UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO DI RICERCA
Scienze e Biotecnologie
dei Sistemi Agrari e Forestali
e delle Produzioni Alimentari

Biotecnologie microbiche agrolimentari

Ciclo XXVIII

Study of microbial consortium for table olives production

Dr.ssa Cinzia Porru

Direttore della Scuola	Prof. Antonello Cannas
Referente di Indirizzo	Dott. Severino Zara
Docente Guida	Prof.ssa Marilena Budroni
Correlatore	Prof.ssa Ilaria Mannazzu
Correlatore	Dott. Francisco Noé Arroyo López

Anno Accademico 2014/2015

Cinzia Porru -"Study of microbial consortium for table olives prduction". Tesi di Dottorato in Scienze e biotecnologie dei Sistemi Agrari e Forestali e delle Produzioni alimentari, indirizzo in Biotecnologie Microbiche Agrolimentari, Università degli Studi di Sassari

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Cinzia Porru gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 – Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.) and the Sardinia Foundation (Prat.2013.1364) for financial support.

SUMMARY

Directly brined black table olives of Bosana variety are a traditional food produced and consumed in Sardinia. Bosana table olives are produced by "natural" fermentation, an empirical process where yeasts involved. To date, the yeast community of Bosana table olives has not been investigated. In this work yeast isolates were identified by random amplified polymorphic DNA with primer M13 and sequencing of D1/D2 domains of rDNA 26S gene. Technological and probiotic properties were evaluated by biochemical and spectrophotometric methods. Biofilm formation between yeasts and *Lactobacillus pentosus* was investigated by crystal violet staining. Moreover, ability to form biofilm *in vitro* and genome sequencing of eight strains of *Candida boidinii* were investigated. Data of multivariate analysis revealed that *Wickeramomyces anomalus* Wa1 exhibited better technological characteristics while *Saccharomyces cerevisiae* Sc24 and *Candida boidinii* Cb18 showed a probiotic potential. Results about biofilm formation showed that presence of *Lactobacillus pentosus* stimulates biofilm formation by *Candida boidinii*. Information about probiotic and technological features of these yeasts can be used to design a potential multifunctional starter in order to improve and optimize natural process and nutritional characteristics of commercialized product of Sardinia.

In addition genomic study of *Candida boidinii* offers a helpful starting point to understand the mechanism of biofilm formation.

RIASSUNTO

Le olive da tavola ottenute mediante fermentazione al naturale della varietà Bosana sono un prodotto tradizionale prodotto e consumato in Sardegna. Fino ad oggi, non sono stati effettuati studi sulla popolazione di lieviti coinvolta nella fermentazione delle olive della varietà Bosana. In questo lavoro gli isolati sono stati identificati mediante analisi RAPD con il primer M13 e tramite sequenziamento dei domini D1/D2 del gene 26S dell'rDNA. Le caratteristiche tecnologiche e probiotiche sono state studiate tramite metodi biochimici e spettrofotometrici; la produzione di biofilm è stata esaminata mediante la colorazione con il cristal violetto. Inoltre è stata esaminata la capacità di produzione di biofilm dei lieviti ed è stato effettuato il sequenziamento del genoma di otto ceppi di *Candida boidinii.* I risultati dell'identificazione molecolare rivelano che le specie dominanti sono *Wickerhamomyces anomalus* and *Nakazawaea molendini-olei*. I dati ottenuti dall'analisi multivariata mostrano che *Wickerhamomyces anomalus* Wa1 possiede le migliori caratteristiche tecnologiche mentre *Saccharomyces cerevisiae* Sc24 e *Candida boidinii* Cb18 mostrano un potenziale probiotico. I risultati sulla produzione di biofilm mostrano che la presenza di *Lactobacillus pentosus* stimola la formazione di biofilm da parte di *Candida boidinii.* Le informazioni acquisite sulle proprietà probiotiche e tecnologiche potranno essere utilizzate per progettare uno starter multifunzionale per migliorare ed ottimizzare il processo di fermentazione al naturale e per migliorare le caratteristiche nutrizionali del prodotto.

Inoltre, lo studio del genoma di *Candida boidinii* offre un punto di partenza per la comprensione dei meccanismi della formazione di biofilm.

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LIST OF ABBREVIATIONS

Abs – Absorbance

AGRIS – Regional agency for agricultural research of Sardinia

ANOVA – Analysis of Variance

BLAST – Basic Local Alignment Search Tool

CFU – colony forming unit

CSIC – Consejo Superior de Investigaciones Científicas

CV – Crystal violet

DNA – Deoxyribonucleic acid

EFTAS – Authority for Land and Agrarian Transformation of Sardinia

EU – European Union

Fa – fractional area

FAO – Food and Agriculture Organization of the United Nations

GC – gastric digestion

GI – gastrointestinal digestion

ISTAT – National Institute of Statistics

ITS – Internal transcribed spacer

LAB – Lactic acid bacteria

LSU – Large Subunit

MEGA – Molecular Evolution Genetic Analysis

MIC – minimum inhibitory concentration

MRS – De Man, Rogosa and Shape

mQ – Milli-Q

NCBI – National Center for Biotechnology Information

NIC – non inhibitory concentration

OD – Optical density

PBS – Phosphate – buffered saline

PCA -Principal Component Analysis

PCR – Polymerase Chain Reaction

PD – pancreatic digestion

PDO – Protected Designation of Origin

RAPD-PCR – Random Amplification of Polymorphic DNA

rep-PCR – Repetitive extragenic palindromic PCR

RNA – Ribonucleic acid

rpm – revolution per minute

SEM – Scanning Electron Microscope

TOMC – table olive microorganism collection

UNAPROL – National Union of Associations of Producers of Olives

UPGMA – Unweighted Pair Group Method

YM – Yeast mold

YNB – Yeast Nitrogen Base

YPD – Yeast Peptone Dextrose

1. INTRODUCTION

1.1. Olive and historical background

The olive belongs to *Oleacee* family, genus *Olea,* species *Olea europaea* (L.). The origin of olive tree cultivation is lost in legend and tradition. Olive tree had origin approximately 6,000 to 7,000 years ago in the region corresponding to ancient Persia and Mesopotamia. The first historical mentions of olive's presence were observed in Ebla tablets found in ancient city of Ebla, in Syria. The tablets provide information about olive oil production in III millennium BC indicating the value of olive oil was five times that of wine and two and a half times that of seed oils (Vossen, 2007). Records indicate the introduction of olive trees into Greece, Egypt, and western Turkey. Olive cultivation was reported in the Bible books such as Genesis, where the flight of the dove with an olive branch announce the end of the flood (Garrido-Fernández *et al,* 1997). Olives continued to move westward into Sicily, Sardinia, Italy, France, Spain, Portugal, Algeria, Tunisia, and Morocco. In these areas, there are many archaeological sites with olive-related findings (Vossen, 2007).

In Sardinia, Neolithic evidences of Cave of Refuges in Oliena show presence of olive tree. The archaeologist Giovanni Lilliu hypothesized that a little room found in nuragic village of Barumini was used as laboratory for olive oil preparations used to lighting. The expansion and prosperity of Roman Empire contributed to spread olive plantings and olive processing. Many archaeological findings and toponyms of Latin origin in Sardinia testify presence of olive, such as regions of Parteolla and Ogliastra or villages of Dolianova, Oliena and Ollastra Simaxis (Arca *et al.*, 2010). During the Middle Age olive tree cultivation declined. The greatest expansion of olive tree cultivation in Sardinia came in XIII century under Spanish rule, which contributed significantly to spread olive and to introduce Iberian varieties. The House of Savoy in Sardinia continued the work of protection and diffusion of olive granting title of nobility to people that planted olive trees. Recently, agrarian reform performed by EFTAS (Authority for Land and Agrarian

Transformation of Sardinia) increased cultivation of olive and introduced new varieties such us Leccino, Frantoio and Biancolilla (Arca *et al.*, 2010).

1.2 Table olives

Table olives are defined as "product prepared from the sound fruits of varieties of the cultivated olive tree which are chosen for their production of olives particularly suited to curing, and which are suitably treated or processed and offered for trade and for final consumption" (*Olive International Council*, 2015).

Olive fruit is a drupe consisting of epicarp, or olive skin, a mesocarp, or flesh, and an endocarp. The epicarp accounts for 1-3% of fruit, the mesocarp accounts for 84-90% while the endocarp contains the seed. The seed contains 2–4 g oil /100 g. The drupe is mainly made up of water (50%), oil (22%), carbohydrates (2%), phenolic compounds (1-3%) and inorganic substances (1.5%) (Ghanbari *et al.*, 2012).

Fats present in table olives include oleic (C18:1; 75-80%), followed by palmitic (C16:0; 10-12%), linoleic (C18:2; 5-7%), stearic (C18:0; 2-3%), linolenic (C18:3; 0.5-1%) and palmitoleic (C16:1; 0.5-1%) acids (Lanza, 2012). Glucose is the most abundant simple sugar, followed by fructose and sucrose. Amount of these components depends from olive variety and maturity degree (Garrido-Fernández *et al.*, 1997).

Olive fruits contain bitter components (oleuropein and phenolic compounds) that make it unpalatable. The main goal of table olives processing is to remove the bitter taste of freshly harvested fruits by oleuropein hydrolysis, obtaining preservation and improvement of organoleptic characteristics of final product (Corsetti *et al.*, 2012). The most common table olives preparations are the Spanish or Sevillan style with about 60% of production, the Greek style and the Californian style. Spanish-style method consist of lye treatment of green olives (1.8–2.5 NaOH, w/v) followed by washes with tap water in order to remove excess of NaOH solution. After washes olive are immersed in brine (10–11% NaCl, w/v) where occurs partial or complete lactic acid fermentation (Garrido-Fernández *et al.*, 1997). After fermentation, olives are packaged in new brine and sold. This process is

conducted by lactic acid bacteria, which degrade carbohydrate and produce lactic acid and bacteriocins, contributing to safety and quality of table olives.

In Greek style method, drupe are immersed directly in brine after harvesting. Usually, brine has a salt concentration of 6-10% (Corsetti *et al.*, 2012). This process is slow and partial, oleuropein hydrolysis is due to enzymatic activities of esterase and β-glucosidase produced by indigenous microbiota (Garrido-Fernández *et al.*, 1997; Tassou *et al.*, 2002). In this olives processing, debittering is also produced by diffusion of oleuropein from fruits to the surrounding brine. The fermentation process can last 8–12 months and it is mainly conducted by mixed population of LAB and yeasts (Botta and Cocolin, 2012). The fermentation time depends on the physic-chemical conditions, the cultivar, the salt content, and the temperature (Tassou *et al.*, 2002). Olive fruits are subjected to spontaneous fermentation performed by natural microbiota that consist of different microorganisms such as Gram negative bacteria, LAB and yeasts (Nisiotou *et al.*, 2010). After initial phase of vigorous fermentation, prevailing microorganisms are LAB and yeasts that compete for nutrients (Tassou *et al.*, 2002; Nisiotou *et al.*, 2010). Presence of microorganisms depends on different factors such as salt concentration, oxygen availability, initial pH, temperature of fermentation and the presence of microorganisms contaminating the fruit (Tassou *et al.*, 2002). Concentration of NaCl used in natural processing affects growth of LAB and improves development of fermentative yeasts (Tassou *et al.*, 2002).

Olives prepared with Californian style method are obtained from ripe olives and are treated with NaOH solution and then darkened by oxidation with iron salts (Garrido-Fernández *et al.*, 1997; Arroyo-López *et al.*, 2008).

The Mediterranean diet pyramid recommends the consumption of table olives on a daily basis due to nutritional benefits associated with this fruit (Bach-Faig *et al.*, 2011). Among vegetable foods, table olives are the most important and well-known with an estimate world production of 2,660.5 million of tons per year (*Olive International Council*, 2015). In the world, the main producing area is the European Union (EU), which produces

794.0 million of tons per year. Spain is the leading country for table olives production in EU (793.9 tons), followed by Greece (130.0 tons) and Italy (69.3 tons) (*Olive International Council*, 2015).

1.3 Italian table olives processing

In Italy, table olives production is concentrated in central and southern regions (Figure 1). The Italian table olives labelled with Protected Designation of Origin are "Nocellara del Belice" (Sicilia), "La Bella della Daunia" (Apulia) and "Oliva Ascolana del Piceno" (Marche and Abruzzo) (Lanza, 2012).

The main Italian table olive preparations are the following.

1.3.1 Itrana style

The Itrana variety is grown mainly in Lazio region, in Latina district. This variety is used to produce the famous "Oliva nera di Gaeta" by natural method. Harvested olives are immersed in water for about 1 month to stimulate the growth of specific microflora that contributes to the debittering of the fruits. After 10-30 days salt is added to the liquid, in quantities not exceeding 7 kg per 100 kg of fresh olives. After 4-6 months of storage in brine the olive flesh shows a typical red-wine colour and acidic taste probably due to the contribution of hetero fermentative bacteria and yeasts (Lanza, 2012).

1.3.2 Cracked olives

This type of processing is typical of the Calabria, Apulia and Campania regions. The olives fruits harvested at the green stage of ripening, are crushed with a wooden hammer. Then the olives are put in an earthenware pot with water for at least 2 weeks changing the water at least twice a day. The finished product is seasoned with garlic, pepper, oregano and other spices (Lanza, 2012).

Figure 1. Distribution of table olives production in Italy. PDO: Protected Designation of Origin (Lanza, 2012).

1.3.3 Castelvetrano style

This method is used in Sicilia, almost exclusively in the Castelvetrano district using the Nocellara del Belice cultivar. Selected olives are put into plastic vessels and covered with NaOH solution (1.7-2.4% w/v). Eight hours after the lye treatment begins, 6-7 kg of salt is added. The olives are kept in this "alkaline brine" for 8- 10 days (Lanza, 2012).

1.3.4 Ferrandina style

Olives of Majatica cultivar are treated with an ancient method, Ferrandina method, whose name derives from the town Ferrandina, in Basilicata region. Initially the olives are blanched in water at 90°C for 3 min to make the skin more permeable and facilitate the osmotic processes. After blanching, the olives are salted with grinded NaCl (10:1 w/w) for 3 days and finally they are oven-dried in an air-oven at 50 °C. Blanching and salting steps

cause drupe debittering while oven drying contribute to preservation of final product (Lanza, 2012).

1.3.5 Salt dried olives

Black ripe olives are put in alternating layers with coarse salt (equivalent to 10–20% w/w of the weight of olives), orange peel and spices like oregano, fennec and garlic. The resulting olives, or "date olives", are shrivelled in appearance and have a salty bitter-sweet taste.

1.3.6 Ascolana style

The stuffed olives by Ascolana-style are prepared from treated green olives in brine from Ascolana tenera cultivar. (PDO "Oliva Ascolana del Piceno). The meat (beef 40-70%; pork 30-50%; chicken/turkey max 10%) is triturated and browned with onion, carrot and celery in olive oil and cooked on low heat with the addition of dry white wine and salt. When cooked, meat and add-ingredients are shredded and combined with beaten egg, grated cheese and ground nutmeg. The pre-pitted olives are filled with the mixture, are dipped in flour, then beaten egg and finally in breadcrumbs (Lanza, 2012).

1.3.7 Sardinian preparations

Natural olives placed in the brine is the most important preparation of Sardinian table olives. After harvesting, sorting and washing, drupes are immersed in brine (8-14% w/v of NaCl) (Piga e*t al.,* 2002). Another traditional preparation of table olives is "Scabecciu olives". The ripe olives Tonda di Cagliari and Pizz'e Carroga varieties are harvested and are engraved in three points. Then the olives are immersed in brine for three days, washed with water, blanched in vinegar-water and dried in the sun. Finally, they are fried with garlic and parsley and placed in oil. After about 1 month of preparation they can be consumed as appetizer.

1.4 Production of table olives

In Italy, table olives production is concentrated in the southern regions, led by Sicily (43%) and Apulia (25%), and followed by Calabria (18%), Sardinia (4.1%), Basilicata (4.1%), Lazio (3.6%) and Campania (1.9%). In Italy there are several varieties of olive trees (*Olea europaea*). The most widespread variety is "Coratina", followed by minor varieties such as "Ogliarola Salentina", "Cellina di Nardò", "Ogliarola Barese", "Moraiolo", "Bosana" and "Cima di Mola" (Source UNAPROL).

Olive tree cultivation plays an important role in economy, landscape and tradition of Sardinia. Cultivation is widespread in the island, with a patchy distribution and concentrated in the area of Sassari, Parteolla, Oliena, Montiferru and Linas (Arca *et al.*, 2010). The data reported by the Census of Agriculture (ISTAT, 2010) showed that about 39,075 hectares are planted with olive trees, of which 1,660 hectares for the production of table olives. The province of Sassari is the major cultivating area, accounting for 12,000 hectares followed by the provinces of Cagliari and Nuoro (Bandino and Dettori, 2001).

According to data released by the Regional agency for agricultural research of Sardinia (AGRIS), Sardinia produced about 10,000 quintals of table olives, contributing approximately for 1.5% to Italian production. This data is far below the average for the producer regions and shows that, despite being a major consumer, the table olive sector is somewhat of a sideline for Sardinia. In the island, production of naturally table olives is very common homemade. Most of producers are occasional producers that do not able to provide a constant quality and amount. Only ten medium companies are specialized in olives processing of drupes harvested in Sardinia. These companies use the natural method to produce table olives and are concentrated in South Sardinia.

Sardinian varieties (Bosana, Nera di Villacidro, Tonda di Cagliari, Nera di Gonnos, Pizz'e Carroga, and Semidana) include six cultivar. Bosana is the most widespread cultivar in Sardinia, particularly in northern and central Sardinia.

Figure 2. Bosana fruiting (LAORE).

Presumably it has a Spanish origin and it also called Palma, Tondo Sassarese and Olieddu. The areas of greatest Bosana olive concentration are Nurra and Sassarese areas, with an estimated number of tree approximately of 3 million (Bandino and Dettori, 2001).

Fruits with elliptical shape has an average weight of approximately 3 g, which have maturated take on a very bright black colour. This variety is appreciated for the quality of olive oil; big size drupes are used to make black naturally table olives (Bandino and Dettori, 2001). Bosana table olives are a typical product of North Sardinia. After harvesting, sorting and washing, drupes are immersed in brine (8-14% of NaCl) (Piga *et al.*, 2002).

1.5 Microbiology of table olives

1.5.1 Role of lactic acid bacteria in table olives fermentation

Lactic acid bacteria play an important role in Spanish-style table olive fermentation (Garrido-Fernández *et al.*, 1997), in fact they produced antimicrobial substances, bacteriocins and lactic acid from fermentable substrates resulting in pH decrease that

enhance table olives preservation (Ruiz-Barba and Jimenez-Diaz, 1995). LAB and yeasts were able to form mixed biofilm on olive surface during fermentation (Domínguez-Manzano *et al.*, 2012; Arroyo-López *et al.,* 2012). In addition, they also improve the aroma and flavour of final product (Corsetti *et al.,* 2012).

Lactic acid bacteria species commonly found in table olives are *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus paraplantarum* and *Lactobacillus brevis* both in Spanish style fermentation and in directly brined olives (Campaniello *et al.,* 2005; Hurtado *et al.,* 2008. In natural olive fermentation these species are usually observed after at least 10 days from the beginning of technological process (Tassou *et al.,* 2002).

The two most representative species of LAB ecology in table olives are *L. pentosus* and *L. plantarum*. Hurtado *et al.,* (2010) reported that in case of the co-inoculation of strains of *L. pentosus* and L. *plantarum* in Aberquina fermentation only the first dominated. Panagou *et al.,* (2008) observed better performance of *L. pentosus* starter than *L. plantarum* inoculated separately as starter cultures in black olive fermentations.

1.5.2 Role of yeasts in table olives production

Sometimes, yeasts can be dominant microorganisms during table olive fermentation, causing a milder taste and less preservation of final product. This fact was reported by (Tassou *et al.,* 2002).

In the first phase of fermentation, yeasts could produce gas pocket as CO_2 causing formation of blisters, a spoilage known as "Alambrado" (Lamzira *et al.*, 2005; Hernández *et al.*, 2007). During phase of packing yeasts could cause clouding of brines, swollen containers, off flavours, odours and resistance to preservatives (Turantaş *et al.*, 1999).

Recently, role of yeasts table olives production has been re-considered by different researchers. The presence of yeasts during table olives processing is linked to raw materials and related to type of fermentation process (Botta and Cocolin, 2012). Concerning fermentation processing, the role of yeasts is very important in directly brined

olive processing (Garrido-Fernández *et al.,* 1997). Bautista-Gallego *et al.,* (2011) reported high amount of yeast in directly table olive than in Spanish style olives. The most important yeast species found in table olive belong to *Aureobasidium, Candida, Cryptococcus, Issatchenkia, Pichia, Rhodotorula, Saccharomyces,* and *Zygotorulaspora* genera (Bevilacqua *et al.,* 2013).

In relation to growth matrices (brine and drupe), Hernández *et al.,* (2007) found higher count of yeasts in brine than fresh fruit. The dominant species detected in the brine belong to *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* species while mains species detected in the drupe was *Cryptococcus laurentii.*

During fermentation, yeasts produce volatile compounds and organic acids such as such as ethanol, glycerol, higher alcohols and acetaldehyde that define organoleptic characteristics and maintenance of texture during table olive processing (Fernández *et al.,* 1995; Arroyo-López *et al.,* 2008). Aponte *et al.,* (2010) detected several aromatic compound synthesized by catabolism of fatty acids during green Sicilian table olive fermentations dominated by yeasts. Yeast improve the growth of lactic acid bacteria. Tsapatsaris and Kotzekidou, (2004) reported improvement of growth of *L. plantarum* after inoculation of *Debaryomyces hansenii* in same olive juice.

Regarding organoleptic characteristics of table olives, yeasts exhibit important enzymatic activities such as catalase, esterase, lipase, and β-glucosidase which are strains-specific (Botta and Cocolin, 2012). Catalase positive yeasts contribute to preserving olives against unsaturated fatty acid oxidation and peroxide formation (Hernández *et al.,* 2007). Esterase and lipolytic activities are desirable because they can enhance the flavour of olives through the formation of volatile compounds that can be generated by the catabolism of free fatty acids (Bautista-Gallego *et al.,* 2011). *Candida boidinii, D. hansenii,* and *Torulaspora delbrueckii* showed high lipase activity in assays *in vitro* (Psani and Kotzekidou, 2006; Rodríguez-Gómez *et al.,* 2010).

Enzymatic activities are also important for polyphenols degradation. Spanish style processing produced a large amount of wastewater and water with high concentrations of phenols. β-glucosidase produced by yeasts may lower the level of phenols in wastewater produced by lye treatment of olives. So, β-glucosidase can be used to design an eco-friendly olive process to replace lye treatment.

Yeasts produce toxic proteins or glycoproteins, called killer toxins, which are able to decrease or inhibit the growth of bacteria, fungi and non-desirable yeasts (Bevilacqua *et al.,* 2015). Therefore, yeast can be used as biocontrol agents in olive fermentation in order to inhibit development of spoilage and pathogen microorganisms. For example, Hernández et al., (2008) studied killer activity of strains isolated from green seasoned table olives against spoilage microorganisms. Moreover, Psani and Kotzekidou, (2006) found strains of *D. hansenii* and *Torulaspora delbrueckii* with action against food-borne pathogen.

Moreover, yeasts can synthesize vitamins (Ruiz-Barba and Jimenez-Diaz, 1995), compounds with killer activity (Psani and Kotzekidou, 2006). The vitamins synthesized or accumulated by yeasts are thiamin, pantothenic acid, nicotinic acid and pyridoxine (Abbas, 2006).

Table olives can be considered itself as a functional food, because of their high content in dietary fiber, antioxidant compounds, vitamins, anticancer substances (Rodriguez-Gomez *et al.*, 2014). The functional value of table olives can be improved by turning this fermented vegetable in a carrier of beneficial microorganisms to the human body. In this sense, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO, 2001). So addition of probiotic microorganisms is useful to produce functional foods.

Probiotic characteristics of LAB was extensively reviewed in the past. Lavermicocca *et al.,* (2005) used table olives as a vehicle to incorporate probiotic bacteria species into the human gastrointestinal tract. Many LAB belong to *L. pentosus, L. plantarum, Lactobacillus*

paracasei isolated from table olives exhibited probiotic characteristics in *vitro* (Argyri et al., 2013; Bautista-Gallego et al., 2013). Moreover, LAB with probiotic properties can be used in the packing stage for table olive fortification. In fact, *L. pentosus* TOMC 2 survived under packing conditions for long time (Rodríguez-Gómez *et al*., 2014). Recent study showed that use of probiotic *L. pentosus* as starter for fermentation of table olives by Spanish style method led to decrease growth of *Enterobacteriaceae* (Rodríguez-Gómez *et al.,* 2013).

In addition to LAB, many authors focused on probiotic traits of yeast such as adhesion to intestinal mucosa, survival and/or persistence into the gut, antimycogenic and antibacterial activity, production of vitamin B-complex, biodegradation of phytate complexes and reduction of cholesterol levels. *Saccharomyces boulardii* is a probiotic yeast isolated for the first time from litchi fruit in Indochina in 1923 (Moslehi-Jenabian *et al.,* 2010). It is the only yeast whose use has been recommended for its clinical effects (Hatoum *et al.,* 2012). In fact, several authors described the effect of *S. boulardii* on antibiotic-associated diarrhoea (D'Souza *et al.,* 2002; Erdeve *et al.,* 2004). Moreover, administration of *S. boulardii* with antibiotics showed beneficial effect on *Clostridium difficile* associated diarrhoea. Several case works reported that some patients with recurrent *C. difficile* diarrhoea treated with *S. boulardii* showed improvement. This effect is due to production of a 54 kDa protease that degrade toxin A and toxin B secreted from *C. difficile,* leading to a reduction on effects of *C. difficile* infection (Castagliuolo *et al.*, 1996, 1999). Finally, the effect of *S. boulardii* in prevention of traveller's diarrhoea has been demonstrated (McFarland, 2010).

Numerous studied showed benefits of *S. boulardii* to patients affected with inflammatory bowel disorders (Hatoum *et al.,* 2012). Mechanism of action of *S. boulardii* is showed in Figure 3.

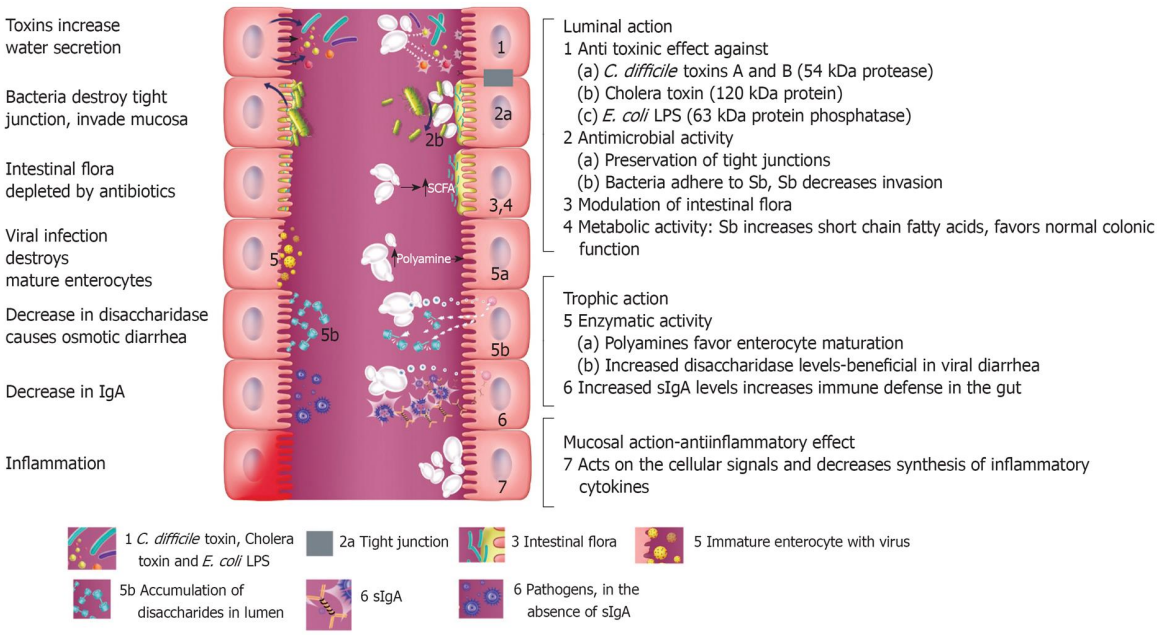

Figure 3. Potential mechanism of action of *S. boulardii* on intestinal tract. On the left, effects of different pathogenic microbes are showed. On the right, seven different protective effects of *S. boulardii* are showed. Within the lumin of the intestine, *S. boulardii* may degrade toxins of pathogens, interfere with pathogenic adherence, modulate normal microbiota and preserve normal intestinal physiology (McFarland, 2010).

Regarding yeasts associated to table olives, only Psani and Kotzekidou, (2006), Silva et al., (2011) and Bonatsou, *et al.*, (2015). Psani and Kotzekidou, 2006 and Silva *et al.*, (2011) reported that D. *hansenii*, *T. delbrueckii, Candida oleophila* and *S. cerevisiae* tolerated 0.3% (w/v) bile salts and low pH. Finally, Bonatsou *et al.*, (2015) in a study about selection a multifunctional starter recorded high resistance to simulated digestion of *Pichia guilliermondii*.

Phytase is an enzyme that catalyses the phytic acid hydrolysis, an indigestible form of phosphorus present in vegetables and cereals. Therefore, phytase activity enhances adsorption of iron, zinc, magnesium and phosphorus. These enzyme has been common detected in several microorganisms. Among yeasts, *Candida krusei* (*Issatchenkia orientalis*), *Schwanniomyces castellii*, *Debaryomyces castellii*, *W. anomalus*, *Pichia rhodanensis*, *Pichia spartinae*, *Cryptococcus laurentii*, *Rhodotorula gracilis*, *S. cerevisiae*, *Saccharomyces kluyveri*, *T.*

delbrueckii, *Candida* spp. and *Kluyveromyces lactis* have been identified as phytase producers (van der Aa Kühle *et al.,* 2005). This activity was detected for the first time in *P. guilliermondii*, *Pichia kluyveri*, and *Metschnikowia pulcherrima* isolated from Greek natural table olives (Bonatsou *et al.,* 2015).

Yeasts synthesize folic acid (vitamin B9) but mammals lack the ability to synthesize them and therefore are dependent on their intake in the diet. Folates are involved in the synthesis of nucleic acids and metabolise amino acids necessary to cell division, therefore folate producing yeasts can be used in table olives fortification (Arroyo-López *et al.,* 2012).

High level of cholesterol in blood causes hypercholesterolemia, and it is related to risk factor in coronary heart disease. Low cholesterol and saturated fatty acid diet is suggested to people who have high serum cholesterol level. Decrease of cholesterol is a trait exhibited by several yeast strains such as *K. lactis*, *S. cerevisiae*, and *I. orientalis*, usually found in table olives (Arroyo-López *et al.,* 2012).

Candida and *Saccharomyces* species produce natural antioxidants such us carotenoids, citric acid, D-erythro ascorbic acid, tocopherols and glutathione with interesting antioxidant properties (Abbas, 2006). The synthesis of bioactive antioxidants can reduce the oxidative degeneration of fatty substances improving human health (Arroyo-López *et al.,* 2012).

Mycotoxins are toxic secondary metabolites produced by fungi belonging mainly to the *Aspergillus*, *Penicillium* and *Fusarium* genera. The presence ochratoxins A, citrinin and aflatoxin B has been recently reported in table olives by El Adlouni *et al.,* (2006). Some yeasts exhibited detoxification ability of mycotoxins. For example, *S. cerevisiae* showed ability to inhibit ochratoxin A production in *Aspergillus carbonarius* and *Aspergillus ochraceus* (Cubaiu *et al.,* 2012). Therefore, *S. cerevisiae* could be used for mycotoxins biodegradation in table olives.

1.5.3 Molecular methods to study table olives microbiota

In the past, characterization of microorganisms associated to table olives processing was performed by biochemical and morphological methods. Recently, molecular methods have revolutionized approaches used to study microorganisms of fermented products. In particular, two strategies were used. In culture-dependent approach, in which microorganisms are isolated from food matrix by traditional microbiological techniques and are subsequently studied by molecular method. In culture independent method, DNA or RNA are extracted and analysed directly from food matrix. This method allows to investigate complexity of table olives microbiota, detecting the not cultivable microorganisms. On the contrary, culture independent methods are not adapt to select new starter culture (Botta and Cocolin, 2012).

As far as yeasts, the restriction analysis of the ITSs (ITS1 and ITS2) and the 5.8S rRNA gene described by Esteve-Zarzoso *et al.,* (1999) has been used to identify a total of 132 species of yeasts (Botta and Cocolin, 2012). Restriction analysis of 5.8 rRNA gene and internal transcribed spacers ITS1 and ITS2 and sequence analysis of 26S rRNA gene (Kurtzman and Robnett, 1998) allowed the identification of yeast species such as *I. occidentalis*, *Geotrichum candidum, Hanseniaspora guilliermondi, S. cerevisiae* and *C. boidinii* (Arroyo-López et al., 2006). *W. anomalus*, *C. boidinii* and *Debaryomyces etchellsii,* predominant species in French naturally black olives, were identified by rDNA ITS method and sequencing of the D1/D2 region of the 26S rRNA gene (Coton *et al.*, 2006). Hurtado *et al.,* (2008) identified the species *C. boidinii*, *Candida sorbosa*, *Candida diddensiae*, *Candida membranifaciens*, *K. lactis*, *Pichia membranifaciens*, *W. anomalus*, *P. kluyveri*, and *Rhodotorula glutinis* from of Arbequina table olives. However, in 2011, sequencing of domains 1 and 2 (D1/D2) of the LSU rRNA gene and/or the ITS1-5.8SITS2 region were proposed as universal barcode for fungi (Schoch *et al.*, 2012).

With reference to molecular typing, molecular method commonly used both for LAB and yeasts in fermented food are rep- PCR and RAPD-PCR (Andrighetto *et al.,* 2000; Tofalo

et al., 2013; *Mari et al.*, 2016). Molecular typing was used to group the isolates from olive fermentations in relation to their similarity and subsequently to choose the representative strains, which are identified using the techniques described above (Torriani et al., 2001; Gardini *et al.*, 2006).

1.6 Biofilm

Biofilms are defined as structured communities of microbial cells enclosed in a self-produced polymeric matrix adherent to abiotic or biotic surfaces (Steenackers *et al.*, 2012). Presence of biofilms consists of yeast or LAB or both microorganisms in fermented food such as wine and beer is well documented. Regarding yeasts, biofilm formation of *S. cerevisiae* and its mechanism was described from several authors (Budroni *et al.*, 2005; Zara *et al.*, 2009; Legras *et al.*, 2016).

An essential requirement for potential probiotic/human-healthy olive yeasts is that they must be able to adhere to olive skin to survive during storage/packaging and to be finally ingested by consumers at elevated numbers (Arroyo-López *et al.*, 2012). For this reason, recently attention of researchers focus on biofilm formed on olive skin during the fermentation (Domínguez-Manzano *et al.*, 2012; Arroyo-López *et al.*, 2012; Grounta and Panagou, 2014; Grounta *et al.*, 2015; León-Romero *et al.*, 2016). Imagines obtained by Scanning Electronic Microscope of biofilm revealed presence of yeasts and LAB embedded in an extracellular matrix. Arroyo-López *et al.*,(2012) and Domínguez-Manzano *et al.*, (2012) reported presence of mixed species biofilm formed by yeasts and LAB on the epidermis of Gordal and Manzanilla fruits processed according to Spanish style. *Pichia galeiformis*, *C. sorbosa* and *G. candidum* for the yeast species, and *L- pentosus* for LAB on olive skin. In a scanning electron microscopy study, Grounta and Panagou, (2014) showed the formation of biofilms between *L. pentosus* and *P. membranifaciens* on oxidized Greek black olives, while Benítez-Cabello *et al.*, (2015) described the formation of microbial biofilms on the epidermis of directly brined "natural" green olives. Recently, Grounta *et al.*, (2015) have investigated the formation of biofilm on abiotic surface of fermentation

vessels and observed that the most abundant species were *W. anomalus, D. hansenii* and *P. guilliermondii.*

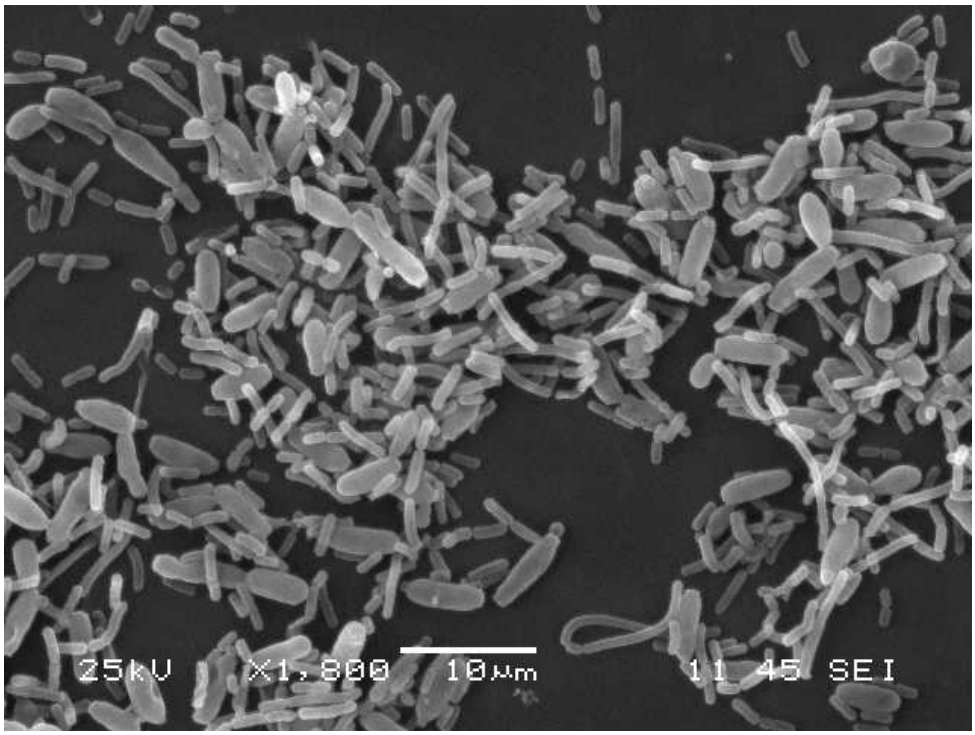

Figure 4. Scanning electron microscopy (SEM) image of mixed biofilm of LAB and yeast on olive surface. (Olifilm)

Probably, the formation of this mixed biofilms improves their viability during the passage through the gastrointestinal tract (Arroyo-López *et al.,* 2012). However, biofilm formation have an impact on table olive processing.

In a recent work about *in vitro* ability of *L. pentosus* and yeast isolated from table olives to form biofilm, was reported strong biofilm production in *Candida boidinii- L. pentosus* mixed cultures (León-Romero *et al.*, 2016).

1.7 Aim of thesis

The aim of this PhD thesis were:

- to design an autochthonous multifunctional starter for table olive processing in order to improve and optimize the fermentation process and the quality and safety of the final product.
- to identify genes related to biofilm formation by genome sequencing of different strains of *C. boidinii*.

In order to obtain the first objective, characterization of the yeast biodiversity of Bosana brines using RAPD-PCR analysis with primer M13 and sequencing of D1/D2 domain of 26S gene was carried out. Moreover, qualitative and quantitative technological properties and probiotic features were investigated. Subsequent bioinformatic analysis was applied for obtaining dendrogram of yeast population and selecting representative strains to evaluate:

- enzymatic activities (esterase, β-glucosidase and lipase) and assimilation of oleuropein by quantitative spectrophotometric assays;
- quantitative resistance to NaCl by modelling techniques;
- probiotic properties *in vitro:* resistance to simulated gastric and pancreatic digestion and cholesterol removal;
- *in vitro* biofilm formation in mono-culture and mixed culture with *Lactobacillus pentosus*.

Data were analysed by ANOVA and multivariate analysis to select yeast strains with the best probiotic and technological characteristics.

To achieve the second objective, genotyping of eight strains o*f C. boidinii* and biofilm-forming ability *in vitro* were assessed. Finally, sequencing of whole genomes of all *C. boidinii* strains was performed.

2. MATERIALS AND METHODS

2.1 Strains

This study was conducted on 72 isolates of yeasts obtained from Bosana table olives brines, a widespread traditional variety of Northern Sardinia, collected during the 2003/2004 season (Pinna, 2005). Drupes of Bosana cultivar were harvested during the 2003/2004 season at black ripening stage. After sorting, sizing, and washing, the olive fruits were dried and were put into barrels. Each barrel had 20 L of total capacity, and was filled with 10.5 kg of olive fruits and 9.5 L of brine (6% NaCl w/v), with a ratio/brine drupe of 1:1.1. Spontaneous fermentation started when olives were placed in the brine. Yeasts were isolated as described by Pinna (2005) and stored for long term in yeast extract, peptone, glucose and glycerol (YEPGly) at -80°C. Reference strains are listed in Table 1.

Saccharomyces cerevisiae strain BY4741 was used in β-glucosidase assay and *Yarrowia lipolytica* 1 in esterase assay. *Yarrowia lipolytica* 1 belong to UNISS collection of University of Sassari. *Wickerhamomyces anomalus* strain Y45 was used to assess reproducibility of RAPD-PCR. Strains of *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG were used as reference strain for probiotic test. *Lactobacillus pentosus* TOMC 2 was used in biofilm assay because it is capable to produce biofilm. Strain Y45, TOMC 2 and *L. rhamnosus* GG belongs to Table Olives Microorganisms Collection (TOMC) of Institute of la Grasa (CSIC, Seville). Eight strains of *C. boidinii* in single culture and combined culture with the LAB *L. pentosus* TOMC 2, *L. plantarum* TOMC 9 and *Pediococcus pentosacesus* TOMC 56, were studied for their capability to form biofilm *in vitro*. Strains DBVPG 6799, DBVPG 7578 and DBVPG 8035 were purchased from Industrial Yeasts Collection of University of Perugia. Strain NDK27A1 was provided by Giuseppe Blaiotta (University of Naples). All isolates and the reference stains were stored at -80 ° C in a Yeast extract Peptone Dextrose medium (YPD) or YM containing 30% (v / v) glycerol until use. *Lactobacillus pentosus* TOMC 2, *L. plantarum* TOMC 9 and *Pediococcus pentosaceus* TOMC 56 were maintained in MRS broth supplemented with 25% (v/v) of glycerol.

Species	Strain	Origin	Characteristics
Candida boidinii	Cb18	UNISS culture, direcly brined table olives	Biofilm assay
Candida boidinii	Cb60	UNISS culture, directly brined table olives	Biofilm assay
Candida boidinii	DBVPG 6799	Industrial Yeasts Collection of University of Perugia, Cactus *Opuntia*	Biofilm assay
Candida boidinii	DBVPG 7578	Industrial Yeasts Collection of University of Perugia, soil	Biofilm assay
Candida boidinii	DBVPG 8035	Industrial Yeasts Collection of University of Perugia, fresh water lake	Biofilm assay
Candida boidinii	NDK27A1	University of Naples, wine	Biofilm assay
Candida boidinii	TOMC Y13	Table olives, Table olives microrganisms collection of Institute de LaGrasa, (CSIC, Seville)	Biofilm assay
Candida boidinii	TOMC Y47	Table olives, Table olives microrganisms collection of Institute de La Grasa, (CSIC, Seville)	Biofilm assay
Lactobacillus pentosus	TOMC 2	Table olives, Table olives microrganisms collection of Institute de La Grasa, (CSIC, Seville)	Biofilm assay
Lactobacillus plantarum	TOMC 9	Table olives, Table olives microrganisms collection of Institute de La Grasa, (CSIC, Seville)	Biofilm assay
Lactobacillus rhamnosus	GG	Table olives microrganisms collection of Institute de La Grasa, (CSIC, Seville)	Probiotic control for *in vitro* simulated GD and PD
Pediococcus pentosaoceus	TOMC 56	Table olives, Table olives microrganisms collection of Institute de La Grasa, (CSIC, Seville)	Biofilm assay
Saccharomyces boulardii		Zambon	Probiotic control for *in vitro* simulated GD and PD
Saccharomyces cerevisiae	BY4741	Laboratory strain deposited at UNISS collection Parental strain for the International Systematic Saccharomyces cerevisiae Gene Disruption Project	Negative control for β-glucosidase assay
Yarrowia lipolytica	1	Laboratory strains deposited at UNISS culture collection	Positive control for esterase assay

Tab 1. Reference strains used in this study. CSIC, Consejo Superior de Investigaciones Cientificas; GD, gastric digestion; PD, pancreatic digestion.

2.2 Culture media

Media used for culture maintenance and development are shown below.

YEP medium supplemented with glycerol (YEPGly)

Yeast extract	10.0 g/L
Glucose	20.0 g/L
Peptone	20.0 g/L
Glycerol	20%

Yeast Mold Agar (YM, Difco)

Yeast Extract	3.0 g/L
Malt Extract	3.0 g/L
Peptone	5.0 g/L
Dextrose	10.0 g/L
Agar	20.0 g/L

YM Broth (Difco)

Yeast Extract	3.0 g/L
Malt Extract	3.0 g/L
Peptone	5.0 g/L
Dextrose	10.0 g/L

YPD agar

Yeast extract	10 g/L
Dextrose	20 g/L
Peptone	20 g/L
Agar	20 g/L

YPD Broth

Yeast extract	10.0 g/L
Dextrose	20.0 g/L
Peptone	20.0 g/L

Man, Rogosa and Sharpe broth (MRS, Oxoid)

Magnesium sulphate	0.2 g/L
Dipotassium hydrogen phosphate	2.0 g/L
Triammonium citrate	2.0 g/L
Yeast Extract	4.0 g/L
Sodium acetate 3 H_2O	5.0 g/L
Lab-Lemco Powder	8.0 g/L
Peptone	10.0 g/L
Sorbitan mono-oleate	1 mL
Dextrose	20.0 g/L

Yeast Nitrogen Base (YNB, Difco)

Ammonium sulphate	5.0 g/L
Monopotassium Phosphate	1.0 g/L
Magnesium Sulphate	0.5 g/L
Sodium Chloride	0.1 g/L
Calcium Chloride	0.1 g/L
L-Histidine Monohydrochloride	0.01 g/L

Arbutin agar (β-glucosidase activity)

Arbutin	5 g/l
(hydroquinone-β-D-glucopyranoside)	
Yeast extract	10 g/l
Ferrous Ammonium Citrate	20 drops

Agar	15g/L

Tributyrin Agar (esterase activity)

Special peptone	5 g/L
Yeast extract	3 g/L
Tributyrin	10 g/L
Agar	12 g/L

YNB-CG (cholesterol removal)

Yeast Nitrogen Base	0.67 g/L
Glucose	10 g/L
Cholesterol	182 mg/L

YNB-CGO (cholesterol removal)

Yeast Nitrogen Base	0.67 g/L
Glucose	10 g/L
Oxgall	3 g/L
Cholesterol	182 mg/L

YNB-CO (cholesterol removal)

Yeast Nitrogen Base	0.67 g/L

Oxgall	3 g/L
Cholesterol	182 mg/L

2.3 Isolates identification

2.3.1 Getyping and identification of yeast isolates

Genomic DNA extraction was performed in accordance with the protocol of Senses-Ergul *et al.* (2012).

Yeasts were cultured overnight in YPD broth at 28°C in an orbital shaker at 150 rpm. Cultures were centrifuged at 14,000 rpm for 5 minutes. After removing of the supernatant, the pellet obtained was washed and re-suspended in 1 mL of sterile ultrapure water. The cells were lysed with 200 μL of breaking buffer (2% Triton X v / v, 1% SDS v / v, 100 mM NaCl, 10 mM Tris-HCl, 1 mM Na_2EDTA) and treated with 200 μL solution of phenol-chloroform-isoamyl alcohol (25: 24: 1, v / v) and 0.3 g of glass beads of diameter <106 μm (Sigma-Aldrich, USA). Samples were vortexed for 3 minutes and centrifuged for 5 minutes at 14,000 rpm. After centrifugation, the DNA contained in the supernatant was precipitated with the addition of 3 volumes of 96% ethanol, washed with 70% ethanol, dried and re-suspended in sterile water. Extracted DNA was quantified using the Spectrostar Nano spectrophotometer (BMG LABTECH, Germany) through the absorbance reading at 260 nm.

2.3.2 Molecular identification of yeast species

In the present work, to identify yeast isolates at species level, two methods were applied:

i. random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) with primer M13;

ii. sequencing of D1/D2 region of 26S rDNA gene.

2.3.2.1 Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) with primer M13 was used for discrimination at strain level. Reaction mixture was carried out

in 25 µl containing 80-100 ng/µL of genomic DNA, 5 µl of 5X buffer MyTaq™ (Bioline, USA), 1 µL (25 pmol/ µL) of M13 primer (5 'GAGGAGGGTGGCGGTTCT 3', (Huey and Hall, 1989), 0.1 µL (0.5 U) of MyTaq™ DNA polymerase (Bioline, USA).

The PCR was conducted in a MasterCycler Pro (Eppendorf, Germany) and the program consisted of the following steps: 1) initial denaturation step at 95 ° C for 5 minutes; 2) 45 cycles of: 93 ° C for 45 seconds, 44.5 ° C for one minute, 72 ° C for one minute; 3) final extension was performed at 72 ° C for 6 minutes (Andrighetto *et al.,* 2000). Negative control PCR mixture without DNA was included in all experiments.

2.3.2.2 PCR products electrophoresis

RAPD-PCR products were separated by electrophoresis on 2% (w/v) agarose gel. Electrophoresis was carried out at 70 V for 90 minutes in TAE buffer (Promega, USA). Ladder 1 kb (Invitrogen, USA) was used as size marker of DNA fragments. After electrophoresis, gel was stained with ethidium bromide solution (10 mg/L). Gel was visualized with a transilluminator Enduro™ GDS (Labnet International, Inc., USA). The RAPD profiles were captured and analysed using BioNumerics 6.6 software (Applied Maths, Belgium). Dendrogram was built with UPGMA (Unweighted Pair Group Method) method and Pearson correlation (optimization 0.5%, curve smoothing 0%). Reproducibility and sensibility of RAPD-PCR were evaluated by comparing the profiles of strain Y45 obtained from different DNA extractions, different PCR and different gels performed in different days.

2.3.2.3 Sequencing of D1/D2 region of 26S rDNA gene

To corroborate the clustering analysis and for identification of strains, the 26S rDNA gene of all isolates was further sequenced. The genomic DNA was amplified by using the primers NL1 and NL4 (Kurtzman and Robnett, 1998). Sequence of primers were:

NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′

NL4 5′-GGTCCGTGTTTCAAGACGG-3′

PCR reactions were carried out in a volume of 25 µL containing 1 µL of DNA, 1.25 µl (25 pmol/ µl) of each primer, 5 µl of 5X buffer (Bioline, USA) and 0.1 µL (0.5 U) of Taq polymerase (Bioline, USA). All amplification reactions were carried out in a thermocycler MasterCycler Pro (Eppendorf, Germany) programmed as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 ° C for 1 minute, 55.5 ° C for 2 minutes, 72 ° C for 2 minutes followed by a final extension at 72 ° C for 10 minutes.

2.3.5 DNA purification and sequencing

Amplification products were analysed by electrophoresis on agarose gel (1% w/v) stained with ethidium bromide (10 mg/L). Molecular weight of amplified DNA fragments was evaluated by comparison with a DNA ladder 1 kb ladder plus (Invitrogen, USA). Purification of the PCR products was carried out using Isolate DNA kit (Bioline, USA) according to the manufacturer's instructions. Quality and amount of purified PCR product was evaluated by agarose gel electrophoresis (1% w/v). An amount of 10 µL of purified product with forward primer NL1 was used for sequencing by Stab Vida (Lisbon, Portugal).

2.3.6 Sequences analysis

The alignment of sequences was performed with software MEGA 5.0 (Molecular Evolution Genetic Analysis). Sequences were compared with sequences deposited in data bank Gene Bank (http://www.ncbi.nlm.nih.gov/) using software BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information, USA).

2.4 Qualitative technological characterization

For qualitative technological characterization, yeast were cultured in YPD agar and incubated at 28°C for two days. Each experiment was carried out in duplicate.

2.4.1 Qualitative salt resistance assay

Growth in presence of NaCl was evaluated on YPD agar supplemented with different concentration of NaCl (Microbiol, Italy): 6%, 8%, 10% and 12% (w/v). Strains were streaked

on plates and incubated at 25°C for 5 days. Results were expressed with the follows: "-" no growth, "+" growth.

2.4.2 β-glucosidase assay

Evaluation of the β-glucosidase activity was assayed on Arbutin agar (Caridi *et al.*, 2005). Yeasts were streaked on the surface of the plates and incubated at 25 ° C for 7 days. The strain *Saccharomyces cerevisiae* BY4741 was used as negative control. The β-glucosidase activity was indicated by a darkening of the medium and was expressed according as follows: 0 = colourless; 1 = light brown; 2 = brown; 3 = dark brown.

2.4.3 Esterase activity

The esterase activity was evaluated on Tributyrin Agar (Sigma-Aldrich, Milan) (Hernández *et al.*, 2007) supplemented with an emulsion of Tributyrin (Sigma-Aldrich, Milan) and Tween 80 (Acros Organics, Belgium). The yeasts were seeded on TAT and incubated at 25 ° C for five days. Esterase activity is highlighted by a clear halo around the colonies and expressed as follows: "-" (no halo), "+" (alone defined). Strain 1 of *Yarrowia* lipolytica was used as positive control.

2.4.4 Catalase activity

The catalase activity of yeasts was assayed by the method of Whittenbury (1964), by adding 3% (v / v) of hydrogen peroxide (H_2O_2) on the colonies grown on YPD agar. Catalase activity was indicated by the development of oxygen bubbles due to H_2O_2 decomposition indicated the presence of catalase activity. The results were expressed as "-" (no activity) or "+" (activities).

2.4.5 Production of biogenic amines

The production of biogenic amines was evaluated as decarboxylase activity by the method described by Gardini *et al.* (2006). Briefly, was used YNB containing 1 g / l of amino acids precursors of biogenic amines (L-lysine, L-proline, glycine, alanine, L phenylalanine, L-tyrosine, L-histidine, ornithine and arginine hydrochloride) with violet

purple as indicator. Strains were grown on medium containing 0.67 g of YNB, 1 g / l of amino acids precursors of biogenic amines (L-lysine, L-proline, glycine, alanine, L phenylalanine, L-tyrosine, L-histidine, ornithine and arginine hydrochloride) and 0.06 g of bromocresol purple (Sigma-Aldrich, Milan). Medium was sterilized by filtration. Plates were incubated at 25 ° C for 72 hours. Medium without amino acids inoculated with yeast was used as a control. If yeasts have a decarboxylase activity, pH increases and a colour change of medium from yellow to purple occurs.

2.5 Quantitative technological characterization

Assays performed on representative strains chosen from each cluster are described in this section.

2.5.1 Quantitative salt resistance assay

Resistance to NaCl of yeast strains was monitored by Bioscreen C automated spectrophotometer (Labsystem, Finland) with a wideband filter (420-580 nm) (Bonatsou *et al.*, 2015). Briefly, overnight culture were centrifuged at 10,000 rpm and collected cells were re-suspended in sterile saline solution reaching an initial OD approximately 0.2. An amount of 20 μL of yeast inoculum was dispensed into wells of Honeycomb plate with 330 μL of sterilized YM broth (pH 4.5) supplemented with 0, 10, 30, 50, 70, 80, 90, 100, 110, 120,130, 150 and 180 g/L of NaCl. Uninoculated wells were included in Honeycomb plate to subtract noise signal. Microplates were incubated at 28°C with shaking of 3 seconds before each OD measurements. All experiments were performed in triplicate. Measures of OD were used to obtain 351 growth curve (9 yeast strains x 13 NaCl concentrations x 3 replicates). The curves were used to calculate the fractional area (*fa*) by comparison of area of OD/time curve of positive control (strain growth in absence of salt) with area of OD/time curve of the samples:

$fa = area_{test}/area_{cont}$

where $area_{test}$ is the area of yeast strains and $area_{cont}$ is the area of positive control. Integral of areas under OD curves were calculated with OriginPro software 7.5 (OriginLab Corporation, USA).

Results in form of fractional area *vs* logarithm of concentration of NaCl were analysed using the modified Gompertz equation (Bonatsou *et al.*, 2015):

$$y= \exp(- (x/\ln(MIC)/\exp(- (\ln(\ln(NIC)/\ln(MIC))/2.71828))))^{(-2.71828/\ln(\ln(NIC)/\ln(MIC))))}$$

where y is the *fa*, x is logarithm of NaCl concentration, MIC is the minimum NaCl concentration (g/L) that inhibits yeast growth and NIC is NaCl concentration where a negative effect on yeast growth begin to be detected.

2.5.2 Quantitative enzymatic assays

Quantitative enzymatic assays of β-glucosidase, esterase and lipase were determined in accordance with the protocol described by Bonatsou *et al*. (2015).

Enzymatic activities were measured using a spectrophotometric assay based on the ability of β-glucosidase, esterase and lipase to catalyse hydrolysis of 4-nitrophenyl-β-D glucoside and 4-nitrophenyl palmitate and *p*-nitrophenyl stearate to 4-nitrophenol, respectively. The concentration of 4-nitrophenol released from each chromogenic substrate was determined by reading the absorbance at 410 nm. A calibration curve was plotted by measuring the absorbance of 12 solution containing different concentration (10-200 μmol/L) of nitrophenol (Figure 5).

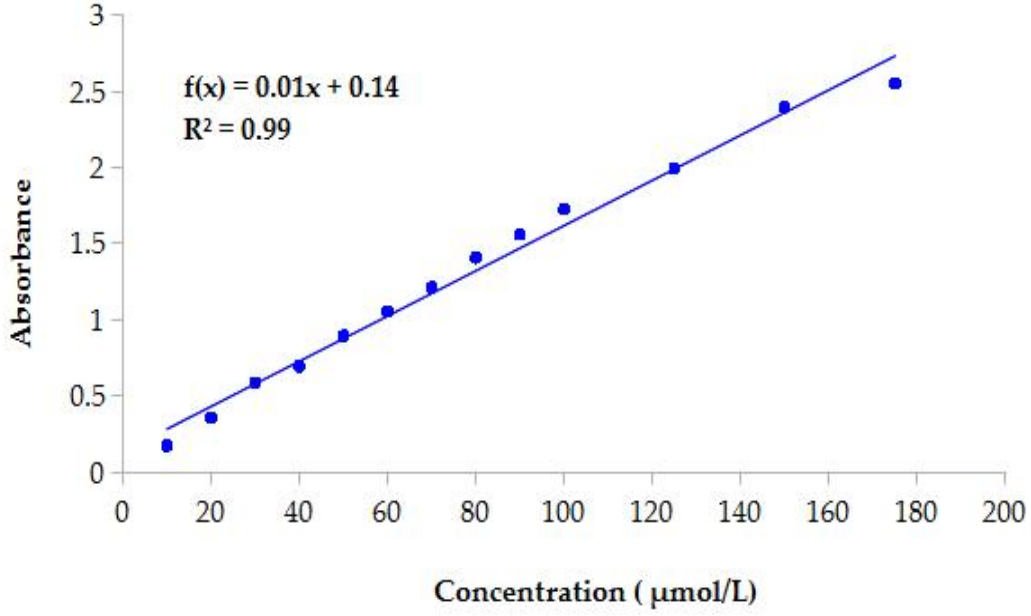

Figure 5. Calibration curve of nitrophenol for enzymatic assay.

The coefficient obtained from the slope of the calibration curve was used to calculate the molar extinction coefficient (ε).

Molar extinction coefficient allows to determine concentration of nitrophenol in according to Beer-Lambert law:

$$A = \varepsilon c l$$

where A is the absorbance of sample, ε is the molar extinction coefficient, c is the 4-nitrophenol concentration and l is the path length. The equation of calibration curve was $A = 0.01c + 0.14$. The correlation coefficient (R^2) was 0.99 and indicated a good linearity between concentration and absorbance.

Before each quantitative enzymatic assays, yeasts were cultured in YM broth and incubated at 28°C for 72 hours at 150 rpm. Yeasts culture were centrifuged at 10,000 rpm for 15 minutes to collect cells. Then, supernatant was sterilised by filtration with 0.2 µm filter (Millipore Co., USA) and used as extracellular fraction. Harvested cells were washed

twice with 50 mM sterile phosphate buffer (pH 7.0) for esterase and lipase activity and 100 mM of sterile citrate phosphate buffer at pH 5.0 for β-glucosidase assay. Finally, pellet was re-suspended in same buffer and used to perform enzymatic activities. Each assay was carried out in duplicate. The data were expressed in $nmolh^{-1}*ml^{-1}$ under assay conditions. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.5.2.1 β-glucosidase activity

The reaction mix was composed of 200 μL of sample and 200 μL of *p*-nitrophenyl-β-D glucoside 3mM. After incubation of 1 hour at 40°C, reaction was stopped by adding 1.5 mL of 0.2 M carbonate buffer (pH 10). Samples were read against the blank in a Cary 1E UV spectrophotometer (Varian INC., Palo Alto, USA) at 400 nm.

2.5.2.2 Esterase and lipase activity

The reactions to perform esterase and lipase activities were started by addition of 1 mL of corresponding substrate dissolved in undecane (1 mM) to 1 mL of cellular and extracellular fractions. Samples were incubated for 24 hours at 40° C. Reaction was stopped by adding 0.2 M carbonate buffer (pH 10) to 1 mL of aqueous phase. The absorbance at 410 nm was measured in Cary 1E UV spectrophotometer.

2.5.3 Growth in presence of oleuropein

To determine the capacity of the yeast strains to use oleuropein as carbon source, overnight cultures was centrifuged for 10 minutes at 10,000 rpm and pellet was re-suspended in sterile saline solution (0.9 g/L). Then, 20 μL of yeast inoculum (OD ~ 0.2) was added to 330 μL of sterile YNB broth (pH 4.5) supplemented with 1% (w/v) of oleuropein (Extrasynthese, France). Samples were incubated at 28°C for 7 days in a Bioscreen spectrophotometer. Measurements of OD were taken every 2 hours and, before each measurement, samples were automatically shaken for 3 seconds. Uninoculated wells were used as negative control. Each test was carried out in triplicate.

2.6 Probiotic characteristics

2.6.1 I*n vitro* simulated gastric and pancreatic digestion

The assay was performed according with the procedure described by Bonatsou *et al.* (2015). The GI digestion was reproduced in 100 ml plastic tube maintained under stirring (250 rpm) at constant temperature of 37° C. The GI digestion was divided into two steps: gastric and pancreatic digestion. For gastric digestion, 30 ml of O/N preculture was washed with sterile saline solution (0.9 g/L) and subsequently suspended in 30 ml of synthetic gastric juice at pH 2 including NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), KCl (0.37 g/L), ($CaCl_2$) 0.11 g/L, 0.0133 g/L of pepsin, 0.01 g/L of lysozyme (Sigma-Aldrich,). To simulate stomach conditions, samples were incubated at 37°C for 2.5 hours at 250 rpm. Serial dilutions of samples were streaked on YM agar (Difco) before and after gastric digestion for plate count and incubated at 28°C for two days.

At the end of gastric digestion, cells were immediately washed with 10 mL of saline solution and suspended in synthetic pancreatic juice at pH 8 including Na_2HPO_4 (50.81 g/L), NaCl (8.5 g/L), bile (3 g/L) and pancreatin (0.1 g/L). After 3.5 hour of incubation at 250 rpm, serial dilutions were seeded on YM agar. Petri dishes were incubated at 28°C for two days. Each experiment was performed in triplicate.

2.6.2 *In vitro* cholesterol removal

The ability of yeast strains to remove cholesterol was determined in different media, namely YNB, YNB-CG, YNB-CGO and YNB-CO. Culture media composition are listed in table 2.

Medium	YNB (g/L)	Cholesterol (mg/L)	Glucose (g/L)	Oxgall (g/L)
YNB	0.67			
YNB-CG	0.67	182	10	
YNB-CGO	0.67	182	10	3
YNB-CO	0.67	182		3

Tab 2. Media formulations for study ability of selected yeast strains to remove cholesterol

Yeast cells were harvested by centrifugation at 10,000 rpm for 10 minutes and re-suspended in sterile saline solution for 2 hours. 20 μL of yeast suspension was added to 230 μL of each medium. After an incubation of 48 hours at 37° C, surnatant was used to check removal of cholesterol with a kit from BioSystems (Spain). Briefly, 120 μL of surnatant was added to 450 μL of Reagent A, mixed thoroughly and incubated for 5 minutes at 37°C. Cholesterol quantification was performed by UV spectrophotometric analysis. Absorbance was measured at 500 nm against the blank.

A calibration curve was made from analysis of 6 solutions with known cholesterol concentration (Figure 6).

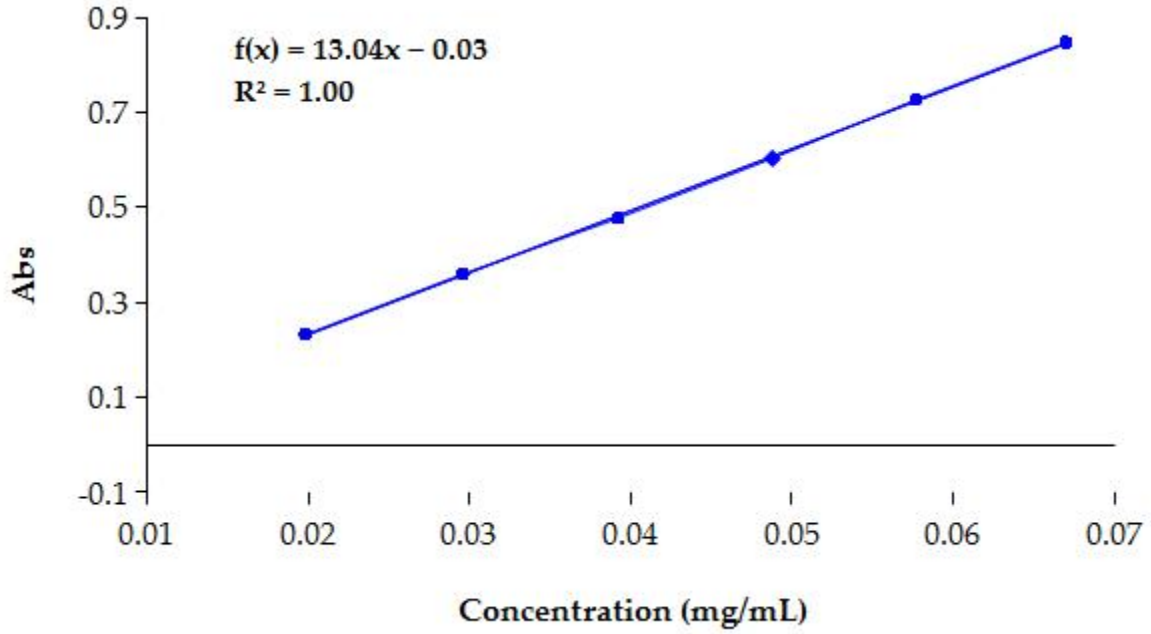

Figure 6. Calibration curve for spectrophotometric analysis of cholesterol. Abs, absorbance.

Regression coefficient (R) line was 1.00 for cholesterol ranging between 0.02 mg/mL and 0.07 mg/mL and indicated a good linearity. The molar extinction coefficient, that coincides with the angular coefficient of the right line, was 13.04.

The tubes without yeast inocula were used as negative control. Each experiment was performed in triplicate for each medium.

2.7 Interaction between yeasts and lactic acid bacteria

2.7.1 *In vitro* biofilm production

To assess the ability of eight selected yeast strains and *S. boulardii* to develop biofilm *in vitro* the protocol described by (Toledo-Arana *et al.*, 2001) was used. The ability to form biofilm was evaluated by quantification of biomass on the bottom of microtiter plate wells with crystal violet (CV) staining. The crystal violet absorbed by the biofilm is then dissolved. The absorbance of the obtained solution is proportional to the amount of biofilm on the bottom of well.

In particular, the wells of 96-well microplate (Nunclon, Thermofisher Scientific) were inoculated with 100 μL of overnight culture of each strain. To perform mixed culture experiment, wells were filled with 100 μL of *L. pentosus* TOMC 2 culture and 100 μL of yeast suspension. Control were YM and MRS alone. After a 48 hours incubation at 28°C, liquid was removed from wells and wells was washed twice with sterile saline solution. Subsequently, 200 μL crystal violet solution (0.8% w/v) was added to each well. Plates were incubated at room temperature for 30 minutes and wells were washed twice with sterile distilled water. Finally, 200 μL of an ethanol-acetone mixture (80:20, v/v) was added in order to extract crystal violet bound to biofilm. After an incubation of 30 minutes at room temperature, the absorbance at 595 nm was determined with a spectrophotometer Spectrostar Nano (BMG Labtech, Germany). Wells containing medium without microorganisms were used as negative control. The experiment was carried out with six replicates for each sample. The OD_{595} values obtained were processed and the strains were classified as follows:

- non biofilm forming $OD_{595} \leq 1$
- weak biofilm $1 < OD_{595} \leq 2$
- medium biofilm $2 < OD_{595} \leq 3$
- strong biofilm $OD_{595} > 3$

2.7.2 Biofilm assay of *Candida boidinii-Lactobacillus pentosus* mixed cultures

To investigate if microorganisms contact is involved in *C. boidinii-L. pentosus* biofilm formation, a mixed culture experiment on 24-well polystyrene plate (CELLSTAR, Greiner Bio-One, USA) was performed in according to the protocol of León-Romero *et al.* (2016). Each well of plate contained a 0.4 μm pore size (ThinCert – TC Inser membrane) that allowed to obtain a two-compartment device. Two hundred μL of overnight culture of *C. boidinii* (strain 18 and strain 60) was inoculated into the downer compartment and filled with YM broth until reaching a volume of 2 mL. Then, 200 μL overnight culture of *L. pentosus* TOMC 2 was added in the upper compartment and filled until 2 mL. Plate was incubated at 28° C for 48 hours.

Subsequently, biofilm were observed under scanning electronic microscope by method described from León-Romero *et al.*, (2016). The biofilm was fixed with 2.5 % glutaraldehyde in PBS overnight. The slides were washed two times in PBS for 15 minutes and then dehydrated in a series of increasing concentrations of ethanol (50 %, 70 %, 80 %, 90%, 95% and 100%) for 15 minutes. Finally, the slides were treated in *tert*-butyl alcohol. The samples were coated with gold and observed using a Jeol JSM-6460LV scanning electron microscope (Jeol USA, Inc., Peabody, MA) at the Centro de Investigación, Tecnología, e Innovación of Seville University (CITIUS, Seville, Spain).

2.7.3 Biofilm production in mixed cultures of *Candida boidinii* and lactic acid bacteria

Ability to form biofilm of eight strains belonged to *C. boidinii* species and three strains of lactic acid bacteria was tested with microtiter plate method described above. The strains of *C. boidinii* and LAB used in this study were obtained from different sources: four strains from table olives, one strain from wine, one from *Cactus Opuntia*, one from soil and one from lake water. Biofilm formation assay was performed as mentioned above. The assay was carried out for single culture and every combination of each strain of *C. boidinii* with each strain of lactic acid bacteria. Strains of *C. boidinii* were isolated from different sources

and different countries. Lactic acid bacteria strains TOMC 2, TOMC 9 and TOMC 56 were isolated from table olives.

2.8 Statistical analysis

Microbiological data and concentrations were expressed as mean of replicates ± standard deviation. To compare differences among yeast strains subjected to technological, probiotic and biofilm experiments, analysis of variance ANOVA followed by *post-hoc* Scheffé test were applied. Scheffé test was chosen because is considered one of conservative post-hoc tests. Moreover, Scheffé's procedure can be used with unequal sample sizes. Data of quantitative enzymatic activities, simulated gastric and pancreatic digestion, quantitative resistance to salt and assimilation of oleuropein were compared with one-way ANOVA. For cholesterol removal and biofilm production factorial ANOVA was applied. Factors analysed were yeast strain (for cholesterol removal and biofilm assay), growth medium (YNB-CG, YNB-CGO and YNB-CO for cholesterol removal) and mixed culture yeast- *L. pentosus* (biofilm assay).

To select yeast strain with desirable technological and probiotic properties, multivariate analysis was applied. Principal Component Analysis is a multivariate method used to manage and reduce a large dataset of quantitative variables by orthogonal transformation. This method describes relations between cases (8 selected strains and *S. boulardii*) and variables (probiotic and quantitative technological characteristics). The obtained values were called principal components. The Kaiser criterion was applied to select number of Principal Component and only factors with eigen-values higher than 1.00 were maintained. The considered variables were:

- percentage of isolation (% Isolation);
- cellular lipase (L-Ce);
- extracellular lipase (L-Ex);
- cellular esterase (E-Ce);
- extracellular esterase (E-Ex);

- cellular β-glucosidase (B-Ce);
- extracellular β-glucosidase (B-Ex);
- gastric and pancreatic digestions (GD and PD, respectively);
- cholesterol assimilation in medium supplemented with cholesterol and glucose, cholesterol, glucose and Oxgall and cholesterol and Oxgall (RC CG, RD CGO and RC CO, respectively);
- susceptibility and resistance to NaCl (NIC and MIC values, respectively);
- oleuropein degradation (Oleu);
- production of biofilm *in vitro* of monoculture and in presence of *L. pentosus* TOMC 2 (Auto-aggregation and Co-aggregation LAB2, respectively).

Statistica software version 7.1 (StatSoft Inc, OK, Tulsa, USA) was used for data processing, statistical analysis and generation of graphic.

2.9 *Candida boidinii* genome sequencing

2.9.1 Genomic DNA extraction of *Candida boidinii* strains

DNA extraction of strain of *C. boidinii* was performed according to Borelli *et al.,* (2016) with slight modifications. First, yeasts strains were grown in YM broth overnight at 28°C and then yeast cells were washed with 1 mL of sterile mQ ultrapure water. Washed cells were collected at 15,000 rpm for 10 minutes. After removal surnatant, 200 µL of lysis buffer (2% Triton-X-100, 1% SDS v/v, 100mM NaCl, 10mM Tris-HCl pH 8, 1mM EDTA pH 8), 0.3 g of glass beads and 200 µL of Phenol + Chloroform +Isoamyl alcohol (25:24:1 v/v) were added to pellet. After vortexing for 2 minutes, 200 of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added. It was followed by centrifugation at 15,000 rpm for 10 minutes. The surnatant was transferred in another tube, 3 µL of RNase (10 µg/mL) was added and mixture was incubated at 37°C for 30 minutes. After incubation, DNA was precipitated

with 18 μL of Sodium Acetate (3M, pH 5.3) and 400 μL of cold ethanol 100%. After centrifugation for 15 minutes at 15,000 rpm surnatant was discarded and DNA pellet was washed with ethanol 70%. DNA pellet was suspended in 50 μL of TE buffer. The concentration and quality of extracted DNA were checked using Spectrostar NANO spectrophotometer.

2.9.2 Genotyping of *Candida boidinii* strains

For genotyping of *C. boidinii* strains with primer M13, the method described by Andrighetto *et al.,* (2000) with slight modification was used. The reaction mix for a volume of 25 μL was: 2 U of MyTaq™ DNA polymerase (Bioline, USA), 2.5 μL of 5X buffer MyTaq™ (Bioline, USA), 1μM of primer and 80 ng/μL of DNA. Amplification was performed using the following amplification conditions: initial denaturation step at 95 ° C for 5 minutes; 45 cycles of: 93 ° C for 45 seconds, 44.5 ° C for one minute, 72 ° C for one minute; final extension was performed at 72 ° C for 6 minutes.

3. RESULTS

3.1 Study of the yeast biodiversity in Bosana brines

A total of 72 isolates from Bosana brines were genotyped by RAPD-PCR analysis with M13 primers and further identified by sequencing analysis D1/D2 domains belonging to 26S rDNA gene. At first, reproducibility of RAPD technique was tested using a strain of *W. anomalus* TOMC Y45 as control. Having observed a similarity of 85.6% from 7 different DNA extractions, 7 different amplifications and 7 different running gels of the control the same technique was applied to 72 isolates. Amplification profiles produced by RAPD-PCR contained from a minimum of 2 bands to a maximum of 6 bands and the size of amplified fragments ranged between 400 and 1,650 bp. Eight different profiles were obtained from cluster analysis. Profiles were analysed with BioNumerics 6.6 software (Applied Maths, Belgium) and they were used to build the dendrogram showed in Fig. 7 by UPGMA method. All strains of the same species clustered together and clusters were named with numbers I-VIII. All clusters showed a similarity higher than similarity of control *W. anomalus* TOMC Y45.

All isolates were subjected to 26S rDNA amplification and sequencing for species identification. Sequencing of ITS1-5.8S-ITS2 regions and D1/D2 domain of 26S region were proposed as universal barcode for fungi by (Schoch *et al.*, 2012). Amplification produced fragments of approximately of 600 bp. The sequences obtained then were compared to NCBI GenBank database using BLAST software. Results of species identification are reported in Table 4. Sequences identity ranged from 99 to 100%.

Cluster I (90.3% of similarity) included 28 isolates that belonged to *W. anomalus* species. Cluster III (84.7% of similarity) was made of 27 isolates of *Candida molendinolei* (recently reclassified as *Nakazawaea molendini-olei* from Kurtzman et al. 2014). Five isolates of *C. diddensiae* were assigned to cluster IV (95.7% of similarity). Cluster V and VI were made of 6 isolates of *C. boidinii* (94.8% of similarity) and 4 isolates of *Zygotorulaspora mrakii* (97.7%

of similarity), respectively. Surprisingly, two isolates belonging to *C. diddensiae* and *C. boidinii* species did not cluster in group IV and V but grouped in clusters II and VII. Finally, one isolates of *S. cerevisiae* was assigned to group VIII. In figure 8 the distribution percentage of genotypes is shown. The most abundant genotype was *W. anomalus* (38.5%) followed by *N. molendini-olei* (34.7%). *C. boidinii* and *C. diddensiae* showed 2 genotypes, while *Z. mrakii* and *S. cerevisiae* had frequencies of 4.2% and 1.4% respectively. One representative strain from each cluster was chosen to be subject quantitative analysis of technological and probiotic properties together *S. boulardii*. Selected strains are indicated with red asterisks.

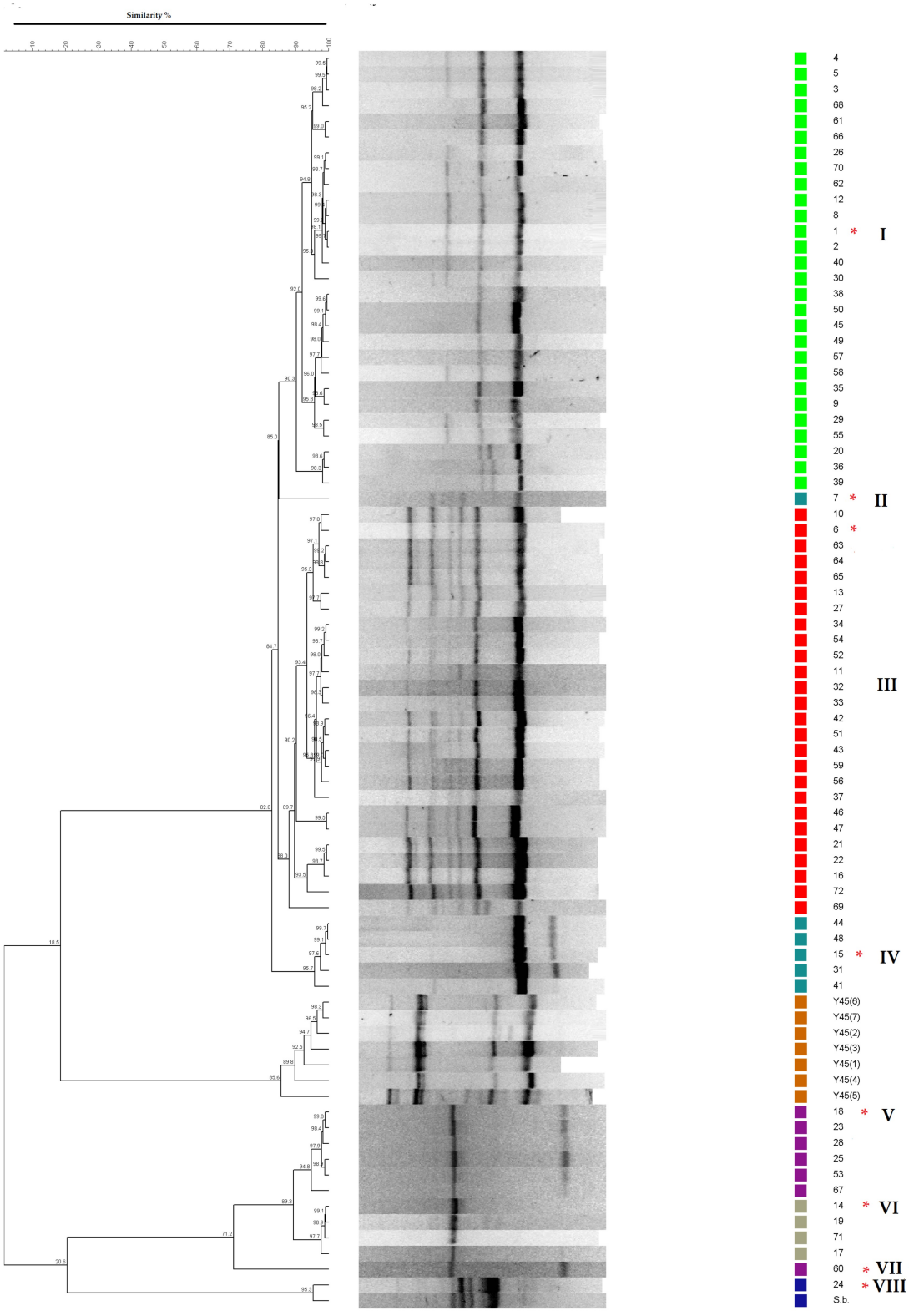

Figure 7. Dendrogram obtained by RAPD-PCR pattern of 72 isolates of Bosana brines. The dendrogram was built using UPGMA algorithm. The red asterisks indicate representative strain. The right column shows Roman numerals (I- VIII) of identified clusters. Y45: control strains used to assess reproducibility of RAPD-PCR. S.b: *S. boulardii*.

Cluster	Strain	Species	Identity percentage	Cluster	Strain	Specie	Identity percentage
I	1	*Wickerhamomyces anomalus*	100%	III	37	*Nakazawaea molendini-olei*	100%
I	2	*Wickerhamomyces anomalus*	99%	I	38	*Wickerhamomyces anomalus*	99%
I	3	*Wickerhamomyces anomalus*	99%	I	39	*Wickerhamomyces anomalus*	99%
I	4	*Wickerhamomyces anomalus*	99%	I	40	*Wickerhamomyces anomalus*	99%
I	5	*Wickerhamomyces anomalus*	100%	IV	41	*Candida diddensiae*	100%
III	6	*Nakazawaea molendini-olei*	99%	III	42	*Nakazawaea molendini-olei*	99%
II	7	*Candida diddensiae*	100%	III	43	*Nakazawaea molendini-olei*	99%
I	8	*Wickerhamomyces anomalus*	100%	IV	44	*Candida diddensiae*	100%
I	9	*Wickerhamomyces anomalus*	99%	I	45	*Wickerhamomyces anomalus*	99%
III	10	*Nakazawaea molendini-olei*	98%	III	46	*Nakazawaea molendini-olei*	100%
III	11	*Nakazawaea molendini-olei*	99%	III	47	*Nakazawaea molendini-olei*	100%
I	12	*Wickerhamomyces anomalus*	99%	IV	48	*Candida diddensiae*	100%
I	13	*Nakazawaea molendini-olei*	99%	I	49	*Wickerhamomyces anomalus*	100%
VI	14	*Zygotorulaspora mrakii*	100%	I	50	*Wickerhamomyces anomalus*	100%
IV	15	*Candida diddensiae*	100%	III	51	*Nakazawaea molendini-olei*	99%
III	16	*Nakazawaea molendini-olei*	99%	III	52	*Nakazawaea molendini-olei*	99%
VI	17	*Zygotorulaspora mrakii*	100%	V	53	*Candida boidinii*	100%
V	18	*Candida boidinii*	100%	III	54	*Nakazawaea molendini-olei*	99%
VI	19	*Zygotorulaspora mrakii*	100%	I	55	*Wickerhamomyces anomalus*	100%
I	20	*Wickerhamomyces anomalus*	99%	III	56	*Nakazawaea molendini-olei*	99%
III	21	*Nakazawaea molendini-olei*	99%	I	57	*Wickerhamomyces anomalus*	99%
III	22	*Nakazawaea molendini-olei*	99%	I	58	*Wickerhamomyces anomalus*	99%
V	23	*Candida boidinii*	100%	III	59	*Nakazawaea molendini-olei*	100%
VIII	24	*Saccharomyces cerevisiae*	100%	VII	60	*Candida boidinii*	100%
V	25	*Candida boidinii*	100%	I	61	*Wickerhamomyces anomalus*	100%
I	26	*Wickerhamomyces anomalus*	99%	I	62	*Wickerhamomyces anomalus*	99%
III	27	*Nakazawaea molendini-olei*	99%	III	63	*Nakazawaea molendini-olei*	99%
V	28	*Candida boidinii*	100%	III	64	*Nakazawaea molendini-olei*	99%
I	29	*Wickerhamomyces anomalus*	99%	III	65	*Nakazawaea molendini-olei*	99%
I	30	*Wickerhamomyces anomalus*	99%	I	66	*Wickerhamomyces anomalus*	99%
IV	31	*Candida diddensiae*	100%	IV	67	*Candida boidinii*	100%
III	32	*Nakazawaea molendini-olei*	99%	I	68	*Wickerhamomyces anomalus*	99%
III	33	*Nakazawaea molendini-olei*	99%	III	69	*Nakazawaea molendini-olei*	99%
III	34	*Nakazawaea molendini-olei*	100%	I	70	*Wickerhamomyces anomalus*	100%
I	35	*Wickerhamomyces anomalus*	99%	VI	71	*Zygotorulaspora mrakii*	100%
I	36	*Wickerhamomyces anomalus*	99%	III	72	*Nakazawaea molendini-olei*	99%

Tab 3. Identification of isolates by sequencing D1/D2 domain. The homology obtained by comparison of strains sequence with reference sequence of GenBank is reported.

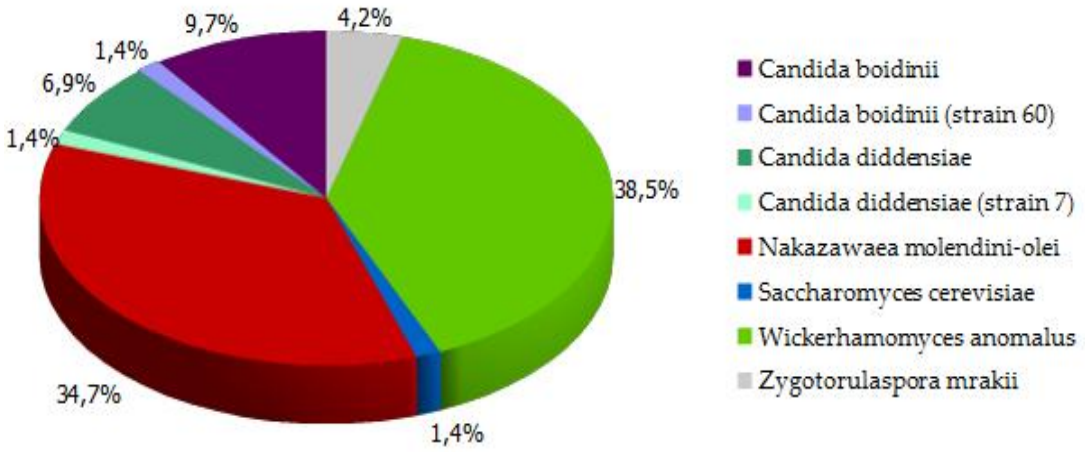

Figure 8. Distribution of genotypes of yeasts isolated from Bosana brines.

3.2 Qualitative technological characterization

Desirable properties (resistance to salt, β-glucosidase, catalase and esterase activities) and production of biogenic amines were qualitatively determined for all strains. Results are reported in Table 4.

Species	β-glucosidase	Catalase	Esterase	Biogenic amines
Candida boidinii	0	7	6	7
Candida diddensiae	1	6	6	6
Nakazawaea molendini-olei	2	26	26	0
Saccharomyces cerevisiae	0	1	1	0
Wickeramomyce anomalus	24	28	25	25
Zygotorulaspora mrakii	0	4	4	0

Tab 4. Enzymatic activities and biogenic production of yeast isolated from Bosana brines. Number indicates number of strains that exhibited each activity.

Figure 8 shows β-glucosidase activity. β-glucosidase activity was noticed in *W. anomalus* (24 isolates, 33.3%), *N. molendini-olei* (2 isolates, 2.78%), *C. boidinii* (one isolate, 1.39%) and *C. diddensiae* (one isolate, 1.39%).

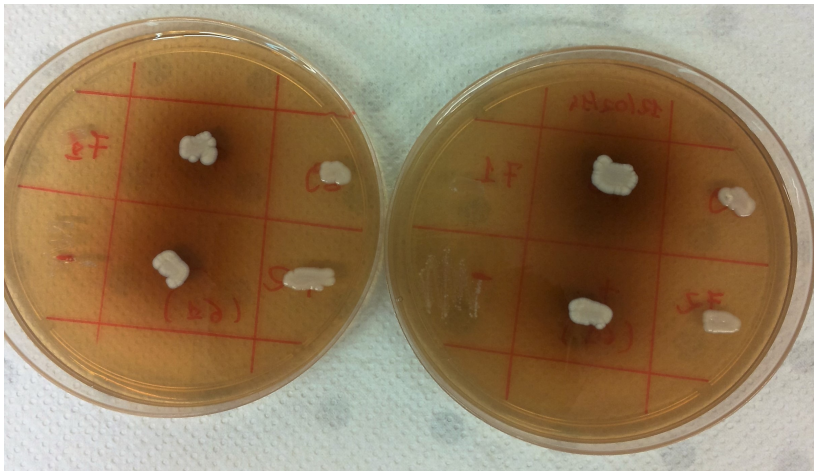

Figure 9. β-glucosidase activity. Brown halo indicated β-glucosidase production.

Catalase activity was exhibited by all isolates.

Esterase activity was exhibited by most of strains. Only 4 strains belonging to *W. anomalus* (3 isolates, 4.17%) and *C. boidinii* (one isolate, 1.39%) species did not produced esterase in Tributyrin agar.

Production of biogenic amines was detected for the species *W. anomalus* (26 strains), *C. diddensiae* (5 strains) and *C. boidinii* (7 strains). Strains of *N. molendini-olei*, *Z. mrakii* and *S. cerevisiae* did not produce biogenic amines *in vitro*.

Strain	6% of NaCl	8% NaCl	10% NaCl	12% NaCl
C. boidinii	7	7	7	0
C. diddensiae	1	1	1	1
N. molendini-olei	26	26	26	0
S. cerevisiae	1	1	1	1
W. anomalus	28	28	24	24
Z. mrakii	3	3	3	0

Tab 5. Growth of yeast strains on YPD supplemented with 6%, 8%, 10% and 12% of NaCl . Data are expressed as number of strains growth on medium.

Regarding the effect of NaCl on yeast strains assayed in YPD agar, all strains were able to grow at concentrations of 6, 8 and 10% with exception of four strains of *W. anomalus* and five strains of *C. diddensiae* that were not able to grow in YPD supplemented with 10% of NaCl. *C. diddensiae* (one strain), *S. cerevisiae* and *W. anomalus* (24 strains) were able to grow on YPD agar supplemented with 12% of NaCl. Results are summarized in Table 5.

3.3 Quantitative technological characterization

3.3.1 Salt resistance assay

The effect of different concentration of NaCl was evaluated on the selected yeast strains and on S. *boulardii* used as a control. For this purpose, a total of 351 growth curves (9 strains x 13 salt concentrations x 3 replicates) obtained in Bioscreen C spectrophotometer were analysed. Data were transformed into fractional areas, compared with control consisting of yeast strain growth in absence of salt and fitted with Gompertz equation. In figure 10 an example is reported of reparametrized Gompertz curve that describes relation between *fa* (fractional area) and ln of NaCl concentration. Curve can be split into three main sections: a first region corresponding to NaCl concentration below the NIC where no effect of salt was detected; a second section between MIC and NIC (a progressive inhibitory effect of NaCl is noticed) and a third section above the MIC, where no growth of control yeast was noticed.

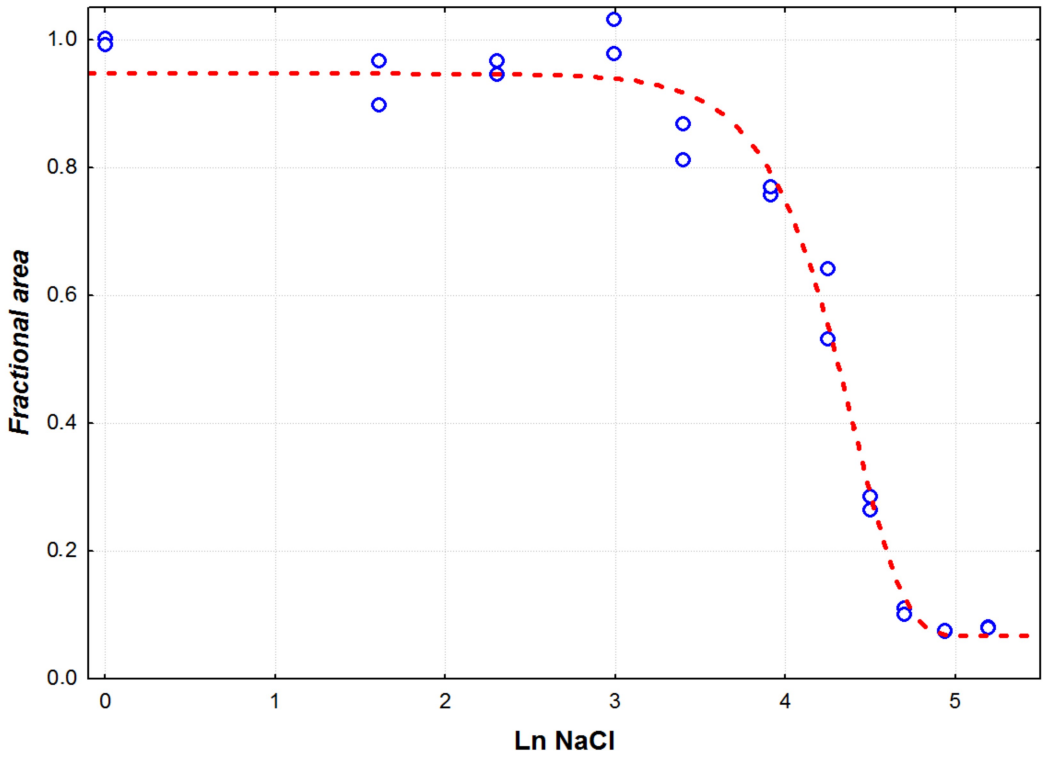

Figure 10. Example of reparameterized curve of Gompertz used to calculate MIC and NIC of *C. boidinii* Cb60.

Data of ANOVA carried out for MIC and NIC obtained for growth in presence of different concentrations of salt were resumed in Table 5 and Figure 11-12. MIC value, regarding resistance of yeast strains to NaCl, ranged from 86.2 g/L (*S. cerevisiae* Sc24) to 171.47 g/L (*W. anomalus* Wa1) while NIC value, related to susceptibility of yeast to NaCl, varied from 24.54 g/L of *S. boulardii* to 114.80 of *W. anomalus* Wa1. Graphics represented in Figure 11 and 12, show clearly that *W. anomalus* Wa1 exhibited the highest values of MIC and NIC while strains of *S. boulardii* and of *S. cerevisiae* Sc24 showed low resistance to salt.

Yeast reference	**MIC (g/L)**	**NIC (g/L)**
Sbo	86.39 (6.88) a	24.54 (4.36) b
Wa 1	171.47 (11.74) c	114.80 (6.26) d
Nm 6	118.92 (0.96) a, b	67.2 (2.35) a
Cd 7	98.95 (21.9) a	64.58 (7.16) a
Zm 14	96.74 (4.39) a	40.55 (2.14) b
Cd 15	140.12 (3.21) b, c	93.67 (7.40) c
Cb 18	110.11 (1.91) a, b	68.48 (1.11) a
Sc 24	86.29 (6.74) a	24.82 (4.13) b
Cb 60	115.43 (0.50) a, b	80.94 (1.60) a, c

Tab 6. MIC (minimum inhibitory concentration) and NIC (non inhibitory concentration) values obtained using growth model. Data was reported as mean and standard deviation in parenthesis. Different letters after each value indicate significant differences according to Scheffé test. Sbo, *S. boulardii*; Wa1, *W. anomalus*; Nm6, *N. molendini-olei*; Cd7 and Cd15, *C. diddensiae*; Zm14, *Z. mrakii*; Cb18 and Cb60, *C. boidinii*; Sc24, *S. cerevisiae*.

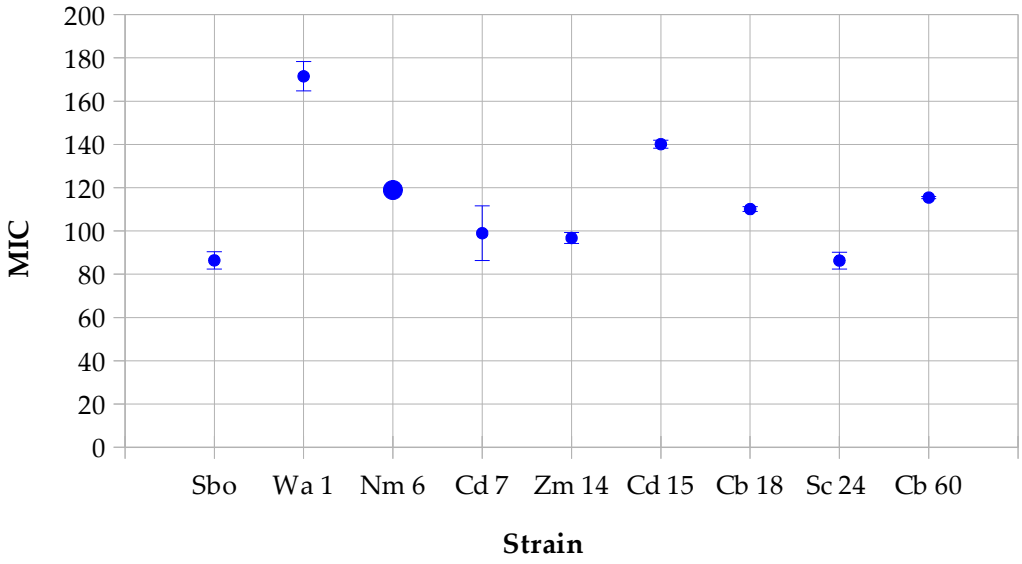

Figure 11. MIC values of selected strains isolated from Bosana brines. Standard error is indicate by vertical bars.

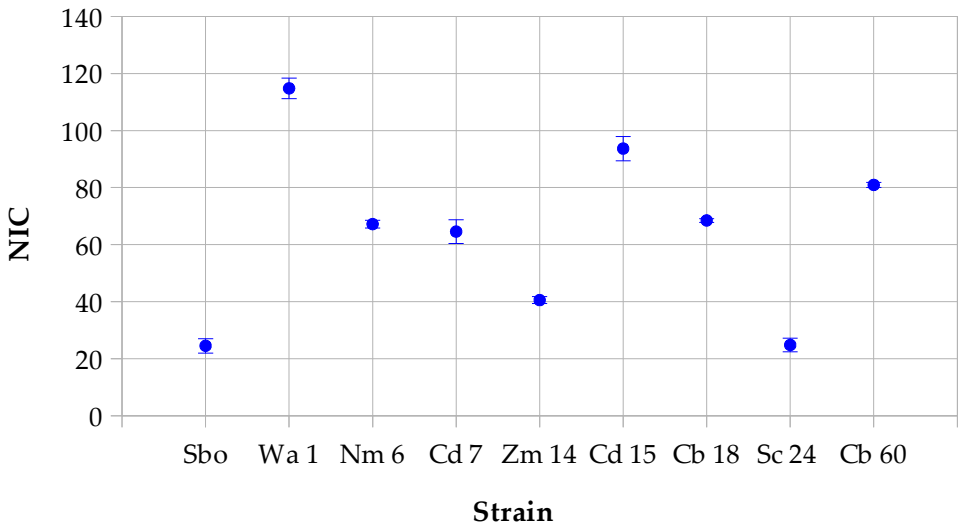

Figure 12. NIC values of selected strains isolated from Bosana brines. Vertical bars indicate standard error.

3.3.2 Enzymatic activities

In this study, the ability of yeast strains to exert characteristics of technological interest was investigated. The Table 7 reports the results of quantitative enzymatic activities.

Yeast reference	B-C	B-E	E-C	E-E	L-C	L-E
Sbo	1280.85 (1164.60) a, b, c	1147.03 (458.80) d, e	402.89 (126.36) a, b	267.24 (85.14) a, b, c	0.00(0.00) a	0.00(0.00) a
Wa 1	3271.64 (475.64) c,d	634.14 (123.39) b,c, d	1790.82 (198.47) d	910.52 (87.20) d	131.00 (107. 93) b	95.65 (24.68) b
Nm 6	2307.73 (329.146) b, c	37.97 (7.38) a, b	39.50 (14.35) a	27.01 (4.32) a, b	0.00(0.00) a	7.02 (1.23) a
Cd 7	0.00(0.00) a	0.00(0.00) a	1076.31 (279.07) c	150.49 (97.67) a, b	41.49 (19.08) a, b	0.00(0.00) a
Zm 14	902,21 (142,40) a, b	1337.92 (215.63) e	452.66 (93.39) a, b	343.42 (83.32) b, c	0.00(0.00) a	0.00(0.00) a
Cd 15	10986.50 (1516.55) e	473.99 (131.87) a,b, c	304.38 (83.92) a, b	183,21 (17,79) a, b	0.00(0.00) a	1.19 (1.62) a
Cb 18	0.00 (0.00) a	683.80 (237.69) c, d	408.31 (141.21) a, b	182.09 (73.68) a, b	2.84 (0.91) a	0.00(0.00) a
Sc 24	5054.71 (743.12) d	3720.90 (126.41) f	716.38 (60.95) b, c	540.79 (254.80) c	0.00(0.00) a	0.00(0.00) a
Cb 60	94.82 (52.15) a	190.84 (90.48) a, b,c	82.77 (34.56) a	15.04 (5.5) a	0.00(0.00) a	0.00(0.00) a

Tab 7. Data of cellular and extracellular enzymatic activities. Data was reported as mean and standard deviation in parenthesis and are expressed in nmol mL-1 h-1. Each value followed by a letter, are significantly different in accord to Scheffé test. B-C: β-glucosidase cellular, B-E β-glucosidase extracellular, E-C esterase cellular, E-E esterase extracellular, L-C lipase cellular, L-E lipase extracellular.

Most of selected biotype produced β-glucosidase both in cellular and extracellular fraction with exception of *C. diddensiae* Cd7 and *C. boidinii* Cb18 (cellular fraction). For B-C the highest value was 5054.71 nmol mL^{-1} h^{-1} while for B-E was 3720.90 (*S. cerevisiae* Sc24).

All strains were able to produce esterase in both cellular and extracellular fractions. Values of cellular fraction varied from 39.50 nmol mL^{-1} h^{-1} of *N. molendini-olei* to 540.79 mol mL^{-1} h^{-1} of *S. cerevisiae* while values of extracellular fraction ranged from 15.04 nmol mL^{-1} h^{-1} of *C. boidinii* Cb60 to 910.62 nmol mL^{-1} h^{-1} of *W. anomalus* Wa1.

With regard to lipase activity, *W. anomalus* Wa1, *C. diddensiae* Cd7 and *C. boidinii* Cb18 produced lipase in cellular fraction while in extracellular fraction only *W. anomalus* Wa1 (95.65 nmol mL^{-1} $h^{-1)}$, *N. molendini-olei* Nm6 (7.02 nmol mL^{-1} $h^{-1)}$ and *C. diddensiae* Cd15 (1.19 nmol mL^{-1} $h^{-1)}$ showed lipase activity. For all enzymatic activities investigated, the results were significantly higher for *W. anomalus* Wa1.

3.3.3 Growth in presence of oleuropein

Oleuropein is responsible for the bitter taste of olive fruit and its hydrolysis performed by yeasts is a very important feature for debittering process in Greek style fermentation. In this study, growth of examined yeast strains and *S. boulardii* in presence of oleuropein was monitored by Bioscreen C analyser. Most of strains showed low levels of maximum specific growth rate (μ_{max}), ranging from 0.04 and 0.32. Only the strain *W. anomalus* Wa1 exhibited high μ_{max} with a value of 0.32. The strain Zm14 of *Z. mrakii* showed the lowest μ_{max}. Data were reported in Table 8 and Figure 13.

Yeast reference	μ_{max}
Sbo	0.07 (0.00) a,b
Wa 1	0,32 (0.03) d
Nm 6	0.1 (0.01) b, c
Cd 7	0.04 (0.01) a
Zm 14	0.04 (0.01)a
Cd 15	0.09 (0.00) a,b, c
Cb 18	0.06 (0.01)a, b, c
Sc 24	0.13 (0.03) c
Cb 60	0.09 (0.00) a,b, c

Tab 8. Values of maximum specific growth rate (μ_{max}) of selected yeasts grown in presence of oleuropein. Data are reported as mean of three replicates followed by standard deviation in parenthesis. Values followed by letter denote significant differences according to Scheffé test.

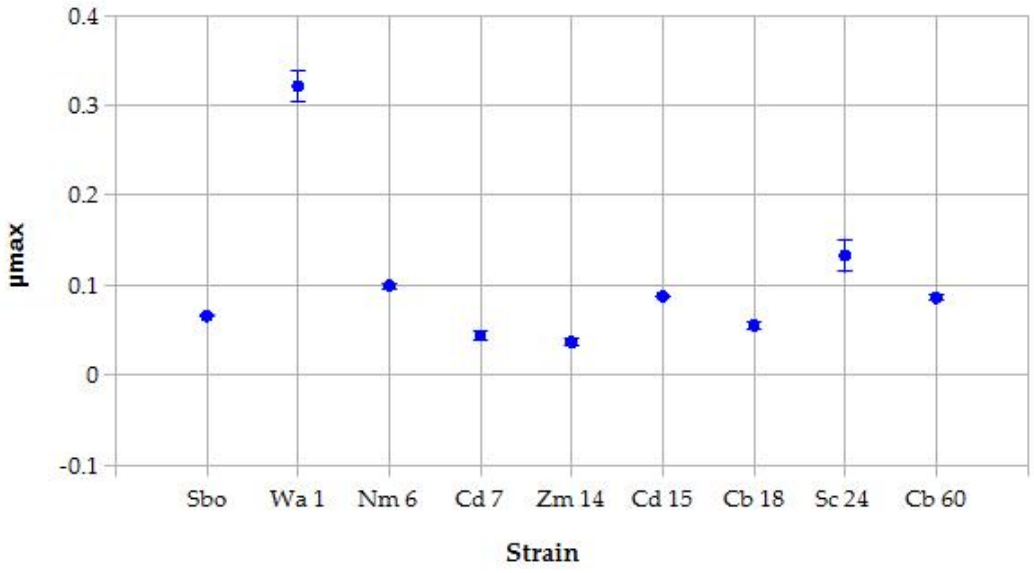

Figure 13. Oleuropein assimilation. Vertical bars indicate standard error.

3.4 I*n vitro* simulated gastric and pancreatic digestion

Resistance to gastric and pancreatic juices secreted during passage through digestive system is a very important feature of probiotic strains. For this purpose, the capability of yeast strains to survive to simulated gastric and pancreatic conditions was investigated. The results of gastric and pancreatic digestions *in vitro* are expressed in percentage of survival and are reported in Table 9. Probiotic microorganisms *L. rhamnosus* GG and *S. boulardii* were used as control. All strains exhibited a survival percentage to gastric synthetic juice higher than that of *L. rhamnosus* GG (6.19%). Compared to probiotic yeast *S. boulardii* (95.88% of survival), selected strains showed lower survival percentage to simulated gastric digestion. *C. diddensiae* strains Cd15 and Cd7 and *S. cerevisiae* Sc24 exhibited a high percentage of survival. On the contrary, *C. boidinii* C18 and *N. molendini-olei* Nm6 were considerably affected by gastric digestion.

Yeast reference	Gastric digestion	Pancreatic digestion
LGG	6,19 (0,33) a, b	7,92 (2,16) a,b,
Sbo	95,88 (13,16) c	10,99 (1,02) a, b, c
Wa 1	49,15 (8,4) a, b, c	5,28 (3,6) a, b
Nm 6	15,43 (3,47) a, b	0,02 (0,00) a
Cd 7	64,94 (1,2) a, b, c	24,76 (0,31) a, b, c
Zm 14	19,54 (0,51) a, b	0,10 (01) a
Cd 15	66,4 (16,4) a, b, c	38,49 (6,45) b, c
Cb 18	4,19 (3,26) a	3,53 (2,6) a, b,
Sc 24	79,68 (4,65) b, c	46,60 (0,63) c
Cb 60	55,38 (21,76) a,b,c	22,10 (13,81) a, b, c

Tab 9. Survival (%) of yeast strains to gastric and pancreatic digestion in vitro. Results followed by letter indicate significant differences according to Scheffé post-hoc test. LGG refer to strain of *L. rhamnosus* GG used as probiotic control.

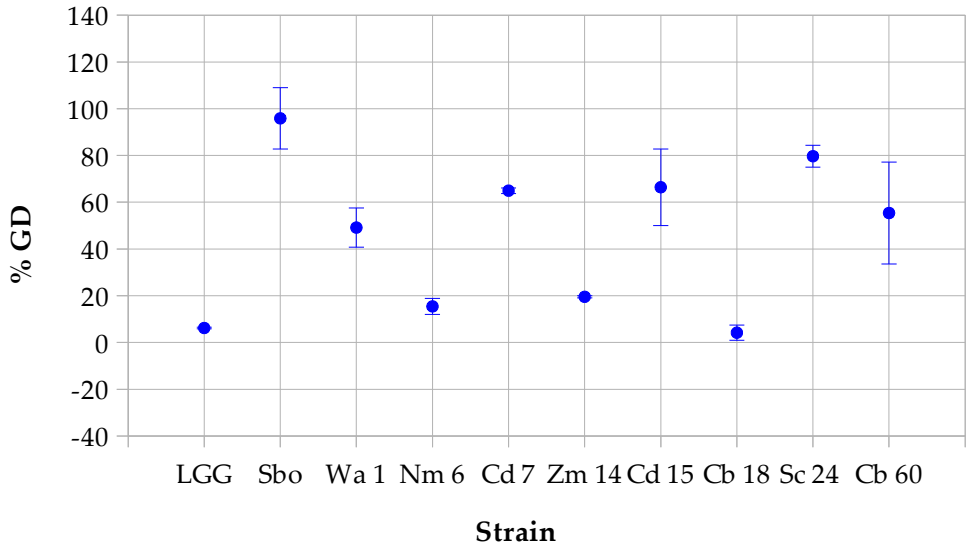

Figure 14. Percentage of survival of selected strains, *L. rhamnosus* GG and *S. boulardii* during *in vitro* gastric digestion. Vertical bars indicate standard error.

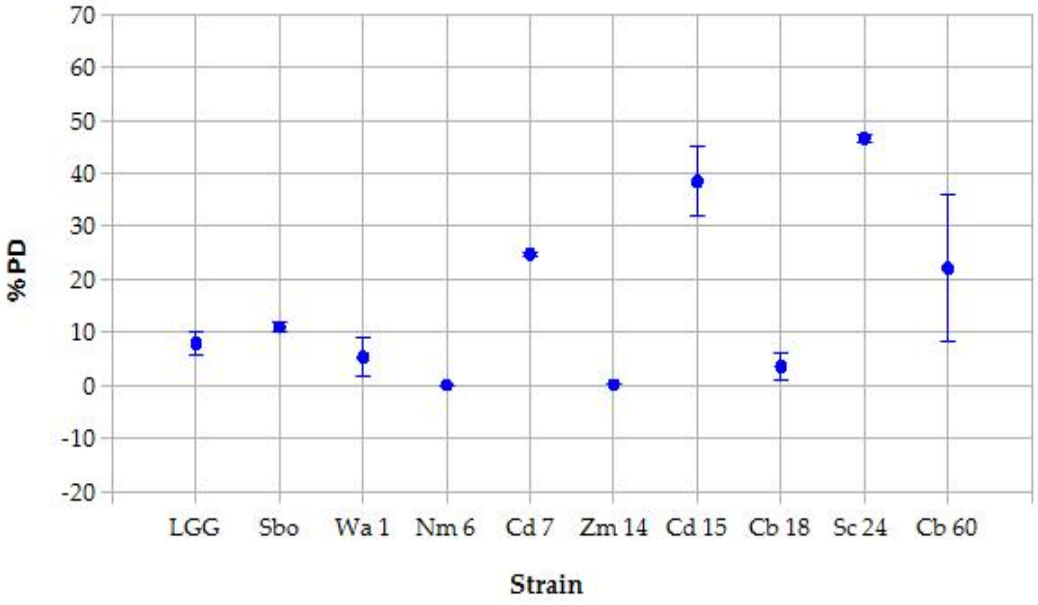

Figure 15. Percentage of survival of selected strains, *L. rhamnosus* GG and *S. boulardii* during *in vitro* pancreatic digestion. Vertical bars indicate standard error.

A decrement of resistance of yeast strains after simulated and sequential pancreatic digestion was observed. In particular, selected biotypes showed percentage values higher of *L. rhamnosus* GG with exception of the strain *N. molendini-olei* Nm6, *Z. mrakii* Zm14 and *C. boidinii* Cb18 that exhibited a high mortality. On the other hand, *C. diddensiae* Cd7 and Cd15, *S. cerevisiae* Sc24 and *C. boidinii* Cb60 showed higher survival percentages than probiotic yeast *S. boulardii.*

3.5 *In vitro* cholesterol removal

Another important feature of probiotic microorganisms is degradation of cholesterol. For this purpose, the ability of *S. boulardii* and examined yeast strains to remove cholesterol from 3 different media was tested. Free and esterified cholesterol in the supernatant forms a pink complex (Figure 16) that can be measured spectrophotometrically by the following reactions (Allain *et al.*, 1974; Meiattini *et al.*, 1978).

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Chol. esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + \tfrac{1}{2}\, O_2 + H_2O \xrightarrow{\text{Chol. oxidase}} \text{Cholestenone} + H_2O_2$$

$$2H_2O_2 + 4 - \text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + H_2O$$

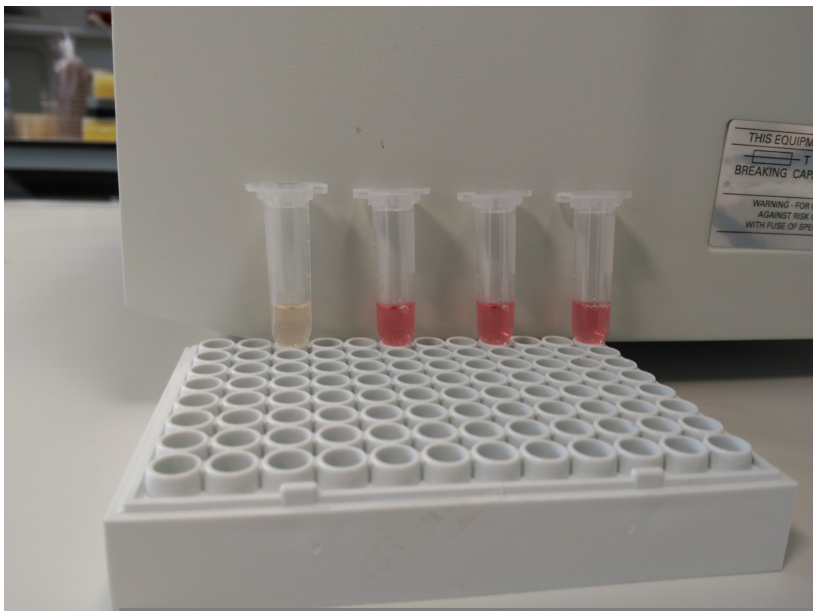

Figure16. Spectrophotometric assay for cholesterol degradation. Tube 1, sample without cholesterol, tube 2, 3 and 4 tubes with cholesterol.

Results are reported Table 10 and Figure 17. Data revealed differences in yeast ability to remove cholesterol *in vitro* in YNB-CG, YNB-CGO and YNB-CO media. Strains of *C. boidinii* Cb18 and *S. cerevisiae* Sc24 exhibited greatest ability to degrade cholesterol. These strains showed higher percentage of cholesterol degradation than probiotic control *S. boulardii*.

Cholesterol removal increased in YNB supplemented with cholesterol and glucose and it ranged from 20.4% of *C. boidinii* Cb60 to 25.57% of *C. boidinii* Cb18. Compared to probiotic control *S. boulardii*, all strains with exception of *C. boidinii* Cb60 showed high degradation of cholesterol.

Yeast strains in medium with cholesterol and Oxgall exhibited lower ability to remove cholesterol than medium supplemented with cholesterol and glucose with range of percentage of degradation between 18.7% and 13.1%. The highest percentage of cholesterol degradation was recorded for *C. boidinii* Cb60 while *N. molendini-olei* Nm6 removed less cholesterol.

Medium supplemented with cholesterol, glucose and Oxgall had a strong impact on ability to degrade cholesterol of yeast strains, this ranged from 10.83% to 15.67%.

Yeast reference	YNB CG	YNB CGO	YNB CO
Sbo	21.35 (0.78) a, b, c, d, e, f, g	10.83 (0.93) a	13.83 (1.89) a, b, c, d, e
Wa 1	22.2 (2.80) a, b, c, d, e, f, g	12.37 (1.97) a, b	14.4 (3.52) a, b, c, d, e, f
Nm 6	24.63 (1.61) d, e, f, g	12.23 (0.12) a, b	13.1 (1.91) a, b, c
Cd 7	24.77 (2.06) e, f, g	15.23 (1.99) a, b, c, d, e, f, g	16.13 (1.53) a, b, c, d, e, f, g
Zm 14	26.3 (1.56) g	14.8 (1.14) a, b, c, d, e, f	14.67 (4.49) a, b, ,c, d, e, f,
Cd 15	24.3 (1.47) c, d, e, f, g	13.37 (2.84) a, b, c, d, e, f, g	17.0 (1.35) a, b, c, d, e, f, g
Cb 18	25.57 (1.72) f, g	15.67 (2.30) a, b, c, d, e, f, g	17.33 (0.35) a, b, c, d, e, f, g
Sc 24	23.53 (1.40) b, c,d, e, f, g	13.43 (2.99) a, b, c, d, e	17.47 (1.96) a, b, c, d, e, f g
Cb 60	20.4 (1.08) a, b, c, d, e , f, g	14.93 (3.26) a, b, c, d, e, f, g	18.7 (0.98) a, b, c, d, e, f, g

Tab 10. Cholesterol removal (%) of assayed yeast strains. Data were expressed as mean of three replicates followed by strandard deviation in parenthesis. Each values followed by letters indicate significant differences in accordance with Scheffé test. YNB-CG, Yeast Nitrogen Base supplemented with cholesterol and glucose; YNB-CGO Yeast Nitrogen Base supplemented with cholesterol, glucose and Oxgall; YNB-CO Yeast Nitrogen Base supplemented with cholesterol and Oxgall.

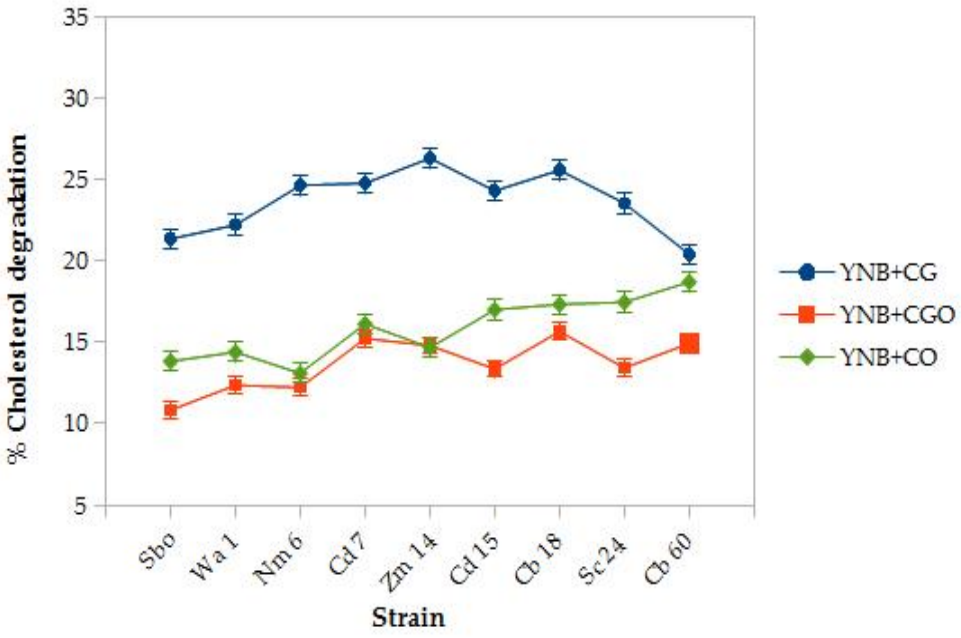

Figure 17. Percentage of cholesterol removal. Vertical bars indicate standard error.

3.6 Interaction between yeasts and lactic acid bacteria

To investigate possible interactions between yeasts and LAB, selected yeast strains and *S. boulardii* were tested for biofilm formation assay in pure culture and mixed culture with LAB. The biofilm formation was quantified as capability of yeast strains to adhere to polystyrene 96-well plate in monoculture and mixed culture with *L. pentosus* TOMC 2. Results of biofilm quantification by crystal violet (CV) staining are showed in Table 11 and Figure 18. Data show that some yeast strains showed higher biofilm in mixed culture than in mono culture. In fact, most yeast strains were not able to produce biofilm in monoculture. *Z. mrakii* Zm14 (0.32), *S. boulardii* (0.35) and *N. molendini-olei* Nm6 (0.35) were the lowest biofilm producers. Only *W. anomalus* Wa1, *C. diddensiae* Cd15 and *C. boidinii* Cb60 showed high production of biofilm. The highest ability to form biofilm in mixed culture with *L. pentosus* TOMC 2 was noticed in mixed cultures made of TOMC 2-*S. boulardii* (3.05), TOMC 2-Wa1 (2.93), TOMC 2-Cb18 (3.38) and TOMC 2-Cb60 (3.23). Surprisingly, mixed cultures of *L. pentosus* TOMC 2 and both *C. boidinii* strains (Cb18 and Cb60) produced a biofilm on the well (Figure 19). Biofilm and cells morphology of monoculture of *C. boidinii* strains and surface of biofilm of mixed culture of *C. boidinii-L. pentosus* TOMC 2 were observed by SEM. As shown in Figure 20 SEM examination revealed presence of *C. boidinii* cells and absence of cells of *L. pentosus* TOMC 2 in biofilm surface of mixed culture. Concerning the question whether contact between *C. boidinii* and *L. pentosus* is involved in biofilm formation, results showed that presence of filter did not affect biofilm development.

Strains reference	Abs CV
Sbo	0.35 (0.09) a,b
Wa 1	1.15 (0.31) b, c, d, e, f
Nm 6	0.35 (0.11) a,b
Cd 7	0.43 (0.12) a, b
Zm 14	0.32 (0.15) a
Cd 15	1.60 (0.43) e, f
Cb 18	0.45 (0.26) a, b, c
Sc 24	0.58 (0.27) a, b, c, d
Cb 60	3.11 (0.26) g
Lab 2	1.27 (0.14) d, e, f
Sbo+Lab 2	3.05 (0.29) g
Wa1+Lab 2	2.94 (0.36) g
Nm 6 + Lab 2	0.69 (0.09) a,b, c, d
Cd 7 + Lab 2	1.36 (0.22) d, e, f
Zm 14 + Lab 2	0.85 (0.07) a, b, c, d, e
Cd 15 + Lab 2	1.25 (0.30) c, d, e, f
Cb 18 + Lab 2	3.38 (0.00) g
Sc 24 + Lab 2	1.69 (0.18) f
Cb 60 + Lab 2	3.23 (0.19) g

Tab 11. Biofilm formation of tested yeast strains in microtiter plate. Data are expressed as mean of six replicates followed by standard deviation in parenthesis. Letter after each value indicate significant differences according to Scheffé test.

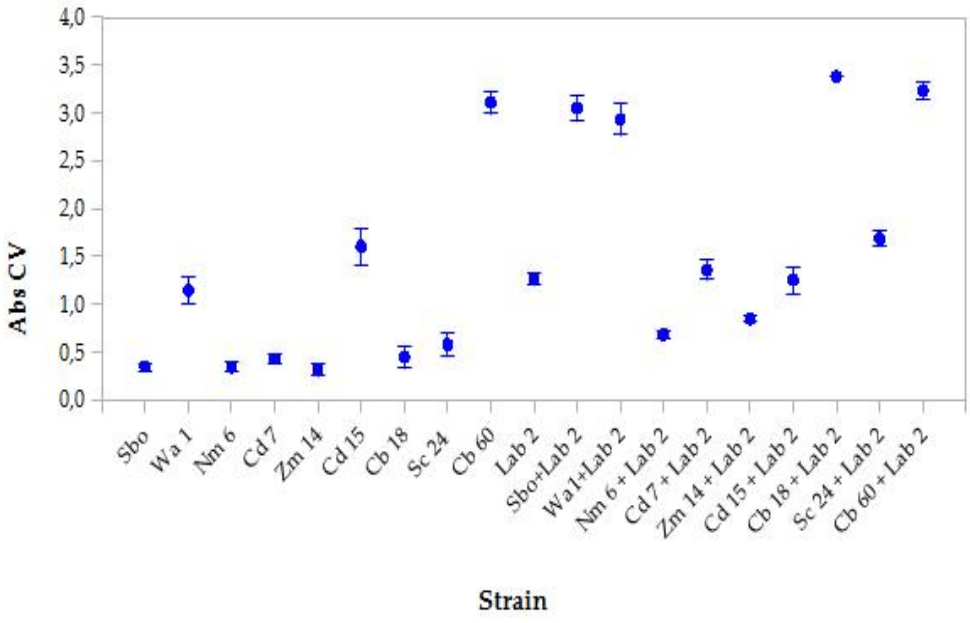

Figure 18. Biofilm formation (expressed as absorbance of CV) of selected yeast strains and *S. boulardii* in single and mixed cultures with *L. pentosus* TOMC 2. Vertical bars indicate standard error.

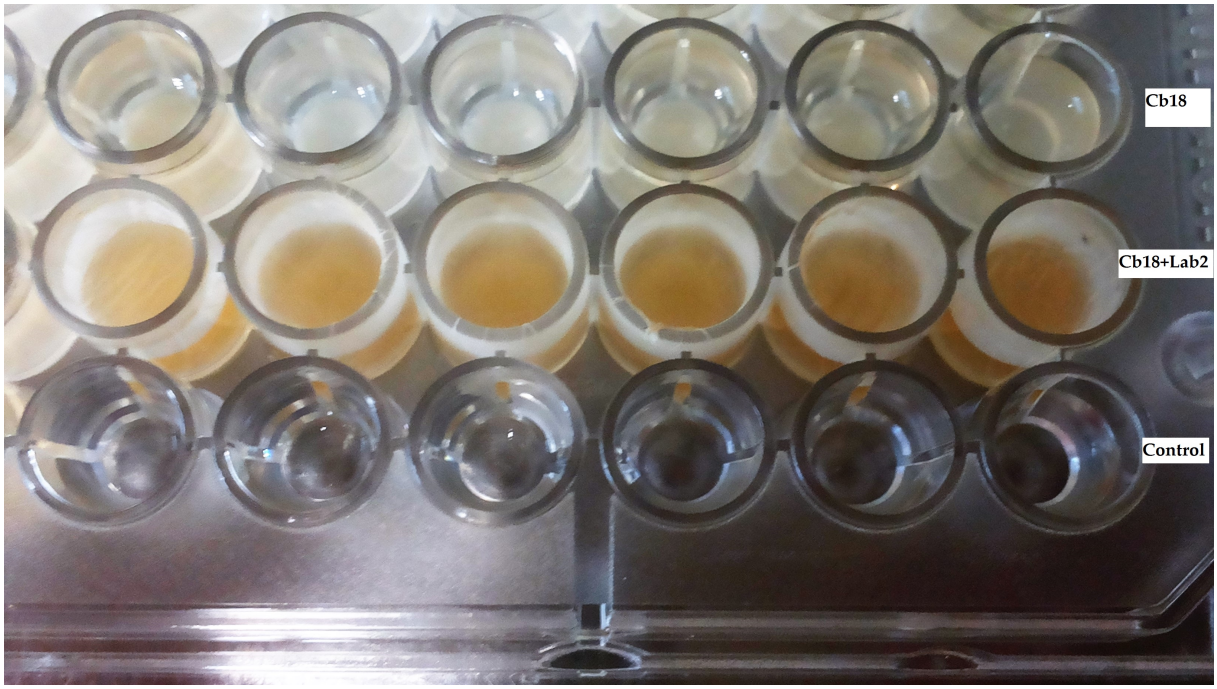

Figure 19. Biofilm production in microtiter plate of *C. boidinii* Cb18 and mixed culture *C.boidinii-L. pentosus* TOMC 2. Control wells contained uninoculated medium.

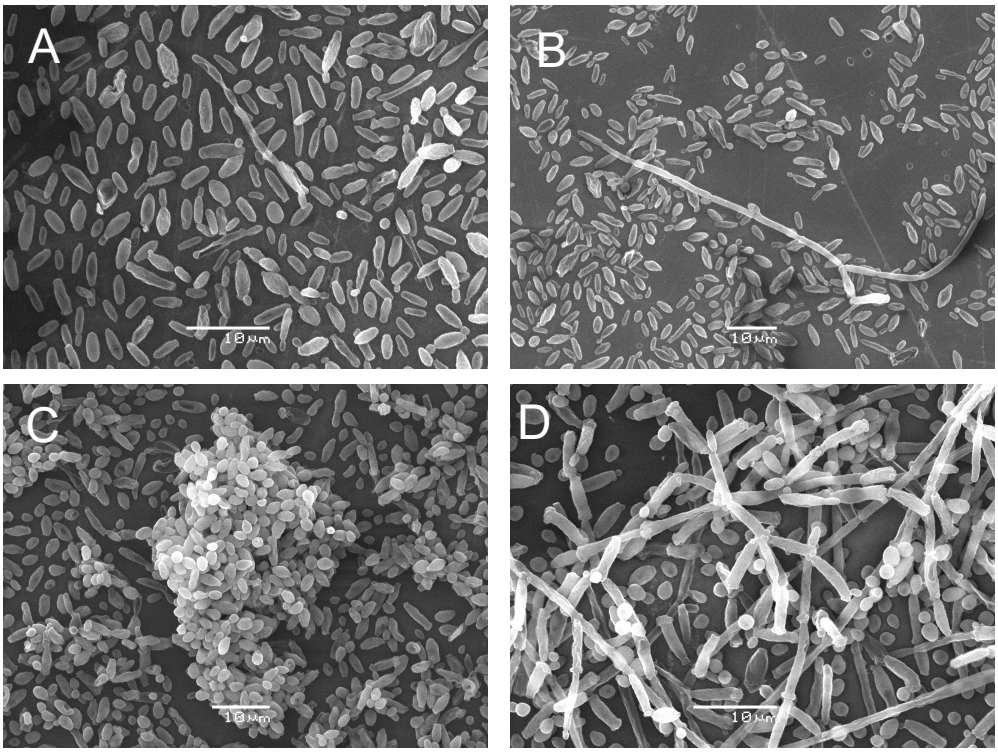

Figure 20. Scanning Electronic Microscopic images of biofilms formed by *C. boidinii*. A and B single culture of *C. boidinii* 18 and 60; C and D, combined cultures of *C. boidinii* strain 18 and 60 with TOMC 2.

3.7 Statistical analysis

To reduce the number of variables into a smaller number of Factors, quantitative data obtained from quantitative technological and probiotic tests were subjected to PCA analysis. Five eigen values higher of 1 were calculated, showing that 17 variables can be grouped into five Factors that explained the 92.47% of variance. This Factors accounted for 36.35%, 19.12%, 16.22%, 10.89% and 9.89% of the total variance. Figure 21 showed relation between the major factors (Factor 1 and Factor 2) and original variables. Factor 1 was linearly related to percentage of isolation and removal of cholesterol (RC CG) while Factor 2 had positive correlation with esterase activity (E-Ex and E-Ce), lipase activity (L-Ex and L-Ce), resistance to salt (MIC and NIC), assimilation of oleuropein (Oleu), production of biofilm in mono-culture and mixed culture with TOMC 2 (Auto aggregation and Co-aggregation TOMC 2) and cellular β-glucosidase (B-Ce). The projection of cases (yeast strains) onto the planes formed of two major Factors (Factor 1 and Factor 2) that explained the majority of variance (55.47%), generated the graphs illustrated in the bottom of Figure 21. In this way, strains were discriminated in three groups. Two groups, nearly a positive axis of Factor 1 were consisted of Sc24, Cb60, Cd15 and *S. boulardii,* while lower group was formed of Cd7, Cb18, Zm14 and Nm6. The third group, near to Factor 2 and far from other two groups, included only *W. anomalus* Wa1 strain. This strain had interesting technological characteristics.

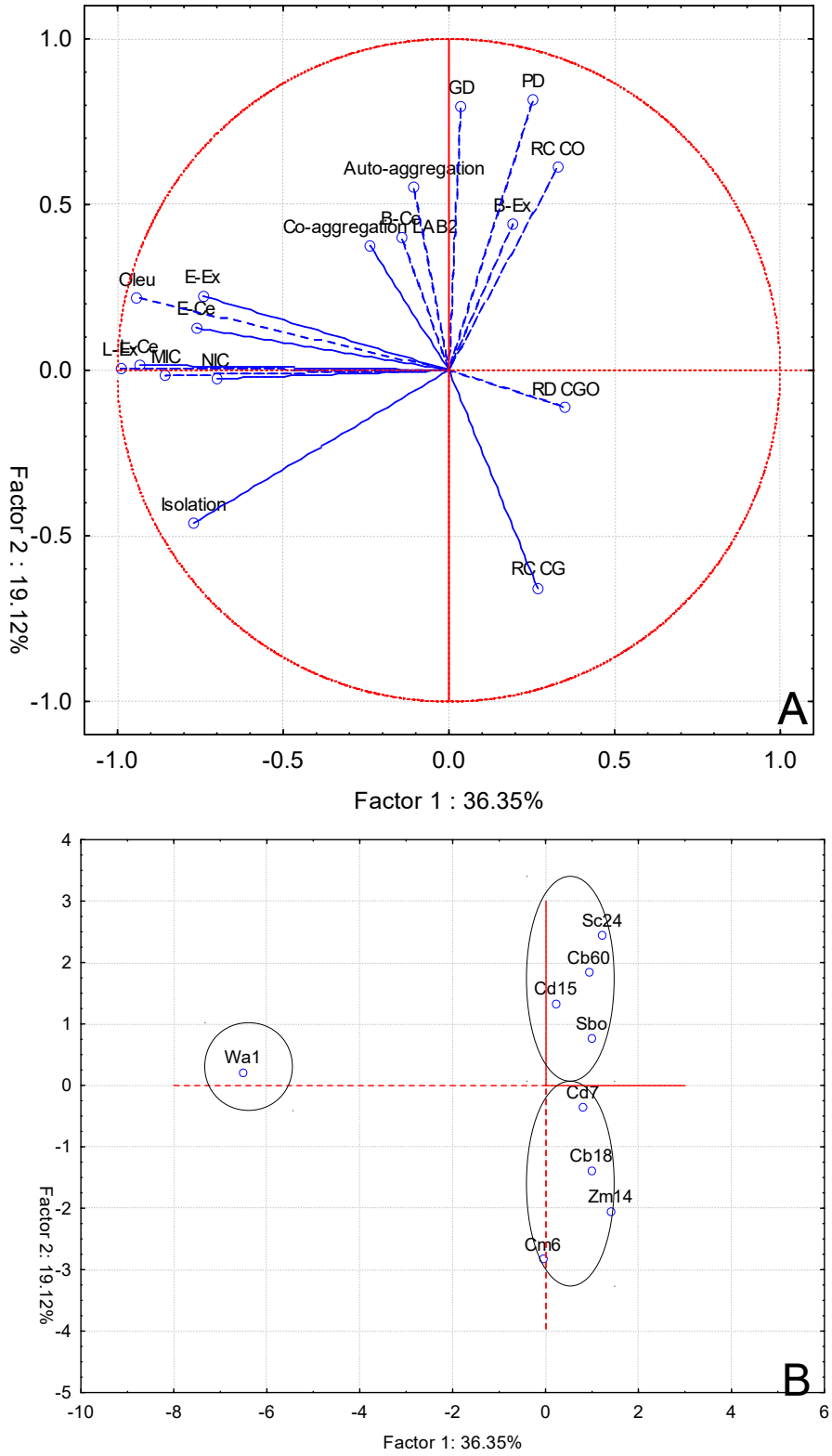

Figure 21. PCA analysis of yeasts variables for evaluation of technological and probiotic characteristics. **A**, Projection of the variables onto the plane of the first two Factors. **B**, Projection of the two major factors as function of yeast strains.

3.8 Genotyping of *Candida boidinii* strains

Eight strains of *C. boidinii* isolated from different sources were tested using DNA fingerprinting with primer M13. Sixteen patterns, containing from two to six bands were achieved. In addition, to assess reproducibility of technique, different repetition of RAPD analysis of strain control TOMC Y45 (*W. anomalus*) were performed. Repetion of RAPD-analysis of TOMC Y45 strain produced pattern of 85% of similarity according to Pearson coefficient. Dendrogram built by cluster analysis of sixteen patters of *C. boidinii* is shown in Figure 22.

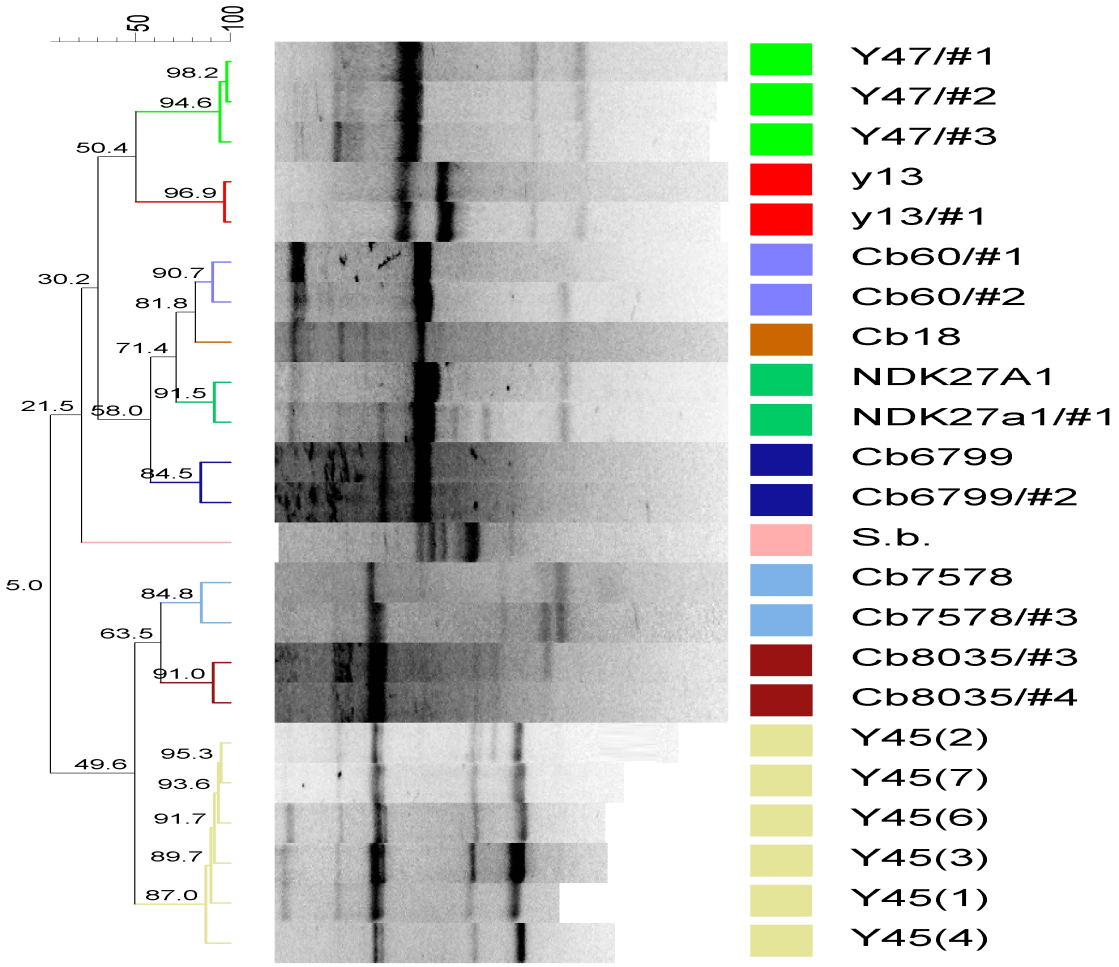

Figure 22. Dendrogram generated by comparison of RAPD-PCR patterns of eight strains of *C. boidinii*. Y45, *W. anomalus* TOMC Y45.

3.9 Biofilm production *in vitro* in mixed culture with different combinations of strains of *Candida boidinii* and acid lactic bacteria

Because of the apparent higher ability of *C. boidinii* strains to form biofilms with *L. pentosus*, ability of eight *C. boidinii* strains to produce biofilm in mixed culture with three strains of lactic acid bacteria was investigated. Multifactorial ANOVA was used to compare absorbance values obtained by CV staining. Results of multifactorial ANOVA were resumed in Figure 23. Table 12 shows that production of biofilm is higher in mixed culture than in mono-culture. Regarding formation of biofilm of mixed cultures, results indicate that *C. boidinii* strains Cb18, Cb60 and NDK27A1 exhibit the highest ability to develop biofilm *in vitro* when cultured with *L. pentosus* TOMC 2.

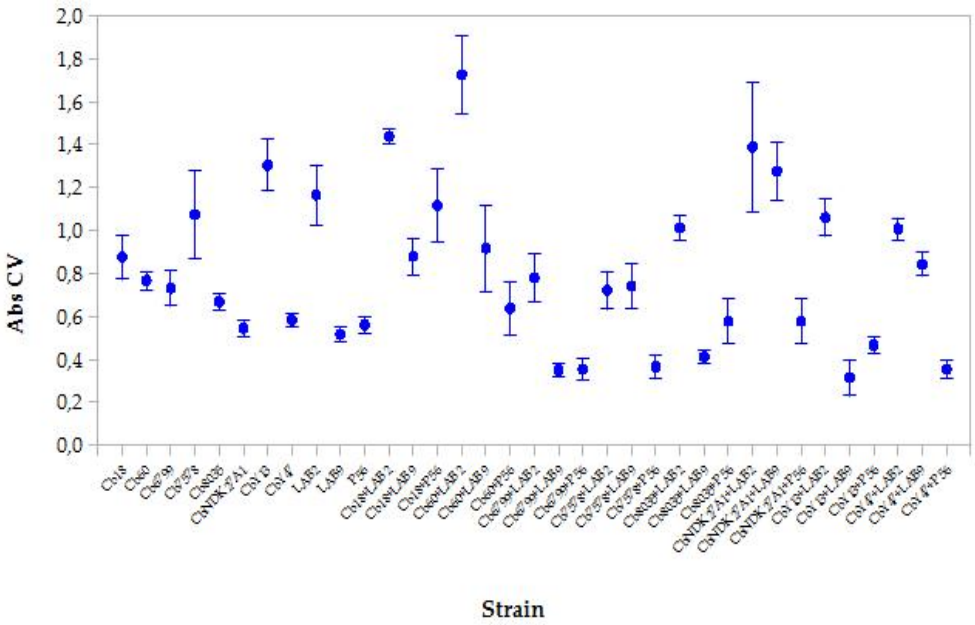

Figure 23. ANOVA representation of formed biofilm in mono and mixed culture of strains of *C. boidinii* and LAB. Vertical bars indicated standard error.

Culture	Abs CV
Cb18	0.88 (0.10) a, b, c
Cb60	0.77 (0.04) a, b, c
Cb6799	0.73 (0.08) a, b, c
Cb7578	1.07 (0.21) a, b, c
Cb8035	0.67 (0.04) a, b, c
CbNDK27A1	0.54 (0.04) a, b
CbY13	1.30 (0.12) a, b, c
CbY47	0.58 (0.03) a, b
LAB2	1.17 (0.14) a, b, c
LAB9	0.52 (0.03) a, b
P56	0.56 (0.04) a, b
Cb18+LAB2	1.44 (0.03) b, c
Cb18+LAB9	0.88 (0.09) a, b, c
Cb18+P56	1.12 (0.17) a, b, c
Cb60+LAB2	1.73 (0.18) c
Cb60+LAB9	0.92 (0.20) a, b, c
Cb60+P56	0.64 (0.13) a, b, c
Cb6799+LAB2	0.78 (0.11) a, b, c
Cb6799+LAB9	0.35 (0.03) a, b
Cb6799+P56	0.35 (0.05) a, b
Cb7578+LAB2	0.72 (0.09) a, b, c
Cb7578+LAB9	0.74 (0.10) a, b, c
Cb7578+P56	0.37 (0.06) a, b
Cb8035+LAB2	1.01 (0.06) a, b, c
Cb8035+LAB9	0.41 (0.03) a, b
Cb8035+P56	0.58 (0.10) a, b
CbNDK27A1+LAB2	1.39 (0.30) a, b, c
CbNDK27A1+LAB9	1.28 (0.13) a, b, c
CbNDK27A1+P56	0.58 (0.10) a, b, c
CbY13+LAB2	1.06 (0.09) a, b, c
CbY13+LAB9	0.32 (0.08) a
CbY13+P56	0.47 (0.04) a, b
CbY47+LAB2	1.01 (0.05) a, b, c
CbY47+LAB9	0.84 (0.05) a, b, c
CbY47+P56	0.35 (0.04) a, b

Tab 12. Biofilm production, measured as absorbance of CV released. Standard deviation in parenthesis. Letters after each value indicate significant differences according to Scheffé test.

4. DISCUSSION

4.1 Yeast biodiversity in Bosana olive fermentations

Presence of yeasts is related to raw materials (Botta and Cocolin, 2012) and associated to processing of drupes. Concerning fermentation processing, (Bautista-Gallego *et al.*, 2011) reported higher yeast number in directly brined fermented olives than in Spanish style fermented olives. Indeed, knowledge about table olives microbiota and in particular of yeasts concurring to fermentation is very important to improve organoleptic quality of table olives. In fact, yeasts produce volatile compounds and metabolites that affect aroma and taste of the final product. Moreover, yeasts can be reduce olives wastewater by degradation of polyphenols and they can be used as biocontrol agents. Yeasts have also a beneficial effect on human health. These effect include probiotic features, biodegradation of phytate complexes and mycotoxins, reduction of cholesterol level, biofortification with folate and vitamins, and production of bioactive compounds (Arroyo-López *et al.,* 2012). On the other side it should be taken into account that yeasts can also be responsible of formation of gas, softening of drupes tissue, clouding of brine and production of odors (Arroyo-López *et al.*, 2008) thus underlining the importance of the management of the microbiota that is involved in table olive processing.

This work describe for the first time a yeast community collected from Bosana brines. Bosana variety is widespread in Sardinia and used for olive oil production and table olives processed with Greek style fermentation. The six species highlighted by sequence analysis of D1-D2 domain of the 26S rDNA namely *Wickerhamomyces anomalus*, *Nakazawaea molendini-olei*, *Candida boidinii*, *Candida diddensiae*, *Zygotorulaspora mrakii* and *Saccharomyces cerevisiae* were observed in several table olives preparations. Among these species *W. anomalus* and *N. molendini-olei* were dominant in Bosana brines. *W. anomalus* was observed in several studies of table olives (Hernández *et al.*, 2007; Arroyo-López *et al.*, 2008; Hurtado *et al.*, 2008; Nisiotou *et al.*, 2010; Muccilli *et al.*, 2011; Doulgeraki *et al.*, 2012; Mateus *et al.*, 2016). This microorganism is related to bioactive antioxidants production that retard

oxidation of fatty acids (Arroyo-López *et al.*, 2008). De Angelis *et al.,* (2015) reported that growth of a strain of *W. anomalus* was inhibited by lactic acid bacteria strains. Moreover, Hernández *et al.,* (2008) observed that strains of *W. anomalus* had killer activity against spoilage and pathogen microorganisms, thus can be used as biocontrol agent in table olives processing.

N. molendini-olei was detected for the first time by (Čadež *et al.*, 2012) in virgin olive oil and its products. This species was observed with low frequency by Mateus *et al.,* (2016) in cracked green table olives. In addition, Mari *et al.,* (2016) reported presence of strains *N. molendini-olei* in extra virgin olive oil in Italy.

Other yeasts species detected with lower frequency in Bosana brines were *C. boidinii*, *C. diddensiae*, *Z. mrakii* and *S. cerevisiae*. Presence of C. boidinii in all table olives preparations is very common. Arroyo-López *et al.,* (2008) referred presence of *C. boidinii* both in green and black directly brined olives. Farris et al., (1986), isolated strains of *C. boidinii* in directly brined black olives cv. Tonda while Pereira *et al.,* (2015) reported presence of *C. boidinii* in spontaneous fermentation of green olives. However, Bautista-Gallego *et al,* 2011 isolated strains of *C. boidinii* in Manzanilla olives processed with Spanish style.

C. diddensiae was reported as strongly fermentative yeast producing nicotinic and pantothenic acids, biotin, and vitamin B6 (Ruiz-Barba and Jimenez-Diaz, 1995). This microorganism is associated to decrease of pH during fermentation process in *Arbequina* table olives (Hurtado *et al.,* 2008). Presence of *C. diddensiae* in directly brined olives was reported also by Farris *et al.,* (1986); Muccilli *et al.,* (2011); Mateus *et al.,* (2016). Chakri *et al.,* (2007) referred ability to degrade polyphenols in olive oil mill wastewater exhibited by two *C. diddensiae* strains isolated in digestive tract of larvae of fly *Bactrocera oleae.*

Z mrakii, was observed in natural table olive processing by Alves *et al.,* (2012) and (Bautista-Gallego *et al.,* 2011). Alves *et al.,* (2012) described *Z. mrakii* and *S. cerevisiae*,

dominant species in green cracked olives cv Manzanilla, as responsible of instability of packed olives.

Occurrence of *S. cerevisiae* in table olives preparations is well documented. In fact, *S. cerevisiae* was observed in Spanish (Bautista-Gallego *et al.*, 2011), Portuguese (Alves *et al.*, 2012; Pereira *et al.*, 2015; Mateus *et al.*, 2016), Italian (Farris *et al.*, 1986; Muccilli *et al.*, 2011; Tofalo *et al.*, 2013) and Greek (Nisiotou *et al.*, 2010) cultivars. *S. cerevisiae* produced many gases causing problems in table olives package, so the role of this yeast in table olives processing is not clear (Tofalo *et al.*, 2013).

Genotyping of all isolates by RAPD-PCR with primer M13 lead to their characterization at the strain level. RAPD-PCR was used in different studies to investigate genetic variability of yeasts in fermented food (Andrighetto *et al.*, 2000; Vasdinyei and Deák, 2003; Tofalo *et al.*, 2013; Mari *et al.*, 2016) but not often applied to the characterization of yeasts associated to table olives or olives environment (Arroyo-López *et al.*, 2006). Mari *et al.*, (2016) discriminated seventeen yeast species isolated from olive oil and its products. In another study, yeast isolated from six Italian cultivar of table olives were discriminated by RAPD PCR with primer M13 (Tofalo *et al.*, 2013). In agreement with these authors, efficacy of RAPD PCR was confirmed as a useful tool discriminate yeasts at strain level. Results showed that assessed strains were grouped according to species into eight groups at a similarity higher than 85.6% and permitted to detect two genotypes of *C. boidinii* and *C. diddensiae*.

4.2 Multifunctional features of the yeast biotypes

Research of NaCl influence on yeast helps to control development of desirable or spoilage microorganisms. Moreover, NaCl is commonly used in natural table olives processing at high concentrations. *S. cerevisiae* Sc24 showed a MIC value (86.29 g/L) lower than MIC values reported by Bautista-Gallego *et al.*, (2008); Bonatsou *et al.*, (2015) and Romero-Gil *et al.*, (2013) while *W. anomalus* Wa1 and *C. boidinii* strains Cb18 and Cb60

showed MIC values higher than values reported by Bautista-Gallego *et al.,* (2008) and Romero-Gil *et al.,* (2013). Respect to susceptibility to NaCl, Romero-Gil *et al.* (2013) Bonatsou *et al.,* (2015) and Bautista-Gallego *et al.,* (2008) reported values slightly different of NIC for one strain of *S. cerevisiae*, *W. anomalus* and *C. boidinii*. They determined NIC of 31 g/L (S. *cerevisiae*), 99.9 g/L (*W. anomalus*) and 57.72 g/L (*C. boidinii*). The results obtained for *W. anomalus* Wa1 are agreement with literature. In fact, Arroyo-López *et al.,* (2006) observed that W. *anomalus* showed high resistance to salt.

For the first time in this study, values of MIC and NIC of *C. diddensiae*, *N. molendini-olei* and *Z. mrakii* were determined by mathematical growth modelling. Data achieved in this study showed that these had low resistance to NaCl.

4.2.1 Enzymatic activities and assimilation of oleuropein

Several studies were previously conducted on β-glucosidase, catalase, esterase and lipase activity of yeast species involved in table olives processing (Hernández *et al.,* 2007; Rodríguez-Gómez *et al.,* 2010; Bautista-Gallego *et al.,* 2011; Bevilacqua *et al.,* 2013; Bonatsou *et al.,* 2015). These technological features influence sensory characteristics of table olives. Moreover, metabolites produced by yeasts improved growth of lactic acid bacteria and can inhibits development of undesirable microorganisms.

Catalase activity was exhibited by most of strains. This characteristic is important for table olives preservation, because inhibits oxidation of fatty acids and consequent production of peroxide. These results are congruent with those reported by Hernández *et al.,* (2007). Moreover, *W. anomalus*, *P. galeiformis* and *K. lactis* are interesting yeast species with a high catalase activity (Bevilacqua *et al.,* 2015).

Production of esterase and lipase is important for the breakdown of fatty acids producing volatile components that affect flavour and aroma of table olives (Hernández *et al.,* 2007; Rodríguez-Gómez *et al.,* 2010; Bevilacqua *et al.,* 2013). In fact, drupes have high fat concentration, thus yeasts with lipase can affect nutritional composition of table olives. *W.*

anomalus showed highest esterase and lipase activity. Bonatsou *et al.,* (2015) reported similar results for yeasts isolated from Green black table olives. Production of lipase by examined yeast strains is very low, in fact only *C. diddensiae* Cd7 and *C. boidinii* Cb18 showed weak lipase activity. Presence of lipase is a widespread feature of *C. boidinii* but in this study a strain of *C. boidinii* Cb60 did not exhibited lipase activity. This result is in agreement with Ciafardini *et al.,* (2006) that described a lipase negative strain of *C. boidinii* isolated from extra virgin olive oil. Moreover, in contrast with results reported in this work, Bonatsou e*t al.,* (2015) and Rodríguez-Gómez *et al.,* (2010), Ciafardini *et al.,* (2006) Hernández *et al., (*2007) referred presence of lipase-producing strain of *S. cerevisiae* in virgin olive oil and table olives. Other yeast species with high lipolytic activity are *D. hansenii* and *T. Delbru* that enhanced aromatic profile of Greek table olives (Psani and Kotzekidou, 2006).

Esterase activity is a widespread property of yeast strains isolated from table olives. Bautista-Gallego *et al.,* (2011) found esterase activity in strains of *C. diddensiae* and *W. anomalus*. Esterase production for *S. cerevisiae* isolated from table olives was reported by Hernández *et al.,* (2007). On the contrary, this feature did not find in *S. cerevisiae* strains associated to table olives by Silva *et al.,* (2011) and Tofalo *et al.,* (2013). In addition, esterase activity could be involved in cholesterol catabolism of yeasts (García *et al.,* 2012).

β-glucosidase and esterase catalyses hydrolysis of oleuropein (Figure 23). Oleuropein is responsible of bitter taste of drupes. Oleuropein can be hydrolysed by chemical method (treatment with NaOH) or biological method. β- β-glucosidase and esterase could be used in controlled olives processing in order to avoid lye treatment that cause removing of soluble compounds. Several authors described β- glucoside activity in *W. anomalus*. Bautista-Gallego *et al.,* (2011), Bevilacqua *et al.,* (2013) and Tofalo *et al.,* (2013) detected β- β-glucosidase activity on Arbutin agar. Concerning spectrophotometric assay, Bonatsou, *et al.,* (2015) observed β-glucosidase production in *W. anomalus* and *S. cerevisiae* isolated from Greek table olives. Restuccia *et al.,* (2009) characterized a β-glucosidase produced by a

strain of *W. anomalus* isolated from naturally table olives which exhibited the maximal activity at alkaline pH and at 35˚C.

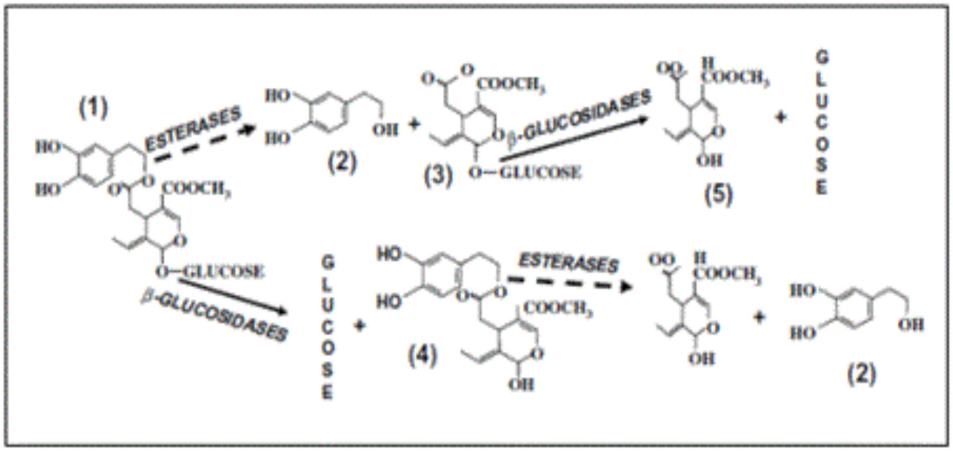

Figure 24. Scheme for enzymatic hydrolysis of oleuropein and products formed according to the reaction type: (1) oleuropein, (2) hydroxytyrosol, (3) glucosyl derivate, (4) aglycone, and (5) elenolic acid. (Ozdemir *et al.*, 2014)

W. anomalus revealed higher ability to degrade oleuropein as carbon source. From a technological point, the dominance of *W. anomalus* and its high level of oleuropein assimilation and production of β-glucosidase and esterase, suggests its use as starter in fermentation of olives. In last years, researchers focused on study of oleuropein degradation performed by yeasts. In particular, Silva *et al.*, (2011) found 5 strains of *Candida oleophila* with oleuropeinolytic activity while ability to hydrolyse oleuropein by strains of *D. hansenii and T. delbrueckii* was reported by Psani and Kotzekidou, (2006).

Hydroxytyrosol and elenolic acid produced by oleuropein hydrolysis have antimicrobial and antioxidant activities, lowing blood pressure and risk of cardiovascular diseases. In addition, it is described as antitumoral compounds inhibiting proliferation human leukemia cells.

4.3 Probiotic characteristics

Until few years ago, human gut was the main source of probiotic strains, but recently studies focused on microorganisms isolated from fermented foods. The attention of many researchers is currently focused on the study of microorganisms' probiotic features in order to prepare functional foods as probiotic table olives (Lavermicocca *et al.*, 2005). For example, Lavermicocca *et al.,* (2005) showed that probiotic *L. paracasei*, *L. rhamnosus* and *Bifidobacterium bifidum* can colonise olive skins. Abriouel *et al.,* (2011) isolated from brines of naturally Aloreña green table olives a strain of *L. pentosus* with interesting probiotic traits as inhibition of human pathogenic bacteria, survival at low pH (1.5) and bile salt tolerance (3%). Some promising strains were found from several researchers as *L. plantarum* and *Leuconostoc mesenteroides* isolated from Italian table olives and *L. pentosus* found in Spanish table olives (Bevilacqua *et al.,* 2010; Bautista-Gallego *et al.,* 2013; Botta *et al.*, 2014); these strains showed promising technological characteristics and probiotic traits and can be used as multifunctional starters in table olives processing (Rodríguez-Gómez *et al.,* 2013; Rodríguez-Gómez *et al.,* 2014). Several yeasts (*D. hansenii, T. delbrueckii, K. lactis, Kluyveromyces marxianus, Kluyveromyces lodderae, S. boulardii*) exhibited probiotic properties such as inhibition of growth of pathogen microorganisms, acid and bile tolerance and resistance through gastrointestinal tract. For example, a strain of *S. cerevisiae* reduces growth of *Escherichia coli* in a dynamic gastrointestinal model (Etienne-Mesmin *et al.,* 2011). However, *S. boulardii* is the only yeast with clinical effects and proven probiotic efficiency in double-blind studied (Moslehi-Jenabian *et al.,* 2010).

According to the guidelines of Food and Agriculture Organization (FAO 2001) for the evaluation of probiotics for human food applications, resistance to gastrointestinal conditions is one of most important characteristic of probiotic strains. Results obtained in this study showed that *C. diddensiae*, *S. cerevisiae* and one strain of *C. boidinii* had higher survival percentage to gastric and pancreatic digestion than probiotic strains *S. boulardii* and *L. pentosus*. Similar results were achieved by Bonatsou *et al.,* (2015) that reported high

resistance of S. cerevisiae in both gastric and pancreatic digestion than *L. rhamnosus* GG. These results are also in line with the findings by Pennacchia *et al.,* (2008), that observed high ability to survive conditions simulating gastrointestinal tract of strains of *S. cerevisiae* isolated from different food. On the contrary, Silva *et al.,* (2011) reported low survival to acidic conditions of *S. cerevisiae* isolated from Portuguese table olives.

Assimilation of cholesterol is important for yeasts development. In fact, sterols play an important role in formation of eukaryotic membrane (Şanlidere Aloğlu *et al.,* 2016). Many studies reported ability of lactic acid bacteria to remove *c*holesterol from media supplemented with cholesterol and bile salts (Pereira and Gibson, 2002; Kimoto *et al.,* 2016; Şanlidere Aloğlu e*t al.,* 2016). Regarding yeasts, it was reported that yeasts removed cholesterol from medium supplemented with cholesterol micelles (Psomas *et al.*, 2003). Kourelis *et al.,* (2010) described ability of *S. cerevisiae*, *S. boulardii* and *Candida* strains isolated from feta cheese or gastrointestinal tract of infants to reduce cholesterol *in vitro*. In addition, Şanlidere *et al.,* (2015) observed that low amount of lyophilized yeasts administered with animal feed reduced the level of cholesterol of rat at the rate of 25%.

Indeed, this is the first study that assess ability of yeast strains isolated from table olives to remove cholesterol *in vitro*. In this work, examined yeast strains exhibited maximum percentage of cholesterol degradation in medium supplemented with cholesterol and dextrose while they showed low degradation in presence of Oxgall. Various mechanisms of cholesterol-lowering has been hypothesized. The best studied mechanism of probiotic microorganisms described enzymatic deconjugation of bile acids by bile-salt hydrolases of probiotics bacteria (Lambert *et al.*, 2008). Produced bile acids are less soluble and cholesterol was used to form new bile resulting in a decreasing of cholesterol level. Another process proposed is related to the capability of probiotic microorganisms to bind cholesterol. This mechanism is growth and strain dependent. However, it has been observed that dead cell can remove cholesterol from medium, indicating that cholesterol could be absorbed by the surface of probiotic cells (Usman and Hosono, 1999). However,

Psomas *et al.,* (2003) reported that cholesterol removed from laboratory medium was not degraded but it was assimilated by yeast cells. This hypothesis should be confirmed in further studies.

4.4 Interaction between yeasts and lactic acid bacteria

4.4.1 *In vitro* biofilm production

Production of biofilm in fermented foods is reported by several authors. Concerning table olives, Nychas *et al.*, (2002) described for the first time presence of biofilm on olive skin during fermentation. Subsequently, different studies mentioned presence of poly-microbial community on table olives skin processed by Spanish and natural method (Domínguez-Manzano *et al.,* 2012; Arroyo-López *et al.,* 2012; Benítez-Cabello *et al.,* 2015). For this reason, ability to form biofilm in vitro of yeast strains selected by DNA fingerprinting in presence of *L. pentosus* TOMC 2 was evaluated.

Among combined cultures, Cb18-Lab2 and Cb60-Lab2 showed the highest biofilm production followed by Sbo-Lab2 and Wa1- Lab2. These results are in line with the findings by (Grounta *et al.,* 2015), that reported presence of *L. plantarum*, *L. pentosus*, *W. anomalus*, *P. guilliermondii* and *D. hansenii* in biofilm on vessels used for Spanish-style olive processing. In recent years, several studies about table olives elaborations have indicated presence of poly-microbial community on olive skin (Domínguez-Manzano *et al.,* 2012; Arroyo-López *et al.*, 2012; Rodríguez-Gómez *et al.,* 2013). These communities are formed by yeasts and bacteria species embedded in complex exopolysaccharide matrix (Arroyo-López *et al.,* 2012; Domínguez-Manzano *et al.*, 2012). The main yeast species found in poly-microbial biofilm of table olives are *C. sorbosa*, *G. candidum* and *P. galeiformis*. Grounta and Appanage, (2014) observed biofilm formed by *L. pentosus* and *P. membranifaciens* on surface of Greek black olives while Benítez-Cabello *et al.,* (2015) described biofilm of natural green cv. Gordal olives consisted of same microorganisms.

Among the combined culture, *C. boidinii* strains Cb18 and Cb60 exhibited highest amount the biofilm. SEM analysis revealed that biofilm consisted of only *C. boidinii* cells. Moreover, the contact between *C. boidinii* and *L. pentosus* is not necessary for biofilm production of *C. boidinii.* León-Romero *et al.*, (2016) achieved similar results and demonstrated that biofilm formation of *C. boidinii* in mixed with *L. pentosus* is inhibited by D -(+) mannose.

4.2.1 Biofilm production in mixed culture of *Candida boidinii* and lactic acid bacteria

From eight strains of *C. boidinii* and three of LAB, 3 strains of *C. boidinii* Cb18, Cb60 and NDK27A1 and one strain of *L. pentosus* were the highest producer of biofilm. These results suggested that ability of *C. boidinii* to form biofilm could be limited to *L. pentosus.*

Several studies indicate that some mixed culture of LAB and yeast isolated from food increase biofilm formation (Furukawa, 2016; León-Romero *et al.*, 2016). For example, it was showed that several combinations of yeast-LAB increased biofilm production such as co-culture of *S. cerevisiae–L. casei*, *S. cerevisiae -L. plantarum* (Kawarai *et al.*, 2007; Furukawa *et al.,* 2010). Interspecies cell-cell co-aggregation is important for mixed biofilm formation between *S. cerevisiae* and *L. plantarum*. The co-aggregation is inhibited by heat, proteinase K and D-(+) mannose.

In attempt to isolate the factors that induces biofilm development, the genome of the eight strains of *C. boidinii* isolated from different source were sequenced and will be analysed in depth in further studies. Preliminary results obtained from bioinformatic analysis and RAPD-PCR shows that the eight selected *C. boidinii* strains are different biotypes of the same species. Lin *et al.,* (1996), shows using karyotyping and chemical analysis that strains ascribed to *C. boidinii* species are characterized by great biodiversity.

5. CONCLUSIONS

In this study it has been shown that yeast strains isolated from Bosana brines exhibited interesting technological and probiotic properties. In fact, it was found that strains belonging to *W. anomalus*, *C. boidinii* and S. *cerevisiae* species have promising probiotic and technological potential that for improvement of table olive processing and sensory characteristics of final product. These findings will help to design a single or mixed multifunctional culture starter that could enhance preservation, texture, aroma, and flavour of final product. Moreover, the application of multifunctional starter could be useful to decrease costs of olives processing and design an "eco-friendly" process. Data of biofilm production in vitro highlighted the strong ability of *C. boidinii* strains Cb18, Cb60 and NDK27A1 to form biofilm in presence of *L. pentosus*. Future work will involve transcriptomic, proteomic and epigenetic studies to clarify mechanism of biofilm formation of *C. boidinii.*

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