la resistenza di tutti i ceppi in terreno MRS sia acidificato a pH 2.5 sia in presenza di sali biliari fino al 2%. Sette ceppi hanno, inoltre, prodotto esopolisaccaridi. I risultati ottenuti *in vivo* hanno mostrato che tutti i ceppi sopravvivono e/o sviluppano nelle confetture fino ad oltre 2 mesi dall'inoculo. Alla luce dei risultati ottenuti è possibile proporre l'impiego di tali ceppi per l'ottenimento di confetture di frutta probiotiche.

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FABIO LICCIARDELLO, SERENA MUCCILLI, IOLE PITINO, GIUSEPPE MURATORE, CINZIA L. RANDAZZO, CRISTINA RESTUCCIA, CINZIA CAGGIA

EFFETTO DI *LACTOBACILLUS RHAMNOSUS* SULLE CARATTERISTICHE CHIMICO-FISICHE DI CONFETTURA PROBIOTICA DI PESCHE

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INTRODUZIONE

I probiotici sono una preparazione alimentare contenente cellule microbiche vive e vitali che, se somministrati in quantità elevata, sono in grado di esercitare un effetto benefico sulla salute dell'uomo (FAO/WHO, 2002). Dal momento che le caratteristiche probiotiche sono considerate ceppo-specifiche numerosi ceppi sono continuamente proposti come probiotici, soprattutto tra i lattobacilli di origine casearia (Mattia e Merker, 2008). Affinché un microrganismo possa essere impiegato nella preparazione di alimenti esso deve soddisfare i requisiti di sicurezza, di funzionalità e tecnologici (Saarela *et al*., 2000). I requisiti tecnologici riguardano la capacità di sviluppare e di mantenersi ad elevate concentrazioni nel prodotto fino al momento del consumo e la capacità di non alterarne le caratteristiche fisico-chimiche e sensoriali. In Europa i probiotici sono commercializzati generalmente sottoforma di latte fermentato e yogurt, tuttavia la presenza di lattosio e di colesterolo ne limitano il consumo e recentemente nuovi alimenti probiotici a base di succhi di frutta, cereali, dessert e cioccolato sono apparsi sul mercato (Mattila-Sandholm *et al*., 2002; Saxelin, 2005). Ad oggi tuttavia nessuna informazione esiste circa confetture probiotiche che, abbinando l'immagine salutistica della frutta con i vantaggi dei probiotici, potrebbero estendere il consumo di probiotici ad adolescenti e a consumatori che seguono particolari regimi dietetici.
Nel presente lavoro confetture pesche (*Prunus persica* L. Batsch) sono state inoculate con 6 ceppi probiotici di *Lb. rhamnosus* allo scopo di valutarne la sopravvivenza, fino a 78 giorni di conservazione a 7° e 25°C.

MATERIALI E METODI

Confettura di pesca: è stata utilizzata confettura prodotta da un'azienda locale che trasforma "Pesche tardive di Leonforte" (*Prunus persica* L. Batsch) un'Indicazione Geografica Protetta (IGP) (Regolamento CE n. 510/06), proveniente da agricoltura biologica.
Inoculo dei ceppi: i ceppi di *Lb. rhamnosus* (Tab. 1) impiegati sono stati precedentemente isolati da formaggio tradizionale e caratterizzati per le attitudini probiotiche (Pitino *et al*., 2009). Colture fresche sono state centrifugate e sospese in soluzione fisiologica (0,9% w/v NaCl) fino ad ottenere una concentrazione finale di 10^8 UFC/g di confettura di pesche. I campioni sono stati conservati a 7° e 25 °C.

Analisi microbiologiche: i campioni ottenuti sono stati analizzati per seguire la sopravvivenza dei ceppi a 0, 15, 30 45 e 78 giorni di conservazione. La conta della carica mesofila aerobia totale e dei lattobacilli mesofili è stata condotta su 10 g di campione.
Analisi chimico-fisiche: il pH, l'imbrunimento, i parametri CIE Lab del colore sono stati determinati. L'imbrunimento è stato valutato per via spettrofotometrica (Abs a 420 nm) su 3 g di campione diluiti in 7 mL di acqua distillata e addizionati di 0,5 g di bentonite, centrifugati e filtrati.
I parametri del colore L*, a* e b* sono stati misurati con un colorimetro portatile (Nippon Denshoku Ind. Co. Ltd, Giappone) su circa 10 g di campione posti in piastre di vetro.

RISULTATI E DISCUSSIONI

Perché un microrganismo possa essere considerato probiotico è fondamentale che rimanga vitale nell'alimento fino al momento del consumo (Salminen *et al*., 2006) ad una concentrazione compresa tra 10^6 e 10^{12} UFC/g (Oliveira *et al*., 2002).
Nel presente lavoro tutti i campioni di confettura hanno evidenziato, durante il periodo di conservazione, carica mesofila aerobia totale nulla (dati non mostrati) così come nulla è stata la conta dei lattobacilli mesofili nei campioni non inoculati (controllo). I campioni inoculati hanno evidenziato conte in MRS variabili in funzione della temperatura di conservazione (Tab. 1).

Tabella 1 - Sviluppo dei batteri lattici durante la conservazione in confetture di pesche inoculate.

	Giorni	Conta microbica (espresso come log di UFC/g) e deviazione standard (SD)					
		R61	H25	H12	N24	D44	D13
25°C	0	7.50±0.3	8.39±0.5	7.69±0.0	7.47±1.1	8.77±0.0	8.35±0.6
	15	8.97±0.2	10.37±0.0	12.14±0.9	11.60±0.7	15.43±0.0	13.11±1.2
	30	7.95±0.9	14.22±1.4	9.07±0.5	8.14±0.9	9.51±1.8	9.34±0.3
	45	9.44±1.4	10.69±0.9	2.00±0.0	6.44±0.0	7.94±0.6	6.81±0.4
	78	6.32±0.1	4.92±0.9	2.30±0.0	5.14±0.3	5.56±0.9	1.00±0.0
7°C	0	7.50±0.2	8.39±0.4	7.69±0.4	7.47±0.9	8.77±0.0	8.35±0.3
	15	9.73±0.2	8.00±0.8	11.34±0.8	9.54±0.4	9.96±0.9	10.63±0.4
	30	7.88±0.1	12.12±1.1	10.27±0.9	7.83±0.5	11.02±1.8	11.35±0.0
	45	9.78±0.3	9.44±0.0	8.83±0.6	8.18±0.0	8.27±0.5	10.44±0.0
	78	7.75±0.7	7.80±0.2	7.81±0.9	7.48±0.0	8.57±0.2	7.80±0.3

A 25°C i ceppi hanno esibito un rapido ed elevato sviluppo con cariche batteriche superiori a 10 Log UFC/g già al 15° giorno di conservazione, ad eccezione del ceppo R61 che ha mostrato concentrazioni mediamente più basse rispetto agli altri ceppi studiati. A 7°C i campioni hanno evidenziato conte prossime a 10 Log UFC/g tra il 15° e il 30° giorno di conservazione. Cariche superiori a 7 Log UFC/g sono state ottenute dopo 78 giorni di conservazione.
In Tab. 2 sono riportati i valori di pH dei campioni studiati.

Tabella 2 - Andamento del pH durante la conservazione in confetture di pesche inoculate.

	Giorni	Controllo	Ceppi di *Lactobacillus rhamnosus* inoculati					
			R61	H25	H12	N24	D44	D13
25°C	0	4.50±0.0	4.41±0.2	4.46±0.1	4.44±0.0	4.37±0.0	4.46±0.0	4.43±0.0
	15	4.00±0.0	3.75±0.1	3.63±0.2	3.62±0.1	3.61±0.0	3.60±0.0	3.59±0.0
	30	4.31±0.0	3.59±0.1	3.49±0.1	3.44±0.0	3.56±0.0	3.50±0.0	3.51±0.0
	45	4.31±0.0	3.50±0.0	3.38±0.0	3.42±0.1	3.46±0.0	3.39±0.8	3.40±0.4
	60	3.51±0.0	3.46±0.0	3.36±0.0	3.38±0.0	3.44±0.0	3.37±0.0	3.35±0.3
	78	3.58±0.0	3.42±0.0	3.36±0.5	3.38±0.0	3.43±0.2	3.35±0.0	3.26±0.2
7°C	0	4.50±0.0	4.41±0.2	4.46±0.0	4.44±0.0	4.37±0.1	4.46±0.0	4.43±0.0
	15	4.05±0.0	4.20±0.0	4.26±0.0	4.27±0.0	4.10±0.0	4.32±0.0	4.08±0.0
	30	4.32±0.0	4.11±0.0	4.02±0.0	3.99±0.0	4.31±0.0	4.17±0.0	4.07±0.0
	45	4.30±0.0	4.01±0.3	3.94±0.0	3.89±0.0	4.13±0.1	4.02±0.0	3.96±0.0
	60	4.32±0.0	3.98±0.0	3.91±0.0	3.86±0.0	4.06±0.0	3.98±0.0	3.91±0.0
	78	4.34±0.0	3.95±0.0	3.96±0.0	3.89±0.0	4.04±0.0	3.98±0.0	4.00±0.1

Una lieve riduzione del pH è stata registrata solo per il controllo a 25°C. La massima riduzione del pH si è osservata per i campioni mantenuti a 25°C. Per i campioni mantenuti a 7°C le maggiori riduzioni di pH sono state riscontrate per i ceppi H12, H25 e D44.
I parametri chimico-fisici determinati non hanno evidenziato differenze significative tra i campioni inoculati e il controllo (dati non mostrati). Differenze sono state osservate per i parametri L* e b* nei campioni mantenuti a 25°C, che hanno subito una lieve riduzione. Per i campioni mantenuti a 7°C si è registrato solo un lieve aumento dei parametri L* e b*, e una diminuzione del parametro a*. Il valore di imbrunimento (Abs 420 nm) è aumentato, rispetto al controllo, per tutti i campioni inoculati. Questo andamento può essere dovuto, da un lato, alla produzione di metaboliti secondari da parte dei microrganismi inoculati, dall'altro alla maggiore esposizione all'ossigeno determinata dalla miscelazione della sospensione di probiotici.

CONCLUSIONI

Come emerge dai risultati ottenuti, 5 ceppi di *Lb. rhamnosus* hanno mostrato capacità di mantenere la loro vitalità in campioni di confettura a livelli commercialmente accettabili fino a 78 giorni di conservazione a 7°C e fino a 30 giorni di conservazione a 25°C.
La produzione di confetture probiotiche potrebbe rappresentare una valida strategia sia per aumentare il consumo di derivati di frutta tra adolescenti e consumatori costretti a seguire particolari regimi dietetici, per valorizzare le Pesche Tardive di Leonforte e ampliarne le occasioni di consumo.

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RIASSUNTO

Nel presente lavoro 6 ceppi di *Lactobacillus rhamnosus* probiotici, isolati da formaggio pecorino tradizionale, sono stati impiegati per la formulazione di una confettura probiotica di pesche. I campioni di confettura inoculata (conservati a 25° e a 7°C) sono stati monitorati per 78 giorni per valutare la sopravvivenza dei ceppi e l’andamento dei parametri fisico-chimici quali pH e colore.
Cinque ceppi sono rimasti vitali al di sopra di 6 Log UFC/g, a 25°C fino al 45° giorno di conservazione mentre a 7°C e tutti i ceppi hanno esibito conte superiori a 7 Log UFC/g dopo 78 giorni di conservazione.
Questo lavoro dimostra che ceppi di *Lb. rhamnosus,* isolati da formaggio possono essere impiegati per la produzione di confettura probiotica.

SUMMARY

EFFECTS OF LACTOBACILLUS RHAMNOSUS *STRAINS ON PHYSICO-CHEMICAL PARAMETERS ON PROBIOTIC PEACH JAM*

Survival of six wild strains of Lactobacillus rhamnosus*, previously isolated from traditional chesses, into commercial peach jam, was followed up to 78 days of storage both at 25° and 7°C. Changes in pH, colour parameters and viable cell counts during storage were monitored. In all samples, after 78 days of storage at 7°C, all strains highlighted a Log CFU/g higher than 7, with the strain D44 showing a Log CFU/g value of 8.6.*

This work revealed that strains of Lb. rhamnosus*, isolated from cheese, are suitable microorganisms to achieve probiotic jam*.

To:

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Prof. Marco Gobbetti
IJFS Co-Editor

Bari, June 08, 2010

Dear Dr. Cristina Restuccia,

the manuscript **"**Employment of *Saccharomyces* hybrids as a tool for improving quality of Moscato di Siracusa DOC wine" has been reviewed once again by reviewer 2.

I am delighted to inform you that the manuscript has been found suitable for publication in our journal. I will send back the manuscript also to the editor in chief Prof. P. Fantozzi who shall take care to send it to the Technical Editor for further steps. Please, during revision, check once again carefully the manuscript for English language.

Best regards,

Marco Gobbetti

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SACCHAROMYCES HYBRIDS AS A TOOL FOR IMPROVING THE QUALITY OF MOSCATO DI SIRACUSA DOC WINE

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ABSTRACT

The study aimed to evaluate four *Saccharomyces cerevisiae* strains, one intraspecific *S. cerevisiae* hybrid, and five interspecific *S. cerevisiae*×*S. uvarum* hybrids with respect to the quality of Moscato di Siracusa DOC wine by comparing them with a commercial *Saccharomyces cerevisiae* strain.

Most of the interspecific hybrids maintained volatile acidity (VA) at very low levels, produced high concentrations of glycerol, malic and succinic acid, and yielded the highest concentration of positive sensory attributes.

On the basis of the results of these experimental fermentation trials, a real opportunity to produce special wines employing *S. cerevisiae*×*S. uvarum* hybrids is proposed.

- Key words: acidic components, intraspecific and interspecific *Saccharomyces* hybrids, Moscato di Siracusa DOC wine, sensory analysis -

INTRODUCTION

The fermentation of high-sugar grape musts, such as in the production of iced or dried grape wines, can give rise to stuck or sluggish fermentations due to the high osmotic pressure and toxicity of ethanol to yeast cells (BISSON, 1999; BISSON and BUTZKE, 2000). When the fermentation of these musts stops prematurely, wines of low quality and stability are produced due to high volatile acidity (VA) from the growth of acetic acid bacteria, heterofermentative lactic acid bacteria, non-*Saccharomyces* yeasts (CARIDI et al., 1999; FLEET and HEARD, 1993), and/or *Saccharomyces cerevisiae* wine yeasts used as starter cultures (MURATORE et al., 2007). The latter seem to be a major contributor of VA in high °Brix musts because previous studies demonstrated that sugar stress regulates the expression of structural genes involved in the synthesis of acetic acid from acetaldehyde (SHIMAZU and WATANABE, 1981; ATTFIELD, 1997; BAUER and PRETORIUS, 2000; ERASMUS et al., 2003), and the production of VA is inversely correlated with the maximum cell concentration and the assimilable nitrogen concentration (BELY et al., 2003). Concerning this problem, MURATORE et al. (2007) demonstrated the successful use of a *S. uvarum* strain as a starter culture in the production of Malvasia delle Lipari wine; '*uvarum*-type' strains found in the fermenting yeast biota of sweet wines, which are usually osmotolerant and psychrotolerant, are frequently able to overgrow *Saccharomyces cerevisiae* by the end of the fermentation (SIPICZKI et al., 2001; NAUMOV et al., 2002). As an alternative approach, a recent study of BELY et al. (2008) suggested the use of a mixed culture of *Torulaspora delbrueckii* and *S. cerevisiae* as the best combination for improving the analytical profile of sweet wine, particularly volatile acidity and acetaldehyde production.

However, the degree of acetic acid formation is yeast-strain dependent (REMIZE et al., 1999) because different yeast strains react to the same osmotic pressure by producing different concentrations of acetic acid and glycerol. For this reason, the selection and genetic

improvement of the starter yeast should consider other more specific properties in addition to the basic oenological traits for the type of wine desired.

At present, wine yeast selection is based mainly on screening wild yeast populations. However, the likelihood of identifying a strain expressing all of the optimal properties for winemaking is very low. An alternative approach to obtaining a strain with numerous oenological properties without using recombinant DNA technology is breeding. Crossing *Saccharomyces* species is considered to be a useful tool for obtaining improved wine yeast strains combining fermentative features of both parents (ROMANO et al., 1985; ZAMBONELLI et al., 1997; RAINIERI et al., 1998; MARULLO et al., 2004; 2006; GIUDICI et al., 2005). Interspecific hybrids between cryotolerant *S. uvarum* and non-cryotolerant *S. cerevisiae* strains have been successfully employed in oenology because they possess higher fermentation competitiveness than the parental strains as well as characteristics of the parents in new and interesting combinations (CASTELLARI et al., 1994; ZAMBONELLI et al., 1997). However, a noteworthy study by SOLIERI et al. (2008) demonstrated that the type of mtDNA is an important trait for constructing new improved hybrids for winemaking. Hybrids with *S. uvarum* mtDNA have a higher tendency to ferment and a lower tendency to respire than those with *S. cerevisiae* mtDNA, suggesting that mtDNA type and fermentative:respiratory performance are correlated in *S. cerevisiae* × *S. uvarum* hybrids.

The aim of this study was to compare four *S. cerevisiae* yeast strains, one intraspecific *S. cerevisiae* hybrid, and five interspecific *S. cerevisiae*×*S. uvarum* hybrids with respect to the acidic component, glycerol formation, ethanol tolerance, and sensory characteristics in a very high sugar content must for the production of Moscato di Siracusa from sun-dried grapes, which is one of the most ancient wines produced in Italy. In such a high osmotic must, the practice of inoculating a *Saccharomyces* strain to carry out the vinification requires the

previous selection of a suitable wine yeast strain, both for maintaining volatile acidity below the legal limit fixed by COUNCIL REGULATION (EC) No. 479/2008 and for improving overall quality (2008).

MATERIALS AND METHODS

Yeast strains and fermentation trials

Four strains of *S. cerevisiae*, one *S. cerevisiae*×*S. cerevisiae* hybrid, and five *S. cerevisiae*×*S. uvarum* hybrids bearing *S. cerevisiae* mtDNA (SOLIERI et al., 2008) were used. The list of the strains and origins is reported in Table 1. The strains were cultured for 48 h at 25°C on fresh YPD medium (w/v: yeast extract 1%, peptone 2%, glucose 2%, agar 2%) before their use in fermentation experiments.

The strains' ability to perform alcoholic fermentation of special wines was tested in Muscat grape must. Moscato di Siracusa wine is produced from dried grapes in Sicily in the Siracusa area, and its production is regulated by the controlled origin wine appellation system, known in Italy as DOC (Denominazione di Origine Controllata), which was instituted in 1973 (DPR, OFFICIAL GAZETTE OF ITALIAN REPUBLIC, 1973).

Lightly dried Muscat Blanc grapes produced on sandy soil and siliceous clay in the Siracusa area and surroundings (Sicily, Italy), undamaged and without *Botrytis cinerea*, were used.

The grape berries were crushed and clarified by pressurised filtration through a sack-filter (Spagni s.n.c., Reggio Emilia, Italy); then, 1.5 l was poured into 2-l glass fermentors and inoculated in triplicate with a 48-h yeast pre-culture (5% of volume) that had been prepared in the same sterilised must. Pre-cultures were inoculated independently with the different

yeasts to reach an initial population of 7 log CFU ml^{-1}; yeast population densities were estimated by direct count using a haemocytometer.

Fermentation was carried out in a conditioned room where the temperature was maintained at 18±1°C. The fermentation was monitored by daily measuring of the weight loss associated with the liberation of carbon dioxide. Fermentation was considered complete when the weight loss was negligible.

At the end of fermentation, the wine was filtered, poured into 0.375-l glass bottles, corked, and stored at 4°C.

Analytical determinations

Ethanol content, total acidity, volatile acidity, reducing sugars, and pH were determined for the musts and wines according to the official methods of the Office International de Vigne et du Vin (OIV, 1990).

Wine colour was determined using a spectrophotometer set at 420 nm.

L-malic acid, succinic acid, and glycerol were quantified using enzymatic assay kits (K-LMALR, K-SUCC and K-GCROL, Megazyme International iland Ltd, Bray, Co. Wicklow, Ireland).

All analyses were performed in triplicate. The data shown are the average of all repetitions with standard deviations.

Principal component analyses (PCA) and analysis of variance (ANOVA) were performed by STATGRAPHICS® Plus version 4.0 (Manugistics, Scottsdale, AZ, USA) on chemical and physical data to ascertain significant differences among mean values.

Sensory analysis

To define the attributes of the products and to investigate the differences among the samples, the sensory profile method (ISO 13299, 2003) was used. The wines were evaluated by a panel of ten (six female and four male) trained judges experienced in wine sensory analysis and ranging in age between 20 and 30 years selected from among the Food Science and Technology Department staff members of the University of Catania.

A preliminary session was performed using several commercial Moscato di Siracusa wines to develop a common vocabulary, and this allowed the assessors to use the same terms for describing their perceptions. Descriptors with at least a 70% frequency of citation were chosen. The nineteen descriptors used included: two for appearance (yellow intensity and golden reflection), seven for aroma (fruity, exotic fruit, raisins, wood, honey, sourdough, alcohol), two for taste (sour and sweet), one for mouth feel (sharp), one for rheological properties (viscosity), and six for flavour (fruity, exotic fruit, raisins, wood, honey, sourdough). All evaluations were conducted in individual testing booths at the sensory laboratory (ISO 8589, 1988) at 20 °C, asking the judges to quantify the intensity of each attribute by assigning a score between 1 (absence of perception) and 9 (extremely intense). A data collection program was used (FIZZ Software® solutions for sensory Analysis and consumer Tests, Ver.2, Biosystemes, Couternon, France). Samples were evaluated in five sessions (two by two) using 20 ml of wine in approved wine glasses (ISO 3591, 1977) labelled using a 3-digit code and covered with a plastic lid to minimise the loss of volatile compounds.

Data were statistically processed using STATGRAPHICS® Plus version 4.0 (Manugistics, Scottsdale, AZ, USA). Each attribute was analysed by one way analysis of variance (ANOVA) to verify significant differences among the samples. The significance was evaluated by means of the F test; the mean values were subjected to the multiple comparison

test using the LSD procedure (least significant difference), which allows the attributes differentiating the samples to be determined. Principal component analysis was applied to the sensory data.

RESULTS AND DISCUSSION

Different yeast strains were screened for their vinification properties in must from partially dried Muscat Blanc grapes to select the most appropriate strain for this kind of product. Fermentations of high-sugar grape musts are often sluggish, taking months to reach the desired ethanol level, and they usually have high levels of volatile acidity.

The sugar content of freshly squeezed Muscat must was 319 g l^{-1}, comparable with those reported by NICOLOSI ASMUNDO et al. (1990) for similar grape musts (280-340 g l^{-1}). Total acidity was 7.0 g l^{-1} of tartaric acid; malic acid content was estimated at 0.5 g l^{-1}, and the pH was 3.34.

Fermentation with the different yeast strains resulted in widely variable residual sugar content. The sample inoculated with strain LS9 presented the highest sugar content (98.3 g l^{-1}), while the strains LS7, 522, and AL41 showed a strong ability to reduce the initial amount, and therefore to perform alcoholic fermentation, in musts with high sugar content (Table 2).

Except for the LS9 strain, which probably was influenced by both ethanol and sugar stressors (TROLLMO et al., 1988; PIPER, 1995), ethanol content (Table 2) was about 15% vol for all the hybrid strains, as required by the disciplinary regulations of production; among them, LS7 yielded the highest value (16.2% vol).

In spite of the very low levels of reducing sugars at the end of the alcoholic fermentation, strains AL41 and 522 exhibited lower ethanol levels (14.3 and 14.6% vol, respectively) than

the interspecific hybrids, which could be explained by the diversion of sugar metabolism into by-products other than ethanol.

Except for LS9 and LS8, which produced the highest total acidity values, the total acidity of the wine samples at the end of fermentation was largely unaffected by the identity of the yeast strain (Table 2). The ability to increase the acidic component is considered particularly favourable, especially for wines produced in warm climates such as southern Italy, because advanced ripening leads to products with very low acidity that lack the sensory characters of freshness and vivacity. In addition, acidity contributes resistance to oxidative and microbial spoilage.

VA content was below 1 g l^{-1} for most of the wine samples, except for LS9 and 16003, which produced the highest VA levels (2.06 and 1.14 g l^{-1} respectively). In particular, strains LS3, LS6, and LS7 showed a marked ability to maintain volatile acidity at very low levels (Table 2). VA has a negative effect on the quality of wines and is considered one of the principal problems for the marketability of wines produced from dried grapes. The results of the present study are particularly interesting if compared with those found by MALACRINÒ et al. (2005) in wines from partially dried Corvina grapes fermented with selected *S. cerevisiae* (0.81-1.24 g l^{-1}), and with those reported by MURATORE et al. (2007) in Malvasia delle Lipari wines fermented with *S. uvarum* strains during two consecutive vintages (0.64-0.85 g l^{-1}).

The malic acid content of the grape must was very low due to the over-ripening of the grapes. The production of malic acid during the fermentation process was variable among the *S. cerevisiae* strains, with a minimum content of 0.89 g l^{-1} and a maximum of 1.46 g l^{-1}. On the other hand, the interspecific hybrids produced higher amounts of malic acid, with LS9 producing the highest level of 2.00 g l^{-1} (Table 2).

Succinic acid is not usually present in grape must, and its origin is related to the yeast strain employed. *S. cerevisiae* strains produced the lowest concentrations, with a minimum of 0.23 g l^{-1}. The interspecific hybrids, except for LS9, showed a good ability to synthesise succinic acid as reported in the literature (ZAMBONELLI et al., 1997; RAINIERI et al., 1998), with a peak content of 1.20 g l^{-1} for LS8 (Table 2). This was probably due to stimulation of succinic acid production by sugar stress, as the transcription of all genes involved in the production of succinic acid is enhanced under these conditions (ERASMUS et al., 2003).

Enzymatic assessment of glycerol levels showed wide variability among the strains. The lowest values were synthesised by the *S. cerevisiae* strains and the intraspecific hybrid A3B. The highest concentrations of glycerol were produced by the interspecific hybrids, as demonstrated by EUSTACE and THORTON (1987), with a maximum value of 11.63 g l^{-1} for LS8 (Table 2). This is a considerable amount if compared with what is usually formed by *S. cerevisiae* in wine, which is in the range of 4–9 g l^{-1} (RIBEREAU-GAYON et al., 1972 ; GRAZIA et al., 1995). This finding confirms a previous study that demonstrated a direct correlation between succinic acid and glycerol production (GIUDICI et al., 1995). Glycerol is a non-volatile compound with no aromatic properties, but it significantly contributes to wine quality by providing sweetness and fullness (MALACRINÒ et al., 2005; RIBEREAU-GAYON et al., 1972). Wine yeasts generally adapt to increased osmotic stress by enhanced production of intracellular glycerol, which is the main compatible solute that counter-balances the osmotic pressure (NEVOIGT and STAHL, 1997). Due to the favourable impact of glycerol on wine quality, the benefits of increasing glycerol production to improve the sensory characteristics of wines lacking in body have been emphasised (PRETORIUS and VAN DER WESTHUIZEN, 1991; BARRE et al., 1993; DEGRE, 1993; BISSON, 1996).

The absorbance values at 420 nm, indicating the influence of the yeast strains on the yellowness of the wines, are reported in Table 2. The ability of the yeast strains to delay

browning has recently been investigated by several authors such as LOPEZ-TOLEDANO et al. (2006), who ascribed this characteristic to an inhibitory effect on the formation of coloured compounds. Spectrophotometric analysis revealed a different yellow intensity for each sample. The results of the experimental trials indicated no correlation between browning prevention and the type of yeast strain, as strains LS7, AL41, and A3B, which all showed a good ability to maintain the pale yellow colour of Moscato di Siracusa wine, are (respectively) an interspecific hybrid, a *Saccharomyces cerevisiae*, and an intraspecific hybrid.

The relationships among the chemical parameters are shown in Fig. 1, where the first two principal components explained 82.76% of the variance. Sugar, total acidity, and volatile acidity had the highest negative loading on PC1 (explained variance 53.48%), and the sample LS9 had the highest values for these parameters. Ethanol content, succinic acid, and glycerol had the highest positive loading on PC2 (explained variance 29.28%); in particular, the sample produced by the strain LS7 (unlike the other samples) was characterised by a high level of ethanol and a low sugar content. Except for the interspecific hybrid LS3, all the *S. cerevisiae* strains and the intraspecific hybrid were grouped as a separate cluster, which was characterised by lower levels of acid and glycerol.

Among the 19 sensory attributes considered, only 11 (Table 3) significantly contributed to the character of the wines: yellow intensity ($p \leq 0.001$), golden reflection ($p \leq 0.05$), raisin aroma ($p \leq 0.01$), honey aroma, sourdough aroma ($p \leq 0.05$), sour aroma ($p \leq 0.01$), sweetness ($p \leq 0.001$), viscosity ($p \leq 0.01$), fruity flavour, ($p \leq 0.05$), raisin flavour, and honey flavour ($p \leq 0.001$).

The relationships among the eleven significant attributes were explicated by PCA analysis; the first two principal components explained 90.43% of the variance. As can be observed from the PCA plot (Fig. 2), the wines were distinct in terms of sensory attributes. Moving

from left to right along the first component (explained variance 80.72%), the samples produced with LS3, 522, and AL41 are distinct from the others. The second component (explained variance 9.71%) distinguishes the LS7 sample (in the upper left corner) from the other wines. Raisin aroma, sweetness, raisin flavour, and honey flavour (positive loading), as well as acid (negative loading) are on PC1; sourdough aroma and golden reflection had the highest positive loading on PC2, while the attribute viscosity was equally positively loaded on PC1 and PC2.

The sample fermented with LS7 was characterised by a more intense sourdough aroma, while LS6, LS8, and LS9 provided wines with intense yellow colour and golden reflection, high viscosity and sweetness, raisin and honey flavours, and a negative correlation with undesirable attributes.

The sensory profiles of six of the wine samples selected on the basis of the previous PCA (those fermented with 522, 16003, A3B, FRENCH, LS6, and LS8) were defined by the mean values of the eleven significant attributes. Except for the sour attribute, the wine fermented with the commercial 522 strain had the least intense sensory attributes in comparison with the other five samples, while strains LS8 and LS6 showed a richness of aroma and flavour compounds in addition to intense yellow colour, golden reflection, and high viscosity. An intermediate sensory profile was found for the A3B sample.

CONCLUSIONS

To make wines of high quality, starter cultures are usually used to induce effective and rapid fermentation. Thus, strains of *S. cerevisiae* characterised with respect to their principal technological traits are normally used. However, in some cases, such as in the production of special wines from dried grapes, strains that match the specific characteristics of the particular

wine should be selected. The results of the present study demonstrate that hybrids between cryo- and non-cryotolerant *Saccharomyces* strains may be technologically promising for the production of Moscato di Siracusa wine.

Their ability to perform alcoholic fermentation in grape must with high sugar content was comparable (LS8) or higher (LS3, LS6, LS7) than that of a *Saccharomyces cerevisiae* reference strain. In addition, they were able to increase total acidity, malic acid, succinic acid, and glycerol, and they strongly reduced VA.

The sensory analysis performed on the wine samples by experienced judges revealed (especially for the interspecific hybrids LS6 and LS8) a greater richness of aroma and fruity, raisin, and honey flavour compounds relative to the commercial yeast strain, in addition to intense yellow colour, golden reflection, and high viscosity; most of these attributes are reported in the production disciplinary of Moscato di Siracusa DOC as characterising this precious wine.

The PCA plot of chemical and sensory parameters revealed that the *Saccharomyces* strains employed in this study strongly differ in their chemical and sensory properties.

Among the interspecific hybrids, LS9 produced the highest VA content, while LS7 and LS3 yielded a sourdough aroma and a sour taste, respectively, that were considered to be negative traits. Thus, LS6 and LS8 provided the best fit and might be of use for the production of this quality wine.

This study proved and clearly confirmed the contribution that newly selected yeast strains can make to improve the quality of wine produced according to the most ancient traditions.

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FIGURE LEGENDS

Fig. 1 - Principal component analysis of mean chemical data from the Moscato di Siracusa DOC wine samples.

Fig. 2 - Principal component analysis of mean significant sensory attributes of the Moscato di Siracusa DOC wine samples.

1

Table 1 - *Saccharomyces* strains used in this study.

Strain	Species	Origin	Characteristics
LS3	*S. cerevisiae*×*S. uvarum*	Dipartimento di Scienze Agrarie e degli alimenti (University of Modena and Reggio Emilia, Italy)	*S. cerevisiae* mtDNA
LS6 (4003-1A×7877-10A)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS7 (4003-1B×7877-10B)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS8 (6167-3A×7877-9B)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS9 (6167-8C×7877-6C)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
FRENCH	*S. cerevisiae*	"	Parental A3B
16003	*S. cerevisiae*	"	Parental A3B
AL41	*S. cerevisiae*	DOFATA (University of Catania, Italy)	β-glucosidase positive strain (RESTUCCIA et al., 2002)
522	*S. cerevisiae*	Davis (California)	Commercial reference strain
A3B	*S. cerevisiae*×*S. cerevisiae*	Dipartimento di Scienze Agrarie e degli alimenti (University of Modena and Reggio Emilia, Italy)	High fermentation power

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Table 2 – Chemical and physical parameters of the Moscato di Siracusa DOC wine samples.

	16003	**522**	**A3B**	**AL41**	**FRENCH**	**LS3**	**LS6**	**LS7**	**LS8**	**LS9**
Sugar (g l^{-1})	46.00 ^{c} ±5.00	2.00 ^{e} ±0.00	18.58 ^{d} ±4.00	2.90 ^{e} ±0.25	43.40 ^{c} ±3.00	19.65 ^{d} ±2.00	48.40 ^{c} ±6.00	3.20 ^{e} ±0.50	60.40 ^{b} ±8.00	98.30 ^{a} ±4.00
Ethanol (% vol)	14.80 ^{ab} ±0.00	14.55 ^{bc} ±0.95	14.01 ^{c} ±0.81	14.30 ^{c} ±0.20	14.12 ^{c} ±0.92	15.20 ^{ab} ±0.50	15.85 ^{ab} ±0.25	16.20 ^{a} ±0.00	14.58 ^{bc} ±0.99	12.50 ^{d} ±0.30
Total acidity	8.50 ^{bc} ±0.00	6.45±0.05^{e}	7.23±0.53^{de}	6.70±0.00^{e}	8.08±0.40^{c}	6.85±0.75^{e}	7.80±0.20^{cd}	7.90±0.00^{cd}	9.25±0.45^{b}	10.90±0.00^{a}
Volatile acidity (g l^{-1})	1.14 ^{b} ±0.02	0.51 ^{cd} ±0.13	0.60 ^{cd} ±0.18	0.65 ^{cd} ±0.01	0.81 ^{bc} ±0.12	0.38 ^{ef} ±0.15	0.44 ^{de} ±0.06	0.25 ^{f} ±0.02	0.88 ^{bc} ±0.29	2.06 ^{a} ±0.26
Malic acid (g l^{-1})	1.05 ^{ef} ±0.04	0.89±0.01^{fg}	0.72±0.05^{g}	1.29±0.06^{cd}	1.46±0.13^{c}	1.21 ^{de} ±0.16	1.39 ^{cd} ±0.09	1.72 ^{b} ±0.06	1.74 ^{b} ±0.14	2.00 ^{a} ±0.12
Succinic acid (g l^{-1})	0.23 ^{d} ±0.08	0.50 ^{c} ±0.09	0.51 ^{c} ±0.02	0.49 ^{c} ±0.03	0.46 ^{c} ±0.09	0.54 ^{c} ±0.09	0.74 ^{b} ±0.06	1.15 ^{a} ±0.10	1.20 ^{a} ±0.09	0.45 ^{c} ±0.11
Glycerol (g l^{-1})	6.71 ^{de} ±0.70	6.61 ^{de} ±0.61	6.37 ^{de} ±0.93	5.71 ^{e} ±0.65	7.90 ^{bc} ±1.00	7.53 ^{cd} ±0.19	9.60 ^{ab} ±0.35	9.24 ^{ab} ±1.05	10.39 ^{a} ±1.05	9.47 ^{ab} ±0.71
Colour intensity (Abs)	0.37 ^{c} ±0.04	0.53 ^{b} ±0.03	0.23 ^{d} ±0.04	0.21 ^{d} ±0.05	0.26 ^{d} ±0.03	0.23 ^{d} ±0.06	0.37± ^{c} 0.06	0.19 ^{d} ±0.01	0.67 ^{a} ±0.04	0.47 ^{b} ±0.05

Means in rows followed by the same letter are not significantly different ($p \leq 0.05$)

Table 3 - Analysis of variance of sensory attributes (F values). Mean scores of the nineteen sensory attributes for the ten samples.

		LSD Samples									
Attribute	**F value**	**16003**	**522**	**A3B**	**AL41**	**FRENCH**	**LS3**	**LS6**	**LS7**	**LS8**	**LS9**
Yellow colour	10.19***	7.0^{b}	4.9^{a}	6.9^{b}	4.9^{a}	6.8^{b}	4.1^{a}	6.8^{b}	4.8^{a}	7.6^{b}	7.7^{b}
Golden reflection	2.47*	4.2^{abcd}	3.1^{a}	5.3^{cde}	4.2^{abcd}	4.3^{abcde}	3.3^{ab}	5.2^{bcde}	3.7^{abc}	6.1^{de}	6.2^{e}
Fruity aroma	1.11 n.s	5.1	4.7	5.4	5.0	5.2	6.0	4.3	3.4	5.1	4.7
Exotic fruit aroma	1.79 n.s	4.5	4.1	4.6	4.8	4.8	5.8	4.6	2.7	5.5	4.4
Raisin aroma	3.36**	5.2^{b}	4.8^{b}	6.0^{bc}	4.4^{ab}	5.2^{b}	4.3^{ab}	5.3^{b}	2.8^{a}	5.7^{bc}	7.4^{c}
Wood aroma	1.25 n.s	3.7	3.2	3.5	3.1	4.5	3.7	5.0	4.7	5.1	5.0
Honey aroma	2.20*	4.7^{bc}	3.7^{ab}	4.4^{bc}	3.9^{bc}	4.2^{bc}	3.4^{ab}	4.2^{bc}	2.2^{a}	4.4^{bc}	5.3^{c}
Sourdough aroma	2.18*	2.9^{a}	3.2^{a}	2.9^{a}	3.0^{a}	2.6^{a}	3.8^{ab}	3.9^{ab}	5.2^{b}	3.4^{a}	2.7^{a}
Alcohol aroma	0.59 n.s	4.8	5.0	4.9	4.4	4.7	5.0	6.0	5.2	5.3	4.7
Sourness	3.11**	4.8^{abc}	6.0^{cd}	4.4^{abc}	5.4^{bcd}	4.1^{ab}	6.0^{cd}	4.5^{abc}	6.6^{d}	4.5^{abc}	3.3^{a}
Sweetness	13.03***	4.5^{b}	2.1^{a}	4.9^{bc}	2.3^{a}	5.5^{bc}	2.7^{a}	5.3^{bc}	2.4^{a}	5.9^{cd}	7.0^{d}
Sharpness	1.70 n.s	4.6	4.9	3.8	3.8	5.1	5.3	4.0	5.4	3.7	2.7
Viscosity	3.38**	4.0^{abcd}	3.4^{ab}	3.9^{abc}	2.7^{a}	4.1^{abcd}	3.3^{a}	4.8^{bcd}	2.9^{a}	5.2^{cd}	5.4^{d}
Fruity flavour	2.22*	4.0^{bcd}	3.5^{ab}	3.7^{abc}	4.0^{bcd}	5.2^{d}	4.0^{bcd}	4.3^{bcd}	2.5^{a}	5.1^{cd}	4.6^{bcd}
Exotic fruit flavour	1.23 n.s	4.1	3.4	3.2	4.0	4.5	3.6	4.3	2.9	4.9	3.6
Raisin flavour	4.32***	5.3^{cde}	4.3^{abcd}	4.4^{bcd}	3.4^{ab}	5.4^{cde}	3.8^{abc}	5.9^{de}	2.6^{a}	6.0^{de}	6.8^{e}
Wood flavour	1.10 n.s	3.5	3.5	4.4	2.6	4.1	3.7	4.8	4.4	3.9	5.1
Honey flavour	5.09***	4.0^{cde}	2.9^{abc}	3.8^{cde}	3.5^{bcd}	5.0^{ef}	2.4^{ab}	4.4^{def}	2.0^{a}	4.7^{def}	5.4^{f}
Sourdough flavour	0.69 n.s	4.1	3.1	2.9	3.2	2.9	3.6	3.2	4.4	3.2	3.0

Values for each parameter followed by different lower-case letters indicate differences according to the Student-Newman-Keuls test ($P \leq 0.05$).

* $p \leq 0.05$
** $p \leq 0.01$
*** $p \leq 0.001$
n.s not significant