Cyprus University of Technology

Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

Study of microbiological and physicochemical characteristics of fermented table olives in Cyprus and the multifunctional properties of the predominant microbiota

Dimitrios A. Anagnostopoulos

Limassol, July 2020

CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND ENVIRONMENTAL MANAGEMENT

DEPARTMENT OF AGRICULTURAL SCIENCES, BIOTECHNOLOGY AND FOOD SCIENCE

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Approval Form

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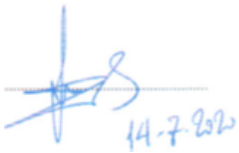

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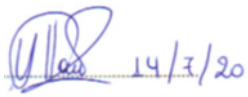

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Ευχαριστίες

Φτάνοντας στο σημείο να γράφω το κομμάτι των ευχαριστιών, σημαίνει πως έφτασα στο τέλος ενός πολύ όμορφου και μαγευτικού ταξιδιού, κατά τη διάρκεια του οποίου απέκτησα πολύ σημαντικές εμπειρίες που θα τις έχω εφαλτήριο για όλη μου τη ζωή. Είναι αλήθεια ότι η δομή και η ιδιοσυγκρασία των διδακτορικών σπουδών είναι κατί παραπάνω από τον τίτλο του Δρ. που απονέμεται. Αποτελεί πηγή έμπνευσης και αλλαγή του τρόπου σκέψης που αντιλαμβανόμαστε τα πράγματα. Νιώθω ευλογημένος που μου δόθηκε η ευκαιρία να περάσω από αυτό το στάδιο και αν γυρνούσα τον χρόνο πίσω, θα το ξαναέκανα χωρίς δεύτερη σκέψη. Όμως, θα ήταν ανέφικτο να ολοκληρώσω τον κύκλο των διδακτορικών μου σπουδών, χωρίς την υποστήριξη και την καθοδήγηση πολλών ανθρώπων, οι οποίοι συνέβαλαν είτε έμμεσα είτε άμμεσα στην επιτυχή ολοκλήρωση αυτού του πολύ σημαντικού κεφαλαίου της ζωής μου.

Ένα ευχαριστώ είναι πολύ λίγο για τον μέντορά μου και επιβλέποντα καθηγητή μου Δρ. Δημήτρη Τσάλτα. Τον ευχαριστώ πολύ για την θέση που μου προσέφερε και την ευκαιρία που μου έδωσε να δουλέψω δίπλα του. Η καθοδήγησή του, η συνεχής παρότρυνσή του, οι συμβουλές του και η ψυχολογική και οικονομική του στήριξη, συνέβαλαν τα μέγιστα στη διαμόρφωση ενός ιδανικού περιβάλλοντος για την επιτυχή ολοκλήρωση των σπουδών μου. Νιώθω πολύ τυχερός που είχα την ευκαιρία να τον γνωρίσω και να δουλέψω υπό την αιγίδα του, καθώς πρόκειται για έναν εξαιρετικό δάσκαλο, αλλά και σπάνιο άνθρωπο.

Ευχαριστώ το Τμήμα Γεωπονικών Επιστημών, Βιοτεχνολογίας και Επιστήμης Τροφίμων του Τεχνολογικού Πανεπιστημίου Κύπρου, που με φιλοξένησε ως υποψήφιο διδάκτορα, καθώς επίσης και το Ευρωπαϊκό Ερευνητικό Πρόγραμμα με το ακρώνυμο «ΑΓΡΟΤΑΥΤΟΤΗΤΑ – INTERREG Ελλάδα-Κύπρος» για τη χρηματοδότηση σε μέρος της διδακτορικής μου διατριβής.

Επίσης, θα ήθελα να ευχαριστήσω τα μέλη της συμβουλευτικής μου επιτροπής, την Δρ. Έφη Τσακαλίδου και τον Δρ. Φώτη Παπαδήμα, για τις χρήσιμες συμβουλές τους καθ' όλη την διάρκεια της διατριβής μου.

Από κει και πέρα θα ήθελα να ευχαριστήσω όλους τους φίλους και συναδέλφους του Tsaltas + Papademas team (Έλενα Καμιλάρη, Ελένη Ξενοφώντος, Μαρία Κοσμά, Δρ. Αγνή Χατζηλούκα, Δρ. Μιχάλης Χριστοφόρου, Δρ. Δέσποινα Μποζούδη, Δημήτρης Αδαμίδης, Χρίστος Βούρας, Δρ. Μαρία Ασπρή, Παναγιώτης Μουσικός), με τους οποίους περάσαμε ατέλειωτες ώρες στο εργαστήριο και μοιραστήκαμε πολλά, τόσο σε επιστημονικό, όσο και σε φιλικό επίπεδο. Εύχομαι στον καθένα ξεχωριστά τα καλύτερα και να επιτύχουν τους στόχους τους.

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Ευχαριστώ μέσα από τα βάθη της καρδιάς μου, τη σύντροφό μου Μαρίνα, η οποία μέσα από την αμέριστη συμπαράσταση και κατανόησή της, μου υπενθύμιζε κάθε στιγμή ότι βρίσκεται δίπλα μου, στα εύκολα και στα δύσκολα. Το μεγαλύτερο κομμάτι της επιτυχίας αυτής οφείλεται σε εκείνη. Σ ευχαριστώ για όλα!!

Τέλος, θα ήθελα να επισημάνω ότι η παρούσα διατριβή αφιερώνεται στον αδελφικό μου φίλο Μιχάλη, ο οποίος ‘έφυγε’ τόσο άδικα πριν λίγα χρόνια, αφήνοντάς με πίσω να εκπληρώσω ένα ένα τα όνειρα που κάποτε κάναμε μαζί. Αδερφέ μου δεν θα σε ξεχάσω ποτέ!!

ABSTRACT

Table olives are one of the most well-known traditionally fermented vegetables and their global consumption is increasing exponentially. According to the directly brining method, table olives are produced spontaneously, without any pre-treatment. This process remains empirical and inconstant, since it is strongly affected by physicochemical attributes of the fruit and the microbiota coming from the olive fruit and the environment. Nowadays, process modernization is extremely recommended worldwide, aiming to innovative products and/or processes. The stabilization of fermentation and thus, increasing the added value of the final product is a highly needed innovation making one of the oldest traditional food processes and products highly modernized. Furthermore, the reduction levels of sodium chloride used during processing is one of the main industry's challenges. In this regard, in the present thesis, the significant contribution of a commercial starter culture containing *L. plantarum* to both Cypriot and Picual table olives fermentation is illustrated, even in lower NaCl concentration (7%), supporting the application of starter cultures in table olives processing. Furthermore, considering microorganism acclimation to local conditions, industrial interest and consumers demand, for the development of indigenous starter cultures, several LAB and yeasts strains were isolated from Cypriot table olives and screened for interesting technological and potential probiotic features. Results indicated that Cypriot olives is a new source of probiotic microorganisms, as a series of them (including LAB and yeast strains) exhibited very promising multifunctional profile and thus, are recommended to be used as starter cultures at industrial scale fermentation process, in order to produce stable, novel and potentially functional table olives. Finally, the present thesis fully supports the concept of local microbial terroir and can potentially fingerprint the products.

Keywords: *Table olives, Fermentation, Starter culture, LAB, Yeasts, Technological properties, Probiotics*

ΠΕΡΙΛΗΨΗ

Η επιτραπέζια ελιά είναι ένα από τα πιο δημοφιλή, παραδοσιακά, ζυμούμενα προϊόντα και η παγκόσμια παραγωγή και κατανάλωσή της αυξάνονται εκθετικά. Σύμφωνα με τη μέθοδο του φυσικού τύπου παραγωγής, οι ελιές υπόκεινται σε απευθείας ζύμωση σε άλμη, χωρίς καμία προ-επεξεργασία ξεπικρίσματος. Η διαδικασία αυτή παραμένει μέχρι και σήμερα εμπειρική και ασταθής, καθώς επηρεάζεται έντονα από τις φυσικοχημικές ιδιότητες του καρπού και τη μεταβολική δραστηριότητα μικροοργανισμών, οι οποίοι είναι προσαρτημένοι στον καρπό ή μεταδίδονται από το περιβάλλον. Στις μέρες μας, ο εκσυγχρονισμός της διαδικασίας παραγωγής έχει κεντρίσει το ενδιαφέρον τόσο της βιομηχανίας όσο και της επιστημονικής κοινότητας, με στόχο τη σταθεροποίηση της ζύμωσης και συνεπώς, την αύξηση της προστιθέμενης αξίας του τελικού προϊόντος. Επιπλέον, η μείωση του επιπέδου χλωριούχου νατρίου που χρησιμοποιείται κατά τη διάρκεια της ζύμωσης, είναι μία από τις κυριότερες προκλήσεις της βιομηχανίας και απαιτήσεις της αγοράς. Για τον λόγο αυτό, στην παρούσα διατριβή, εφαρμόστηκε και μελετήθηκε η χρήση μιας εμπορικής καλλιέργειας εκκίνησης (*L. plantarum*) στη ζύμωση τόσο της κυπριακής όσο και της ποικιλίας Picual, ακόμη και σε χαμηλότερη συγκέντρωση NaCl (7%). Από τα αποτελέσματα προκύπτει η σημαντική συμβολή της καλλιέργειας εκκίνησης και προτείνεται η μόνιμη εφαρμογή της σε βιομηχανική κλίμακα. Επιπλέον, λαμβάνοντας υπόψη τόσο το βιομηχανικό ενδιαφέρον όσο και την απαίτηση των καταναλωτών για την ανάπτυξη νέων λειτουργικών τροφίμων, εξετάστηκε το τεχνολογικό και προβιοτικό προφίλ αυτόχθονων οξυγαλακτικών βακτηρίων και ζυμών, που απομονώθηκαν κατά τη ζύμωση της κυπριακής ελιάς. Τα αποτελέσματα έδειξαν ότι οι κυπριακές ελιές θα μπορούσαν να θεωρηθούν ως μία νέα πηγή προβιοτικών μικροοργανισμών, καθώς μια σειρά από απομονώσεις έδειξε ενδιαφέροντα

βιοτεχνολογικά χαρακτηριστικά. Συνεπώς, συνίσταται η χρήση τους ως καλλιέργειες εκκίνησης σε μελλοντικές ζυμώσεις βιομηχανικής κλίμακας, με σκοπό την παραγωγή σταθερών, καινοτόμων και πιθανώς λειτουργικών επιτραπέζιων ελιών. Τέλος, η παρούσα διατριβή υποστηρίζει πλήρως την έννοια του τοπικού μικροβιακού terroir και μπορεί δυνητικά να αποτυπώσει το προϊόντα.

Λέξεις-κλειδιά: *Επιτραπέζιες ελιές, Ζύμωση, Καλλιέργεια εκκίνησης, Οξυγαλακτικά βακτήρια, Ζύμες, Τεχνολογικές ιδιότητες, Προβιοτικά*

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If you deconstruct Greece, all you could see remaining is an olive tree, a grapevine, and a boat remain.

Which means, with as much, you could reconstruct her.

Odysseas Elytis

Chapter 1: General Introduction

Modified parts of this chapter have been published as book chapters

Anagnostopoulos, D.A., & Tsaltas, D. (2019). Fermented foods and beverages. In C. M. Galanakis (Ed.). Innovations in traditional foods, Chapter 10 (pp. 257–291). Woodhead Publishing https://doi.org/10.1016/B978-0-12-814887-7.00010-1

Anagnostopoulos, D.A, Bozoudi, D., & Tsaltas, D. (2017). Yeast ecology of fermented table olives: A tool for biotechnological applications. Yeast - industrial applications (pp. 135–152). Intech Chapter 6. DOI: 10.5772/intechopen.70760

1.1 Fermentation and fermented food products

Fermented foods and beverages, coming both from plants or animals, are essential components of the food culture and intangible heritage of each community in the world, playing a vital role in human's health, culture and history (Tamang et al., 2016). The use of fermentation processes comes from the ancient times by different communities all around the globe. In many cases of fermented products, the fermentation process did not change at all since their first report (Bell et al., 2017). As a result, the tradition of the fermentation processes of many communities have passed through generations and have been reported in various documents of historical, religious, scientific or technical context. The fermentation process can be considered as a biological method for food preservation, giving desirable properties such as good organoleptic properties in the final product (Ray and Joshi, 2014). Its contribution in the nutritional value and variety of sensory attributes, has made this process popular in many cultures in which fermented foods are part of the human daily intake (Xiang et al., 2019). In general, it is a low cost reproductive process, aiming to provide products with acceptable organoleptic attributes, ensuring both safety and sustainability, as well (Liu et al., 2011). Among others, taste, texture and color are the most desirable characteristics given to the final product, which are totally unlike of those presented in the raw materials. For instance, raw olives are inedible without the microbial (or chemical) induced removal of bitter tasting, provoked by phenolic compounds (Cocolin et al., 2013). Additionally, the growth of yeasts, alone or with lactic acid bacteria (LAB), achieves dough leavening during bread manufacture or the transformation of grape must to wine (Gobbetti and Gänzle, 2013). Even though both science and technology provided small modifications, while novel manufacturing technologies

arisen last decades, many of these fermentation processes are still outdated (Plessas et al., 2011).

Fermented products are a part of human nutrition since the ancient times. Their fermentation is achieved by the activity of microorganisms. As a result, foods obtain sensory characteristics, which are generally accepted by consumers (Şanlier et al., 2019). In other words, fermentation provides a biological way to produce edible products with desirable tasty and health benefits (Simango, 1997). Except from fermentation advantages, there are some disadvantages that have to be mentioned. On the one hand, eating fermented products could improve the immune system and have enhanced uptake of vitamins, phenolic and antioxidant compounds etc. (Melini et al., 2019) but, on the other hand, many of them, especially those with high salt content, could be harmful, especially for vulnerable groups (Pino et al., 2019).

1.2 Fermentation history

Fermentation process was discovered a long time ago. From an archeological point of view, fermentation is an alcoholic drink made from fruit, rice, and honey, dates from 7000 to 6600 B.C, in the village of Jiahu (Mcgovern et al., 2004) and winemaking dates from 6000 B.C, in Georgia, in the Caucasus area (Pretorius and Høj, 2004). There is strong evidence that people were fermenting alcoholic drinks in Babylon at 3000 B.C, Ancient Egypt at 3150 B.C, pre-Hispanic Mexico at 2000 B.C and at 1500 B.C (Chojnacka, 2009). Many processes (fermentation, smoking, drying etc.) were invented by ancient civilizations worldwide, in order to convert raw products to edible for consumption, a remarkable step in the food culture history of human beings (Tamang et al., 2015).

1.3 Table olives

1.3.1 General introduction

Oleaceae family comprises 600 species in 24 genera. Among 30 species of the genus Olea, *Olea europea L.* is the most well-known and closely related to Mediterranean history (Hashmi et al., 2015). It is the only member of its genus which is intended for consumption. Botanically, olives are classified as drupes including three main component tissues, the epicarp or skin (rich in chloroplasts), the mesocarp or flesh and the endocarp or pit (Ghanbari et al., 2012). The mesocarp (rich in protoplasm), constitutes the majority of fruit weight and surrounds the endocarp which progressively sclerifies during fruit maturation (Connor and Fereres, 2010). Furthermore, mesocarp consists of parenchymal cells in the middle of which there are vacuoles, which contain soluble polar essential elements (sugars, organic acids, proteins etc.).

Both geographic origin and spread of olive, are still under consideration, while there are many dissents between scientists and historians. According to a legend, the olive tree was a gift from the goddess Athena to the inhabitants of Athens, who gave her name to their city afterwards, as a sign of gratitude. Athena then taught them how to cultivate the olive tree, which then was spread to other regions of the Mediterranean. However, according to scientists, the most likely origin of the olive tree is the region of present-day Syria and then was subsequently spread to different parts of the Mediterranean. This is scientifically based on the existence of great genetic variability of olive tree in the region of Syria, while Greece is considered as a secondary region of origin of the olive. Although the first olive cultivation is estimated around 6000-5000 B.C, the earliest report of olive cultivation on the planet is in the village of Fylia in

Cyprus, in 4800 B.C (Trump, 1980). However, there are strong indications of the olive tree existence in much older times (Connor and Fereres, 2010). Nowadays, olive cultivars have spread globally (Fig. 1.1), while table olive is considered as the most important fermented vegetable worldwide, with a gross production exceeding 2.7 million tons/year, while the main producers are the Mediterranean countries (Spain, Greece, Italy, Algeria, Turkey, Egypt) (IOC, 2018).

Fig. 1. 1. Distribution of olive tree worldwide (reproduced from Cimato, 2015).

1.3.2 Olive's nutritional value

Olives are one of the main elements in daily Mediterranean diet (Fig. 1.2). Many studies have noted that Mediterranean's residents exhibit higher healthy rates, regarding to heart disease and many types of cancer (Trichopoulou et al., 2014; Dernini and Berry, 2016). Based on this finding, experts report that Mediterranean dietary type feeding contribute to longevity (Lăcătușu et al., 2019).

Fig. 1. 2. Mediterranean diet pyramid (reproduced from D'Alessandro et al., 2019)**.**

Nutrient components are present at the highest percent in olives mesocarp. They are represented by a high level of water and lipids and a low level of sugars and protein. The values of each nutrient may significantly vary depending on the cultivar, degree of maturation and post-harvest treatments (Rallo et al., 2018). They contain rich lipid (mainly oleic, palmitic, linoleic and stearic acids) and carbohydrate contents (mainly soluble decreasing sugars including glucose and fructose and non-reducing sugars such as mannitol), depending of olive breed. Their sugars concentration is lower in comparison with any other drupes since they act as precursors for fatty acids synthesis during fruit growth (Russo et al., 2012). The remaining sugar content is used as the main carbon source by microorganisms during fermentation (Lanza, 2013). Complex sugars (lignin, hemicellulose, cellulose and pectin) are also distributed in the olive fruit, playing crucial role in the structural characteristics of olive flesh (Lanza, 2013). Even

though the protein content is low, it is of great importance, due to the presence of essential amino acids like threonine, valine, leucine, and lysine, histidine, tyrosine etc. (Russo et al., 2012). Phenolics are also present in remarkable amounts, from which Oleuropein, a secoiridoid glucoside, is the main representative phenolic compound and responsible for the bitter taste of the fruit, making it inedible for consumption (Johnson and Mitchell, 2018). Its hydrolysis during processing; via microorganism's enzymatic activity (Fig. 1.3); leads to the formation of other less bitter compounds, namely oleuropein derivatives, which are contribute not only to sensory and aromatic characteristics of the olive, but also to human health via several pharmaceutical and physiological benefits (Ozturk, 2014; Gouvinhas et al., 2017).

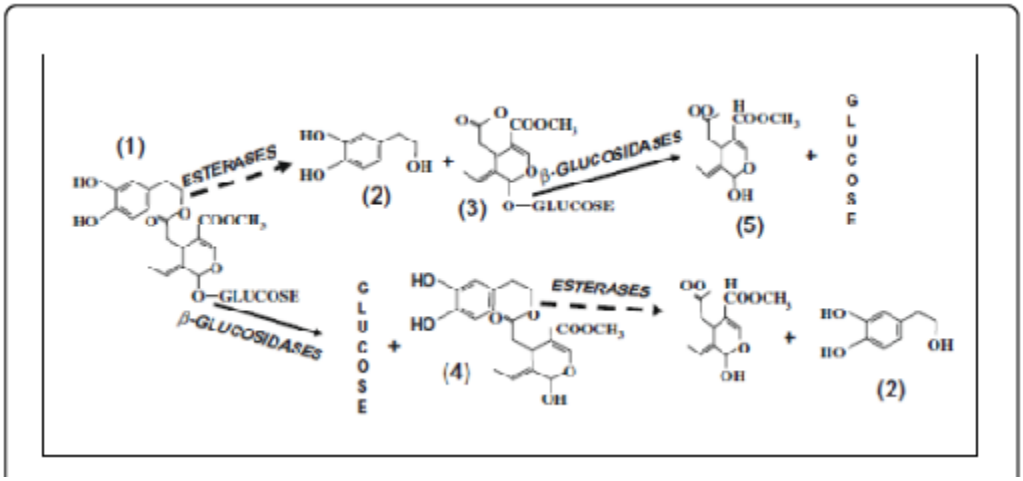

Fig. 1. 3. Enzymatic degradation of oleuropein according to the 2 different biochemical pathways a) esterase and b) β-glucosidase, producing the oleuropein derivatives: (1) oleuropein, (2) hydroxytyrosol, (3) glucosyl derivate, (4) aglycone, and (5) elenolic acid (reproduced by Ozturk, 2014).

1.3.3 Table olive processing

Table olives fermentation is one of the oldest methods applied. Despite its socio-economic importance, it is mainly craft-based and empirical process, which has not changed, since antiquity. During this process, olives obtain desirable organoleptic characteristics, enhanced technological and nutritional properties, as well as health benefits to consumers. The Trade Standard Applying to Table Olives (International Olive Oil Council, 2004) defined table olives as "the sound fruit of varieties of the cultivated olive trees (*Olea europaea L.*) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing to remove their bitterness and preserved by natural fermentation".

Among other styles, a) treated green olives (Spanish style), b) naturally black olives (Greek style) and c) ripe olives processed by alkaline oxidation (Californian style), are the main processing methods (Fig. 1.4) (International Olive Oil Council, 2004). The Spanish processing method includes treatment with sodium hydroxide solution for biochemically hydrolysis of oleuropein, washing, brining, fermentation and packaging. The Greek-style is milder than Spanish method including washing, natural fermentation in brine and packing. The Californian method involves lye treatment, washing, iron salt treatment and air-oxidation, canning and heat treatment. In the past, the method which would be applied, was depended on the olives color, that is, the harvesting time from the tree (Panagou et al., 2008). However, in our days, olives can be processed as treated or natural independently of their color (green, turning color or black). The fermentation process beased on the cultivar itself and/or on local industrial and agricultural practices (Perpetuini et al., 2020). Although some cultivars can be harvested at different stages of maturity and processed by both treated and natural

methods, normal practice is to prepare each cultivar using a single procedure and well-established local practices (Barbosa-cánovas et al., 2017). In general, processing methods not only eliminate the natural bitterness of the fruit, caused by oleuropein, for making the final product edible, but also ensure that the olives obtain specific organoleptic characteristics, generally accepted from consumers (Panagou and Tassou, 2006; Sánchez Gómez et al., 2006).

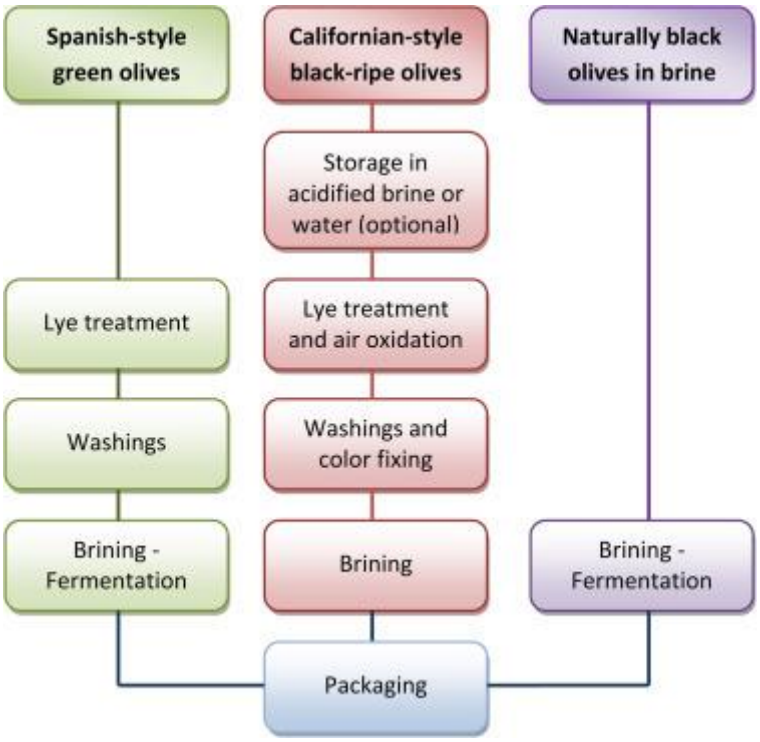

Fig. 1. 4. Flow diagram for the 3 different processing of table olives (reproduced by Papadaki and Mantzouridou, 2016).

Fermentation is considered to be over when the diffused sugars from fruits to brines are consumed by microorganisms, completely. The necessary time for this process depends on several factors, including region, variety and olive size, the involving microbiota, the salt concentration and the temperature (Romeo, 2012). Other

important factor indicating the end of fermentation is oleuropein hydrolysis, which means the total or partial debittering of olives. Nevertheless, there are neither physicochemical nor microbiological markers to objectively determine the end of process. Thus, producers decide, according to personal criteria, when olives are ready for consumption (Hurtado et al., 2008).

The abovementioned aspects reflect the fact that the final product significantly vary according to regional and national practices. Thus, table olives are considered an unstable and not reproductive product, until nowadays. In Cyprus, the main traditional method for table olive production is the directly brining, according to which treated and untreated olives are washed, put into containers and then filled with freshly prepared brine (10% v/v), in combination with an acid (usually citric acid). They are allowed to ferment for about 4 to 8 months. For enhancing their safety extent, pH is required to be 4.5 or below according to the international standard (IOC, 2011). The fruits are maintained in the brine until losing most of their natural bitterness, where they undergo the fermentation process, whose characteristics depend on the cultivar and on the applied conditions. When process finishes, the olives acquire typical characteristics of final products. Additionally, another remarkable processing method applied in Cyprus, is the directly brining with an increased salt content (~15%), which is added once a week, in combination with an acid (mainly citric acid). The penetration of excessive sodium chloride and acid into the olive fruit causes a breakdown of its tissue, permitting the endogenous hydrolase enzymes (β-glucosidase and esterase) to act on the oleuropein molecule (Ramírez et al., 2016). According to this method, olives are ready for consumption in a short-term period (ca. 1 month), although the final product may

lag to both organoleptic characteristics and safety, while it is disputed whether this process may be considered as fermentation.

1.3.3 Microbiota associated with olives fermentation

As table olive fermentation is a spontaneous process, the involving microbiota vary somewhat from year to year, cultivar to cultivar and the type of olive processing. Microbial communities of processed brines and/or olives include several species belonging to *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Staphylococcus*, LAB, yeasts and to a lesser extend molds (Heperkan, 2013; Bonatsou et al., 2017). The growth of *Enterobacteriaceae* family members taking place at the beginning of fermentation (Alves et al., 2012; Randazzo et al., 2012). These groups should be eliminated during fermentation, being undetectable at the end of the process. *Enterobacteriaceae* levels range from 2 to 4.5 log cfu/ml in the brine at the initial stage, but no viable counts (<10 cfu/ml) should be observed at the end of fermentation (Alves et al., 2012). *Clostridium* and *Pseudomonas* species may be found at the beginning of fermentation, as well. However, *Clostridium* is unable to survive till the end of a successful process, mainly due to the low pH (Heperkan, 2013). Indeed, *Clostridium botulinum* was isolated only from heat treated /conserved olives (Pereira et al., 2008) where the pH of the jar was above 4.6 (Cawthorne et al., 2005). *Pseudomonas savastanoi* isolated only from raw olives, caused the endemic disease, olive knot or tubercle (Tassou et al., 2010). Therefore, it should be noted with confidence that both *Pseudomonas* and *Clostridium* is not a problem in fermented olives under suitable conditions.

The abovementioned microorganisms presented during the first days of fermentation and progressively decrease. By the end of the primary stage, LAB become

the predominant microflora. The main genus involving in table olives is *Lactobacillus*. Other genus such as *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Lactococcus* have also been found, but to a lesser extent (Hurtado et al., 2012a). *L. plantarum* and *L. pentosus* are the most abundant species in most fermentations but, depending on the olive cultivar, the processing method and the geographical origin, other lactobacilli or genera can play an essential role or even be the main species. The substrates are converted through several metabolic pathways, making the enzymatic reactions of LAB much more complex than the name of their group suggests. Thus, the lactic fermentation of the table olive is based on the ability of LAB to produce lactic acid by soluble sugars convert, lowering the pH and increasing free acidity of the brines. The lactic acid produced, is effective for inhibiting the growth of other bacteria which can decompose and spoil the olives. While the heterofermentative *Streptococcus* and *Leuconostoc* are the least acid-producing species; due to the production of Co_2, acetic and other acids; the majority of *Lactobacillus* group is homofermentative and produce the highest amounts of acid. In addition, *Pediococcus* and *Enterococcus* are somewhere between the two abovementioned genus (Liu, 2002). In terms of a proper fermentation, the presence and the fast growth of LAB, are required (Hurtado et al., 2012a). Although the initial conditions are inconstant, it is well-established that LAB grow spontaneously during olives processing, but in some cases they can be substituted by yeasts. LAB can convert carbohydrates into CO_2, lactic acid and other organic acids without the presence of oxygen in the medium. Thus, the changes that take place in this conditions do not modify the composition of olives to any great extent.

When the process is completed, olives are maintained in brines until proceed to market. Brines with properly fermented olives contain mainly lactic and acetic acids,

whereas the presence of succinic, citric, malic and tartaric acid has also been reported to lower concentrations (Bleve et al., 2015; Papadelli et al., 2015). During the storage period, the development of "zapatera" spoilage is of great concern. Metabolites such as formic, propionic, butyric, valeric, caproic and caprylic acids in the brine have been associated with this type of spoilage. The first off odors appear have been described as "cheesy" and as the spoilage progress fecal odor is developed. Mainly *Propionibacterium* species via propionic and highly acetic acid production and *Clostridium* species are the microorganisms associated with this condition. For avoiding the growth of such bacteria, the pH should be kept below 4.3 and the sodium levels in the brine should be raised up to 8% (Lanza, 2013).

Another major group of microbes involving during olives processing are yeasts. Among other genera, *Candida*, *Pichia*, *Debaryomyces*, *Wickerhamomyces* and *Saccharomyces* are the main yeasts associated with table olive processing, playing a double role; beneficial or prejudicial; (Arroyo-López et al., 2012a). As already mentioned, in a proper fermentation, which demands high levels of free acidity and low pH (pH<4.5) in the brine, LAB should predominate over yeasts. This is generally achieved when using low salt brines. Under high salt brining (~10% w/v NaCl), yeasts may overcome LAB and due to weaker acid produced (mainly acetic acid), results in a final product with milder taste and less self-preservation characteristics (Aponte et al., 2010). Excessive growth of fermentative yeasts has been linked with gas pockets formation due to vigorous carbon dioxide production and fruit softening (Arroyo-López et al., 2012b). Some strains of *R. minuta*, *W. anomalus* and *D. hansenii* have been reported to produce enzymes including proteases, xylanases and pectinases causing softening of the fruits (Hernández et al., 2007; Bautista-Gallego et al., 2011b).

Furthermore, their presence during olives storage may cause clouding of the brines and lactic acid assimilation, raising safety concerns of the final product (Arroyo López et al., 2005). However, recent studies show the other side of coin, reporting that via enzymatic activities, yeasts may produce desirable metabolites such as higher alcohols, esters and other volatile compounds contributing in aroma and flavor development (Arroyo-López et al., 2012a; Bonatsou et al., 2018a; Porru et al., 2018). Furthermore, other interesting enzymatic and biotechnological capacities, may lead to a plethora of desirable traits. Degradation of polyphenols, production of killer toxins and enhancement of LAB growth are among such attributes (Psani and Kotzekidou, 2006; Hernández et al., 2007; Aponte et al., 2010; Silva et al., 2011; Bonatsou et al., 2015).

1.3.4 Starter culture and functional olives

As previously mentioned, olives fermentation occurs spontaneously, influenced by various intrinsic and extrinsic factors (Hurtado et al., 2008). During such an uncontrolled process, development of spoilage microorganisms and deterioration of the product is a possible scenario, leading to economic losses for the table olive industry.

Monitoring and constant adjustment of the salt concentration, as well as the acidification of the brine, are the main industrial practices to control the process and avoid abnormal fermentation (Bonatsou et al., 2017). Nowadays, a promising way to control olive fermentation is the use of starter cultures that decrease the risk of spoilage microorganism growth, accelerating both the course of fermentation and acidification of the brine (Panagou and Tassou, 2006; Corsetti et al., 2012).

Several commercial starter microbes have already been offered in the market. Although the interest in their application is increasing steadily, their use is still

uncommon. The reason is that the desirable control of the process may not be achieved because those allochthonous strains have not been optimized for this particular fermentation and thus, they may not be able to survive (Ruiz-Barba and Jiménez-Díaz, 2012). Although the latter hypothesis should be deeply investigated, recent studies suggest that well-defined autochthonous strains, which are adapted and competitive in olive fermentation matrix, could also be used as starter culture (Tataridou and Kotzekidou, 2015a). Either way, the selection of potential starter cultures is generally based on the following criteria: (i) fast and predominant growth, (ii) homofermentative metabolism, leading high acidification environment, (iii) tolerance to the harsh environment of the brine such as salt, organic acids and phenolic compounds, (iv) minimum nutritional requirements, (v) ability to grow at industrial applied temperatures, and (vi) ability to degrade phenolic compounds, mainly oleuropein (Hurtado et al., 2012a; Heperkan, 2013; Bonatsou et al., 2017).

Different strains from different cultivars and preparation methods have been studied for interesting technological properties, in pursuit to their potential use as starters, with focus mainly on *L. plantarum* and *L. pentosus* as the main species associated with fermented olives (Panagou and Tassou, 2006; Panagou et al., 2008; Sabatini and Marsilio, 2008; Pistarino et al., 2013a; Randazzo et al., 2014, 2017; Pino et al., 2018; Benítez-Cabello et al., 2019; Pino et al., 2019). They have also been employed as single or co- inoculated cultures and their effectiveness is associated with the type of cultivar and processing method used (Panagou and Tassou, 2006). The species of *L. plantarum* is commonly used to ferment plant-based food products, where phenolic compounds are abundant, as this bacterium possesses relevant enzymatic activities to degrade such compounds. Several complex phenolics can be hydrolyzed

by *L. plantarum* to simpler and more biologically active compounds leading to the production of functional foods. The in addition adaptative behavior of *L. plantarum* induced by phenolics under stress conditions, modulates beneficial traits for its gastrointestinal survival (Rodríguez et al., 2009). Thus, *L. plantarum* strains can lead to the development of an autochthonous starter culture, in order to achieve the biological debittering of olives (Kaltsa et al., 2015).

Apart from their use to safely-drive and accelerating the process, other functional properties such as their probiotic potential are desired, based on consumer's demand for the production of functional foods. Towards modern nutrition requirements, the interest in production and consumption of health promoting products, in the altar of the concept "make your food become your medicine", is increasing rapidly. This has led the food industry to the development of a diverse range of food products with enhanced characteristics known as "functional foods" (Bigliardi and Galati, 2013). The term of functional foods was firstly introduced as a concept in Japan, in 1980s (Arai, 1996). They are defined as "foods that beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. They are consumed as part of a normal food pattern and they are not a pill, a capsule or any form of dietary supplement" (Bigliardi and Galati, 2013). With the aim of developing such functional foods, probiotics have received much attention recently. Probiotics can be defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Intake of probiotics contributes to the maintenance of the intestinal microbial balance of the host by inhibiting pathogens, lowering the risk of gastrointestinal diseases. They are associated with

anticarcinogenic, antihypertensive, antimicrobial, antioxidant and immunomodulatory properties while also alleviate certain intolerances, such as lactose intolerance and food allergies prevention (Bautista-Gallego et al., 2013; Oliveira et al., 2017; Perpetuini et al., 2020). So far, the majority of the current functional foods are mainly dairy based (yogurts, cheeses, kefir, etc.). However, due to the increasing number of individuals faced with lactose intolerance and/or milk protein allergy, the need for the production of non-milk based probiotic products has been arisen (Bevilacqua et al., 2015; Bonatsou et al., 2017).

In this regard, table olives have gained much of the scientific attention, as it is a product which acts as a biological carrier of probiotic microorganisms (Perpetuini et al., 2020). Thus, this traditional product could be transformed into a promising novel functional food. The first attempt to incorporate probiotic microorganisms on table olives was applied some years ago (Lavermicocca et al., 2005). In this work, different types of ready to eat table olives were inoculated with seven probiotic strains of *Lactobacillus* and *Bifidobacteria*. The tested strains were able to adhere and colonize the olive surface and one of the strains, *L. paracasei IMPC2.1*, was recovered from human fecal samples of healthy volunteers, in high population amounts. In another work, the same strain was used both as as starter culture in the fermentation process of *cv Bella di Cerignola* green olives, leading to a final product with functional appeal (De Bellis et al., 2010).

During the last decades, a high number of LAB originating from different olives and regions has been characterized for functional properties. For instance, a total of 144 LAB isolates obtained from naturally fermented *Aloreña* green olives were screened for their potential probiotic features (Abriouel et al., 2012). In this study, fifteen *L.*

pentosus strains and one *Ln. pseudomesenteroides* strain had antimicrobial properties and tolerance to low pH and to high bile salt concentration. In another study, Lactobacilli isolated during fermentation of green olives, exhibited interesting probiotic properties, during a series of *in vitro* phenotypic tests (Bautista-Gallego et al., 2013). Furthermore, four well screened probiotic *L. pentosus* strains, were used as starters to better control Spanish-style green olive fermentation, with very satisfied results (Rodríguez-Gómez et al., 2013). Other LAB strains isolated from naturally fermented olives were found to possess desirable in vitro probiotic properties, similar or superior to the reference probiotic strains *L. rhamnosus GG* and *L. casei Shirota*. (Argyri et al., 2013). Finally, it should be noted that despite the nowadays extensive literature for characterization of potentially probiotic LAB strains, the investigation of novel probiotic strains from new sources (varieties, regions, etc.), is always of great interest.

As previously noted, the role of yeasts in table olives processing has been revised (Oliveira et al., 2017). Nowadays, they are considered interesting microorganisms that can be used as starter (single or in combination with LAB), especially due to their potential probiotic effects. For instance, *S. boulardii*, is a yeast species with proven probiotic efficiency in double blind clinical studies (Sazawal et al., 2006). Thus, isolating novel yeast strains with probiotic characteristics, mainly from table olives, is of great interest. In this regard, several studies have been documented the probiotic potential of yeasts isolated from fermented table olives (Silva et al., 2011; Bonatsou et al., 2015; Porru et al., 2018). In particular, Psani and Kotzekidou, (2006), were first screened potential probiotic yeast species, isolated from table olives. They found 2 strains (*T. delbrueckii* and *D. hansenii*) with interesting properties, such as tolerance in high bile salt concentrations and low pH values, as well as antimicrobial

activity against food pathogens. Similar findings have been noted by other works (Bevilacqua et al., 2012; Oliveira et al., 2017; Bonatsou et al., 2018a; Zullo and Ciafardini, 2019). All these studies are summarizing a plethora of interesting properties. For instance, diverse yeast strains showed great ability to reduce cholesterol serum levels, as well as strong phytase activity. The latter is of great importance as phytate has a strong chelating ability to form insoluble complexes with divalent minerals of nutritional importance such as zinc, calcium, magnesium and iron. Due to a lack of some required enzymes, humans cannot degrade phytate complexes in the gastrointestinal tract. It is known that dephosphorylation of phytate is catalyzed by phytases, which are widespread in diverse yeast species (Bonatsou et al., 2018a). Thus, yeasts could be included in humans' diet in order to contribute to this aspect. Continuously, folates (vitamin B9) are considered essential co-factors in the biosynthesis of nucleotides and play an important role in cellular replication and growth. It is common that mammals cannot synthesize folates. As yeasts contain a folate biosynthesis pathway and can produce natural folates, they could play this role for mammals. Some of those species, which have a high folate biosynthesis pathway, are *S. cerevisiae* and *C. glabrata* (Moslehi-Jenabian et al., 2010). Other yeast species, such as *P. membranifaciens* and *P. farinose*, isolated from table olives, exhibit the ability to produce B-complex vitamins (Arroyo-López et al., 2012a). This means that yeasts can synthesize a number of bioactive compounds, which can serve as natural antioxidants. Moreover, researchers are interested in screening yeasts for free radical scavenging activity. For instance, a *S. cerevisiae* strain, isolated from table olives, produced high scavenging capacity (Oliveira et al., 2017). The production of natural antioxidants can improve human health, via the deceleration of fatty acids oxidation, preventing damage of cells, DNA, etc. (Gil-Rodríguez et al., 2015). For all the above-

mentioned perspectives, the study of potential probiotic yeast species, isolated from table olives fermentation is now at the forefront of scientific research.

1.4 Thesis scope and outline

Based on the aforementioned aspects, the aims of the present thesis were 1) to study the microbiological, physicochemical and sensorial attributes of Cypriot **(Chapter 2)** and *cv.* Picual **(Chapter 3)** table olives during spontaneous and inoculated fermentation process, while a reduction of NaCl content was also applied, 2) to determine new potential biochemical markers for fermentation monitoring and 3) to screen the multifunctional characteristics of indigenous LAB **(Chapter 4 & 6)** and yeast **(Chapter 5)** species, isolated during fermentation of Cypriot table olives, regarding interesting technological and potential probiotic properties, in order to be used in the future as starter cultures, to produce functional table olives. Finally, in **Chapter 7** the main findings of this work are summarized and the main conclusions of the thesis are discussed, providing some crucial topics as future perspectives.

Chapter 2: Microbiological, physicochemical and sensorial changes during spontaneous and inoculated fermentation of green Cypriot table olives

A slightly modified version of this chapter has been published

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ABSTRACT

Table olives fermentation remains an empirical and variable process, since it is strongly influenced by physicochemical parameters and microbiota presented in olive drupes. In the present work, Cypriot green cracked table olives were processed directly in brine (natural style), using three distinct methods: non-inoculated/spontaneous fermentation (control), inoculation with lactic acid bacteria at a 7% or a 10% NaCl concentration. Sensory, physicochemical and microbiological parameters were monitored at intervals and differences were detected across treatments. Results indicated that the predominant microflora in the inoculated treatments were lactic acid bacteria, while yeasts predominated in control. Consequently, starter culture contributed to faster acidification and lower pH. This was attributed to a successful lactic acid production, contrasting the acetic and alcoholic fermentation observed in control. Furthermore, it was established that inhibition of enterobacteria growth was achieved in a shorter period, even at a significantly lower salt concentration, compared to the spontaneous fermentation. Even though no significant variances were detected in terms of the total phenolic content and antioxidant capacity, the degradation of oleuropein was achieved faster in inoculated treatments, thus, producing higher levels of hydroxytyrosol. Notably, the reduction of salt concentration, in combination with the use of starter, accented novel organoleptic characteristics in the final product, as confirmed from a sensory panel; hence, it becomes obvious that the production of Cypriot table olives at reduced NaCl levels is feasible.

2.1. Introduction

Table olives are an essential element, which is closely related to Mediterranean history. Nowadays, they are considered as the most important vegetables worldwide, with a gross production exceeding 2.7 million tons/year (IOC, 2018). The main purpose of table olive fermentation is to achieve a preservation effect and, in parallel, enhancing the organoleptic attributes of the processed product, hence, meeting consumer's needs. However, in order to standardize this process and consequently secure the quality of the final product, the study of microbiological and physicochemical descriptors for monitoring the fermentation is a pre-requisit (Bleve et al., 2014).

Natural fermentation is mainly driven by yeasts and LAB, present on olive drupes (Corsetti et al., 2012; Campus et al., 2018). It has been noted that the LAB are responsible for the fermentation of treated olives (Spanish style). However, in a natural process, LAB and yeasts compete, and in some cases, yeasts can exclusively lead the fermentation (Hurtado et al., 2012a). Except from these two dominant groups of microorganisms, diverse microbial populations are also participating during olive fermentation, such as several species of *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Staphylococcus*, and molds (Bonatsou et al., 2017). These microorganisms via their metabolic activities contribute to crucial aspects, such as organoleptic characteristics (color, texture, flavor, etc.) and safe consumption (Heperkan, 2013). In general, LAB activity results in brine acidification, via the production of lactic and other organic acids, using the fermentable substrates, resulting in pH decrease, providing microbiological control to the final product, hence, extending its shelf life (Papadelli et al., 2015; Sorrentino et al., 2016). Oppositely, yeasts contribute to the flavor and aroma formation via the production of volatile and other

desirable compounds, while, at the same time, they enhance LAB growth and the degradation of phenolic compounds, such as oleuropein (Bevilacqua et al., 2015). However, the microbiota formation also heavily depends on olive cultivar type, since different fruit dimension and composition can affect the microbial dynamics responsible for olive fermentation and sway the sensorial attitudes of the product (Valeria, 2012).

During fermentation, major physicochemical changes are taking place. Water-soluble compounds are diffused from olives to the brine, while salt follows the opposite direction, until equilibrium is reached at the end of the brining process (Kiai and Hafidi, 2014). Fermentable sugars are the main source of carbon for microorganisms, metabolizing them into organic acids, which are essential for the stability and succession of the fermentation process.

Although the physicochemical maturation of olives and brines, during processing, has been thoroughly investigated (Panagou et al., 2008; Bautista-Gallego et al., 2010, 2011a; Bleve et al., 2014, 2015; Fadda et al., 2014; Chranioti et al., 2018; Pino et al., 2018), significant organoleptic parameters, such as texture and color, are understudied. Both are the main attributes that most affect the consumer's acceptance and may be strongly affected during processing (Bautista-Gallego et al., 2011a; Fadda et al., 2014).

During olive fermentation, a significant amount of salt is added as a preservative in order to prevent undesirable growth of pathogens or spoilage microbes and improve the organoleptic characteristics of the final product (Pino et al., 2018). However, according to the World Health Organization (WHO, 2012), the daily proposed sodium intake has been set at 5 g. Therefore, one of the main goals of the food industry is to harmonize the global nutritional policies according to this guideline. However, the

potential NaCl replacement depends on a plethora of factors, linked to cultivar type, drupe composition, as well as the processing and technological parameters (Bautista-Gallego et al., 2011a; Rantsiou, 2013). All these parameters should be well inquired before implementation at the industrial scale. Furthermore, the final product must be safe from the microbiological point of view.

Several studies reported the microbiological and chemical changes in table olives during spontaneous or controlled fermentation employing different cultivars (Panagou et al., 2008; Aponte et al., 2010; Tofalo et al., 2012; Bleve et al., 2014; Papadelli et al., 2015); however, the 'fermentation map' of Cypriot green cracked olives have not been charted yet.

For the above cited reasons, the aims of this work were a) to study the microbial and physicochemical changes of Cypriot green cracked table olives during fermentation process at industrial scale, b) to identify potential markers associated with the fermentation progress, c) to evaluate the fermentative process by adding a starter culture, and d) to study the effect of reducing NaCl concentration in combination with starter culture, in order to produce a secure, nutritious, and healthy final product.

2.2 Materials and Methods

2.2.1. Olives Samples and Fermentation Procedure

Olive fruits (*Olea europaea*) were harvested from a commercial orchard (Novel Agro, Nicosia). All fruits were harvested at the green stage of ripening, based on size uniformity criteria and even external color. After the elimination of the defective fruits, drupes were thoroughly rinsed with tap water to eliminate contaminants.

Subsequently, the olives were cracked and subjected to three different types of fermentation, in duplicate (Biological replicate). A particular amount of olive fruits (20 kg) were placed in plastic tanks of 25 L capacity filled with brines supplemented with 0.3% *w*/*v* citric acid. The citric acid was added in accordance with the Cypriot industrial standard production procedure of table olives. The process was monitored for 365 days (23 ± 2 °C). The three types of treatments were: (i) spontaneous fermentation in 10% *w*/*v* NaCl, (Control, Olive 7 [OL7], and Brine 7 [AL7]), (ii) and (iii) fermentation inoculated with a starter culture of *Lactobacillus plantarum* (final concentration 5 $\log_{10}$ cfu/ml) (Vege-Start 60', Chr. Hansen A/S, Copenhagen, Denmark) in (ii) 10% *w*/*v* NaCl (Olive 8 [OL8] and Brine 8 [AL8]) and (iii) 7% NaCl *w*/*v* (Olive 9 [OL9] and Brine 9 [AL9]). The amount of NaCl content to 7% was selected because one of the main aims of the Cypriot olives industry is to reduce the sodium content to those levels.

2.2.2. Microbiological Analysis

Samples were analyzed at regular time intervals (Days 0, 8, 15, 22, 29, 45, 60, 90, 120, 150, 210, 281, 365) throughout fermentation. They were determined for the total viable count (TVC), *Enterobacteriaceae*, LAB, yeasts, *Coliforms*, coagulase-negative *staphylococci* (CoNS), using the standard pour and spread plate methods after serial dilutions in 0.85% *w*/*v* saline water (Table 1)**.** In the case of olives, before serial dilutions, 10 g were aseptically transferred to stomacher bags filled with 90 mL of saline solution (0.85% *w*/*v* NaCl) and homogenized for 2 min using a Stomacher at 220 rpm speed (Bug Mixer, Interscience, Saint Nom, France). Volumes of 0.1 mL or 1 mL (spread and pour plate, respectively) of serial dilutions in saline solution were placed

in Petri dishes for enumeration of the microorganisms. All samples were analyzed in triplicates.

Table 2. 1. Microbiological media used for microflora enumeration.

Growth media	Microorganisms	Method	Incubation conditions
Plate count agar (PCA) (Merck, Darmstadt, Germany)	Total viable count	Spread plate	30 °C/72 h
De Man-Rogosa-Sharpe agar (MRS) (Oxoid, Basingstoke, UK) + natamycin 0.1%	Lactic acid bacteria	Pour plate/Overlay	30 °C/72 h
Sabouraud agar (Oxoid, Basingstoke, UK)	Yeast and Molds	Spread plate	25 °C/5 d
Violet red bile glycose agar (VRBGA) (BD, Sparks, MD)	*Enterobacteriacae*	Pour plate/Overlay	37 °C/24 h
Violet red bile lactose agar (VRBLA) (Oxoid, Basingstoke, UK)	*Coliforms*	Pour plate/Overlay	30 °C/24 h
Mannitol salt agar (MSA) (Oxoid, Basingstoke, UK)	CoNS	Spread plate	30 °C/48 h

2.2.3. Physicochemical Analysis

Titratable acidity (TA) was determined by potentiometric titration with 0.1 mol L^{-1} NaOH up to pH 8.3, and results were expressed as a percentage of lactic acid (*w*/*v*). pH was calculated using a pH meter (Hanna Instruments, UK). The salinity of brines was determined using a salinometer. Electrical conductivity was calculated using a

conductivity meter (Mettler Toledo, Zürich, Switzerland). Finally, water potential was determined using a WP4C dewpoint potentiometer, following the manufacturer's instructions. All measurements were performed in triplicates.

Sugars (glucose and fructose), organic acids (lactic, succinic, tartaric, acetic, citric, and malic), and alcohol (ethanol, glycerol) levels were determined during the fermentation, as described in previous studies (Bleve et al., 2015; Papadelli et al., 2015), with some modifications. In 1 mL of brine, 100 μL of $HClO_4$ was added, and the samples remained at 4 °C for 24 h, following by centrifugation at 12,000 rpm for 60 min at 4 °C. Then, the supernatants were stored at −20 °C for further analysis. Just prior to the analysis, samples were filtered (using 0.22 μm pore diameter filters). Chromatographic analysis conditions were applied as follows: Column: Aminex HPX-87H, 4.6 mm × 250 mm × 3.5 μm (Bio-Rad, Hercules, California, USA), solvent: 4.5 mM H_2SO_4 in H_2O, isocratic flow rate: 0.5 mL min^{-1}, assay temperature: 65 °C; detectors: refractive index detector for sugars and alcohols, and fluorescence at 210 nm for organic acids, injection sample volume in high-pressure liquid chromatography (HPLC) (Waters 1525): 20 μL. Quantitation (mM) was performed by standard curves generated by chromatographic analysis of the standard solutions of the respective substances at various concentrations.

Total polyphenols and antioxidant capacity of brines and fruits were quantified, followed by the identification and quantification of the main polyphenols (oleuropein, hydroxytyrosol) by HPLC (Waters 1525) analysis at regular times throughout fermentation. The extraction of the phenolic compounds was carried out, as reported by Tataridou and Kotzekidou, (2015). The determination of total phenolic components using the Folin–Ciocalteu (F.C) reagent was based on the method described previously

(Uylaşer, 2015a). The reaction products were measured spectrophotometrically at 765 nm. The results were expressed as mg/g or mg/mL of gallic acid equivalent (GAE).

The antioxidant activity was determined using the stable radical 2,2-diphenyl-1-picrylhydrazyl DPPH (Sigma-Aldrich, Taufkirchen, Germany), according to a procedure described previously (D'Antuono et al., 2018). Trolox equivalent antioxidant capacity (TEAC) was used as standard. The results were expressed as mg/g TEAC fresh weight, using the standard curve of Trolox. The measurements were performed three times.

Chromatographic analysis in the extracts was performed using HPLC (Waters 1525). The solvents (mobile phase) used were: Solvent A: 1% acetic acid HPLC grade, Solvent B: 100% acetonitrile HPLC grade, Solvent C: 100% methanol grade HPLC. Chromatographic analysis conditions were as follows: Column C18, 4.6 mm × 250 mm × 5 μm (Sigma-Aldrich, Taufkirchen, Germany), 0–20 min: 95% solvent A + 5% solvent B, 20–40 min: 75% solvent A + 25% solvent B, 40–50 min: 50% solvent A + 50% solvent B, 50–60 min: 5% solvent A + 95% solvent B, 60 min: 95% solvent A + 5% solvent B. Phenolic compounds (oleuropein and hydroxytyrosol) were estimated at the ultraviolet spectrum (254 and 280 nm) using the respectively standards. Results were expressed as means (mg/g or mg/mL) and standard deviations of three replicates.

2.2.4. Color and Texture Analysis

Color determination of table olives was performed during the whole process using a CR200 Chroma Meter (Konica Minolta, Nicosia, Cyprus). The instrument was set to the standard white color (Y = 93.9, X = 0.313 and y = 0.3209 or L * = 94.11, a * = −0.99 and b * = 0.89). The olive color was assessed by taking at least 10 random

measurements from the surface of different olives (Chranioti et al., 2018). The color was expressed as L * (bright, dark-low dark color values), a * (negative values indicate green, while positive values indicate redness), and b * (negative values indicate blue, and positive values indicate yellow). Furthermore, reduction in parameter hue angle (h *) corresponded to change in color from green to yellow. Finally, an increase in C * corresponded to a stronger color.

Texture analysis was monitored in whole fermentation by taking at least 10 random measurements of different olives, using a texture analyzer (Stable Micro Systems TA.XTplusC, Vienna Court, Lammas Road, Godalming, Surrey GU7 1YL, United Kingdom) carrying a 9.5 mm (length) and 3.2 mm (diameter) piston with a 2 mm cylindrical probe (Fadda et al., 2014). The test speed was 1.5 mm/s, and the penetration force was expressed in Newton (N).

2.2.5. Sensory Evaluation

Olive samples were evaluated organoleptically after 4 months of fermentation by a thirteen-member taste-certified panel (5 males and 8 females, aged from 20 to 45 years old) according to International Olive Oil Council (Regulation COI/OT/MO No 1/Rev.1). Texture, flavor, saltiness, bitterness, acidity, off flavors, and overall acceptance were assessed. Each of these features was rated as follows:

- Texture: 0 = soft, 5 = intermediate, 10 = coherent
- Flavor: 0 = absence, 5 = moderate, 10 = strong
- Salty: 0 = no, 5 = moderate, 10 = very much
- Bitterness: 0 = No, 5 = moderate, 10 = high
- Acidity: 0 = no, 5 = moderate, 10 = high

- Off flavors: 0 = absence, 5 = moderate, 10 = strong
- Overall acceptance: 0 = reject, 5 = moderate, 10 = strongly accept

2.2.6. Isolation of the Predominant Microflora

Representative colonies growing on De Man-Rogosa-Sharpe agar (MRS) (LAB) and Sabouraud (yeasts) agar plates were isolated at different stages of fermentation. The isolates were purified by streaking twice on the same medium after phenotypic observation using a light microscope. Pure bacterial and yeast cells were stored at −80 °C using glycerol (20%) for future use.

Finally, in order to detect the presence of the starter culture, rep-PCR genomic fingerprinting was performed on 17 random strains isolated from MRS agar, from brines AL7, AL8, and AL9 at 120 days of fermentation, using the $(GTG)_5$-primer (5′-GTG GTG GTG GTG GTG-3′). DNA from each strain was obtained according to Bautista-Gallego (Bautista-Gallego et al., 2013) and stored at −80 °C. PCR reaction and amplification conditions were applied following a method previously described (Bonatsou et al., 2018b).

2.2.7. Statistical Analysis

Data were subjected to an analysis of variance (one-way ANOVA), using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA), in order to identify statistically significant differences of microbiological, physicochemical, and sensory characteristics across fermentation treatments. Differences between means were determined by the statistical LSD test at $p \leq 0.05$. In order to study the correlations between variables and

treatments, principal components analysis (PCA) was performed (SPSS 20). Furthermore, two of Pearson's correlation matrices (among components and between components-treatments) were calculated, and an optimal Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy was established. A hierarchical clustering analysis (HCA) of the correlation coefficients was depicted using the gplots version 3.0.1 (heatmap.2 command; R Foundation for Statistical Computing, Vienna, Austria). Finally, matrices of the original component data were standardized in order to depict (via a hierarchical clustering analysis heatmap) differences in the content of the relative variables.

2.3. Results and Discussion

2.3.1. Microbiological Analyses

Microbial enumeration was determined in all treatments during fermentation (Fig. 2.1); In general, LAB and yeast numbers were steadily increased and predominated across treatments. On the contrary, *Enterobacteriaceae* and *coliforms* species were decreased, while CoNS were not detected during the whole process.

The population size of *Enterobacteriaceae* and *coliforms* was very similar (no statistical differences were detected) between brines AL8 and AL9 and differed compared to the control during the first days of the fermentation. Specifically, they were detected at an average of 3.5 log cfu/mL at the beginning of the process, but they decreased rapidly and could not be detected after 15 days of fermentation in AL8 and AL9, and after 22 days in AL7, indicating the usefulness of the starter. Indeed, according to Rodriguez-Gomez et al. (Rodríguez-Gómez et al., 2013), the use of selected *Lactobacillus pentosus* strains as starters decreased the *Enterobacteriaceae*

population faster than in the control treatment. The inoculation contribution to the inactivation of *Enterobacteriaceae* has also been noticed by other studies (Panagou and Tassou, 2006; Papadelli et al., 2015; Chranioti et al., 2018). However, it is obvious that in the present study, *Enterobacteriaceae* decreased more swiftly (about half the time). This could be justified by the use of citric acid at the beginning of the process that led to an early pH decrement at the initial stage of the process, resulting in *Enterobacteriaceae* suppression.

The population of LAB in brine samples changed significantly across the different treatments during fermentation. More specifically, there was an initial increase in LAB counts of AL7 (control) until the 22nd day of fermentation, reaching an average value of 3.95 log cfu/mL. After that peak, a slight decrease was observed, and numbers were retained until the end of the process. The low values of the LAB population in control is in accordance with the literature. LAB populations were at low levels in spontaneous fermentation, and this was linked to several factors that could have limited the adaptation of LAB in naturally fermented table olives. Some of these factors were the ambient temperature, high salt content, the availability of a source of energy, and natural inhibitory compounds presented in drupes, since the fruits were not subjected to lye treatment (Aponte et al., 2010). On the other hand, in AL8 and AL9, a slight decrease was observed during the first 8 days, followed by a major increase of LAB population, reaching a maximum rate at 120 days (7.67 log cfu/mL and 7.6 log cfu/mL, respectively). No significant differences between these two treatments were observed, except that during the first days of fermentation, LAB populations in AL9 were initially higher. This could be related to the higher diffusion of sugars from olives to the brines due to the reduced NaCl concentration. Moreover, it must be mentioned that the reduction of the LAB population for AL8 and AL9 in the first days of the process

indicated an intense competition between the starter with indigenous microflora, as well as lack of nutrient compounds. Indeed, a similar trend was noted in previous studies (Segovia Bravo et al., 2007; Chranioti et al., 2018), attributed to the lack of nutritional substrates, as well as the presence of inhibitor compounds. According to our data, it was demonstrated that the starter culture withstood the competition with the natural microflora and was not affected by a high salt concentration, while LAB population predominated in a short period, in contrast to the control treatment. Still, the prevalence of a population during fermentation is a multifactorial process and cannot be always accurately projected. Contrasting to the data of the current study, Rodríguez-Gómez et al. (2013) reported that LAB population numbers between inoculated and control treatments had no significant differences. Finally, during the second half of fermentation, a decreasing tendency was noted but always over 7 log cfu/mL, while the population in control was close to the detection limit (2 log cfu/mL).

Yeast growth had an initial lag phase in all cases, occurring across treatments and reaching the maximum level approximately at circa 8 days (7 log cfu/mL), which was in agreement with the literature (Rodríguez-Gómez et al., 2013; Bleve et al., 2014; Bonatsou et al., 2018b). Following, a major decrease in AL8 and AL9 was observed, reaching a value of 3.7 log cfu/mL and 3.5 log cfu/mL at day 60, respectively (Figure 1B). From that point, population levels were maintained steadily until the completion of the process. On the other hand, in AL7, a major increase was observed until the 8th day, and after that, the population reached and retained 7 log cfu/mL level up to the end. As a result, yeasts were the predominant microorganisms in the control treatment. According to the literature, the dominance of LAB in Spanish-style olives has been extremely reported. On the other hand, yeasts are the main organisms driving the fermentation of naturally processing olives (Medina et al., 2018), although there are

studies that have reported the presence of LAB (Abriouel et al., 2012). In the current study, LAB growth in AL7 might have been hampered by salt-tolerant yeast species, resulting in a less acidic product, which is in accordance with the literature (Marsilio et al., 2005; Bonatsou et al., 2018b). Nevertheless, yeast growth is not considered to present any consumption risk during the fermentation of green olives. On the contrary, yeast can metabolize ingredients that enrich the sensory palette and determine the quality of the final product (Arroyo-López et al., 2012b). It is worth noticing that the presence of yeasts, especially in the first days of the process, might have led to the enhancement of the starter culture in inoculated treatments due to their potential production of vitamins and other nutrients, which are mandatory for LAB growth (Tufariello et al., 2015).

The microbial composition of TVC in fruits and brines was also depicted (Figure 1E). Overall, the number of microorganisms detected in olive fruits was 1 or 1.5 log lower compared to brines, throughout fermentation. At the early stages, total viable counts ranged from 5.2 (OL7) to 5.7 (OL8, OL9) log cfu/g in pulps and from about 6.3 to 6.6 log cfu/mL in brines. The population was increased in all treatments until the 60th day, reaching a maximum value of 7.1, 7.4, 7.3 log cfu/mL for AL7, AL8, and AL9, respectively, while the populations of brined fruits were about 1 log lower than their brines. This magnitude of deviation, among fruits and brine, was also reported in a study carried out on commercialized table olives in Portugal (Pereira et al., 2008). This finding could be related to the high presence of phenolic compounds in olives, thus, high antimicrobial activity, leading to microbial inhibition, especially in the first 45 days of fermentation.

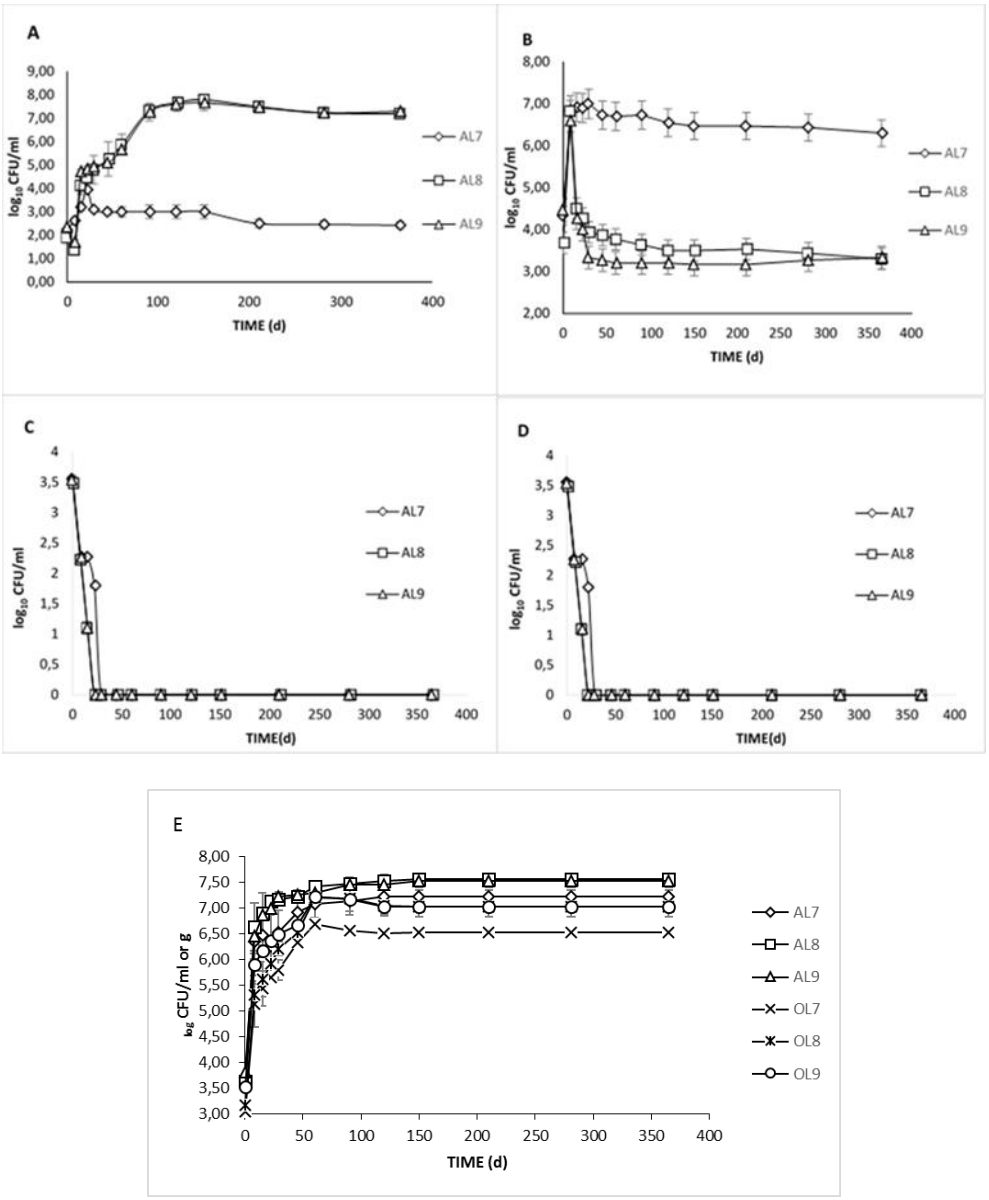


Fig. 2. 1. Evolution of microbial changes of spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) fermentation of Cypriot green cracked table olives. LAB (A), Yeasts (B), *Enterobacteriaceae* (C), *Coliforms*, and (D) TVC (total viable count) (E). Data points expressed as log10 CFU/mL of 3 replicates ± standard deviation.

2.3.2. Physicochemical Analyses

The changes in pH in the brines during fermentation of all varieties are presented in Fig. 2.2A. The initial values (Day 0) in all treatments were very low (ca. 3.3) due to the use of citric acid at the beginning of fermentation. After that, there was an increase of about 1–1.5 units until day 22, followed by a major decrease in inoculated treatments (3.5 and 3.3 for AL8 and AL9, respectively). In control, pH remained stable, reaching finally a value of 4 on the 90th day. In all treatments, a slight increase (4, 3.8, and 3.7 for AL7, AL8, and AL9, respectively) was observed, which was stabilized thereafter at about pH 4 at day 365. No differences between treatments were observed at this time point. It is crucial to mention that the fast acidification in the brine matrix is a crucial preliminary step for the succession of fermentation process. pH in brines below 4.5 preserve table olives from spoilage and pathogen microbial growth during fermentation. Furthermore, it must be noted that the positive effect of the starter in pH drop was profound, especially in the first days, which is in agreement with previous studies (Papadelli et al., 2015; Perpetuini et al., 2018; Pino et al., 2018).

The reverse change was followed on titratable acidity, as expected (Fig. 2.2B). The highest values were recorded in AL8 and AL9 due to the dominance of LAB (0.81 and 0.86% lactic acid, respectively). It is notable that the effect of initial acidification with citric acid was evident for high values of titratable acidity in the brines during the first days. The titratable acidity was higher in AL9 until the 29th day, and, thereafter, no significant differences were observed between inoculated samples. The higher values in AL9 at the first days of fermentation could be explained due to the low salt concentration, which allowed the faster diffusion of sugars from olives to brines, and thus the faster start of fermentation from LAB. The titratable acidity levels found were in accordance with the LAB enumeration and pH values, described above. Moreover, it is noteworthy that a value of more than 0.48% lactic acid in AL7 was not reached at

any time, probably due to the dominance of yeasts, in combination with the weakness of LAB to produce lactic acid due to their low population. In another study (Bleve et al., 2015), fermentation of table olives driven by yeasts attained a final pH close to 4.2–4.3, which was in good agreement with the final pH values of AL7 reported in the present study. However, even though yeasts were the dominant microbial group, the final values for pH and acidity were within the limits of the trade standard applying to table olives of the IOC (2004), where for natural fermentation, the maximum limit for pH and minimum acidity should be 4.3 and 0.3%, respectively. Notably, the higher acidic environment in AL8 and AL9 samples are enough to prevent the growth of spoilage and/or pathogen microorganisms, and thus they may provide an added value to the product. The latter could be confirmed by the faster elimination of such microorganisms, as mentioned above. Thus, our findings suggest that the use of LAB starter culture has a significant effect on the acidification of the brines, achieving a more controllable and successful fermentation.

During fermentation, the production of higher acidity in inoculated treatments caused an increase in electrical conductivity. The pH curve represents the kinetics of the production of H^- ions, while that of electrical conductivity represents the production of all ionic species (Liebeherr, 2006). Fig. 2.2C shows the changes in electrical conductivity during the whole process. As was clearly observed, there was an initial decrease in all treatments until day 22, followed by a major increase until the 60th day. Significant differences were observed in all treatments, while AL9 had the highest values from day 29 to day 60, followed by AL8 and AL7. This was in accordance with pH and acidity scores at this time point, as described above, indicating a clear correlation between the three parameters, which was also confirmed by HCA (Fig. 2.9A), as well. In a previous study (Cais-Sokolińska, 2017), it was also demonstrated a

linear dependence relationship between pH and conductivity during mixed coagulation of milk. However, according to our knowledge, this is the first study indicating the correlation between these parameters in table olives fermentation. Thus, electrical conductivity could be used as a potentially useful tool for table olives monitoring during the fermentation process.

Regarding the water potential of olive fruits, there was a clear difference between OL9 and the other two treatments during the whole process (Fig. 2.2D). The initial values of the three treatments were about −3.9 Mpa. There was a decrease during the first 60 days, where water potential of OL9 was statistically higher compared to OL7 and OL8 olive fruits. After that period, the values of all treatments started to have a slightly increasing trend up to the 120th day and then remained stable until the end of the process. Water potential expresses the tendency of the water to move from the fruit to the brine and is related to the expression of osmosis. Thus, it is clear that osmosis in OL9 was higher than in the other two treatments, allowing the faster diffusion of flesh tissue components (sugars, organic acids, polyphenols, etc.) to the brines. Indeed, Papadelli et al., (2015) reported that the slow extraction of soluble components from the olives to the brine is related to high NaCl concentration. This is the first report proposing the use of water potential as a tool for soluble component kinetic estimations of table olives during the fermentation process.

Finally, salinity in the brines was monitored throughout fermentation, and adjusted to the initial values of 10% and 7% for each treatment, by periodic dry salt additions in the brines. Salt equilibrium was reached in ca. 3 months and until the end of the process, salt concentration was maintained to its initial values (data not shown).

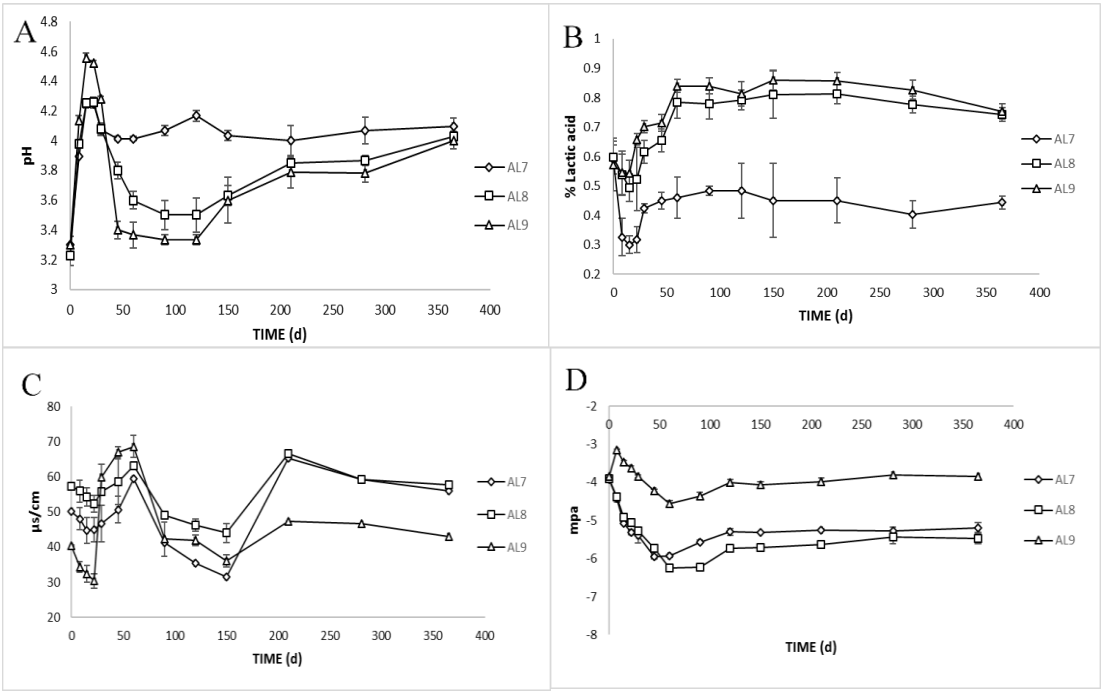


Fig. 2. 2. Changes in pH (A), titratable acidity (B), electrical conductivity (C), and water potential (D) throughout the fermentation of spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) of Cypriot green cracked table olives. Results are expressed as means and standard deviations of three replicates.

The total phenolic evolution of fruits and brine samples is presented in Fig 2.3 A and B, respectively. As clearly observed, the profiles of total phenolic content were quite similar across treatments. Olives exhibited a major loss in total phenolic content during fermentation mainly due to their degradation by LAB and yeasts and secondary due to their diffusion to the brine, as well. A similar trend has been noted by other studies (Álvarez et al., 2014; Kiai and Hafidi, 2014). During the first 45 days of fermentation, the decrease rates of phenolic contents were estimated to 37%, 68%, and 75% for OL7, OL8, and OL9, respectively. After 120 days of brining, phenolic content attained a steady state in traces, with no differences between treatments. The total

reduction of phenolic contents was about 88% for all treatments. In fact, the diffusion of phenolic compounds from olive flesh to the brine depended on several parameters, such as cultivar characteristics, fruit skin permeability, type of polyphenols presented in olive flesh, brine concentration, and their ability to diffuse outside the fruit due to accidental or purposely made fruit damage (cracked or razor slitting). As expected, the total phenolic contents in brines increased gradually in all fermenters to rich maximum concentrations of 3.24, 3.85, and 4 g GAE/g after 29, 22, and 22 days of fermentation for AL7, AL8, and AL9, respectively. After the 29th day of brining, the phenolic content started to decrease. This decline might be due to the degradation of phenolic acids by *Lactobacillus plantarum*. It has been demonstrated that *Lactobacillus plantarum* contains phenolic acid decarboxylases, which decarboxylate different phenolic compounds to their corresponding vinyl derivatives (Rodríguez et al., 2008). However, it is obviously an analogous reduction of total phenols in control treatment, in which, as previously mentioned, yeasts were the leading microorganisms. This indicates a high enzymatic activity of indigenous yeasts in the degradation of phenolic compounds, which is in accordance with the literature, where it has been reported the important role of yeasts in the olive debittering process (Bleve et al., 2014). This finding could explain the similarities of total polyphenols loss between inoculated and control samples from the 60th day of fermentation and thereafter.

Additionally, the loss in phenolic compounds resulted in a remarkable loss of antioxidant capacity in olive fruits, as well (Fig. 2.3C). No significant differences between different fermentations were observed after 60 days. The loss of antioxidant capacity transcended to 90% for all treatments.

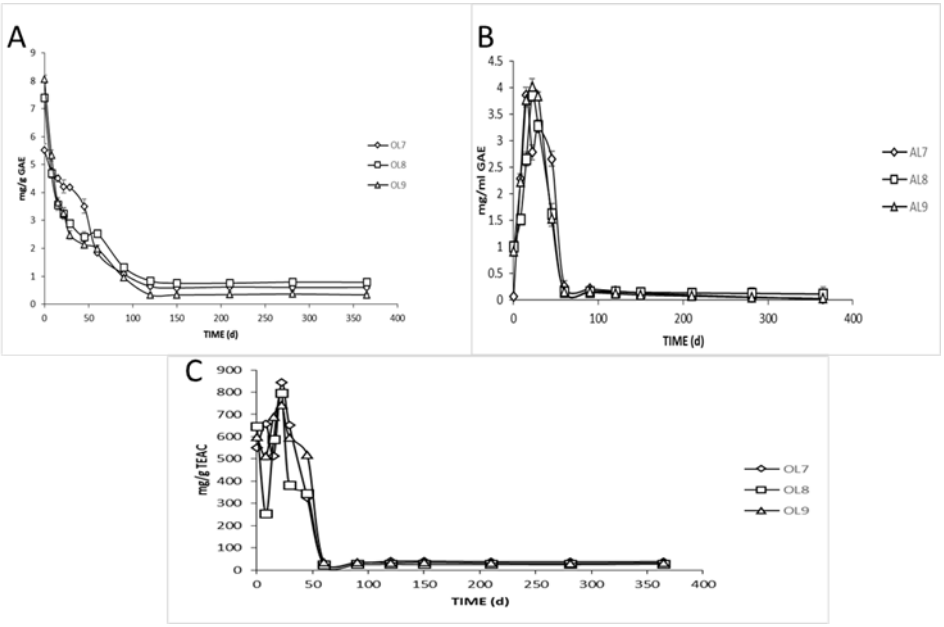


Fig. 2. 3. Total phenolic content of olive pulps (A) and brines (B) and antioxidant capacity of olives (C) during spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) fermentation of Cypriot green cracked table olives. Results are expressed as means and standard deviations of three replicates, equivalent of mg/g or ml.

A major decrease of oleuropein was observed in olive fruits, mainly due to its diffusion to brines and its degradation caused by enzymatic activity (Fig. 2.4A). No significant differences were observed between treatments at the end of the process, which agrees with the trends in total polyphenols values described above. However, the faster-decreasing values of oleuropein in inoculated treatments until the 45th day were notable. At this time point, the reduction reached 62% and 69% for OL8 and OL9, respectively, while, in control, it was no more than 24%. This finding indicated that the inoculated samples were ready to eat in a shorter period. Furthermore, the reduction of oleuropein was also accompanied by an increase in its hydrolysis product in brines

(hydroxytyrosol), where the inoculated treatments had significantly higher values after 90 days of fermentation (Fig. 2.4B). This finding confirmed that the enzymatic activity of the starter culture was higher, affecting the secoroide glucoside and its aglycon derivatives. In line with our findings, in previous studies (Rodríguez et al., 2008; Othman et al., 2009), hydroxytyrosol was referred to as the main phenolic compound found in the brines inoculated with the commercial starter. This compound has been mainly linked to the hydrolysis of oleuropein (Kiai and Hafidi, 2014) being an important biophenol belonging to the odiphenol group with special antioxidant activity (Pistarino et al., 2013b), and it has been considered as a marker for the determination of olives debittering (Randazzo et al., 2011).

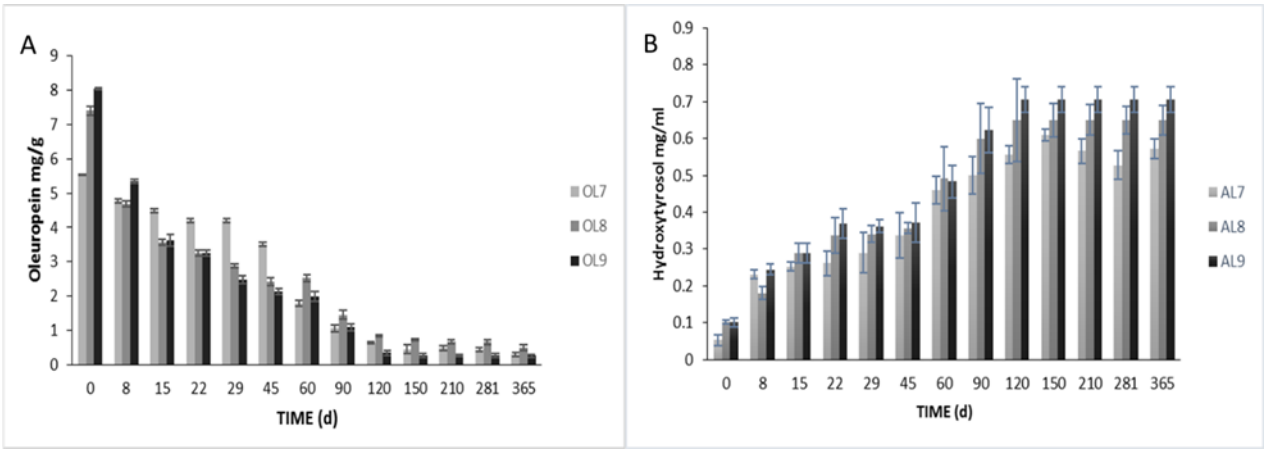


Fig. 2. 4. Evolution of oleuropein (A) and hydroxytyrosol (B) during spontaneous (7), inoculated (10% NaCl) (8), and inoculated (7% NaCl) (9) fermentation of Cypriot green cracked table olives. Results are expressed as means (mg/g or mg/mL) and standard deviations at different times of fermentations.

The changes in the concentration of organic acids in the brines are shown in Fig. 2.5. Significant differences between the three treatments were observed during the whole process, as detected by HPLC analysis. More specific, citric acid was the main

acid at the initial stage in all treatments due to its use at the beginning of the fermentation. After 22 days, in AL7, acetic acid became the main acid with considerable presence, while a slight reduction of citric from day 45 to day 120 was observed. However, the concentration of acetic was increasing until day 120 and then remained stable. Its presence in control could be related to yeast metabolism, as well as to the potential of heterofermentative LAB able to produce acetic acid under particular conditions of environmental stress as well as from the metabolism of citric acid (Laëtitia et al., 2014). Furthermore, malic and tartaric acids were also found in brines and were increased until day 22, indicating their presence in olive fruits and diffusion to the brines the first days of the process. The latter was in line with the literature, as well (Papadelli et al., 2015). Afterward, these two acids remained unchanged until the end of the process. Thus, there was not any metabolic activity for those acids in AL7. Finally, lactic acid was also detected in the brine AL7 in concentrations not exceeding 32 mM throughout the process, which was related to the low populations of LAB found in the microbial enumeration. The low values found for lactic acid are in accordance with previous studies (Bleve et al., 2015; Tufariello et al., 2015). However, as expected, lactic acid was the main acid in inoculated treatments due to the predominance of LAB. Significant differences between these two treatments were observed from day 45 to day 120, where the concentration of lactic acid in AL9 was higher than in AL8. This was in a combination of pH and titratable acidity values described above. Lactic acid presented a gradual increase until day 120, reaching statistically significant higher values in AL9, followed by a steady decline thereafter. This indicated potential assimilation of lactic acid from yeasts after 120th day due to their high population, which was in accordance with the literature (Hurtado et al., 2012a). Citric acid was the main acid at the initial stage due to its use at the beginning of the fermentation. From day 22 to day 60, a major

decrease was observed in both treatments (no significant differences), which was related to its microbial degradation to acetic acid (Papadelli et al., 2015). Succinic acid was also determined in the inoculated treatments, the evolution of which was found to be similar to that of the acetic acid, for the same reason, as well (no differences between treatments). Furthermore, regarding malic and tartaric acids, there was an obvious initial increase during the first 22 days, followed by a major decrease and total disappearance of malic acid in about 120 days, while the tartaric acid remained steady until the end. This finding was in agreement with results reported previously (Panagou et al., 2008; Tofalo et al., 2012), where malic acid detected in traces at the beginning of the process and decreased at the end of the fermentation period. Moreover, the gradual decrease of malic acid in brines observed during the fermentation of green olives is attributed to its microbial degradation to lactic acid and CO_2 (Panagou and Tassou, 2006; Papadelli et al., 2015). Finally, for tartaric acid, it has been reported that yeast and other microorganisms are unable to metabolize it (Chidi et al., 2018).

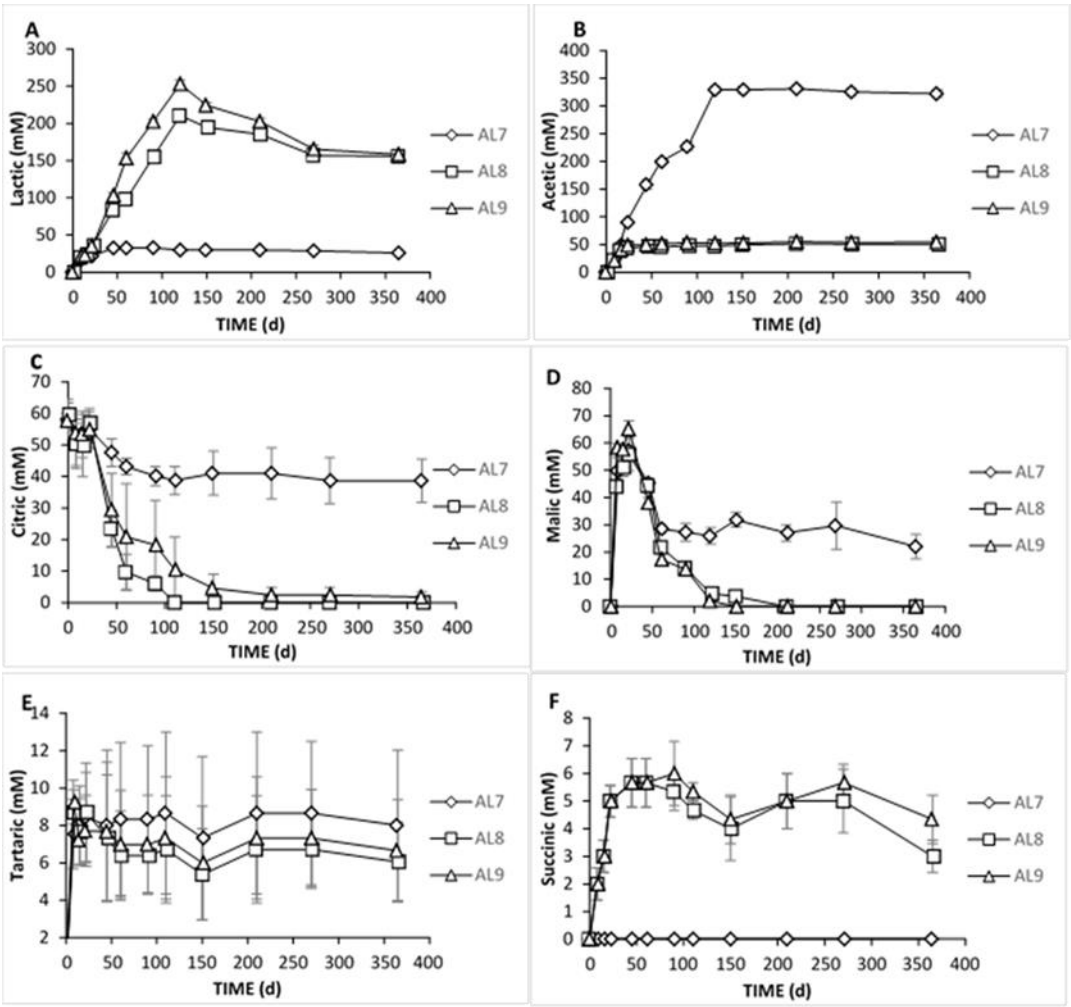


Fig. 2. 5. Changes in the concentration (mM) of organic acids (lactic, A; acetic, B; citric, C; malic, D; tartaric, E; and succinic, F) during spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) fermentation of Cypriot green cracked table olives. Data points are expressed as means and standard deviations of three replicates.

Sugars diffused from fruits into the brine are the main nutritional elements for microbial growth and fermentation. According to our results, glucose and fructose were the main sugars found in the brines as it emerged by HPLC analysis (Fig. 2.6). Glucose was steadily increasing the first days of fermentation, exhibiting the highest value at day 22 for AL9 and day 29 for AL8 and AL7. This could be confirmed by the faster

diffusion of the sugar observed from olives to brine in AL9 because of the lower NaCl concentration. A major decrease was recorded thereafter since it was consumed for microbial growth. In fermentation AL9, this decrease was observed earlier (at day 45) compared to fermentation AL8 (day 60) and AL7 (day 90), which was in accordance with previous research, where it was reported that in the inoculated olives, the decrease of glucose was faster than in control (Papadelli et al., 2015). It was notable that at the end of the process, glucose disappeared, but there was a remaining amount of ca. 0.5 mM in the AL7. A similar trend was found for fructose content in AL7. Its amount never exceeded 12 mM, and it was not found after 120 days of fermentation, while it was detectable in the same concentrations (ca. 7 mM) in AL8 and AL9, until the end of the fermentation. The total depletion of fructose in AL7 could be related to some fructophilic yeast species, a fact that agreed with the results of control treatment in the present study (Papadelli et al., 2015).

Concerning ethanol, it is mainly related to yeast production activity, having a crucial impact on the sensory properties of naturally fermented olives (Bonatsou et al., 2018b). Its concentration in AL7 increased gradually until 90th day of fermentation, reaching values 250 mM, followed by a minor decrease thereafter until the end of the process, where it was maintained at ca 178 mM. Similar trends were previously reported (Bleve et al., 2014). However, ethanol was detected in traces in inoculated treatments because of LAB dominance, confirming that yeast metabolic activity was affected by the inoculation of table olives. Another important product from yeast activity is glycerol (Erasmus et al., 2004). Its presence has been linked with cell protection from osmotic stress (Bonatsou et al., 2018b). According to our results, it was present in high concentration in control, as expected, while it was very limited in the other treatments (ca. 30 mM during the whole process). In AL7, its concentration increased gradually

until the 90th day of fermentation in levels exceeding 207 mM, followed by a decrease afterward, reaching final values of ca. 140 mM. The presence of this compound in naturally fermented table olives has been reported previously (Bleve et al., 2015; Tufariello et al., 2015), which was in good line with the present study. It has been noted that the presence of both compounds (ethanol and glycerol) can, in turn, affect crucial organoleptic characteristics, such as texture maintenance and aroma formation (Arroyo-López et al., 2012b; a).

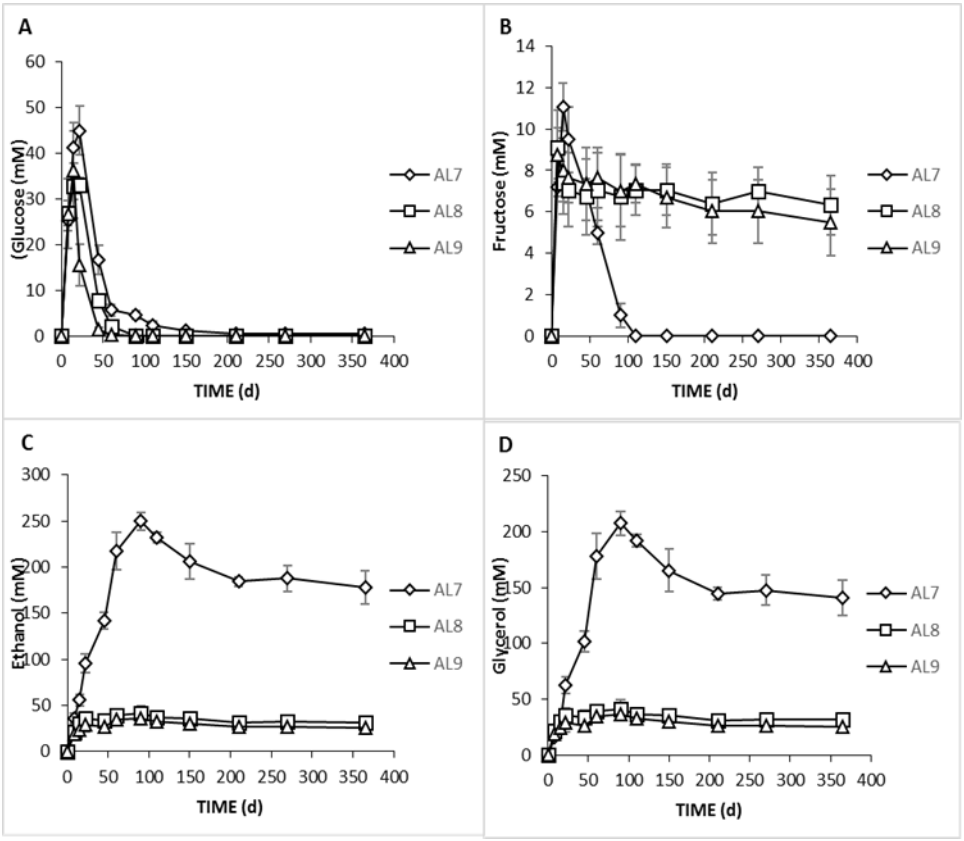


Fig. 2. 6. Changes in the concentration (mM) of soluble sugars (glucose, A; fructose, B) and alcohols (ethanol, C; glycerol, D) in the brines during processing of Cypriot green cracked table olives of Spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) fermentations. Data points are expressed as means and standard deviations in triplicate.

2.3.3. Firmness and Color Evolution of Olives

The texture has a significant impact on consumer's acceptance of a product, while in many cases, it is considered as the most important property (Luckett, 2016). The results of textural analysis during the whole process are presented in Fig. 2.7. It could be observed that the values were being decreased as time passed until the 60th day, and after that remained steady until the end of fermentation, in all treatments. The values of OL9 were significant lower until day 29. This could be explained by the lower NaCl concentration. However, no significant differences were observed thereafter, indicating that neither lower NaCl nor brine inoculation affected the texture profile of the final product. Similarly, Fadda and co-workers (Fadda et al., 2014) investigated the effect of brining time on the texture of naturally fermented green olives, reporting a texture decrease after 30 days of brining. Texture loss is strongly influenced by the enzymatic activity of dominant microflora and, in some cases, may cause softening due to the degradation of pectic substances of the cell wall and middle lamella (Fernández-Bolaños et al., 2002). The latter depends on crucial brine conditions, such as sodium content and pH (Fadda et al., 2014).

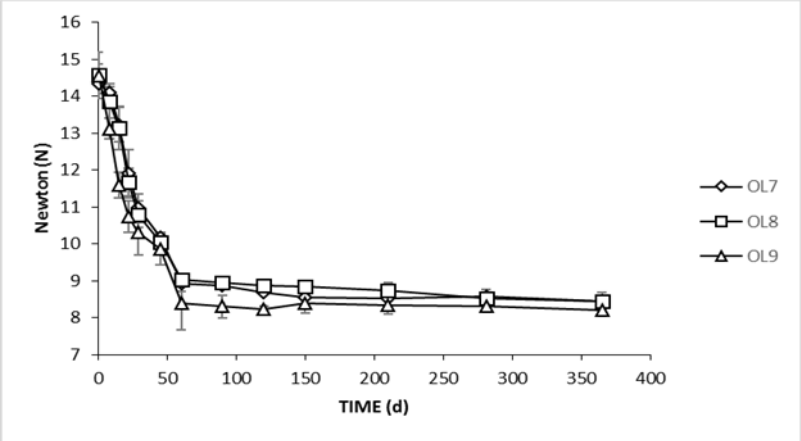


Fig. 2. 7. Evolution of texture of olive fruits during spontaneous (◊), inoculated (10% NaCl) (□), and inoculated (7% NaCl) (Δ) fermentation of Cypriot green cracked table olives. Data points are expressed as means (N) and standard deviations of 10 random measurements.

The color attribute of food products is another crucial factor in the acceptance of a food product. The color parameters of olives are listed in Table 2.2. In general, no significant differences were observed between treatments in any of the parameters. Exceptions were the h* and C* parameters. The latter, in the inoculated treatments, were significantly lower than control. This parameter was related to the volume of color, which accounted for a shift to the dark-green zone. Furthermore, there were no significant differences in b* parameter for control and inoculated samples starting from a value of 33 ± 4.4, reaching a decreased value until 45th day (22.6 ± 6), and thereafter remained unchanged until the end. The decreasing values indicated lowering in yellow color. A similar tendency was observed for the parameter L* (no differences), which decreased until day 45 after the fermentation process. The value of the parameter L* was an indicator of the degree of lightness. However, according to the literature (Ramírez et al., 2015), light color is associated with a low pH value, which is not in agreement with the present study; otherwise, the inoculated samples should have higher lightness than control. This could be explained by the fact that the lightness parameter is mainly variety dependent. Finally, a major increase in a* parameter was observed in all treatments, demonstrating a distinct toning from green to red, starting for values of about −13 at the beginning of the process, while values of about −1.9 ± 1 were reached at 120 days and thereafter no changes were recorded. This effect could be attributed to the presence of chlorophyllase in the first days of fermentation, leading to hydrolysis of phytol or chemical oxidation reactions (Mínguez-Mosquera et al., 1994). In general,

natural green olives had high values for a* parameter, resulting in reddish tones. Finally, the loss of h* was faster in control and lowered significantly until the 150th day, indicating faster brownish coloration. The latter, among other organoleptic characteristics, makes this product less attractive (Ramírez et al., 2015), and thus this is another positive effect of inoculated samples.

Table 2. 2. Evolution of color parameters (a*, b*, L*, h* and C*) of olive fruits during spontaneous (OL7), inoculated (10% NaCl (OL8) and inoculated (7% NaCl) (OL9) fermentation of Cypriot green cracked table olives. Data points are expressed as means and standard deviations of 10 random measurements.

0	8	15	22	29	45	60	90	120	150	210	281	365
						a*						
-13,93±8,32	-7,58±1,11	-5,79±2,90	-3,37±0,92	-2,90±1,75	-1,29±1,04	-1,43±1,09	-1,02±1,43	-0,92±1,13	-1,90±0,99	-1,90±0,99	-1,80±0,92	-1,87±0,98
-13,5±2,31	-8,87±0,96	-8,24±1,58	-4,36±0,75	-3,50±0,41	-1,85±1,40	-1,23±0,88	-1,23±0,88	-0,92±0,50	-1,07±0,70	-1,40±0,38	-1,38±0,39	-1,40±0,38
-13,88±0,81	-7,51±0,76	-5,49±2,75	-4,37±0,04	-3,16±0,08	-1,11±1,07	-1,35±0,92	-1,35±0,92	-1,27±0,81	-1,69±0,46	-1,35±0,74	-1,35±0,62	-1,12±0,89
						b*						
34,84±1,92	39,22±2,52	37,18±3,28	33,15±0,82	32,48±0,41	27,68±6,69	21,67±1,20	22,00±1,00	22,69±11,39	24,26±12,16	23,64±11,83	23,62±11,89	23,31±11,65
34,91±2,40	38,08±3,48	37,98±3,55	36,07±0,84	34,27±1,22	27,19±6,27	22,20±11,27	22,20±11,27	23,39±11,89	24,71±12,37	24,76±12,40	24,71±12,37	24,71±12,37
33,10±1,10	34,43±2,55	36,36±3,13	32,15±1,23	30,43±1,35	27,24±6,61	21,74±11,05	21,74±11,05	23,01±11,57	24,42±12,21	21,8±11,05	23,01±11,57	24,42±12,21
						L*						
53,88±1,17	57,97±3,25	56,76±3,17	58,13±0,17	54,24±0,35	47,75±5,02	44,72±17,38	44,72±17,38	42,02±16,08	41,10±15,67	40,07±15,07	39,93±15,00	39,67±15,06
54,46±0,84	57,60±3,55	54,74±0,89	59,50±0,69	59,23±0,68	49,73±4,87	41,77±16,35	41,77±16,35	40,84±15,70	44,42±17,28	40,30±15,28	40,00±15,18	40,66±15,48
52,98±0,81	52,67±2,35	53,81±2,60	49,35±0,07	50,82±1,25	49,97±6,50	39,51±15,08	39,80±15,18	40,98±15,50	40,66±15,33	40,33±15,19	39,97±15,01	40,10±15,09
						h*						
110,18±9,10	101,00±1,77	92,64±0,59	91,20±0,95	90,80±1,32	89,88±2,60	90,49±3,20	87,58±1,27	87,37±2,59	85,66±2,85	86,57±0,47	86,20±1,27	86,20±0,75
103,68±2,04	103,10±0,79	97,52±1,02	96,32±0,94	96,78±1,29	93,78±2,85	93,36±1,28	92,70±0,97	91,59±0,67	87,89±0,88	87,50±0,65	86,80±0,62	86,07±1,35
104,57±1,25	102,37±1,31	98,03±0,87	98,00±0,04	96,70±0,67	93,71±3,19	93,99±1,34	93,47±1,24	91,83±1,70	89,67±2,24	88,07±0,94	87,43±1,36	86,77±1,09
						C*						
38,70±5,29	39,99±2,48	39,00±3,33	33,68±0,93	32,71±0,48	33,33±2,29	35,26±1,73	30,70±3,50	30,37±3,82	31,67±4,91	29,70±4,47	31,67±4,91	30,37±3,82
35,81±2,86	39,11±3,57	23,77±11,92	25,33±2,19	24,33±1,45	22,24±11,22	22,25±11,29	22,25±11,29	23,41±11,90	22,40±1,47	20± 2,3	19± 1,8	20±3,3
34,21±1,16	35,26±2,54	21,96±11,03	24,00±2,08	24,00±1,15	23,00±1,53	22,00±1,53	20,00±10,02	22,33±0,88	21,7±0,88	23,00±3,06	21,33±1,45	21,00±1,15

2.3.4. Sensory Evaluation

The organoleptic profile of the samples is presented in Fig. 2.8. In general, the samples were characterized by low remaining bitterness, good texture, and satisfactory acidic taste and odor. No off flavors were noticed in any samples. Overall, differences among treatments were detected on a bitterness descriptor, in which OL7 had a higher score from the other two. The higher contents of both ethanol and glycerol in the control sample was in line with the higher score to the bitterness and lower score of the acid descriptor, according to panelist evaluation. A similar trend was observed for the saltiness score, with a lower value scored in OL9 samples. However, no differences were recorded to a flavor descriptor. Regarding texture, OL8 and OL9 had lower scores. However, they received an equal value of acidity, which was higher than control, while they had the highest score for the overall acceptability descriptor. The most important attribute that influenced the judgment of the panelists was salt content, acidity, and bitterness to a lesser extent, as could be concluded by the scores of those parameters, in combination with the overall acceptability scores.

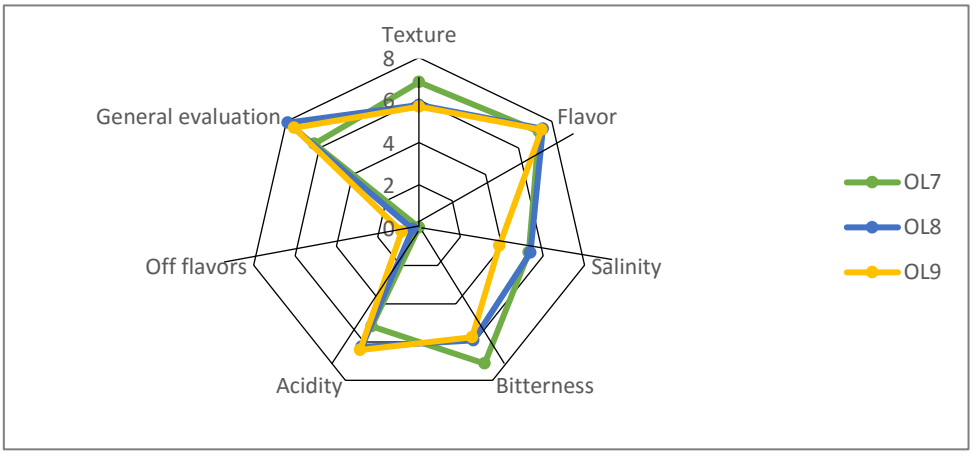


Fig. 2. 8. Sensory profiles of spontaneous (OL7), inoculated (10% NaCl) (OL8), and inoculated (7% NaCl) (OL9) fermentation of Cypriot green cracked table olives at 120 days of fermentation.

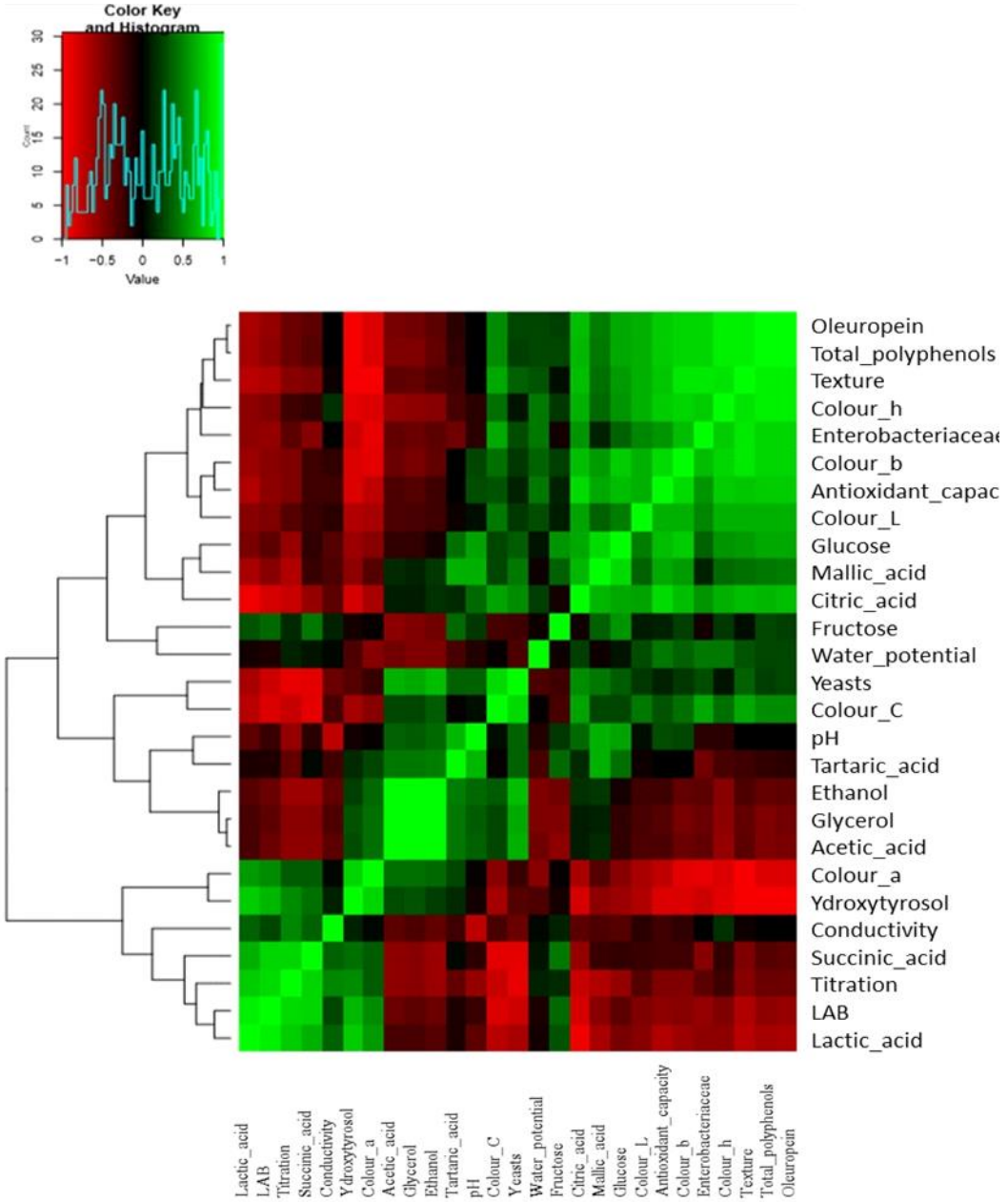
2.3.5. Multivariate Analysis

PCA between all studied variables resulted in four eigenvalues greater than 1, explaining an overall 88.84% of the total variance in the dataset, while the first two components explained 71.5% of the distribution (Fig. 2.9B; Appendix Table S2). PC1 was correlated with LAB, *Enterobacteriaceae*, texture, all color parameters, polyphenols, antioxidant capacity, lactic, citric, succinic acids, and glucose, while PC2 dealt with yeasts, acetic, tartaric acids, ethanol, and glycerol. PC3 was linked with the pH, fructose, malic, and tartaric acids. Finally, PC4 was related to conductivity and water potential. Furthermore, correlations between microbial and physicochemical data are shown in Fig. 2.9A. Among organic acids, lactic and succinic acids were negatively correlated with yeasts and positively correlated with LAB. Oppositely, acetic, tartaric, and malic were positively correlated with yeasts and negatively with LAB. Zooming on the metabolomics, the acetic acid was positively correlated with yeasts, ethanol and glycerol, confirming the results for control treatment, described above. Oleuropein, antioxidant capacity, total phenols, texture, and color parameters L*, b*, h* were firmly related to each other, and all of them were negatively related to LAB and positively related with yeasts. Finally, hydroxytyrosol seemed to be highly correlated with LAB, confirming our results described above.

Regarding correlations between treatments, PCA grouped them into three clusters, clearly characterized based on inoculated treatments versus the control one during fermentation time, as control treatments being separated from inoculated treatments from the 45th day and thereafter (Fig. 2.9B). Inoculation was apparently the most important treatment in sample distribution throughout fermentation. PC1 could be

related to fermentation time since a gradual transition of time was noticeable from the right to the left part of the plot. It is crucial to mention that the reduction of NaCl concentration (AL9) did not affect the groups' distribution. This was a very promising finding, indicating that the NaCl reduction is an achievable goal for the table olives industry.

Furthermore, similarities in the observed microbial and physicochemical profiles between samples are presented in Figure 9C. In detail, in agreement with PCA, inoculated samples had similar profiles to each other after the 45th day, showing a negative correlation with total polyphenols, oleuropein, texture, color h*, color L*, color C*, and malic and citric acid, while they were positively correlated with LAB, titration, lactic and succinic acid, color a*, pH, glucose, and hydroxytyrosol. On the other hand, control treatment was closely related to yeasts, texture, acetic acid, ethanol, and glycerol, while it was negatively related to the positive parameters of inoculated treatments and fructose. Therefore, the multivariate analysis confirmed the different metabolic pathways between non-inoculated and inoculated treatments during fermentation.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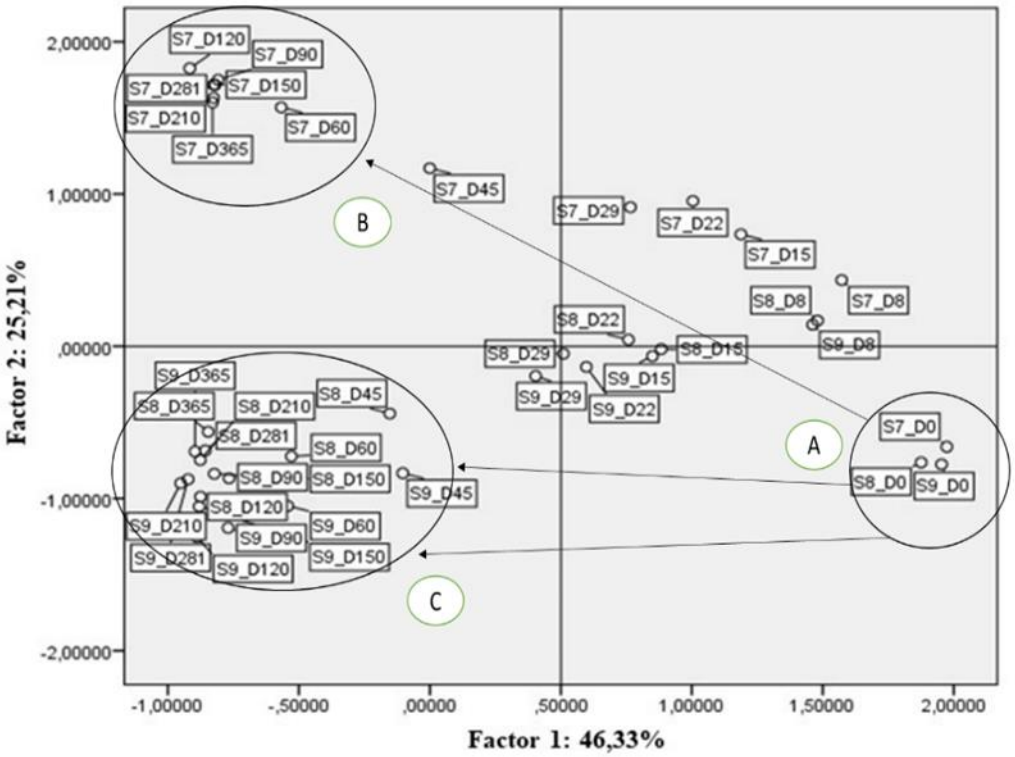
Value
Oleuropein
Total_polyphenols
Texture
Colour_h
Enterobacteriaceae
Colour_b
Antioxidant_capac
Colour_L
Glucose
Mallic_acid
Citric_acid
Fructose
Water_potential
Yeasts
Colour_C
pH
Tartaric_acid
Ethanol
Glycerol
Acetic_acid
Colour_a
Ydroxytyrosol
Conductivity
Succinic_acid
Titration
LAB
Lactic_acid

(**A**)


S7_D120
S7_D90
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S7_D22
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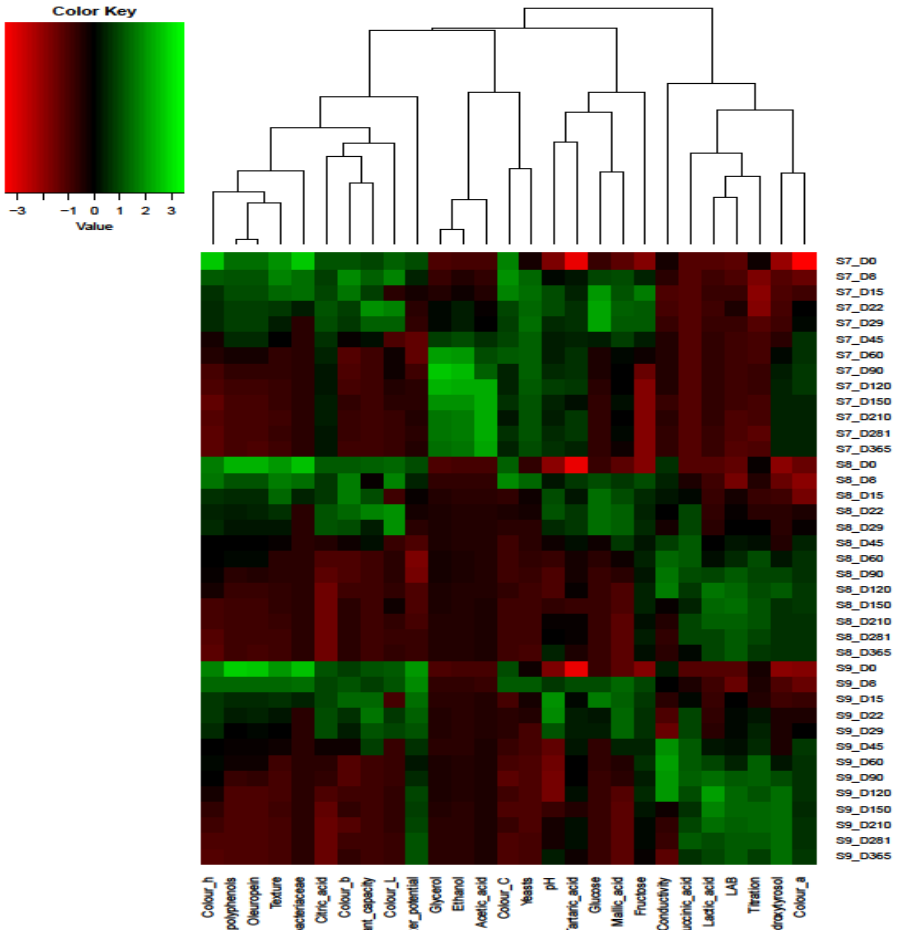
Factor 2: 25,21%
Factor 1: 46,33%

(**B**)



(**C**)

Fig. 2. 9. (A) PermutMatrixEN analysis between microbial and physicochemical profiles of spontaneous, inoculated (10% NaCl), and inoculated (7% NaCl) fermentation of Cypriot green cracked table olives. **(B)** The plot of scores and loadings between treatments formed by the first two principal components from the PCA (principal component analysis) analysis. Labeling of data points indicates the processing treatment of olives (S9: inoculated and 7% NaCl concentration, S8: inoculated and 10% NaCl concentration, S7: control) and fermentation time (D: Days). **(C)** Heatmap showing the similarities in the observed microbial and physicochemical profiles between the three experiments during fermentation days. Labeling of data points indicates the processing treatment of olives (S9: inoculated and 7% NaCl concentration, S8: inoculated and 10% NaCl concentration, S7: control) and fermentation time (D: Days).

2.3.6. Detection of the Presence of the Starter Culture

The presence of inoculated strain was monitored after 4 months of fermentation by rep-PCR on a pool of 17 strains from MRS agar (seven from AL8, eight from AL9, and two from AL7). Preliminarily, the repeatability of the method was confirmed using gDNA from the starter strain (Vegestart 60) as an internal control in four different gels from four different PCR reactions, obtaining a similarity of 91.8%. This value was retained as a threshold to establish the identity of isolates compared to the rep-PCR profile. The produced dendrogram clearly separated the studied strains into three clusters (Fig. 2.10). More specifically, the first cluster related to the starter strain profile containing all isolates from AL9 (7/7, 100%) and many isolates from AL8 (5/8, 62.5%). The isolates of the second cluster belonged to AL8 (3/3), indicating that there were different strains, while isolates belonged to the third cluster came from AL7, in order to prove the distance between indigenous and starter LAB molecular profile.

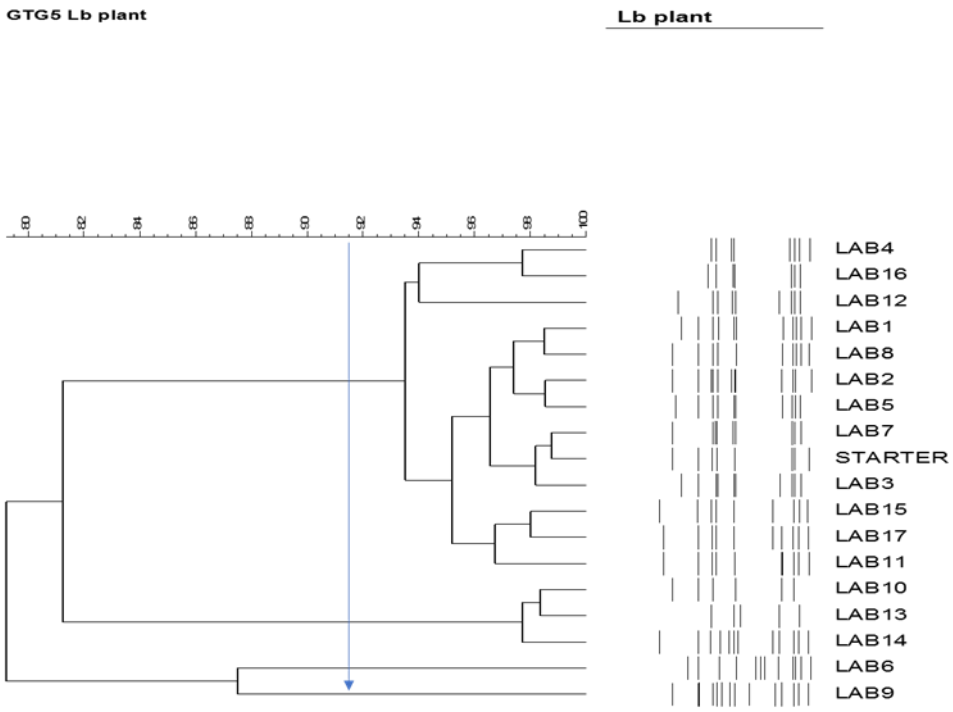


Fig. 2. 10. Dendrogram generated after cluster analysis of the digitized GTG5-PCR fingerprints of LAB (lactic acid bacteria) strains isolated from AL7 (LAB 6,9), AL8 (LAB 1,2,3,4,5,7,8), and AL9

(LAB 10,11,12,13,14,15,16,17) brine samples at 120 days of fermentation. Blue line indicates the similarity threshold (91.8).

2.4. Conclusions

According to the results of the present study, microbial, biochemical, and sensorial attributes were strongly affected by brines inoculation, although a minor influence of salt content was also noted. The use of starter culture changed the microbial dominance and led to faster acidification of brines and faster degradation of oleuropein, indicating the faster fermentation completion. Moreover, the reduction of sodium content resulted in a successful lactic fermentation of Cypriot green cracked table olives. The final products fulfilled microbiological criteria and exhibited more appreciated sensorial characteristics. In addition, the formulation of table olives with low salt content is healthier and more suitable for consumers at risk of hypertension, opening a new era for table olives industry.

It must be mentioned that according to our findings, Cypriot olives were ready to eat after 120 days of fermentation in all treatments. This could be supported by the elimination of oleuropein, as well as the depletion of sugars (glucose and fructose), at this time point. Furthermore, minor changes occurring after 120 days, confirmed the above conclusion. Thus, the subsequent period was considered as preservation stage until selling, which was also important to be studied, in order to avoid any risk regarding the final product.

New methodologies used in olive and brine analysis (water potential and electrical conductivity), provided strong indications that they could be of interest as potential tools for the monitoring of the fermentation progress. However, further studies are required to establish a validated protocol.

Concluding, the effect of the inoculation of table olives on the production of stable quality final product was not dependent on the alternating indigenous microflora. The use of starter culture could lead to the modernization of fermentation, with healthier products of high quality. On this point, the study of the table olive indigenous microflora with additional biotechnological and probiotic potential might lead to further research concerning its beneficial effects during olive processing. Indigenous microorganisms may be more adjusted to the harsh southeast Mediterranean environmental conditions while adding further to locally appreciated organoleptic characteristics.

Chapter 3: Evolution of bacterial communities, physicochemical changes and sensorial attributes of natural whole and cracked *cv.* Picual table olives during spontaneous and inoculated fermentation

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A slightly modified version of this chapter has been published

Anagnostopoulos D.A., Kamilari E. and Tsaltas D. (2020). Evolution of Bacterial Communities, Physicochemical Changes and Sensorial Attributes of Natural Whole and Cracked Picual Table Olives During Spontaneous and Inoculated Fermentation. Front. Microbiol. 11:1128. https://doi.org/10.3389/fmicb.2020.01128

ABSTRACT

In the present work, whole and cracked *cv.* Picual table olives were fermented at industrial scale for 120 days, using three distinct methods (natural fermentation, inoculation with Lactic Acid Bacteria at a 7% or a 10% NaCl concentration). Microbial, physicochemical and sensorial alterations monitored during the whole process and several differences observed between treatments. Results indicated that in all treatments, the dominant microflora were LAB. Yeasts also detected in noteworthy populations, especially in non-inoculated samples. However, LAB population was significantly higher in inoculated compared to non-inoculated samples. Microbial profiles identified by metagenomic approach showed meaningful differences between spontaneous and inoculated treatments. As a result, the profound dominance of starter culture had a severe effect on olives fermentation, resulting in lower pH and higher acidification, which was mainly caused by the higher levels of lactic acid produced. Furthermore, the elimination of Enterobacteriaceae was shortened in time, even at lower salt concentration. Although no effect observed concerning the quantitated organoleptic parameters such as color and texture, significantly higher levels in terms of antioxidant capacity were recorded in inoculated samples. At the same time, the degradation time of oleuropein was shortened, leading to the production of higher levels of hydroxytyrosol. Based on those evidences, the establishment of starter culture driven Picual olives fermentation is strongly recommended. It is crucial to mention that the inoculated treatments with reducing sodium content was highly appreciated by the sensory panel, enhancing the hypothesis that the production of Picual table olives at reduced NaCl levels is achievable.

3.1. Introduction

As previously mentioned, the modernization of table olives fermentation, by the use of appropriate starter cultures, has been extremely supported, aiming to the stabilization of fermentation worldwide (Rodríguez-Gómez et al., 2017). However, in order to standardize this process and consequently secure the quality of the final product, the study of microbial and physicochemical descriptors for monitoring the fermentation is a pre-requisit (Bleve et al., 2014).

The directly brining fermentation, which is among the most popular and established table olive commercial types (Rodríguez-Gómez et al., 2017), is mainly promoted by LAB and yeasts, which are usually coming from olive fruits (Corsetti et al., 2012). Major synergisms and interactions occur between these dominant species until the end of fermentation (Botta and Cocolin, 2012). As already mentioned, diverse microbial populations may be involved throughout fermentation, including many species belonging to *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus* and to a lesser extend *Clostridium* and molds, especially at the initial stage of the process (Bonatsou et al., 2017). All of them affect the course of fermentation and thus contribute to several characteristics of the product (flavor, texture, color and safe consumption) (Heperkan, 2013). However, the microbial consortium formation depends on olive cultivar, fermentation style and technology processing type. This complex matrix can in turn contribute to the microbial consortia formation, responsible for olive fermentation and influence the sensory and safety profile of the final product (Romeo 2012). Thus, both process standardization and an in-depth determination of the normal or abnormal microbial communities at different stages of fermentation are mandatory, in order not only to overpass such problems, but also to identify potential biomarkers responsible

for fermentation; thus learning how to manipulate fermentation conditions to improve the process control (Filippis et al., 2017). On that point, the use of classical molecular methods (culture-dependent techniques) do not offer a complete knowledge about the microbial consortia present in food matrix ecosystem (Cocolin et al., 2013). Last decade, culture-independent techniques have attracted the attention of food scientific community. Nowadays, high-throughput sequencing (HTS) has revolutionized the field of food microbial ecology, as it has been established as a new tool for the quantitative investigation of the microbial communities' structure (Medina et al., 2018). Several applications can be already found in the frontier of food microbiology (Ercolini et al., 2011; Bokulich et al., 2012) and the related crucial contribution; regarding the enhancement of scientific knowledge; has been recently noted (Ercolini, 2013). Next Generation Sequencing (NGS) enables high-resolution studies of microbial composition (raging from phylum to species taxonomical level) in several food system categories, including food spoilage (Parlapani et al., 2018; Parlapani et al., 2019) and food fermentation (Ercolini et al., 2012; Ercolini, 2013; Cocolin et al., 2013). Therefore, this set of techniques has been recommended as a very promising tool for the evaluation of the microbial diversity during fermentation of several products (Zinno et al., 2017). However, up to now, the monitoring of several dairy fermentations are by far the most explored products studied by such analyses (Fuka et al., 2013; Pasquale, 2014; O'Sullivan et al., 2015; Alessandria et al., 2016; De Filippis et al., 2016; Kamilari et al., 2020), while the investigation of other fermented products is more limited (Bokulich et al., 2014; Bokulich et al., 2014), including the monitoring of 'microbial map' of olives fermentation (Randazzo et al., 2017).

Furthermore, despite that the fermentation profile during several olive cultivars processing has been thoroughly investigated (Panagou et al. 2008; Bautista-Gallego et al. 2010; Bleve et al. 2014; Bleve et al. 2015; Chranioti et al. 2018; Pino et al. 2018), limited information is available for the fermentation of Picual table olives. This cultivar is one of the main producing olives in Cyprus and worldwide, exhibiting high level exports, as well.

As mentioned in chapter 2, one of the main aims of the Cypriot industry is to square with the global nutritional policies regarding NaCl reduction. The findings from Cypriot olives fermentation regarding this aspect (Chapter 2) were very promising. However, the potential NaCl reduction depends on plethora of factors, including cultivar type, drupe composition, as well as, other processing treatments (e.g crack olives) (Bautista-Gallego et al., 2011).

Thus, the aims of this work were a) to study the microbial communities and physicochemical changes of Picual (whole and cracked) table olives, during fermentation process at industrial scale, b) to evaluate the process' evolution by adding a LAB starter culture and c) to study the effect of reducing NaCl concentration in combination with starter culture, in order to produce a more secure and healthier final product.

3.2. Materials and methods

3.2.1 Olives samples and fermentation procedure

Olive fruits were supplied from a commercial orchard ("Novel Agro", Nicosia, Cyprus). Green fruits were harvested, based on size uniformity criteria. After visual

screening and abortion of the damaged olives, fruits were thoroughly rinsed with tap water to expulse potential contaminants. Afterwards, 2 types of olives manipulation (whole and cracked) were subjected into three different fermentation treatments, in duplicate (Biological replicate). An amount of olive fruits (~20 Kg) was placed in plastic tanks of 25 L capacity filled with NaCl and citric acid. The citric acid was added in accordance to the Cypriot industrial standard production of table olives. Olives allowed to ferment for 120 days at controlled temperature (23 ± 2 °C). The different types of fermentation are described in Table 3.1.

Table 3. 1. Summary of the experimental design applied in the study.

Olives	Description	Technology
S1	Spontaneous fermentation (10%NaCl, 0.3% citric acid)	Cracked
S2	Fermentation inoculated with starter culture *Lactobacillus Plantarum* (Vege-Start 10/Vege-Start 60) (10% NaCl, 0.3% citric acid)	Cracked
S3	Fermentation inoculated with starter culture *Lactobacillus Plantarum* (Vege-Start 10/Vege-Start 60) (7% NaCl, 0.3% citric acid)	Cracked

S4	Spontaneous fermentation (10%NaCl, 0.3% citric acid)	Whole fruit
S5	Fermentation inoculated with starter culture *Lactobacillus Plantarum* (Vege-Start 10/Vege-Start 60) (10% NaCl, 0.3% citric acid)	Whole fruit
S6	Fermentation inoculated with starter culture *Lactobacillus Plantarum* (Vege-Start 10/Vege-Start 60) (7% NaCl, 0.3% citric acid)	Whole fruit

3.2.2 Microbiological analysis

Brine samples were analyzed at intervals (Days 0, 8, 15, 22, 29, 45, 60, 90, 120), for their Total Viable Count (TVC), *Enterobacteriaceae*, LAB, yeasts, *coliforms* and CoNS, using the standard plate methods after serial dilutions in 0.85% w/v saline water (Table 3.2). Volumes of 0.1 ml or 1 ml (spread and pour plate, respectively) of serial dilutions in saline solution, were placed in petri dishes for enumeration of the microorganisms. All samples were analyzed in triplicates.

Table 3. 2 Microbiological media used for microbial enumeration.

Growth media	Microorganisms	Method	Incubation Conditions
Plate Count Agar (PCA) (Merck, Darmstadt, Germany)	Total Viable Count	Spread plate	30ºC/72h
De Man-Rogosa-Sharpe agar (MRS) (Oxoid, Basingstoke, UK) + natamycin 0.1%	Lactic Acid Bacteria	Pour plate/Overlay	30ºC/72h
Sabouraud Agar (Oxoid, Basingstoke, UK)	Yeast and Molds	Spread plate	25ºC/5d
Violet Red Bile Glycose Agar (VRBGA) (BD, Sparks, MD)	*Enterobacteriacae*	Pour plate/Overlay	37ºC/24h
Violet Red Bile Lactose Agar (VRBLA) (Oxoid, Basingstoke, UK)	*Coliforms*	Pour plate/Overlay	30ºC/24h
Mannitol Salt Agar (MSA) (Oxoid, Basingstoke, UK)	CoNS	Spread plate	30ºC/48 h

3.2.3 16S rRNA meta-barcoding analysis

3.2.3.1 DNA extraction, quality evaluation and sequencing

Microbial genomic DNA from olives (Day 0) and brine samples (Day 60, Day 120) was extracted as described by Medina et al., (2016), using the DNeasy® PowerFood® Microbial Kit (MoBio Laboratories Inc., Carlsbad, CA, US). DNA concentration was measured on a Qubit 4.0 fluorimeter (Invitrogen, Carlsbad, CA) using Qubit dsDNA HS Assay Kit (Invitrogen). The purity of DNA was evaluated by measuring the ratio of absorbance A260/280 nm and A260/230 nm using a

spectrophotometer (NanoDrop Thermo Scientific, USA). Finally, the extracted DNA was stored at −20 °C until further use.

Bacterial diversity was assessed by sequencing the V3–V4 hyper-variable region of the 16S rRNA gene (expected size ~460 bp) using the Illumina's 16S Metagenomic Sequencing Library Preparation protocol (15,044,223b). For the amplification of the V3-V4 region, two universals bacterial 16S rRNA gene amplicon PCR primers were used, including the amplicon PCR forward primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and amplicon PCR reverse primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) with the addition of the overhang adapter sequence. Nextera XT Index Kit (FC-131-2001, FC-131-2002) was used for the multiplexing step. All PCR reactions were performed in a 96-well plate, incorporated in Thermocycler (Biorad), in a final volume of 25µl, containing 12.5µl of 2 × KAPA HiFi HotStart Ready enzyme mix (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.), 10µl of 1µM 16S primer mix and 2.5µl (~5 ng/µl) genomic DNA. PCR conditions were as follow: an initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72°C for 5 min. PCR products were purified to remove unincorporated primers and primer-dimer species using NucleoMag ® NGS Bead Suspension (Macherey-Nagel, Düren, Germany). A second limited cycle index PCR was performed in order to attach dual indices and Illumina sequencing adapters in all PCR fragments. Each index PCR reaction was performed in 50µl final volume, consisting of 5µl PCR product, 25µl of 2 × KAPA HiFi HotStart Ready enzyme mix (KAPA BIOSYSTEMS, Woburn, MA, U.S.A.), 5µl of each Nextera XT index primer (FC-131-2001, FC-131-2002) (Nextera XT Index kit, Illumina Inc, San Diego, California) and 10µl PCR grade water. PCR

reactions were performed using the following program: initial denaturation 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension period of 72 °C for 5 min. Indexed PCR reactions were purified using NucleoMag ® NGS Bead. The DNA concentration of each PCR product was determined using Qubit 4.0 fluorimeter (Invitrogen, Carlsbad, CA), using Qubit dsDNA HS Assay Kit (Invitrogen) and the quality was assessed using a bioanalyzer (Agilent 2200 TapeStation) (expected size ~550 bp). Finally, libraries were loaded at 4 pM in a MiSeq® platform according to the manufacturer's instructions and sequenced using the MiSeq® reagent kit v2 (2 × 150 cycles) (Illumina, San Diego, California).

3.2.3.2 Bioinformatics and data analysis

Bioinformatic analysis was applied using the 16S Metagenomics app (version 1.1.0) from the Illumina BaseSpace platform. Operational Taxonomic Units (OTUs) were created, using Ribosomal Database Project Classifier (RDP) (Wang et al., 2007), against the Illumina-curated version of GreenGenes (v.05.2013) reference taxonomy database (DeSantis et al., 2006), as described previously (Yuan et al., 2018). The classified OTUs were defined at ≥ 97% of sequence homology and converted to percentages (relative abundances), to determine the representation of each microbe among treatments. Alpha diversity indexes were calculated using the EstimateS version 9.1.0 (http://viceroy.eeb.uconn.edu/estimates/index.html), by different metrics (Chao, Shannon, inverse Simpson) after performing a rarefaction analysis. Rarefied OTU to 54,488 sequences (lowest number of reads obtained) were used to obtain these indices. Finally, all sequences were deposited to the National Centre for Biotechnology

Information (NCBI) in Sequence Read Archive (SRA) under the BioProject PRJNA600153.

3.2.4 Physicochemical analysis

The changes of pH were measured using a pH meter (Hanna Instruments), while titratable acidity was estimated by titration of brines with 0.1 mol L^{-1} NaOH up to pH 8.3. Results expressed as a percentage of lactic acid equivalent (% lactic acid w/v). Electrical conductivity was measured using a conductivity meter (Mettler Toledo), while water potential was monitored using a potentiometer dewpoint (WP4C), following the manufacturer's instructions. All analyses were performed in triplicates.

The monitoring of organic acids (lactic, succinic, tartaric, acetic, citric and malic) sugars (glucose, sucrose and fructose), and alcohols (ethanol, glycerol) alternations were applied using HPLC (Waters 1525), as described in Chapter 2 (*Paragraph 2.2.3*).

Total polyphenols and antioxidant capacity of fruits were quantified, followed by the identification and quantification of the main polyphenols (oleuropein, hydroxytyrosol) by HPLC (Waters 1525) analysis throughout fermentation. All methods were carried out as described in Chapter 2 (*Paragraph 2.2.3*).

3.2.5 Color and texture profile analysis

Color and texture monitoring of was performed during the whole process, as described in Chapter 2 (*Paragraph 2.2.3*).

3.2.6 Sensory evaluation

Olive samples were evaluated by sensory analysis after 120 days of fermentation according to International Olive Oil Council (Regulation COI / OT / MO No 1 / Rev.1), as described in Chapter 2 (*Paragraph 2.2.5*).

3.2.7 Statistical Analysis

Microbial, physicochemical and sensorial data were analyzed by analysis of variance (one-way ANOVA), using the SPSS 20 software (StatSoft Inc., Tulsa, OK, USA), to underline any significant difference among treatments. The test used was the Least Significant Difference (LSD) at the significance level of 0.05. Furthermore, Principal Components Analysis (PCA) was performed, using varimax rotation, to determine potential distances between the different treatments, during the fermentation process. Factors exhibiting eigenvalues greater than 1.0 were retained, for the selection of the optimum number of principal components (PCs). Afterwards, scores were projected into plots by the first two components.

3.3. Results

3.3.1. Microbiological Analyses

The population dynamics of TVC, LAB, yeasts, CoNS, *coliforms* and *Enterobacteriaceae* during fermentation are presented in Fig. 3.1. In general, LAB and yeasts increased steadily and became the main members of the microflora in all treatments. Differences were recorded mainly between non and inoculated samples,

regardless olive's technology (cracked or whole). Results indicated that LAB predominated in all treatments, while yeast population was noteworthy, especially in non-inoculated treatments (S1, S4). On the contrary, *Enterobacteriaceae* and *coliforms* species were decreased after the first days, while CoNS were undetectable at any time. More deeply, population size of *Enterobacteriaceae* and *coliforms* was very similar in all treatments. They were detected at an average of 3.5 log cfu/ml at the beginning of the process, but they decreased rapidly and could not be detected after 22 days of fermentation, with exception in S4, where they are detectable until 60th day. It is worth noted the faster elimination of *Enterobacteriaceae* in all inoculated treatments (15 days) compare to the other control sample (S1). Yeast growth had an initial lag phase in all cases, occurring across treatments, reaching the maximum level of approximately 6.5 log cfu/ml at circa 15 days. Following, a decrease was observed in all treatments, until day 60 and then remained steadily. However, the decrease in inoculated olives was significant higher, with an average reduction of about 2.5 log (from 6.5 log cfu/ml at day 15 to 4 log cfu/ml at day 60) (Fig. 3.1B). At the end of fermentation, the population in non-inoculated samples (S1, S4), which had no significant differences to each other, exhibit an average value of 4.7 log cfu/ml, while in the other treatments was lower. Regarding LAB, a steady trend in samples inoculated with starter culture (S2, S3, S5, S6), was recorded, where an initial population of about 2 log cfu/ml followed by a major increase of approximately 4 log at day 60 and thereafter remained steady until the end of fermentation, reaching an average population of 6.6 log cfu/ml. Although there were no differences at the end of fermentation, it is notable the slower increase of LAB population in S2, the first 45 days of the process. Oppositely, un-inoculated samples exhibited significant lower LAB populations, reaching a value of 5 log cfu/ml at 120th day. However, as in inoculated treatments, LAB were the predominant species in S1

and S4 treatments, at the end of the process. Similar behavior was observed for TVC, in which the highest concentration value in all inoculated samples were reached after 45 days of fermentation. The highest value (7 log cfu/ml) was detected in sample S6, while the lower populations were recorded for both control treatments (S1, S4) at the end of the process (4.9 log cfu/ml).

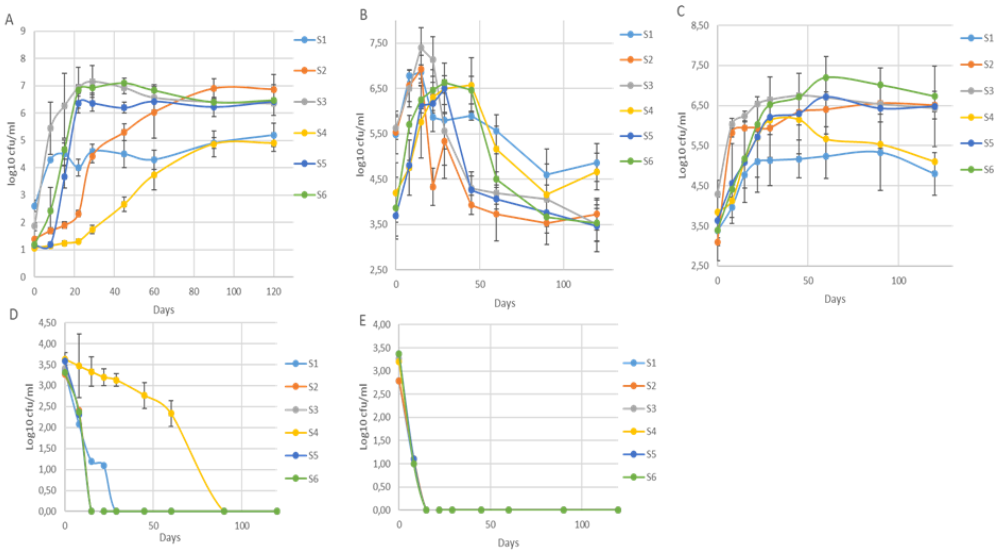


Fig. 3. 1. Evolution of microbial changes throughout the fermentation of different treatments (S1-S6) Results are expressed as means and standard deviations of three replicates. LAB (A), Yeasts (B), TVC (C), Enterobacteriaceae (D) and Coliforms (E). Data points expressed as log10 CFU/ml of 3 replicates ± standard deviation.

3.3.2 Metagenomic analysis

3.3.2.1 Illumina MiSeq data analysis

Overall, a total of 4,170,737 sequences (an average of 231,707 sequences per sample per day) were obtained. Table 3.3 shows the total number of OTUs found in the different samples throughout days and their alpha-diversity indices. In general, higher

biodiversity indicated for treatments S4 and S5, which showed the highest values of Simpson index. It is interesting that in some cases (e.g treatment S4), the highest biodiversity observed at the end of the process. The total number of OTUs assigned ranged from 85 to 272, with an average of 178 detected OTUs per sample. A mean value of ca. 231,707 reads was analyzed, with the highest value recorded (621,655) for the S6 sample at the 120th day and the lowest (54,488) for the S1 sample, at the beginning of fermentation. Regarding chao1 index, our results indicated that most of the bacterial diversity (including singletons and dupletons) was captured by the analysis, since this index was slightly higher compare to the observed OTUs, in all treatments.

Table 3. 3. Number of sequences, OTUs assigned and diversity indices for 16S (bacteria) amplicons according to treatments.

Sample	Number of reads	Number of OTUs	Inv. Simpson	Shannon	Chao1
S1_D0	54488	272	1.71	1.13	436.31
S1_D60	71360	163	1.71	1.12	465.92
S1_D120	102972	133	1.71	1.12	457.36
S2_D0	117071	260	1.71	1.12	451.7
S2_D60	119799	254	1.72	1.13	442.85
S2_D120	130925	210	1.73	1.14	433.44
S3_D0	137191	268	1.73	1.13	425.13

S3_D60	151817	91	1.73	1.13	412.11
S3_D120	157961	97	1.74	1.13	398.4
S4_D0	168025	285	2.08	1.11	266.37
S4_D60	212689	85	2.31	1.11	220.03
S4_D120	241137	215	3.64	1.38	365.81
S5_D0	271235	252	1.81	1.12	340.9
S5_D60	331000	235	1.9	1.14	319.18
S5_D120	363295	129	2.11	1.15	294.78
S6_D0	382718	258	1.75	1.13	387.35
S6_D60	535399	175	1.76	1.12	370.7
S6_D120	621655	90	1.75	1.11	160.02

3.3.2.2 Bacterial diversity

Metagenomic analysis of 16s rRNA indicated that three main bacterial phyla detected in high relative abundances *(Firmicutes, Proteobacteria, Bacteroides*) and another four to a lesser extend (*Actinobacteria, Chloroflexi, Tenericutes, Verrucomicrobia*) (Fig. 3.2). *Proteobacteria* and *Bacteroidetes* were the most abundant phyla at the beginning of the process, following by *Firmicutes*. However, *Firmicutes* predomination was profound at the middle and the end of fermentation. Fig. 3.3 depicts the bacterial genera detected in all treatments. At the beginning of the process, genera

such as *Thermogemmatispora*, *Chitinophaga*, *Agrobacterium*, *Thiomaonas*, *Bosea*, *Bradyrhizobium*, *Heliorestis* and to a lesser extend *Lactobacillus*, were the most frequently detected in all samples On the contrary, mainly *Lactobacillus* and to a lesser extend *Pediococcus* became the most abundant genera at day 60 and 120 in all treatments, while the former genus exhibited higher relative abundance in inoculated treatments. It is crucial to mention that the relative abundance of *Pediococcus* was higher in cracked samples at the end of the process. Finally, *Thermogemmatispora*, *Thiomonas*, *Chitinophaga*, *Enterobacter*, *Sphingomaonas*, *Agrobacterium* and *Heliorestis* were also found at noteworthy abundances in non-inoculated treatment S4 at the end of fermentation.

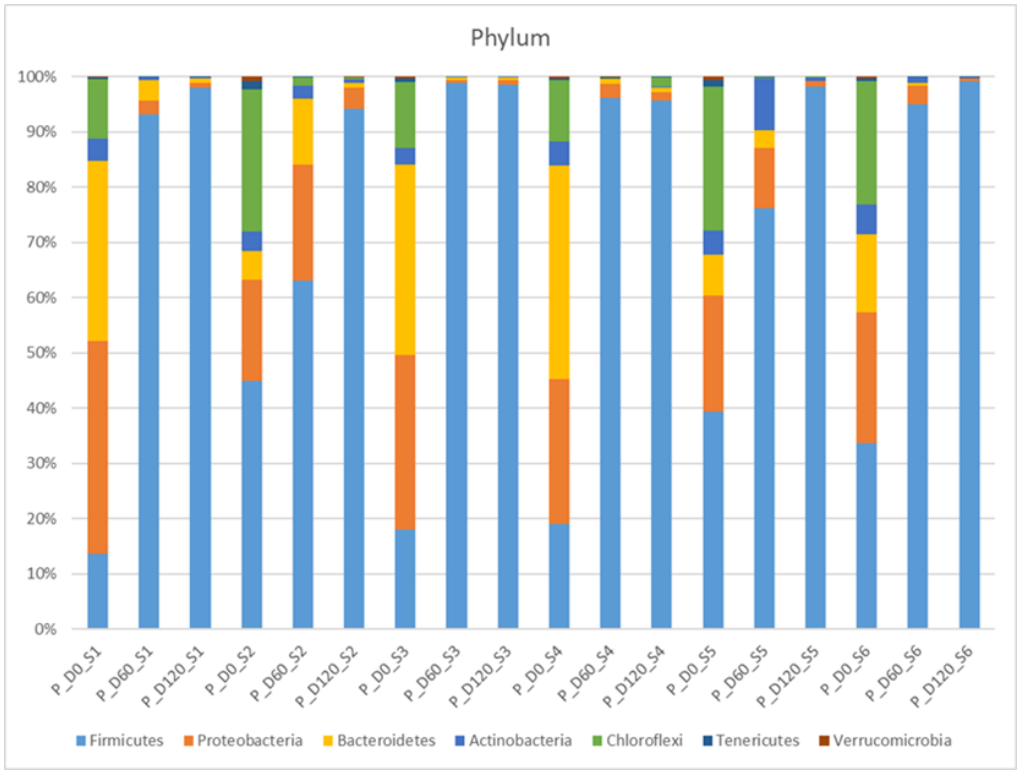


Fig. 3. 2. Relative abundance of bacterial phyla in the different treatments assayed (S1-S6), obtained through metagenetic analysis of 16S rRNA gene at the initial (D0), middle (D60) and the end (D120) of fermentation process.

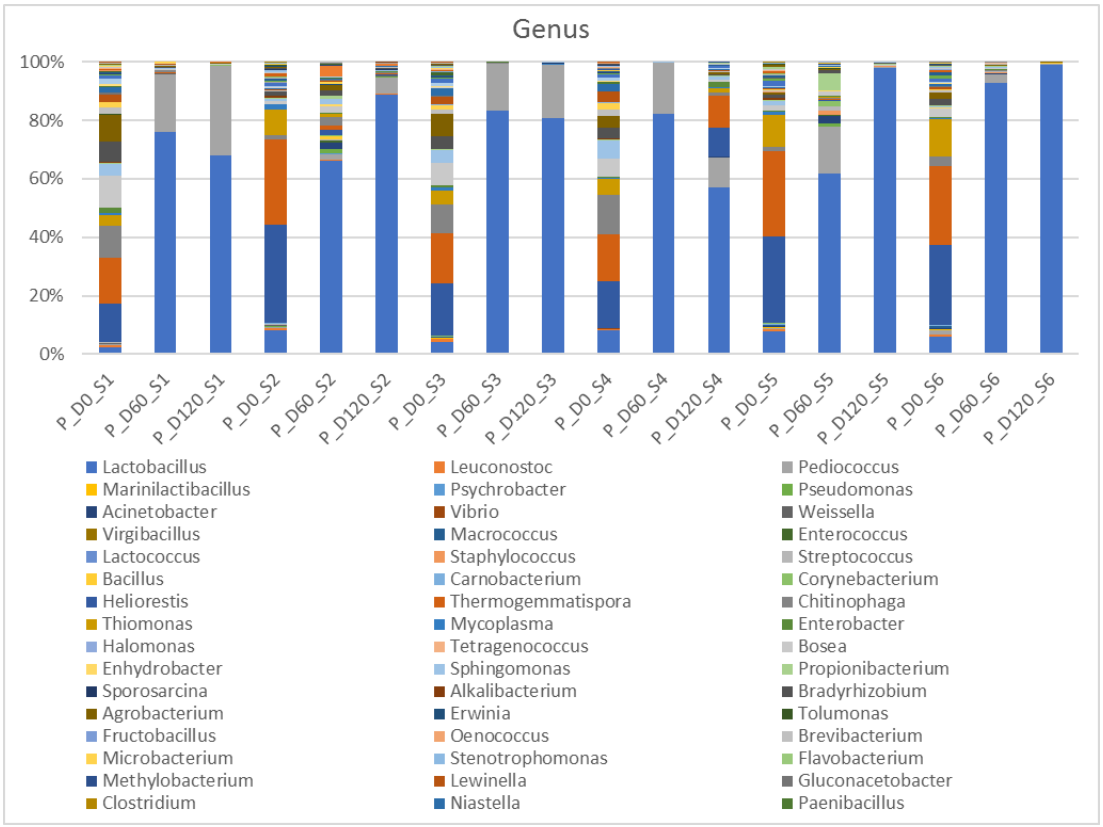


Fig. 3. 3. Relative abundance of bacterial genera in the different treatments assayed (S1-S6), obtained through metagenetic analysis of 16S rRNA gene at the initial (D0), middle (D60) and the end (D120) of the fermentation process.

In the species level, non-inoculated and inoculated treatments recorded differences in their microbiome evolution (Fig. 3.4). NaCl reduction, also had a slight impact on the microbial communities' composition. In day 0, the most abundant species were *Thermogemmatispora acikobensis* and *Chitinophaga soli*, followed by *Thiomonas chermosulfata* and *Bradyrhizobium pachyrhizi*, while *Lewinella lutea*, *Brevibacterium casei*, as well as *Lactobacillus plantarum* group were more limited. However, in the middle of fermentation (day 60), several Lactobacillus spp. and mainly *Lactobacillus plantarum* group and secondly *Lactobacillus manihotivorans* and *Lactobacillus japonicus* became the predominant flora among inoculated treatments. These species

were found to dominate at the 120th day as well, while their relative abundances were equal to day 60. On the contrary, different microbiome profile was observed in S1 treatment, where *Lactobacillus brantae* and *Lactobacillus kefiri* group were by far the most dominant species, while *Lactobacillus plantarum* group and *Lactobacillus manihotivorans* were detected at low levels. As for sample S4, which had the highest biodiversity at the end of the process, it exhibited a quite unique microbial profile. Although *Lactobacillus plantarum* group was the most abundant species, followed by *Lactobacillus manihotivorans*, the presence of *Thermogemmatispora ocikobensis* and *Thiomonas thermosulfata* is noteworthy at the end of the process, while *Lactobacillus japonicus*, *Lactobacillus brantae*, *Lactobacillus parafaraginis* and *Lactobacillus kefiri* group were also detected, at lower levels. Notably, species such as *Enterobacter hormaecei*, *Agrobacterium viscosum* and *Mycoplasma insons* were only detected in this treatment, at the end of the process.

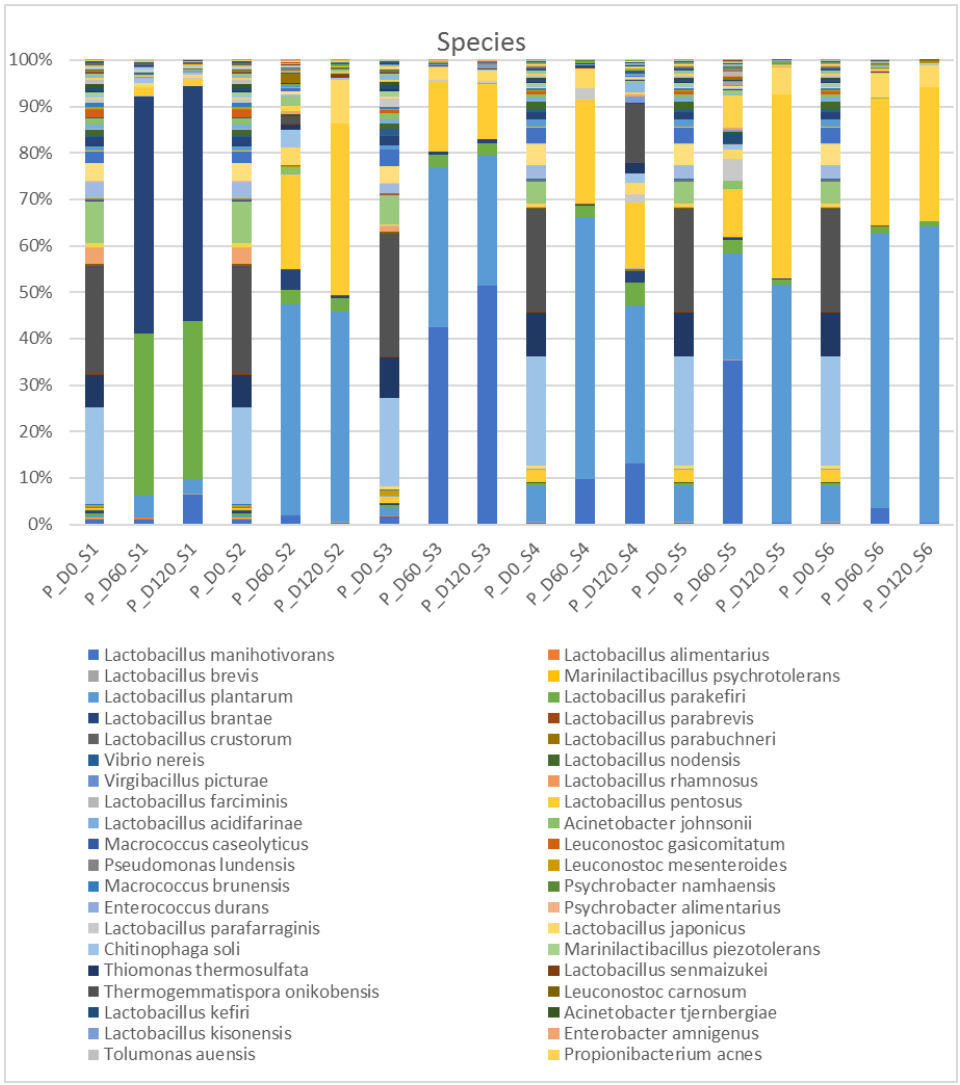


Fig. 3. 4. Relative abundance of bacterial species in the different treatments assayed (S1-S6), obtained through metagenetic analysis of 16S rRNA gene at the initial (D0), middle (D60) and the end (D120) of the fermentation process.

3.3.3 Physicochemical analysis

The changes of pH in the brines during fermentation are presented in Fig. 3.5. As it is clearly observed, the first days of the process (until day 29), there is a differentiation of acidic conditions based on olive's technology (cracked and whole fruit). However, thereafter, it seems that samples grouped based on spontaneous and

inoculated treatments. More specifically, the initial values (Day 0) in all treatments were very low (3.4) due to the use of citric acid at the beginning of fermentation. After that, an increase of approximately 0.4-1 unit until day 8 was observed in cracked olives (S1, S2, S3), while a slight decrease of about 0.3 units was recorded in whole fruits (S4, S5, S6). After that point, major decrease of pH was recorded in inoculated treatments, reaching an average value of 3.4 at day 90, which remained unchanged until the end. On the other hand, between control treatments no significant differences were observed between 60 and 90 days, reaching significant higher pH (4). However, at the end of the process, pH of S4 was significant higher (4.2). Concerning titratable acidity, results indicating that after the first period (ca. 29 days), where it was observed a scarce between treatments, there is a clear differentiation between control and inoculated treatments, which is in agreement with pH findings. At the end of the process, the average acidity of control and inoculated treatments was 0.45 and 0.77 % lactic acid, respectively. In addition, significant differences were observed in electrical conductivity between non and inoculated samples, which were recorded from the first days of fermentation. Inoculated samples exhibited significant higher values during whole process, reaching the highest values at 29^{th} days (87 μs/cm), while values in control treatments were more limited, reaching an average value of 45 μs/cm at the end of the process. Finally, water potential showed significant differences regarding samples with lower NaCl content (S3, S6). The first 8 days water potential of those treatments was significant higher. However, after 90 days of fermentation, treatments with lower NaCl content exhibited the lowest water potential.

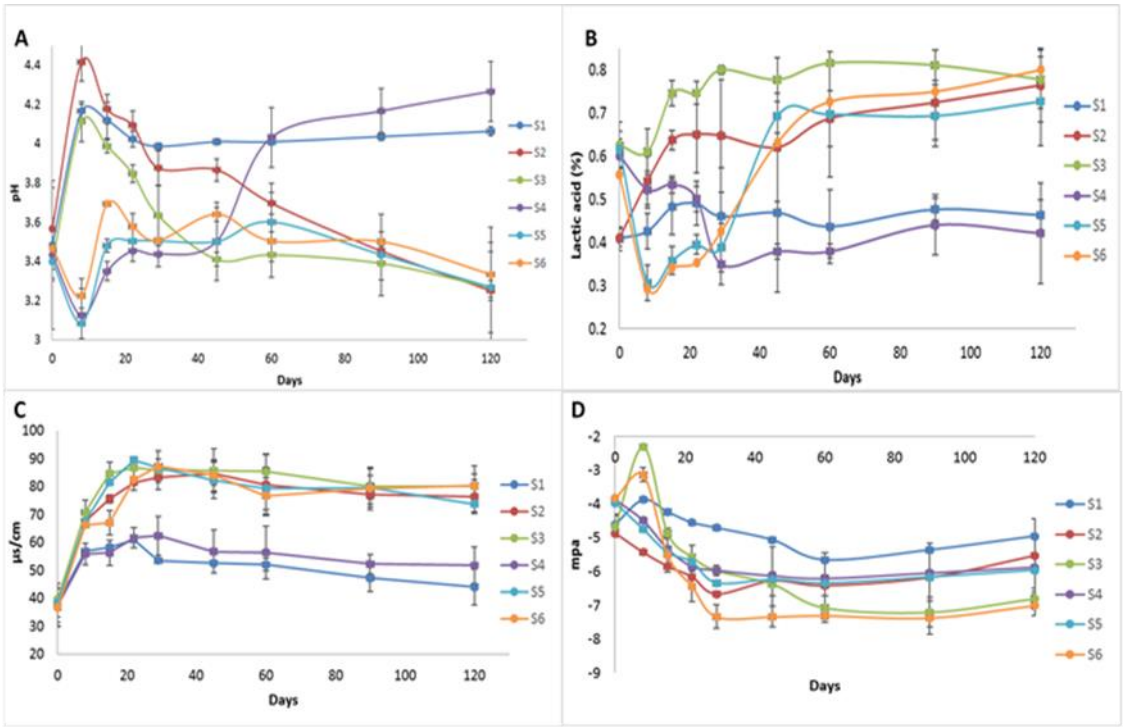


Fig. 3. 5. Changes in pH (A), titratable acidity (B), electrical conductivity (C) and water potential (D) throughout the fermentation of different treatments (S1-S6). Results are expressed as means and standard deviations of three replicates.

The evolution of organic acids in the brines during fermentation is presented in Fig 3.6. Overall, significant differences between un-started and inoculated treatments were observed, as detected by HPLC analysis. More specifically, citric was the main acid at the initial stage in all treatments, due to its use at the beginning of the fermentation. After approximately 22 days, lactic and to a lesser extent acetic became the main acids in all treatments, while an almost total depletion of citric was observed, after 60 days of fermentation. Although lactic acid was the dominant element in all treatments, its levels were significant higher in inoculated treatments, throughout the process, while no differences were recorded for acetic acid. Furthermore, mallic and tartaric acids were also found in traces after 60 days, while the later was totally

disappeared at the end of the process. No differences were recorded between treatments, regarding those acids. Finally, succinic acid was also detected in traces, only in the inoculated treatments, after 90 days of fermentation. No distributions were recorded until the end of the process.

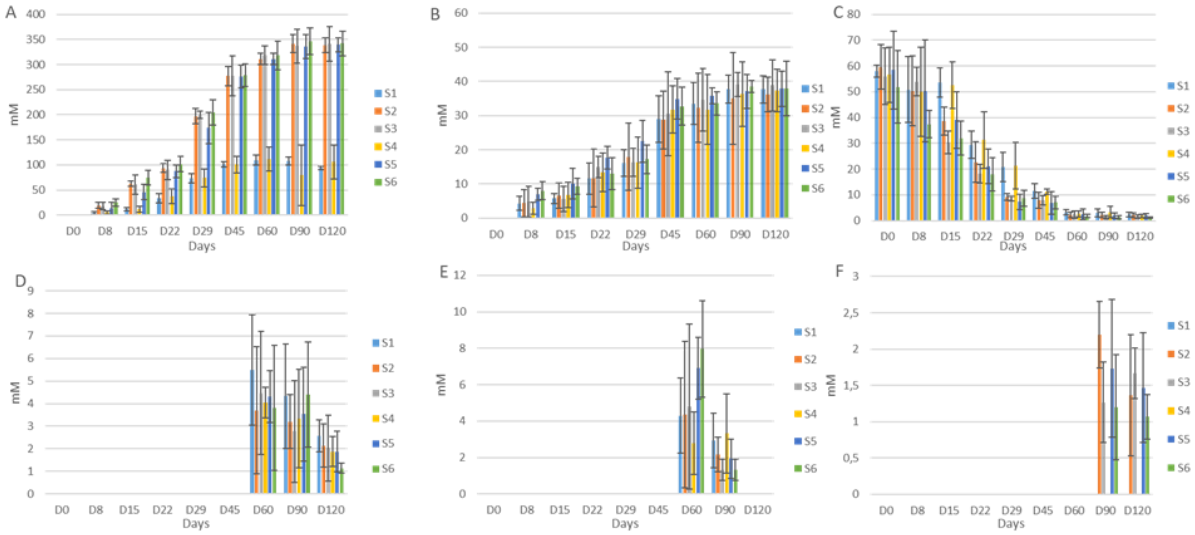


Fig. 3. 6. Changes in the concentration (mM) of organic acids (lactic, A; acetic, B; citric, C; malic, D; tartaric, E; and succinic, F) throughout the fermentation of different treatments (S1-S6). Data points are expressed as means and standard deviations of three replicates.

Concerning soluble sugars, glucose and fructose were the major sugars in the brines detected by HPLC (Fig. 3.7). The content of both rapidly increased at the early stage of fermentation and reached a maximum after 15 days of fermentation. A major decrease was recorded thereafter, while neither glucose nor fructose were detectable at the end of the process. The glucose depletion ascended faster in inoculated treatments, as it was undetectable after 45 days of fermentation. A similar pattern was observed for fructose with two differences. Firstly, no differences were observed between treatments regarding fructose consumption. Secondly, fructose was detectable for 90 days of the

process. Finally, sucrose was never detected in any treatment, indicating the absence of this element in olive fruit. Concerning ethanol and glycerol, the former was detected in all treatments in low amounts (no significant differences) after 90 days of the process, while the latter was observed in traces only in S1 treatment at 120th day (data not shown).

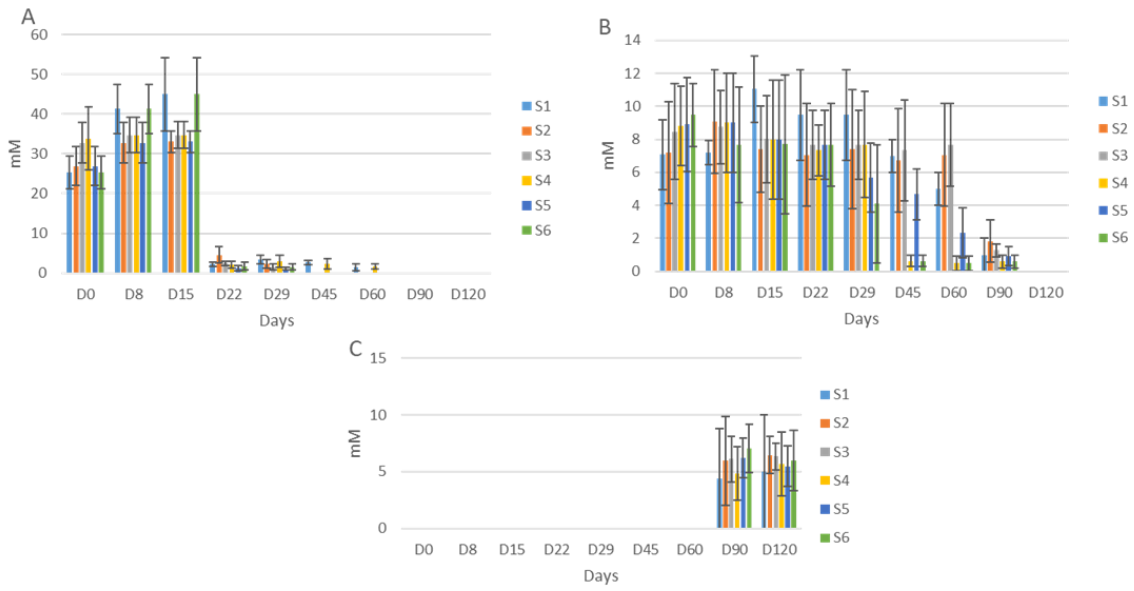


Fig. 3. 7. Changes in the concentration (mM) of soluble sugars (glucose, A; fructose, B) and alcohol (ethanol, C) in the brines throughout the fermentation of different treatments (S1-S6). Data points are expressed as means and standard deviations in triplicate.

Total phenols detected in olive fruits showed quite differences among cracked and whole olives, while within each group similarities on TP content was achieved (Fig. 3.8A). The initial value was close to 5 mg/g GAE in all treatments, but the reduction was faster in cracked olives (S1, S2, S3), reaching an average value of 0.5 mg/g at 45th day, while the TP of S4, S5, S6 was significant higher (ca. 2.45 mg/g) at this time point.

However, at the end of the process, no significant differences were observed in any of treatments. Additionally, the loss in phenolic compounds resulted a remarkable loss of antioxidant capacity in olive fruits, as well (Fig. 3.8B). However, in line with TP reduction, antioxidant capacity reduction was significant faster in cracked olives, while differences between cracked and whole olives were observed until 45th day. Afterwards, it is observed a major decrease (ca. 90%) in whole fruits after 60 days of fermentation. It is crucial to be mentioned that at the end of the process, significant higher values were recorded in inoculated treatments, while S1 exhibited the lowest antioxidant capacity (27.4 mg/g).

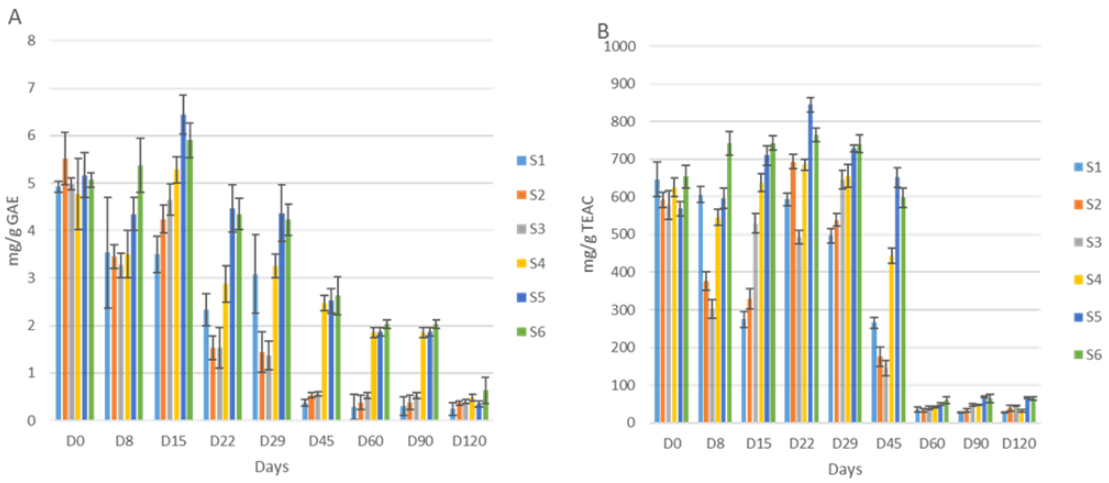


Fig. 3. 8. Total phenolic content (A) and antioxidant capacity of olives (B) during fermentation of different treatments (S1-S6). Results were expressed as means and standard deviations of three replicates, equivalent of mg/g.

Regarding oleuropein monitoring, a major degradation was observed in all treatments as fermentation time passed, which was significant faster in inoculated treatments (Fig. 3.9A). More specifically, the loss of oleuropein in S2, S3, S5 and S6

was close to 92% at day 45, while the equal loss in whole fruits was quite lower (ca. 70%) at the same time point. No differences were observed within these two groups. In addition, at the end of the process, oleuropein was detected in traces, while there is still recorded lower concertation in inoculated samples. Concerning hydroxytyrosol, an exponential increase for all treatments until the end of the process was observed, where higher levels were recorded in samples S3 and S6 (Fig. 3.9B). However, during fermentation, the production of hydroxytyrosol was higher in cracked olives, exhibiting higher levels than in whole fruits until day 29. After that point, it seems that the production of hydroxytyrosol was higher in inoculated treatments; regardless fruit technology. At the end of process, inoculated treatments with 7% NaCl content (S3, S6) exhibited the highest values (ca. 0.64 mg/ml), followed by the other inoculated samples (S2, S5) while the lowest concentrations were recorded for non-inoculated olives, with an average value close to 0.32 mg/ml.

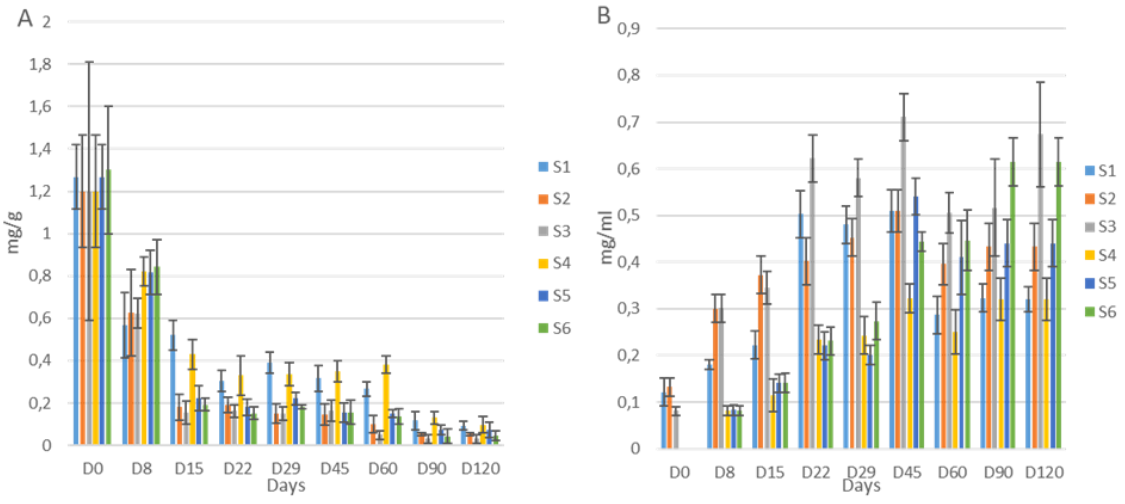


Fig. 3. 9. Evolution of oleuropein in olives (A) and hydroxytyrosol in brines (B) during fermentation of different treatments (S1-S6). Results were expressed as means (mg/g or mg/ml) and standard deviations at different time of fermentations (in Days:D).

Finally, salinity was monitored throughout fermentation and adjusted to the initial values of 10% and 7% for each treatment, by periodic dry salt additions in the brines. Salt equilibrium was reached in ca. 2 months and until the end of the process salt concentration was maintained to its initial values.

3.3.4 Firmness and color evolution of olives

Regarding texture, (Table 3.4), a continuous decreasing tendency was observed, as time passes, until ca. 29 days. After, no changes were recorded in any of treatments, until the end of the process. Samples clearly grouped based on technology, as the firmness of cracked olives were significantly lower during the whole fermentation. No differences observed within groups.

Table 3. 4. Evolution of texture of olive fruits during fermentation of different treatments (S1-S6). Data points are expressed as means (N) and standard deviations of at least 10 random measurements.

DAY	S1	S2	S3	S4	S5	S6
0	19±2	19,66±5,13	20±2,64	49,46±2,9	50,03±1,77	53,5±3,66
8	18,33±2,08	18,33±1,15	19,33±3,05	32,26±4,11	33,62±4,13	32,96±2
15	12,66±1,52	14±2,64	14,66±2,51	20,86±4,55	24,58±3,18	21,83±0,83
22	11,33±1,52	11,66±1,52	12±2,64	18,46±3,13	19,6±1,29	18±3,6
29	7,33±2,08	7±1	8,3±0,3	17,16±3,32	18,9±1,13	18,36±0,56
45	6,23±0,2	5,96±0,55	6,43±0,8	17,3±1,57	18,23±1,68	17,37±0,54
60	6,16±0,15	5,91±0,72	6,53±0,64	17,8±2,3	18,2±1,9	17,26±0,64
90	6,06±0,15	5,6±0,78	6,4±0,65	17,03±1,95	17,6±1,47	17,25±0,64

120	6±0,09	5,53±0,75	5,93±0,55	16,66±1,52	17,54±1,44	17,1±0,78

Regarding color analysis, all parameters are presented in Table 3.5. In general, no significant differences observed for most of color parameters. However, it is crucial to mention that the loss in greenness was higher in samples with reduced NaCl content (S3, S6). Nevertheless, it is notable the close position of all treatments at the end of the fermentation process, with no significant differences.

Table 3. 5. Evolution of color parameters (a*, b*, L*, h* and C*) of different treatments (S1-S6) during fermentation of *Picual* table olives. Data points are expressed as means and standard deviations of 10 random measurements.

Sample	**S1**			**S2**			**S3**			**S4**			**S5**			**S6**		
DAY									**a***									
0	-13.18	±	3.30	-14.44	±	6.14	-15.56	±	1.90	-19.22	±	1.93	-18.87	±	0.61	-19.54	±	1.33
8	-6.85	±	1.34	-6.23	±	1.65	-6.49	±	3.20	-17.61	±	3.71	-16.04	±	2.77	-11.20	±	2.68
15	-3.47	±	0.64	-5.78	±	0.09	-6.49	±	3.20	-6.49	±	3.20	-14.38	±	2.71	-11.20	±	4.15
22	-0.87	±	0.12	-5.78	±	0.09	-2.78	±	0.42	-3.34	±	4.08	-4.13	±	1.78	-2.23	±	2.77
29	-0.33	±	0.53	-3.15	±	2.44	-3.08	±	0.34	-2.37	±	1.39	-3.24	±	0.75	-1.08	±	1.11
45	-0.82	±	1.24	-1.98	±	2.32	-1.43	±	1.09	0.03	±	0.77	-1.31	±	3.07	0.35	±	2.72
60	-0.45	±	0.72	-2.18	±	1.34	-1.31	±	0.89	0.22	±	1.18	1.03	±	1.40	1.24	±	3.51
90	0.37	±	0.55	-2.18	±	1.34	1.47	±	0.45	0.22	±	1.18	-1.92	±	2.86	1.24	±	3.51
120	0.88	±	0.55	-2.30	±	1.72	1.73	±	0.22	0.22	±	1.18	-1.92	±	2.86	1.57	±	2.42
									b*									

0	38.72	±	2.94	37.41	±	4.68	38.70	±	1.62	41.14	±	0.34	36.85	±	1.95	37.21	±	2.25
8	34.34	±	2.72	32.72	±	6.14	35.39	±	3.33	39.06	±	2.31	35.53	±	3.67	37.43	±	2.86
15	31.53	±	2.17	32.62	±	6.11	34.63	±	2.04	39.06	±	2.31	35.53	±	3.67	37.43	±	2.86
22	29.16	±	0.56	34.07	±	0.48	31.68	±	1.29	32.07	±	7.41	33.75	±	1.21	35.02	±	1.72
29	29.13	±	3.94	31.64	±	2.72	32.61	±	3.89	28.72	±	3.91	31.76	±	2.16	33.34	±	0.26
45	28.69	±	2.10	33.04	±	5.27	32.53	±	1.44	29.22	±	5.91	29.47	±	5.64	34.02	±	2.84
60	31.70	±	2.36	32.97	±	4.18	32.64	±	3.66	29.80	±	4.66	30.71	±	0.37	34.85	±	1.86
90	32.11	±	4.92	31.95	±	5.71	31.20	±	4.66	29.27	±	4.26	30.87	±	3.56	34.15	±	3.30
120	32.11	±	4.92	33.13	±	6.32	31.20	±	4.66	29.27	±	4.26	30.87	±	3.56	34.15	±	3.30
									L*									
0	60.01	±	3.55	57.56	±	5.25	57.84	±	2.81	59.25	±	0.95	54.06	±	2.71	56.44	±	3.75
8	57.15	±	5.12	53.85	±	7.55	55.50	±	6.08	56.97	±	1.32	55.61	±	5.52	56.68	±	5.84
15	53.17	±	2.60	54.89	±	7.33	55.30	±	6.23	55.13	±	4.72	52.86	±	0.81	57.08	±	5.50
22	52.23	±	1.70	54.35	±	0.48	52.13	±	0.08	54.75	±	4.36	54.08	±	0.67	53.97	±	0.39

29	53.33	±	2.18	52.14	±	0.83	54.59	±	2.52	53.13	±	3.77	53.23	±	1.94	54.32	±	0.69
45	51.78	±	2.99	54.74	±	2.16	53.56	±	1.48	52.07	±	3.11	53.06	±	6.78	57.53	±	6.24
60	54.15	±	4.24	52.65	±	5.39	55.79	±	4.38	52.15	±	2.38	50.40	±	3.06	58.15	±	5.87
90	54.15	±	4.24	52.65	±	5.39	50.86	±	0.66	52.15	±	2.38	50.40	±	3.06	58.15	±	5.87
120	56.50	±	5.15	55.42	±	6.83	50.86	±	0.66	53.76	±	5.57	51.04	±	4.96	55.76	±	2.11
							C*											
0	40.96	±	3.65	40.26	±	6.39	41.74	±	1.46	45.42	±	1.03	41.40	±	1.98	41.65	±	1.66
8	35.02	±	2.81	33.32	±	6.24	36.04	±	3.89	42.90	±	3.53	39.01	±	4.28	39.71	±	3.61
15	33.59	±	4.78	33.32	±	6.24	35.83	±	4.13	40.94	±	2.86	38.14	±	5.38	39.25	±	4.12
22	29.17	±	0.56	34.84	±	0.49	31.80	±	1.32	34.65	±	8.76	36.20	±	3.29	36.24	±	0.77
29	30.84	±	1.20	30.14	±	1.06	33.38	±	3.15	31.06	±	1.33	32.46	±	1.80	33.09	±	0.75
45	29.24	±	2.86	31.61	±	6.80	32.17	±	1.61	30.93	±	4.42	30.13	±	5.88	33.70	±	3.07
60	31.58	±	2.58	32.16	±	5.58	32.99	±	3.27	31.52	±	1.82	28.38	±	4.24	34.49	±	2.74
90	31.58	±	2.58	35.06	±	2.54	32.99	±	3.27	31.52	±	1.82	28.38	±	4.24	34.49	±	2.74

120	32.89	±	3.64	34.49	±	4.30	29.72	±	2.11	30.97	±	1.57	28.68	±	5.71	34.76	±	3.13
									h*									
0	108.62	±	3.59	110.44	±	6.51	111.92	±	2.78	112.41	±	3.33	117.13	±	0.68	116.67	±	2.77
8	101.25	±	1.91	100.79	±	2.05	100.11	±	3.91	114.08	±	3.54	114.33	±	2.73	109.30	±	2.21
15	93.78	±	4.23	98.91	±	1.82	98.96	±	2.69	96.89	±	2.45	98.51	±	2.19	101.74	±	1.41
22	91.71	±	0.20	98.43	±	2.10	95.79	±	1.73	92.35	±	5.74	97.78	±	2.32	98.88	±	3.66
29	89.46	±	1.43	94.67	±	3.87	95.69	±	1.43	93.16	±	2.60	97.12	±	2.21	93.56	±	2.17
45	91.30	±	2.64	94.24	±	2.44	91.93	±	0.93	88.33	±	2.14	92.51	±	2.80	91.81	±	2.71
60	89.18	±	3.01	93.65	±	1.77	92.97	±	1.49	89.52	±	3.38	90.78	±	3.30	93.54	±	5.03
90	90.05	±	4.45	94.41	±	1.24	90.74	±	3.01	87.55	±	3.16	96.07	±	1.10	93.63	±	2.10
120	88.56	±	2.22	92.61	±	1.28	91.33	±	1.53	88.81	±	6.06	93.86	±	3.00	92.87	±	3.01

3.3.5 Sensory evaluation

The organoleptic profile of olives after 120 days of fermentation is presented in Fig. 3.10. In general, the samples were characterized by low remaining bitterness, good crispness, and satisfactory acidic taste and odor. No off flavor was noticed in any sample as inferred by the low scores of the taste panel for this organoleptic perception. Overall, regarding the gustatory sensations, differences among treatments were detected on bitterness descriptor, in which control treatments had higher score from the inoculated ones. Regarding saltiness score, lower values were scored in S3 and S6 samples, while texture of whole fruits was significantly higher than in cracked. However, inoculated treatments had the highest value of acidity, receiving the highest score for the overall acceptability descriptor, except for S6 sample, which showed an average score between inoculated and control scores. Notably that olives with lower NaCl content exhibited higher score regarding flavors. As a result, it seems that the main parameters affected the preference of the panel were flavor, acidity and bitterness to a lesser extent.

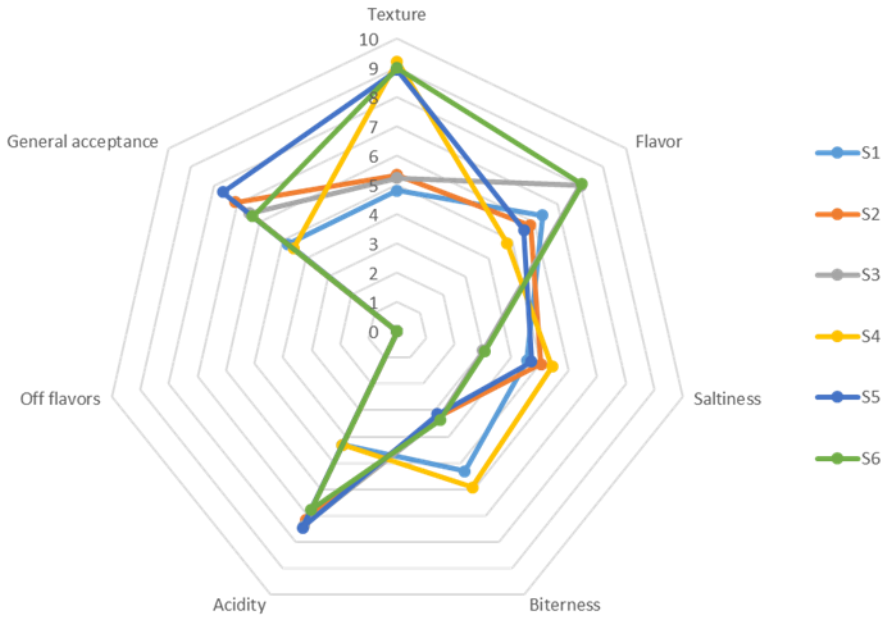


Fig. 3. 10. Sensory profiles of different treatments of Picual table olives after 120 days of fermentation**.**

3.3.6 Multivariate analysis

According to PCA, four factors were retained (eigenvalue more than 1). These factors explained 83.1% of distribution, while the first two factors had a sum of 67.4% of the total variance **(Fig. 3.11).** According to analysis, treatments grouped in four main clusters, based on inoculation. The latter is obvious, especially in the last days of fermentation time (Group C and D, respectively). As clearly observed, non-inoculated treatments were separated from the others from 45th day and thereafter. Thus, it was evident that inoculation was the main factor responsible for the discrimination of treatments during the process. It is crucial to mention that the reduction of NaCl concentration (S3 and S6) did not affect the groups' distribution.

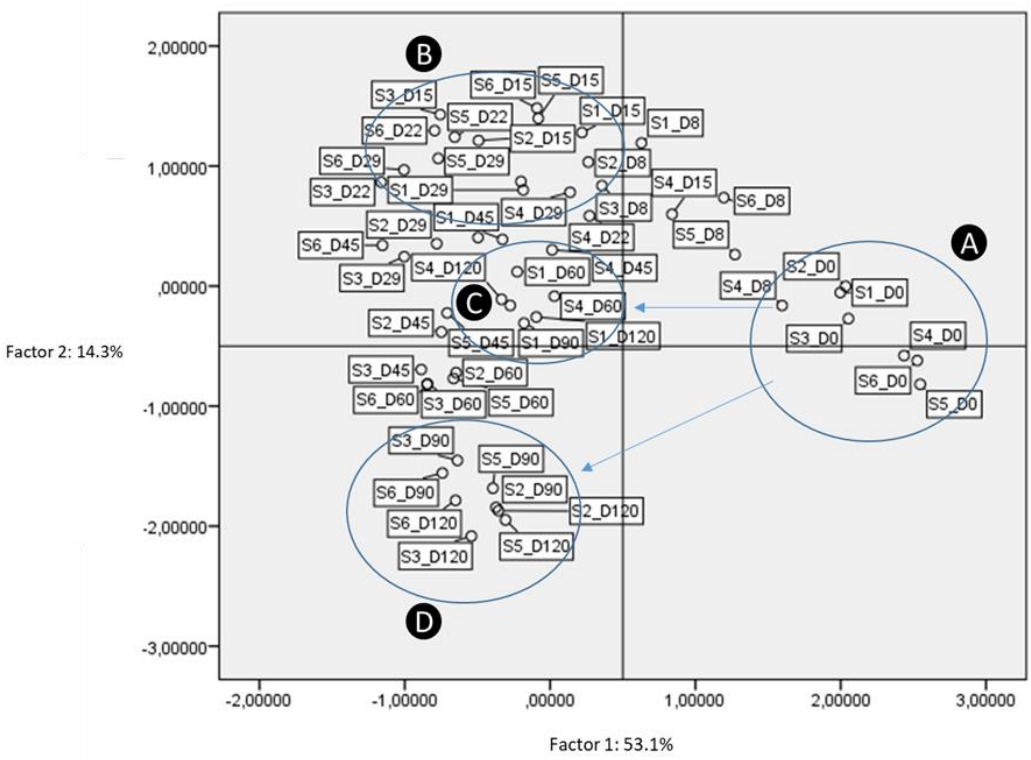


Fig. 3. 11. Plot of scores and loadings between treatments formed by the first two principal components from the PCA analysis. Labelling of data points indicate the processing treatment of olives (S1-S6), and fermentation time (D:Days).

3.4. Discussion

Table olives are fermented fruits with a great impact on the Mediterranean diet. Due to their high nutritional value and their antioxidant capacity, they are considered to be an important functional food. Despite their significant importance, the fermentation is still an empirical method, coming from ancient times. It is driven spontaneously by the indigenous microflora, depending on the year of harvest and processing technology. On this point, a current challenge in the processing technology of table olives, is the establishment of using starter cultures, able to standardize, accelerate and safely drive the fermentation process (Pino et al., 2019). In parallel, a

second challenge for the industry is the reduction of NaCl content, which is in high levels, ranging from 8 to 13% among producer countries, while in Cyprus, the average use of NaCl content is 10%, aiming to reduce the undesirable spoilage and pathogenic microorganisms, ensuring the microbiological safety and quality of the final product. However, nowadays, based on both consumer's demand and regulatory authorities, the reduction of intake sodium is a pre-requisite (Zinno et al., 2017). One of the most widely employed strategies to reduce sodium content in table olives is the use of NaCl substitutes, which can be added alone or in combination with other salts (Bautista-Gallego et al., 2015). Few studies have evaluated the possibility to setup low NaCl table olives without any salt replacement, in combination with the use of starter culture (De Bellis et al., 2010; Pino et al., 2018; Pino et al., 2019). Moreover, to our knowledge, there is limited knowledge concerning fermentation of Picual table olives. Only Tufariello et al., (2019) investigated the fermentation of Picual with the use of yeast starters, however, the study was applied in high NaCl content (11%). In the present study, the application of LAB starter and the reduction of sodium content for Picual table olive production has been investigated for the first time, in order to evaluate if this strategy can potentially ensure and favor the quality and safety of this product.

Overall, according to our results, the main differences observed across treatments, were mainly related to the inoculation with LAB starter, while slight effect were recorded between different salt content (7 and 10%) and the applied processing of olives (cracking vs whole fruit). This was also confirmed by PCA, where treatments were clearly separated based on inoculation. These findings indicate that inoculation could drive the fermentation, regardless salt content and other processing aspects, in more controlled conditions, resulting in a stable and reproducible final product. Based

on this hypothesis, the present study supports that the reduction of salt content in Picual table olives fermentation is feasible, while its inoculation could potentially lead to the production of an added value product.

Initially, the evolution of bacterial communities was studied using NGS analysis. The analysis of 16S rRNA metagenomic has fundamentally enhanced our knowledge regarding bacterial communities during Picual olive fermentation. However, some concerns regarding this analysis have been noted. For instance, a potential preferential amplification could impact the number of OTUs, leading discordance to the real abundance of microbes (Ercolini, 2013). Furthermore, the discrimination between living and dead cells is not feasible, leading to a miscalculation of the real microbiota, presented in a habitat (Emerson et al., 2017). Finally, the reliability up to species taxonomical level using 16S rRNA metagenomics is under dispute by the scientific community. However, a reliable assessment for at least the predominant species can be achieved, in order to proceed to other methodologies, targeting more specific gene regions (e.g recA, DnaK), or full genome sequence, for complete confirmation of the presence of these species. Nevertheless, in the present study, using this culture-independent method, a good assessment of bacterial consortia was achieved up to species level, allowing to a better understanding of this complex matrix. According to our findings, different microbiome profiles were observed in the middle (day 60) and at the end of the process, which are closely linked to the inoculation with the starter culture. More specifically, the dominance of genus *Lactobacillus* and more specifically *Lactobacillus plantarum* group in inoculated treatments was profound, indicating that starter culture withstands the competition with the natural microflora, while was not affected by high salt concentration and predominated in a

short period of time. This is in agreement with the literature, as Pino et al., (2019) reported the dominance of the starter inoculum, while other studies highlighted the successful inoculation during several olive cultivars fermentations, as well (Panagou et al., 2008; Tataridou and Kotzekidou, 2015; Chranioti, Kotzekidou and Gerasopoulos, 2018; Pino et al., 2018). Oppositely, other species of genus *Lactobacillus* (*L. brantae* and *L. kefiri* group) predominated in control S1. To our knowledge, this is the first study reporting these two LAB as predominant species in table olives fermentation. Rodríguez-Gómez et al., (2017), reported the predominance of *Lactobacillus* genera in fresh and cured green *Alorena* table olives at the end of fermentation. Cocolin et al., (2013) found also that after 3 months of fermentation *Lactobacillus* was the main bacterial population present. Both studies are in good line with the present study. However, it was some kind of surprise the microbiome profile exhibited by the second control S4 (spontaneous fermentation, whole fruit). The different profiles between controls (S1 and S4) are obviously linked with the olive's technology (whole fruit). At the end of the process, S4 had the highest biodiversity, with a pool of microorganisms co-existing. Notable that besides the presence of LAB (*Lactobacillus plantarum* group, *L. manihotivorans,* etc.), there is also a worrying presence of *Enterobacteriaceae*, *Chlorobacteria* and other environmental or fresh fruit originating microorganisms, which are not detected with classical microbiology and reported for the first time, indicating the usefulness of HTS analysis. Furthermore, this finding is strongly supporting the hypothesis that inoculation is a must in table olives fermentation, to ensure safety and succession. In a previous study, Medina et al., (2016), using pyrosequencing analysis, reported the presence of undesirable *Celerinatantimonas*, *Pseudomonas*, and *Propionibacterium* as the most abundant genera detected in traditional industrially fermented fruits. Furthermore, it must be mentioned the lower

relative abundance of *Lactobacillus plantarum* group in S3 treatment, indicating high competition between inoculum and indigenous microflora, which were probable able to grow under lower NaCl content, as we are referring to salt intolerant species like *L. manihotivorans* and *L. kefiri* group. Indeed, in previous study, it has been reported a potential competition between starter and indigenous microflora (Chranioti et al., 2018). Finally, as it is clear from literature findings, the determination of microbial communities of table olives fermentation is primarily in early stages, as the results from different works are scarce. Thus, HTS analysis could be a reliable tool to solve this aspect. However, it is strongly recommended the monitoring of microbiome profile during olives fermentation of different years, origin and producers, in order to enhance the hypothesis regarding the stability of fermentation using LAB starter.

The different microbial communities between treatments, led to different microbial populations and biochemical attributes, as revealed from our results. In particular, the higher population levels of LAB in inoculated treatments caused higher reduction of pH values and higher levels of titratable acidity, which were quite lower than the limits proposing by IOC (2004) (4.3 and 0.4 respectively), while the respective values of control treatments were close to those limits. The latter could be an attribute to the higher production of lactic acid in inoculated samples, as revealed by HPLC analysis. The more acidic conditions in inoculated treatments led to the elimination of Enterobacteriacae and coliforms in a shorter period of time, as well. It is crucial to mention that in S4 control, these undesirable microorganisms were detectable for longer period (60 days), enhancing the hypothesis that inoculation is mandatory to ensure the safeness and succeed of fermentation. The profound positive effect of LAB starter in

those aspects is in agreement with the literature (Papadelli et al., 2015; Pino et al., 2018).

Furthermore, the high acidic environment in inoculated treatments increased electrical conductivity. The proportion of pH and conductivity has already been reported (Liebeherr, 2006). This is in line with the findings from Chapter 2, where the relation between pH, titratable acidity and electrical conductivity was noticed. From the same study, this method has been proposed as an alternative tool for table olives fermentation monitoring for the first time. The findings from the present study are strongly enhancing this hypothesis. Notably, this method has been used for fermentation of other products, as well. In particular, Cais-Sokolińska (2017) demonstrated an obvious relationship between pH and conductivity during mixed coagulation of milk.

Regarding water potential, it is clear that in the first days of the process, osmosis in treatments with lower NaCl content was higher than the ones with higher NaCl content. This allowed the faster diffusion of flesh tissue components (sugars, organic acids, polyphenols, etc.) to the brines. Indeed, it has been reported that the slow extraction of soluble components from fruit to brine is closely related to high NaCl concentration (Papadelli et al., 2015). Furthermore, this agrees with our previous study (Chapter 2), where the use of water potential has been proposed as a tool for soluble component kinetic estimations of table olives (diffusion of components from fruit to brine), especially in the first critical days of the fermentation process.

Concerning organic acids, as previously mentioned, lactic was the most abundant acid in all treatments, while its levels were higher in inoculated treatments. This is closely related with the different dominant microbiota between control and

inoculated samples, leading to higher homofermentative metabolism (Papadelli et al., 2015). Furthermore, it is noteworthy the presence of acetic acid, which could be attributed to yeast activity (Bleve et al., 2014; Bonatsou, Paramithiotis and Panagou, 2018), although the contribution of LAB in production of this acid could also be taken into consideration, due to a potentially heterofermentative LAB, such as *L. manihotivorans* (Salvetti et al., 2012) *and L. brantae* (Volokhov et al., 2012), able to generate acetic acid from fermentable material under particular conditions of environmental stress, as well as from the metabolism of citric acid (Laëtitia et al., 2014). Finally, it must be mentioned that the presence of succinic acid only in inoculated treatments, could be related to microbial conversion of citric acid to succinic, via a potential shift from homo- to heterofermentative metabolism of the starter culture (Papadelli et al., 2015).

Moreover, inoculated treatments were richer in antioxidant capacity at the end of fermentation, although during fermentation it was recorded a high impact of olive's physical manipulation (cracked and whole fruit). However, one of the most promising findings in this study, is the faster degradation of oleuropein in inoculated treatments; thus, faster olive's debittering; resulting in parallel to the production of higher levels of hydroxytyrosol. This finding confirms that the enzymatic activity of the starter culture was higher than the respective of indigenous microflora, affecting the secoiridoid glucosides and their aglycon derivatives (Perpetuini et al., 2018). In agreement with our results, Othman et al., (2009) identified hydroxytyrosol as the main simple phenolic compound found in the brine of Tunisian black olives. They observed that spontaneous and inoculated fermentation with a selected strain of *L. plantarum* at ambient temperature using 8% NaCl in the brine resulted to 87.07% and 67.58% of

hydroxytyrosol release, respectively. The accumulation of hydroxytyrosol in the brine, as the main simple phenolic compound of olives of Hojiblanca cultivar at the end of fermentation storage was also confirmed (Pistarino et al., 2013b). The appearance of this compound in the brine is mainly related to the hydrolysis of oleuropein (Kiai and Hafidi, 2014). Hydroxytyrosol is known as an important biophenol belonging to the odiphenol group with special antioxidant activity (Pistarino et al., 2013b), and it has been considered a marker for the determination of oleuropein degradation and diffusion of phenols from drupes to brine (Randazzo et al., 2011).

Texture and color are playing a major role in consumers' acceptance (Luckett, 2016). Since in the present study, no differences were found in texture and color parameters, between non-inoculated and inoculated treatments, we can conclude that the reduction of sodium chloride is advised. Furthermore, the later could be supported by the fact that the starter driven olives were also highly appreciated by the panelists, as indicated by the highest scores in general acceptance. Moreover, based on sensory scores, major differences were found, such as higher acidity and lower remaining bitterness, while no differences were recorded in texture. All those aspects could be, therefore attributed to the added starter culture, which is in line with the literature (Chranioti et al., 2018).

3.5. Conclusions

LAB inoculation has been successfully applied to control the fermentation process of Picual table olives, even in lower NaCl concentration, regardless of olives technology, affecting their microbial growth, the composition of microbial communities, and their biochemical profile. It seems that the time of fermentation was

shortened, as the olives debittering was accelerated, while the process was standardized, and the final products were improved regarding their functional properties, as well as their organoleptic characteristics, as confirmed by sensory analysis. Inoculation was also useful for faster elimination of possible contaminants and undesired microorganisms, leading to a more safe and controlled process. Overall, the findings of the present study are very promising, enhancing the significant contribution of starter culture, raising in parallel the possibility to produce Picual table olives with reduced salt content.

Last but not least, HTS analysis of 16S rRNA can fill in the limitations of culture-dependent methodologies. The bacterial communities during Picual table olives that could not be delivered with classical molecular methods, are now surfaced. New bacterial species have been detected for the first time in table olives, using NGS, indicating the extreme need for such works, to improve our knowledge regarding microbiota formation during table olives fermentation. Consequently, further studies are also necessary to determine the influence of these new microbial species on the sensorial characteristics of table olives.

Chapter 4: Screening of lactic acid bacteria isolated from Cypriot table olives for technological and probiotic properties

ABSTRACT

The present work aimed to evaluate the multivariate characteristics of LAB, previously isolated from naturally fermented Cypriot green cracked table olives, in pursuit to be used as potential starter cultures for the improvement of the traditional fermentation process and the production of newly added value functional olives. A total of 117 LAB strains were screened in a proposed pre-selection protocol and the selected strains (51) were molecularly identified and further studied for their potential technological and probiotic properties. The obtained results indicated that during the simulated gastric and pancreatic digestions, quite a lot of strains were able to reach equal or even higher survival rates to the well-known probiotics *L. acidophilus* LA-5 and *L. rhamnosus* LGG, respectively. Most of them showed high auto-aggregation and DPPH scavenging activity, while hydrophobicity had a strongly scattered distribution; however, Lactobacilli showed similar or higher capacity compared to probiotic controls. None strain showed bile salt hydrolase activities, while they exhibited antimicrobial activity like bacteriocin against at least one indicator strain. Interesting enzymatic profile was observed for the majority of them, especially regarding to lipolytic enzymes, arylamidases, β-glucosidase, α-galactosidase, Naphthol-AS-BI-phosphohydrolase and acid phosphatase. Finally, a multivariate analysis led to the segregation of eight strains belonging to *L. pentosus* (5), *L. coryniformis* (2) and *L. plantarum* (1) with promising technological and potential probiotic properties, enhancing their possible use and establishment as starter cultures (single or combined), at industrial scale fermentation process.

4.1. Introduction

LAB are gram positive, non-spore forming bacteria. They can use their fermentative metabolism to provide energy since they are facultative anaerobes. In general, LAB is a genetically diverse group of bacteria, encompassing rod-shaped bacteria such as *Lactobacilli*, and cocci like *Streptococci*, *Lactococci*, *Enterococci*, *Pediococci*, or *Leuconostoc*. Their contribution during olives processing is of great importance. They are the main group of microbes causing acidified brine environment due to the generation of lactic and other organic acids, aiming to provide stability and safety during fermentation process (Abriouel et al., 2012).

Fermentation procedure is based on spontaneity and thus, it doesn't guarantee stability. However, it constitutes the only official way for the formation of organoleptic characteristics of the final product, as well as the elimination of gram-negative and other undesirable bacteria, which are in abundance the first days of fermentation.

Last decade, several disadvantages have been underlined for this natural process (Heperkan, 2013), while at the same time, the interest in the development and use of indigenous starter cultures for table olive fermentation is exponentially increasing (Pino et al., 2018). Brines inoculation with an appropriate LAB starter culture, could not only reduce the risk of unexpected growth of spoilage strains but also may lead to a more controlled fermentation process and thus a more stable and reproductive final product (Papadelli et al., 2015; Pino et al., 2019). Moreover, the use of starter culture with interesting technological properties, such as high acidification capacity and crucial enzymatic activities, accelerates the whole process (Arroyo-López et al., 2012; Hurtado, Reguant, Bordons, & Rozès, 2012; C. L. Randazzo et al., 2014), contributing significantly to the development of desirable technological and sensory characteristics

of the final product (Randazzo et al., 2017). Finally, the production of an added value and reproducible final product could be achieved by the use of an appropriate starter.

Except from the aforenoted features, there is a high interest for screening a candidate starter microorganism for its potential probiotic properties, based on the increasing consumer's demand, to produce healthier and functional table olives. According to FAO/WHO (2002), probiotics are live microorganisms that are able to surpass the inhospitable environment of the human intestine, conferring some kind of health benefits. Several *in vitro* tests have been settled up, such as resistant to simulated gastric and pancreatic juices, acid and bile tolerance etc. Although all of them need further improvement, they have been established as a first screening for the selection of the most promising probiotic microorganisms (FAO/WHO, 2002). The importance of foods in the efficacy of probiotics have been noted (Ranadheera et al., 2010). New sources of probiotic microorganisms are currently being investigated. Among other traditional fermented foods, table olives are considered as one of the most promising probiotic foods through the use of indigenous microorganisms as starter cultures. Nowadays, they are increasingly recognized as a very promising source of probiotic bacteria, especially those fermented with a non-treated procedure (Greek style or naturally style). Functional bacteria contribute to nutritional or health advantages (Argyri et al., 2013). However, the selection of appropriate strains is quite complex. The brines conditions taking place during the fermentation could play the role of inhibitor for the successful adaption of the starter, while an inappropriate starter culture may cause the production of undesirable metabolites and, thus, the deflection of the fermentation process (Bonatsou et al., 2017). Therefore, a specific selection protocol for the most promising isolates should be established globally. Finally, after the

selection of the most promising strains based on *in vitro* assays, their experimental validation at the industrial scale is pre-required.

Thus, the present work aimed to investigate the technological characteristics and the potential probiotic properties of LAB strains, previously isolated from Cypriot green cracked table olives, during semi-industrial scale spontaneous fermentation. The candidate strains that fulfill the established criteria could, therefore, be potentially used as novel starter cultures with biotechnological properties by the table olive industry.

4.2. Materials and methods

4.2.1. LAB strains and pre-selection

A total of 117 LAB strains were screened in this study. They have been previously isolated from the spontaneous fermentation of Cypriot green cracked table olives (Chapter 2) from distinct phases (early, middle and final stages). All strains were deposited in the culture collection of the Laboratory of Agricultural Microbiology and Biotechnology of Cyprus University of Technology.

In order to establish a pre-selection protocol based on *in vitro* preliminary assays, all strains were tested for their tolerance to a synthetic model brine (SMB) (Bleve et al., 2015). The SMB was formulated based on the results obtained by Chapter 2 in order to be represented; as much as possible; the conditions occurring during the first period of Cypriot olives fermentation (ca. 30 days). More specifically, the SMBs (20 ml) contained 10% (w/v) NaCl, 0.4 mg/ml hydroxytyrosol, 4 mg/ml oleuropein, 50 mM citric acid, 50 mM mallic acid, 8 mM tartaric acid and 40 mM glucose. Bacterial cells from overnight (18 h) cultures were harvested (10,000 x g, 5 min, 4^{o}C), washed

twice with NaCl (0.85% w/v) and resuspended in 1.5 mL of a sterile saline solution (10% w/v) to obtain a final concentration of about 7 log cfu/mL, which was confirmed by plate counting on MRS agar. These bacteria suspensions were used to inoculate the SMBs and incubated at 20^{o}C for 15 days. Enumeration of the bacteria population was applied by plate counting on MRS agar. Results were expressed as survival score [3, high survival (>6 log CFU/ml), 2, medium survival (4.5-6 log CFU/ml, 1, low survival (2-4.49 log CFU/ml and 0 no survival (<2 log CFU/ml)], as they have arisen by means (log CFU/ml) and standard deviations of three replicates. Only strains able to exhibit score 2 or 3 after 15 days of incubation were further screened.

In a second pre-selection step, the acidifying capacity of the selected strains and their ability to degrade oleuropein were also checked in SMBs containing 10% w/v NaCl, 4mg/ml oleuropein and 40mM glucose. Bacteria cells from overnight (18 h) cultures were harvested (10,000 x g, 5 min, 4^{o}C), washed twice with NaCl (0.85% w/v) and re-suspended again in 1.5 mL of a sterile saline solution (10% w/v) to obtain a final concentration of about 7 log CFU/mL (confirmed by plate counting). These re-suspensions were used to inoculate the brines, following by incubation at 20^{o}C for 24h. The acidifying capacity was applied in triplicate and calculated as $\Delta pH = pH\ (0h) - pH\ (24\ h)$. Continuously, oleuropein degradation was determined after 7 days of inoculation by HPLC (Waters 1525) analysis, as reported by Tataridou and Kotzekidou (2015). Results were expressed as percentage (%), as emerged by means (mg/ml) and standard deviations of three replicates. Non-inoculated brines were used as negative control for both assays. Only strains able to reduce pH value less than 4.5 (initial pH 6.5) after 24 h of incubation and degraded at least 50% of oleuropein content after 7 days, were studied for further attitudes.

4.2.2. Identification of selected strains

The selected isolates were examined for Gram staining and catalase. Gram-positive and catalase-negative were presumptively identified as LAB. For rod-shaped isolates, further classification was applied according to the biochemical criteria, such as growth at various temperatures (10^{o}C, 15^{o}C, and 45^{o}C), salt tolerance (2, 4, 6.5, 8% w/v NaCl), production of CO_2 from glucose as unique source of carbon and pH (9.6) (Estifanos, 2014). All rod-shaped isolates which were classified to the genus of *Lactobacillus* based on the biochemical criteria, were applied to DNA extraction. Overnight (18h) bacterial cells grown in MRS broths, were used for total genomic DNA extraction and purification. DNA from each strain was obtained by adding 20 μl lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 65˚C for 24 h. The cell lysate was spin by short centrifugation and diluted with 180 μl buffer (10 mM Tris-HCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000 x g was performed to remove cell debris. Supernatants were checked for their purity (260/280, 260/230) using Nanodrop and stored at -20˚C. Afterwards, a multiplex species-specific PCR targeting the *recA* gene was applied, using the primers paraF (5'-GTC ACA GGC ATT ACG AAA AC-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), planF (5'-CCG TTT ATG CGG AAC ACC TA-3') and pREV (5'-TCG GGA TTA CCA AAC ATC AC-3'), according to Torriani and co-workers (Torriani and Felis, 2001). The isolates that could not be identified with the above method, as well as the remaining cocci-shape strains were analyzed by DNA sequencing of 16S rRNA bacterial locus, using primers 27f (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492r (5’-ACG GCT ACC TTG TTA CGA CTT-3’), according to Barrangou et al., (2002). The DNA sequence was

carried out by Macrogen (Macrogen-Korea, Seoul, Korea). Sequences were identified by a database similarity search in the Genbank Collection using the BLAST software (http://www. ncbi.nlm.nih.gov/BLAST/). Finally, sequences were deposited to Genbank (accession numbers MN935890-MN935898).

4.2.3. Enzymatic assay

The enzymatic activity of the selected strains was studied using the API-ZYM kit (BioMerieux, France), following the instructions of the manufacturer. This kit is designed for the semi-quantification (1. No reaction, 2. Weak reaction, 3. Positive reaction) of 19 different enzymatic activities (alkaline phosphatase, acid phosphatase, esterase, esterase lipase, lipase, lysine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, Naphthol-AS-BI- phosphohydrolase (NAGase), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase). Overnight (18 h) cultures were centrifuged, the supernatant was removed, and the pellets were re-suspended in a saline buffer (0.85% w/v NaCl). Serial dilutions were undertaken to obtain a final concentration of 7 log CFU/ml, which was confirmed by plate counting. Then, 65µL of each bacteria suspension was inoculated in each well of the test kit, followed by incubation in the dark at 37 °C for 4 h. After incubation, a drop of both reagents ZYM A and ZYM B (provided by the kit) was added in every well and after 5 min strips were exposed to light for 20 seconds. Positive, weak or no reaction (score 3, 2 and 1 respectively) was indicated by the production of a specific color according to the manufacturer.

4.2.4. In vitro phenotypic tests related to probiotic potential

The auto-aggregation assay was evaluated based on a protocol described previously (Ogunremi et al., 2015), with minor modifications. More specifically, LAB cultures were centrifuged, washed twice with saline solution to remove the residual growth medium and re-suspended in 10 mL of PBS (pH 7.2). The suspension was vortexed for 10 s and incubated at 37 °C for 24 h. One milliliter of the supernatant was carefully sampled at 0, 2, 4 and 24 h and the absorbance (OD_{600}) was measured using spectrophotometer (Infinite PRO 200, Tecan, Switzerland). The auto-aggregation percentage was expressed, using the formula $1 - (At/A0) \times 100$, where At represents the absorbance at any time (2 ,4 and 24 h), and A0 the absorbance at time t=0 h. Two well-known probiotic strains were used as control (*L. acidophilus* LA-5, *L. rhamnosus* LGG, Chr. Hansen A/S, Copenhagen, Denmark). The analysis was performed in triplicate.

Bacterial hydrophobicity was studied as described by Bonatsou et al. (2018), by measuring microbial adhesion to hydrocarbon (xylene). Overnight (18 h) cultures were centrifuged, the supernatant was removed and the resulting pellet (final concentration ca. 7 log CFU/ml) was washed twice in PBS, resuspended in 3 ml KNO_3 (100 mM) and the A_{600} was measured (A0). One milliliter of xylene was then added to the cell suspension, forming a two-phase system. A pre-incubation of about 10 minutes (~25 °C) was followed and the two-phase system was mixed by vortex vigorously. Then, the two-phase system was separated by incubation for 20 min (~25 °C). Finally, the aqueous phase was removed and the A_{600} was measured (A1) using spectrophotometer (Infinite PRO 200, Tecan, Switzerland). Results was expressed (%) and calculated using the formula $H\% = (1 - A1/A0) \times 100$, where A0 and A1 are the optical density

before and after use of xylene, respectively. Two well-known probiotic strains were used as control (*L. acidophilus* LA-5, *L. rhamnosus* LGG, Chr. Hansen A/S, Copenhagen, Denmark). The assay was performed in triplicate.

Concerning bacteriocin production, the agar well diffusion test was used according to previous work (Maragkoudakis et al., 2006), using the supernatants of each strain after adjusting the pH to 6.5. A lawn of BHI soft agar (10 g/L,) medium containing each indicator microorganism, namely *L. monocytogenes* NCTC 1994, *L. monocytogenes* NCTC 33411, *S. aureus* NCTC 6571, *E. coli* ATCC35150 and *Ps. aeruginosa* NCTC 10662 was poured onto Petri dishes. After solidification, a hole (5 mm diameter) was made in the center of the plate and 50μl of 24 h cell-free MRS broth of the studied strains were placed, after calculated the pH to 6.5. In a parallel independent assay, the same protocol was followed, using strain's supernatants without adjusting the pH to neutral conditions. After incubation at 37 °C for 3-5 days, the plates were examined for halos (mm) around the hole. Two well-known probiotic strains were used as control (*L. acidophilus* LA-5, *L. rhamnosus* LGG, Chr. Hansen A/S, Copenhagen, Denmark). Analyses were applied in triplicate.

The bile salt hydrolase (BSH) was also studied, as described by Zoumpopoulou et al. (2018). Briefly, fresh bacterial cultures were streaked in triplicates on MRS agar containing 0.5% (w/v) taurodeoxycholic acid (Sigma-Aldrich, Taufkirchen, Germany). The hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of anaerobic incubation at 37ºC.

The estimation of antioxidant capacity was also performed, based on the scavenging ability of the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical of each strain

according to a method described previously (Oliveira et al., 2017). Fresh cultures grown at 37 °C for 24 h, corresponding to a cell concentration of about 7 log CFU/mL (confirmed by plate counts), were centrifuged (7000 x g, 10 min, 4 °C) and the resulting pellet was washed twice with a sterile solution 0.9% NaCl (w/v), following by re-suspension in 2 mL of the same buffer. Each test strain (1.6 mL) was then added to 2 mL of a DPPH methanolic solution (0.2 mM). After soft vortex, the mix was incubated for 30 min, in darkness (~25 °C). The scavenging capacity of DPPH free radicals was measured spectrophotometrically (Infinite PRO 200, Tecan, Switzerland) at 517 nm. A solution containing 1.6 mL of 0.9% NaCl (w/v) and 2 mL of DPPH methanolic solution, which was also incubated to the same conditions, was used as blank. Two well-known probiotic strains were used as control (*L. acidophilus* LA-5, *L. rhamnosus* LGG, Chr. Hansen A/S, Copenhagen, Denmark). The percentage of antioxidant activity (A.A) was calculated as follows: % A.A.= [1- (A_{517}(sample) /A_{517} (blank)] x 100.

Simulated gastric digestion (SGD) was performed according to Bonatsou et al. (2018). Results were expressed as survival rate (%) calculated the population after incubation divided the initial population. Simulated pancreatic digestion (SPD) was applied according to Bautista-Gallego et al. (2013). Results were expressed as survival rate (%) calculated the population after incubation divided the survival population after SGD. Finally, overall survival (%) to digestion was obtained by comparison of the initial LAB counts (log CFU/mL) at the beginning of the simulated GD and those cells recovered at the end of the simulated PD. Two well-known probiotic strains were used as control (*L. acidophilus* LA-5, *L. rhamnosus* LGG, Chr. Hansen A/S, Copenhagen, Denmark). The analyses were performed three times.

4.2.5. Safety parameters

4.2.5.1. Haemolytic activity

Freshly prepared cultures were inoculated by spread on Columbia Agar Base (Oxoid Basingstoke, UK), supplemented with 5% (v/v) horse blood and incubated at 37ºC for 24 h. Hemolytic activity was characterized "α" when a green zone was formed round the colonies, "β" when clear zones were created around colonies, and "γ" when no zones were appeared around colonies (Fuka et al., 2017). The analysis was repeated in triplicates.

4.2.5.2. Biogenic amines

All the selected strains were checked for the presence of genes encoding amino acid decarboxylase activity as described in Table 4.1. Five µL of PCR product was electrophorized on a 1% agarose gel and visualized under UV light. A 100 bp DNA ladder (Nippon Genetics, Düren, Germany) was used as marker. The DNA of the reference strain *E. faecalis* ATCC 29212 was used as positive control in the corresponding PCR reactions.

Table 4. 1. List of primers used for the amplification of amino acid decarboxylase related genes.

Target gene	Primers	Annealing temperature (ºC)	Fragment size (bp)	References

Histidine decarboxylase (hdc1)	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46	367	Rivas et al., 2005
Histidine decarboxylase (hdc2)	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	50	534	Rivas et al., 2005
Tyrosine decarboxylase (tdc)	GAYATNATNGGNATNGGNYTNGAYC ARG CCRTARTCNGGNATAGCRAARTCNGT RTG	55	924	Rivas et al., 2005
Ornithine decarboxylase (odc)	GTNTTYAAYGCNGAYAARCANTAYTT YGT ATNGARTTNAGTTCRCAYTTYTCNGG	54	1446	Rivas et al., 2005

4.2.6. Statistical analysis

Statistical analysis of the quantitative characteristics (auto-aggregation, hydrophobicity, DPPH, SGD, SPD and overall digestion), was carried out by One-Way Analysis of Variance ($P<0.05$), by the LSD test, using SPSS v.20 software. In order to study the correlations between strain's technological and probiotic profiles, Principal Components Analysis (PCA) was performed (SPSS v.20), using varimax rotation to identify any underlying relationship. For the selection of the optimum number of principal components (PCs), factors with eigenvalues greater than 1.0 were retained. Then the loadings of the original variables were projected into the subspace defined by the reduced number of PCs (first and second component) to identify potential correlations.

4.3. Results

4.3.1. Pre-selection

The results of the pre-selection assays are summarized in Table 4.2. Results indicated that out of 117 LAB isolates, 76 were able to survive in SMBs, having a survival score more than 2. However, the number of selected strains was further reduced, due to the next pre-selection assays. Acidifying assay showed low capacity for further 25 strains (MITLAB 93-117), as they could not decrease the pH of SMB below 4.5 (initial pH 6.5) after 24h of incubation at 20^{o}C. Continuously, all the remaining strains were able to degrade oleuropein at least 50%. Thus, these strains satisfied all the above parameters and further studied, constituting a total number of 51 strains.

Table 4. 2. Results of the pre-selection protocol. Survival to synthetic model brines (SMB), acidification activity (AC) and oleuropein degradation capacity (OD %) of the 117 strains. Data are means values ± standard deviation of three replicates. Gaps indicating that strain did not satisfied the previous pre-selection assay. Bold face indicates the selected strains.

Strain	SMB	AC	OD (%)	Strain	SMB	AC	OD (%)	Strain	SMB	AC
MITLAB 1	1			**MITLAB 51**	3	2.3±0.26	57.5±5	MITLAB 101	3	1.26±0.58
MITLAB 2	3	2.16±0.35	57.5±6.6	MITLAB 52	1			MITLAB 102	3	1.53±0.56
MITLAB 3	3	2.03±0.05	60±11.4	**MITLAB 53**	3	2.46±0.25	80.8±3.8	MITLAB 103	2	0.36±0.15
MITLAB 4	2	2.13±0.37	52.5±6.6	**MITLAB 54**	3	2.43±0.4	78.3±8	MITLAB 104	2	0.73±0.15
MITLAB 5	3	2.46±0.41	65±5	**MITLAB 55**	3	2.46±0.15	83.3±6.2	MITLAB 105	3	0.8±0.2
MITLAB 6	3	2.3±0.14	65.8±3.8	MITLAB 56	1			MITLAB 106	3	1±0.26
MITLAB 7	3	2.43±0.25	70±2.5	MITLAB 57	1			MITLAB 107	3	1.23±0.32

MITLAB 8	3	2.83±0.15	75.8±8.7	**MITLAB 58**	2	2±0.2	55±6.6	MITLAB 108	3	1.46±0.37
MITLAB 9	3	2.53±0.25	86.6±3.8	**MITLAB 59**	2	2.13±0.15	53.3±10.4	MITLAB 109	3	1.7±0.45
MITLAB 10	1			**MITLAB 60**	2	2.1±0.1	58.3±3.8	MITLAB 110	3	1.3±0.26
MITLAB 11	2	2.3±0.26	61.6±7.6	**MITLAB 61**	2	2.2±0.09	60.8±7.6	MITLAB 111	3	1.33±0.64
MITLAB 12	1			**MITLAB 62**	2	2.06±0.05	57.5±4.	MITLAB 112	3	1.53±0.56
MITLAB 13	2	2.46±0.25	65.8±11.8	**MITLAB 63**	2	2.03±0.05	56.6±6.2	MITLAB 113	3	0.36±0.15
MITLAB 14	2	2.43±0.40	69.1±10.1	MITLAB 64	0			MITLAB 114	2	0.73±0.15
MITLAB 15	2	2.46±0.15	84.1±5.2	MITLAB 65	0			MITLAB 115	2	0.8±0.2
MITLAB 16	1			**MITLAB 66**	2	2.03±0.05	62.5±6.6	MITLAB 116	3	1.03±0.23
MITLAB 17	1			MITLAB 67	1			MITLAB 117	3	1.43±0.45

MITLAB 18	3	2.83±0.15	90±5	MITLAB 68	1		
MITLAB 19	3	2.53±0.25	84.1±5.2	**MITLAB 69**	2	2.1±0.1	52.5±4.3
MITLAB 20	1			MITLAB 70	0		
MITLAB 21	3	2.3±0.26	56.6±7.6	MITLAB 71	1		
MITLAB 22	1			**MITLAB 72**	2	2.03±0.05	60.8±3.8
MITLAB 23	3	2.46±0.25	60.8±3.8	**MITLAB 73**	2	2.1±0.1	83.3±28.8
MITLAB 24	3	2.43±0.4	66.6±3.8	MITLAB 74	0		
MITLAB 25	2	2.46±0.15	77.5±6.6	MITLAB 75	0		
MITLAB 26	1			**MITLAB 76**	2	2.03±0.05	54.1±5.2
MITLAB 27	1			MITLAB 77	1		
MITLAB 28	2	2.83±0.15	78.3±10.1	MITLAB 78	0		
MITLAB 29	3	2.53±0.25	80.8±7.6	**MITLAB 79**	2	2.1±0.1	51.6±2.8
MITLAB 30	1			MITLAB 80	1		
MITLAB 31	3	2.3±0.26	65±7.5	MITLAB 81	0		
MITLAB 32	1			**MITLAB 82**	2	2.1±0.1	55.8±6.2
MITLAB 33	3	2.46±0.25	68.3±3.8	**MITLAB 83**	2	2.03±0.05	52.5±4.3
MITLAB 34	3	2.43±0.4	70±5	MITLAB 84	1		
MITLAB 35	3	2.46±0.15	79.1±8.7	MITLAB 85	0		
MITLAB 36	1			**MITLAB 86**	2	2.03±0.05	59.1±3.8
MITLAB 37	1			MITLAB 87	0		

MITLAB 38	3	2.83±0.15	74.1±5.2	MITLAB 88	0	
MITLAB 39	3	2.53±0.25	78.3±3.8	MITLAB 89	1	
MITLAB 40	1			MITLAB 90	0	
MITLAB 41	2	2.3±0.26	75.8±8	MITLAB 91	1	
MITLAB 42	1			MITLAB 92	1	
MITLAB 43	3	2.46±0.25	58.3±5.2	MITLAB 93	3	0.36±0.15
MITLAB 44	3	2.43±0.4	72.5±6.6	MITLAB 94	3	0.73±0.15
MITLAB 45	3	2.46±0.15	87.5±5	MITLAB 95	3	0.8±0.2
MITLAB 46	1			MITLAB 96	3	0.93±0.35
MITLAB 47	1			MITLAB 97	3	1.23±0.32
MITLAB 48	3	2.83±0.15	83.3±3.8	MITLAB 98	3	1.43±0.4
MITLAB 49	3	2.53±0.25	77.5±6.6	MITLAB 99	2	1.66±0.46
MITLAB 50	1			MITLAB 100	2	1.36±0.32

4.3.2. Identification of selected strains

According to molecular identification, based on 16s rRNA and *recA* gene amplification, the selected strains, constituting a total number of 51 strains, included 36 *L. pentosus*, 6 *L. plantarum*, 4 *L. coryniformis* and 5 *Lc. mesenteroides* (Table 4.3).

Table 4. 3. Identification of the 51 selected strains.

Strain	Species	Strain	Species
MITLAB_2	*L. plantarum*	MITLAB_41	*L. pentosus*
MITLAB_3	*L. plantarum*	MITLAB_43	*L. pentosus*
MITLAB_4	*L. plantarum*	MITLAB_44	*L. pentosus*
MITLAB_5	*L. plantarum*	MITLAB_45	*L. pentosus*
MITLAB_6	*L. plantarum*	MITLAB_48	*L. pentosus*
MITLAB_7	*L. plantarum*	MITLAB_49	*L. pentosus*
MITLAB_8	*L. pentosus*	MITLAB_51	*L. pentosus*
MITLAB_9	*L. pentosus*	MITLAB_53	*L. pentosus*
MITLAB_11	*L. pentosus*	MITLAB_54	*L. pentosus*
MITLAB_13	*L. pentosus*	MITLAB_55	*L. pentosus*
MITLAB_14	*L. pentosus*	MITLAB_58	*L. pentosus*
MITLAB_15	*L. pentosus*	MITLAB_59	*L. coryniformis*
MITLAB_18	*L. pentosus*	MITLAB_60	*L. coryniformis*
MITLAB_19	*L. pentosus*	MITLAB_61	*L. coryniformis*
MITLAB_21	*L. pentosus*	MITLAB_62	*L. coryniformis*
MITLAB_23	*L. pentosus*	MITLAB_63	*Lc. mesenteroides*
MITLAB_24	*L. pentosus*	MITLAB_66	*Lc. mesenteroides*
MITLAB_25	*L. pentosus*	MITLAB_69	*Lc. mesenteroides*
MITLAB_28	*L. pentosus*	MITLAB_72	*L. pentosus*
MITLAB_29	*L. pentosus*	MITLAB_73	*Lc. mesenteroides*
MITLAB_31	*L. pentosus*	MITLAB_76	*L. pentosus*
MITLAB_33	*L. pentosus*	MITLAB_79	*L. pentosus*
MITLAB_34	*L. pentosus*	MITLAB_82	*L. pentosus*
MITLAB_35	*L. pentosus*	MITLAB_83	*L. pentosus*
MITLAB_38	*L. pentosus*	MITLAB_86	*Lc. mesenteroides*
MITLAB_39	*L. pentosus*		

4.3.3. Enzymatic assay

The enzymatic profile of the selected strains is presented in Table 4.4. In general, the majority of strains belonging to the same species evidenced a very similar behavior to one another. In particular, all Lactobacilli strains exhibited leucine and valine arylamidase activity, while all of them plus 3 *Lc. mesenteroides* (MITLAB 63, 66, 69) have been shown to have cysteine and valine arylamidase activities. However, only *Lc. mesenteroides* strains were positively reacted for the enzymes α-galactosidase and alkaline phosphatase, while all strains had weakly positive reaction for the enzyme NAGase. Moreover, *L. pentosus* and *L. plantarum* exhibited weak and strong ability to produce β-glucosidase, respectively, while 2 out of 4 strains of *L. coryniformis* (MITLAB 59, 60) showed weakly positive reaction. No reaction was observed for *Lc. mesenteroides* strains. Concerning β-galactosidase, all *L. pentosus*, 3 out of 6 *L. plantarum* (MITLAB 2, 3, 5) and 2 out of 4 *L. coryniformis* (MITLAB 61, 62) were positive. Furthermore, the majority of strains showed weak acid phosphatase activity, while 10 *L. pentosus* showed strong reaction. It is notable that all *Lc. mesenteroides* strains, exhibited positive reaction for the enzymes esterase and esterase lipase, while 1 *L. plantarum* and 3 *L. coryniformis* were weakly reacted to those enzymes. Continuously, all strains were strongly positive to Naphthol-AS-BIphosphohydrolase. Finally, no reaction was observed for the enzymes lipase, α-glucosidase, trypsin, α-chymotrypsin, β-glucorinidase, α-mannosidase, and α-fucosidase.

Table 4. 4. Enzymatic profile for the 51 selected LAB strains. 1 No reaction, 2 Weak reaction, 3 Positive.

Strain	β-Glucuronidase	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Cystine arylamidase	Trypsin	α-Chymotrypsin	α-Galactosidase	α-Mannosidase	α-Fucosidase	Leucine arylamidase	Valine arylamidase	Naphthol-AS-BIphosphohydrolase	β-Galactosidase	α-Glucosidase	β-Glucosidase	N-Acetyl-βglucosaminidase	Acid phosphatase
MITLAB_2	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	3	2	2
MITLAB_3	1	1	2	2	1	2	1	1	1	1	1	3	3	3	3	1	3	2	2
MITLAB_4	1	1	1	1	1	2	1	1	1	1	1	3	3	3	1	1	3	2	2
MITLAB_5	1	1	1	1	1	2	1	1	1	1	1	2	3	3	3	1	3	2	2
MITLAB_6	1	3	1	1	1	1	1	1	3	1	1	2	3	3	1	1	3	2	1
MITLAB_7	1	3	1	1	1	1	1	1	2	1	1	2	3	3	1	1	3	2	1
MITLAB_8	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_9	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_11	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_13	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_14	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_15	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_18	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_19	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_21	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_23	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_24	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_25	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_28	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_29	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_31	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_33	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_34	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_35	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_38	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_39	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_41	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_43	1	1	1	1	1	2	1	1	1	1	1	2	1	3	3	1	2	2	2
MITLAB_44	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	2	2	3
MITLAB_45	1	1	1	1	1	2	1	1	1	1	1	3	3	3	3	1	3	2	3
MITLAB_48	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_49	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_51	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2

MITLAB_53	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	2	2	2
MITLAB_54	1	1	1	1	1	2	1	1	1	1	1	3	2	3	3	1	2	2	3
MITLAB_55	1	1	1	2	1	2	1	1	1	1	1	3	2	3	3	1	2	2	3
MITLAB_58	1	1	2	2	1	2	1	1	1	1	1	2	2	3	3	1	2	2	1
MITLAB_59	1	1	2	2	1	2	1	1	1	1	1	2	2	3	1	1	2	2	1
MITLAB_60	1	1	2	1	1	2	1	1	1	1	1	2	2	3	1	1	2	2	1
MITLAB_61	1	1	1	1	1	2	1	1	1	1	1	2	2	3	3	1	1	2	1
MITLAB_62	1	1	1	1	1	3	1	1	1	1	1	2	2	3	3	1	1	2	1
MITLAB_63	1	3	3	3	1	1	1	1	3	1	1	1	1	3	1	1	1	2	1
MITLAB_66	1	3	3	3	1	2	1	1	3	1	1	1	2	3	1	1	1	2	1
MITLAB_69	1	3	3	3	1	2	1	1	3	1	1	1	2	3	1	1	1	2	1
MITLAB_72	1	1	1	1	1	2	1	1	1	1	1	1	2	3	3	1	2	2	1
MITLAB_73	1	3	3	3	1	1	1	1	3	1	1	1	1	3	1	1	1	2	1
MITLAB_76	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	2	2	1
MITLAB_79	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	2	2	1
MITLAB_82	1	1	1	1	1	1	1	1	2	1	1	1	1	3	3	1	2	2	1
MITLAB_83	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	2	2	1
MITLAB_86	1	3	3	3	1	1	1	1	3	1	1	1	1	2	1	1	1	3	1
L. acidophilus	1	1	2	2	1	3	1	1	3	1	1	3	3	3	1	1	1	2	3
L. rhamnosus	1	1	2	2	1	3	1	1	3	1	1	3	3	3	1	1	1	2	2

4.3.4. In vitro phenotypic tests related to probiotic potential

The auto-aggregation potential of the selected strains is presented in Fig. 4.1. Auto-aggregation started within the first 4 h of incubation and the results indicated that a quite large number of the studied strains exhibited a strong auto-aggregating phenotype, while some of them had even stronger ability than the probiotic controls (*L. acidophilus and L. rhamnosus*). It is notable the large distribution with significant differences observed between and within species, as well. For example, 2 *L. coryniformis* (MITLAB 59, 60) exhibited values close to 35%, while the other 2 strains (MITLAB 61, 62) were extremely higher, reaching values close to 82%. Similarly, the auto-aggregation potential of some *L. pentosus* strains was even higher than the probiotics controls (70-82%), however some other *L. pentosus* strains exhibited poorly capacity (15-20%). The highest percentages were observed in 8 *L. pentosus* and 2 *L. coryniformis*, reaching values close to 81%. No significant differences were observed between those strains. Oppositely, the poorest capacity was exhibited by 2 strains belonging to *Lc. mesenteroides* (MITLAB 69) and *L. pentosus* (MITLAB 79), with value below to 1%. Finally, *L. plantarum* could be characterized as strains with medium auto-aggregation capacity (18-33%), except from 1 *Lactobacillus plantarum* (MITLAB 2), the value of which was significantly higher (66%).

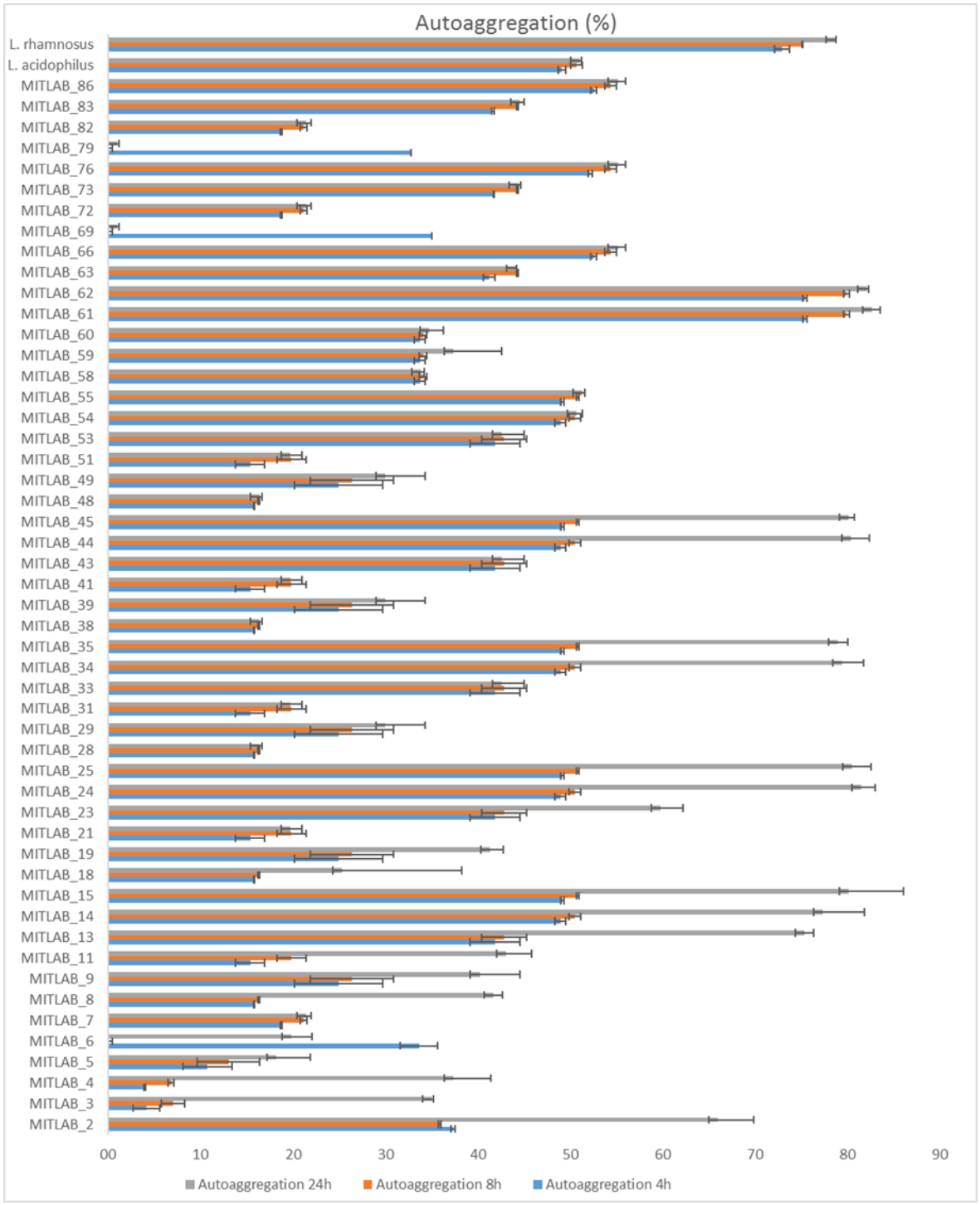


Fig. 4. 1. Autoaggregation capacity (%) of the 51 strains assayed after 2, 4, and 24 h. Data are means values ± standard deviation of three replicates.

Regarding the hydrophobicity test, most of the isolates were weakly hydrophobic, reaching values between 0% and 10% (Fig. 4.2). However, 11 *L. pentosus* (MITLAB 14, 15, 23, 25, 34, 35, 43, 44, 53, 54, 55), 2 *L.plantarum* (MITLAB 2, 4) and 2 *L. coryniformis* (MITLAB 61, 62) showed levels higher than 30%. It is crucial to be mentioned that the 5 out of 11 *L. pentosus* strains exhibited higher hydrophobic levels than the probiotic controls.

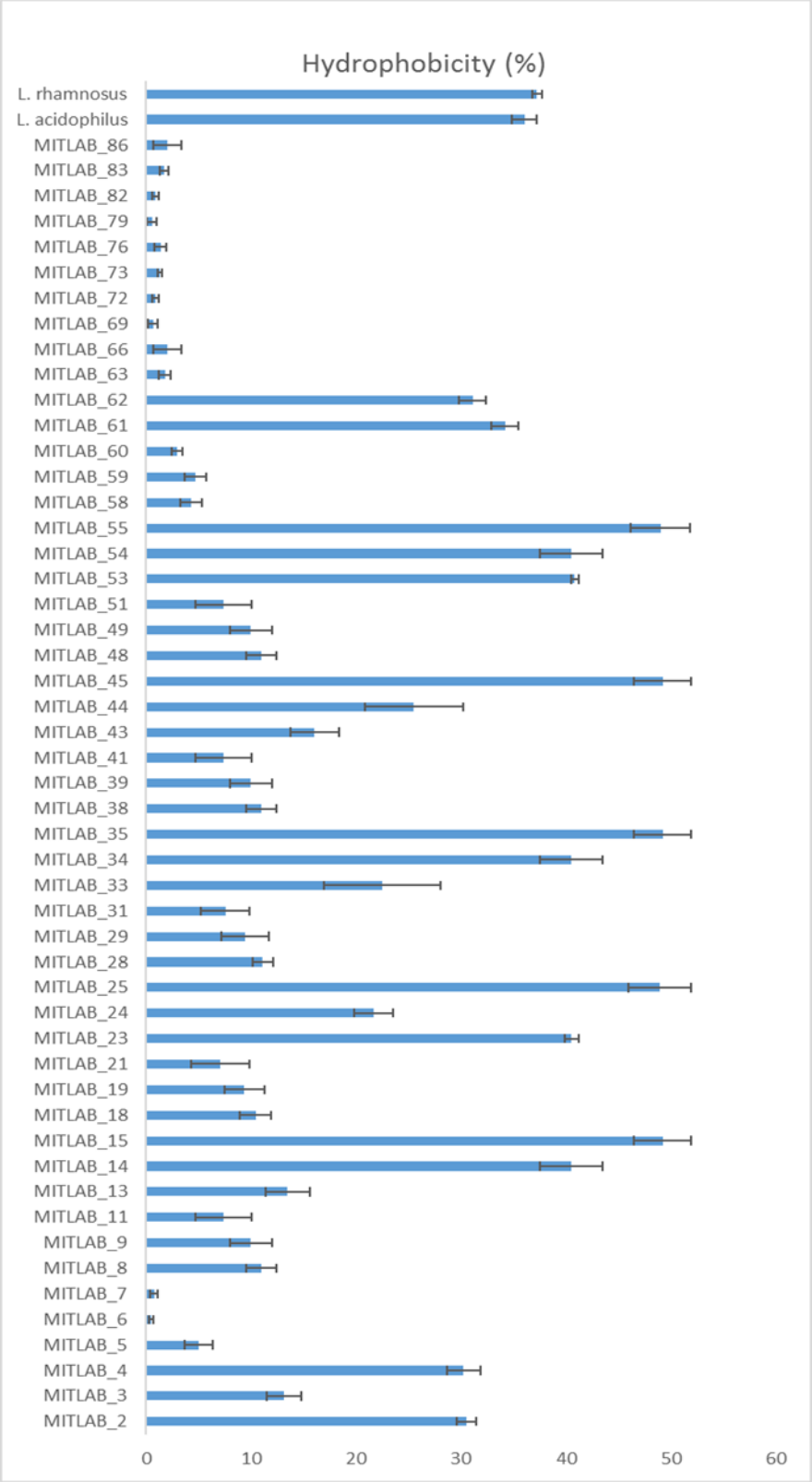


Fig. 4. 2. Hydrophobicity (%) of the 51 strains. Data are means values ± standard deviation of three replicates.

All isolates were screened for bacteriocin production by well diffusion assay, as well. The majority of untreated supernatants (47 isolates; 92%), displayed antibacterial activity against at least one indicator strain (Table 4.5). However, none of the supernatants inhibited the growth of the tested pathogenic strains, when the pH was adjusted to neutral condition (6.5) (data not shown).

Table 4. 5. Inhibition zone (mm) of 51 strains against food pathogens.

Strain	*L. monocytogenes NCTC* 1994	*L. monocytogenes* NCTC 33411	*S. aureus* NCTC 6571	*E. coli* ATCC35150	*Ps. aeruginosa* NCTC 10662
MITLAB_2	2	2	2	3	2
MITLAB_3	4	2	0	3	4
MITLAB_4	0	2	0	2	0
MITLAB_5	4	2	0	4	4
MITLAB_6	0	2	4	2	0
MITLAB_7	0	3	2	0	0
MITLAB_8	4	3	0	4	4
MITLAB_9	0	2	0	0	0
MITLAB_11	4	0	0	4	4
MITLAB_13	4	0	0	4	4
MITLAB_14	4	2	0	4	4
MITLAB_15	4	3	2	4	4
MITLAB_18	4	3	0	4	4
MITLAB_19	0	0	0	2	0
MITLAB_21	2	3	0	2	2
MITLAB_23	0	0	0	2	0
MITLAB_24	0	0	0	0	0
MITLAB_25	0	0	0	0	0
MITLAB_28	0	0	0	0	0
MITLAB_29	0	0	4	0	0
MITLAB_31	2	2	4	2	2

MITLAB_33	2	2	4	2	2
MITLAB_34	2	2	4	2	2
MITLAB_35	0	0	0	0	0
MITLAB_38	2	3	4	0	2
MITLAB_39	0	3	4	0	0
MITLAB_41	0	3	4	2	0
MITLAB_43	0	2	4	2	0
MITLAB_44	0	2	3	2	0
MITLAB_45	0	2	2	0	0
MITLAB_48	0	2	2	0	0
MITLAB_49	0	0	2	0	0
MITLAB_51	0	0	0	2	0
MITLAB_53	0	0	2	2	0
MITLAB_54	2	2	2	2	2
MITLAB_55	2	2	0	0	2
MITLAB_58	0	2	0	0	0
MITLAB_59	0	2	2	4	0
MITLAB_60	2	3	4	2	2
MITLAB_61	0	2	3	0	0
MITLAB_62	0	0	0	2	0
MITLAB_63	0	0	0	1	0
MITLAB_66	0	3	2	1	0
MITLAB_69	0	2	4	2	0
MITLAB_72	0	3	2	0	0
MITLAB_73	0	0	0	1	0
MITLAB_76	0	3	2	1	0
MITLAB_79	0	2	4	2	0
MITLAB_82	0	3	2	0	0
MITLAB_83	0	0	0	1	0
MITLAB_86	0	3	2	1	0
L. acidophilus	3	4	2	3	3
L. rhamnosus	2	3	2	2	2

Fig. 4.3 shows the DPPH free radical scavenging activity for each LAB strain. In general, the majority of *L. pentosus* and *L. plantarum* and some *L. coryniformis* strains exhibited high antioxidant capacity, while *Lc. mesenteroides* activity was more limited. The highest antioxidant activities were obtained by *L. pentosus* MITLAB 14 (53.25 ± 1.2%), MITLAB 15 (52.7 ± 1.83%) and MITLAB 55 (51.2 ± 0.28%), MITLAB 58 (50.4 ± 2.4%), *L. plantarum* MITLAB 2 (51.75 ± 2.33%, as well as *L. coryniformis* 59 (50.65 ± 0.49%), and 60 (51.8 ± 0.7%) which was similar to the reference strains (50.3 ± 1.2%), while the lowest values were obtained for *Lc. mesenteroides*, with exception to MITLAB 63 (47.2 ± 2.68%), the activity of which was significant higher, compare to the other strains in this species.

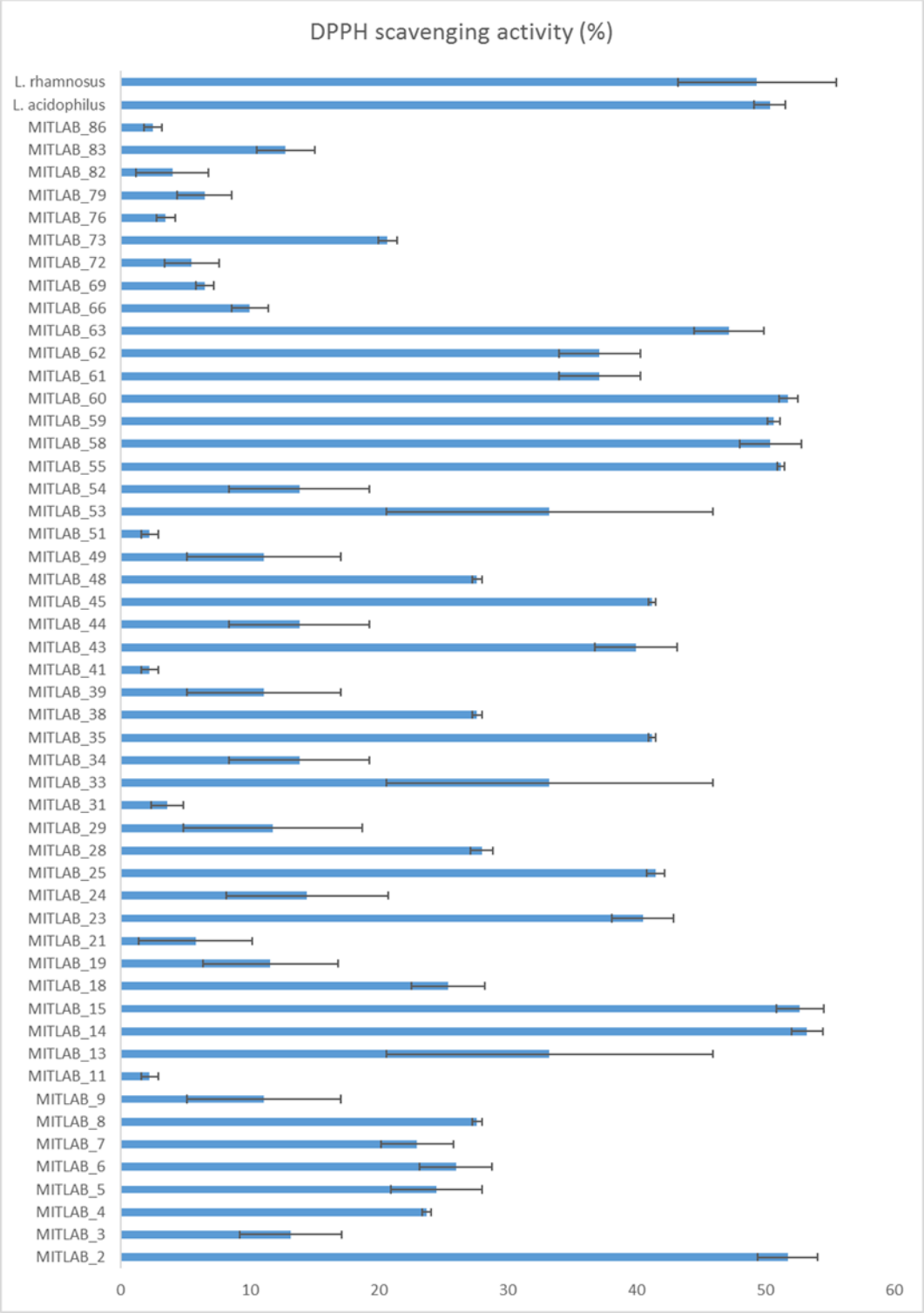


Fig. 4. 3. DPPH radical scavenging activities of the 51 strains. Data are means values ± standard deviation of three replicates.

Most of the isolates showed high survival rates (%) after the SGD. The majority of them exhibited a survival rate between 40% and 60% (Table 4.6). Most of them (96%) showed higher rates than the control strain *L. rhamnosus* (31.8%), while the second probiotic control *L. acidophilus* showed very high survival, close to 72.7%. However, some isolates showed equal or even higher survival rates than the latter control, including 18 *L. pentosus* strains and 1 *L. plantarum*.

After SGD, all isolates were sequentially subjected to the simulated pancreatic digestion. As it is clearly presented in Table 4, an additional survival rate to gastric rate digestion was noticed. The partial survival rate for the pancreatic digestion was generally between 35% and 65%, while most of them exhibited rate equal to *L. rhamnosus* (42.7%), while the survival of *L. acidophilus* (76.9 ± 9.22%), was in line or even lower than the most resistance isolates (e.g., MITLAB 3, 4, 24, 25, 44, 45, 51, 55). The overall survival in the digestive process with respect to the initial population is also shown in Table 4, as well. As it is observed, the overall rates were high and showed that most of the strains had percentages between 20-30%. Notable that the majority of strains showed better survival rates than the probiotic *L. rhamnosus* (21.5%). In line with SGD and SPG results, the probiotic strain L. acidophilus showed higher rates (39.2%), although there were 14 strains, 1 *L. plantarum* and 13 *L. pentosus* with survival values more than 41%.

Table 4. 6. Gastric, pancreatic and overall survival (%) of the 51 strains, during simulating digestion. Data are mean values ± standard deviation of three replicates.

Strain	Gastric Survival (%)	Pancreatic Survival (%)	Overall Survival (%)
MITLAB_2	38.94±0.14	59.65±0.14	23.26±0.09

MITLAB_3	21.99±0.09	94.56±0.44	20.83±0.04
MITLAB_4	26.47±0.44	81.27±0.31	21.64±0.09
MITLAB_5	86.41±1.09	56.11±1.15	48.7±0.69
MITLAB_6	61±0.54	49.52±0.75	30.31±0.59
MITLAB_7	50.68±0.89	48.53±2.77	24.74±1.19
MITLAB_8	66.92±0.94	37.4±4.1	28.5±3
MITLAB_9	66.6±0.89	44.79±8.04	29.95±5.18
MITLAB_11	71.75±1.59	55.2±8.5	43.68±10
MITLAB_13	75.17±8.62	33.27±5.2	23.5±5.55
MITLAB_14	70.58±2.24	60.28±1.69	21.57±0.19
MITLAB_15	75.73±5.93	56.3±8.27	26.43±3.78
MITLAB_18	67.32±1.2	39.81±2.07	26.5±1.19
MITLAB_19	64.6±3.7	61.51±31.68	41.09±20.93
MITLAB_21	74.45±2.69	64.33±17.06	46.59±12.85
MITLAB_23	72.27±5.32	35.16±5.96	27.63±6.17
MITLAB_24	36.26±1.34	75±0.98	45.11±0.49
MITLAB_25	46.83±0.34	75.1±3.81	47.26±5.53
MITLAB_28	62.9±4.54	39.81±2.07	26.5±1.19
MITLAB_29	64.6±4.46	38.6±0.70	25.83±0.64
MITLAB_31	73.87±1.59	43.44±12.46	31.36±8.67
MITLAB_33	77.2±8	35.16±5.96	27.63±6.17
MITLAB_34	70.23±5	57.5±4.94	41.65±2.19
MITLAB_35	76.22±5.63	56.65±6.29	44.79±4.33
MITLAB_38	68.9±2.84	38.21±3.24	24.5±2.9
MITLAB_39	67.4±1.3	39.76±0.93	26.61±0.44
MITLAB_41	72±2	62.4±9.5	47.59±7.8
MITLAB_43	78.3±9.1	38.74±6.1	27.6±6.1
MITLAB_44	70.66±5.53	75.05±3.88	43.77±1.39
MITLAB_45	77.74±3.48	73.35±2.19	44.79±4.33
MITLAB_48	66.92±0.94	39.81±2.07	26.5±1.19
MITLAB_49	66.6±0.89	39.29±0.26	26.24±0.07
MITLAB_51	73.35±3.99	64.33±17.06	46.59±12.85
MITLAB_53	76.1±5	35.16±5.96	27.63±6.17
MITLAB_54	73.12±2.04	49.4±5.51	44.76±0.89

MITLAB_55	76.43±1.64	58.8±3.34	44.79±4.33
MITLAB_58	33.9±0.89	67.16±1.46	24.8±0.19
MITLAB_59	31±0.89	64.2±1.38	22.98±0.19
MITLAB_60	34.6±0.89	68.9±1.29	21.4±0.19
MITLAB_61	46.3±2.1	50.6±3.1	23.86±0.94
MITLAB_62	47.4±1.14	49.±1.38	25.1±4.1
MITLAB_63	46.48±0.44	53.27±1.34	24.85±0.74
MITLAB_66	45.81±1.89	50.26±1.01	23.37±0.94
MITLAB_69	61±0.54	49.52±0.75	30.31±0.59
MITLAB_72	50.68±0.89	48.53±2.77	24.74±1.19
MITLAB_73	44.4±1.4	53.27±1.34	24.85±0.74
MITLAB_76	46.33±2	50.26±1.01	23.37±0.94
MITLAB_79	63.2±4	49.52±0.75	30.31±0.59
MITLAB_82	51.6±1.6	48.53±2.77	24.74±1.19
MITLAB_83	44.9±1.77	53.27±1.34	24.85±0.74
MITLAB_86	44.6±3	50.26±1.01	23.37±0.94
L. acidophilus	72.7±1.14	76.99±9.22	39.28±5.05
L. rhamnosus	31.79±0.09	42.71±0.37	21.5±0.38

Finally, regarding bile salt hydrolysis, none of the strains shown bile salt hydrolase activity, as recorded by MRS plating.

4.3.5. Safety parameters

None of the examined strains exhibited neither α nor β-haemolytic activity when grown in Columbia human blood agar. All strains had γ-haemolytic activity (colonies without halos, i.e. no haemolysis). In addition, regarding the presence of several-amino decarboxylase genes, histidine (hdc1 and hdc2), ornithine (odc), and tyrosine (tdc)

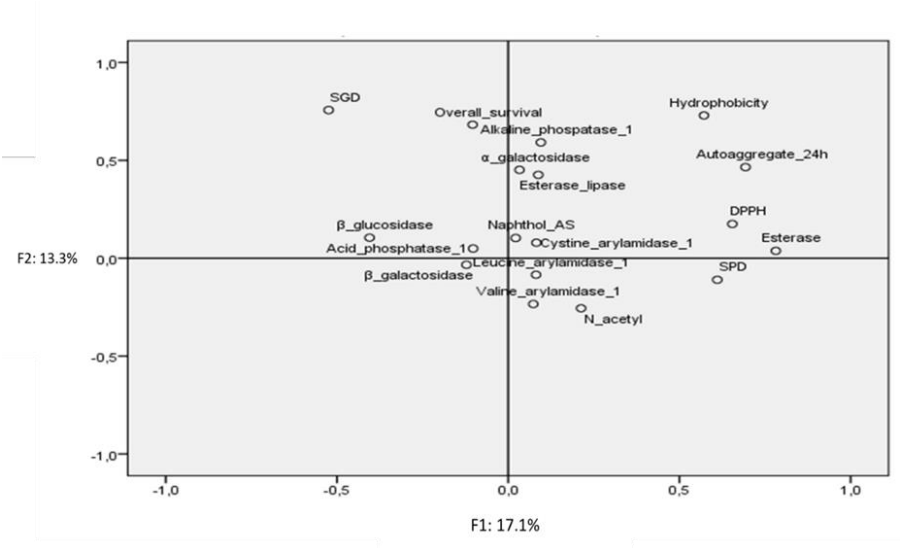
decarboxylase, no amplification occurred, indicating absence of genes encoding amino decarboxylase activity. Thus, none of the isolates should exhibit biogenic amines production regarding histidine, ornithine or tyrosine.

4.3.6. Multivariate analysis

Multivariate analysis between variables is presented in Fig. 4.4. Subjecting all studied variables to PCA (enzymatic activity, auto-aggregation, hydrophobicity, DPPH, antimicrobial activity, gastric digestion survival, pancreatic digestion survival and overall digestion), 8 factors with eigenvalue higher than 1 were obtained. The study of the contribution of variables to the first 2 factors showed that Factor 1 (explained 17.1% of variance) was mainly related to auto-aggregation, DPPH, hydrophobicity and esterase (C4) while Factor 2 (13.3% of variance) explained overall digestion survival and SGD. A projection of the variables on the plane formed by the first two factors (Fig. 4.4A) shows a clear relationship between the variables described above. Regarding strains distribution (Fig. 4.4B), they are quite scattered among them, but they can be distinguished mainly by three groups in which are close to each other. The first group is placed on the negative part of the two factors (Group A) and involves isolates that are related with some enzymatic activities (β-galactosidase, acid phosphatase). However, they are far from probiotic features, as they have low SGD, antioxidant and auto-aggregation capacities and overall survival, as well. A second group (Group B), involves isolated which are close to the first probiotic control (*L. rhamnosus*) and are characterized by high SPD, DPPH and esterase activity, although they are medium hydrophobic, having in parallel medium auto-aggregation capacity. However, this

group is far from crucial enzymatic activities, such as β-glucosidase and acid phosphatase, while they exhibit low SGD and overall digestion. In the third group (Group C), they are strains exhibiting the most promising features, being as well close to the second probiotic control (*L. acidophilus*). Those strains are characterized by the highest values regarding hydrophobicity, auto-aggregation and DPPH, having high SDG and overall survival, as well. Furthermore, they exhibit satisfied enzymatic profile. Apart from three main groups described above, there are also a few isolates separated from the rest, showing specific features, although the combination of them is poor. As a consequence, discrimination criteria were unable to be noticed.

A



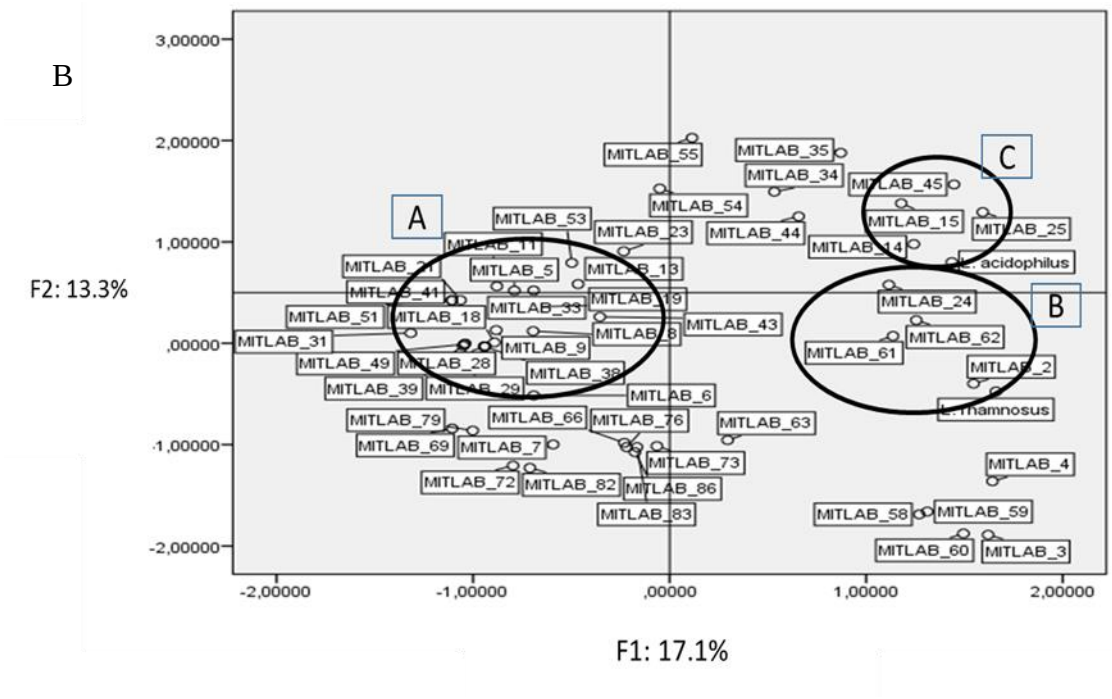


Fig.4. 4. Projection of the variables (A) and isolates (B) onto the plane formed by the first two factors.

4.4. Discussion

Over the years, LAB stand between the most important groups of microorganisms used as starter cultures in fermented foods, especially in dairy ones (Yerlikaya, 2014). However, the increasing number of allergic consumers and/or their demand for production of functional foods from alternative source, has led the investigation to the production of other non-dairy fermented products (Bautista-Gallego et al., 2013). Products traditionally subjected to lactic acid fermentation during their processing are good candidates of such sources. Among others, table olives, are one of the most well-known fermented products in developed countries, with a long time history coming from the ancient time (Marshall and Mejia, 2011). They are associated with the Mediterranean diet and enjoy a healthy and natural reputation, worldwide.

Despite their important economic contribution for the producer countries, the fermentation process is still empirical and uncontrolled. The application of starter cultures in the field of table olives is quite far from reaching its establishment, as it has in other sectors of food industry (e.g., dairy products and alcoholic drinks). However, as previously mentioned, based on the modern consumer's requirements, there is a need for more variation of functional foods, with respect to probiotic frontier. Thus, it is highly probable that presumptive probiotic table olives could be well accepted by consumers.

Except from consumer's demand, there is an industrial high interest in the development of starter cultures for table olive production, which is very increasing, as well (Randazzo, Ribbera, Pitino, Romeo, & Caggia, 2012). Nowadays, we are standing at a cross point between the old fashion and the next generation way of table olives production. LAB play an important role during olives processing (Arroyo-López et al., 2012), due to their interesting biotechnological properties, such as their abilities a) to produce lactic and other organic acids leading to the brine's pH reduction and b) to oleuropein degradation, resulting to a biological debittering of the fruits, constituting appropriate candidates to lead the next level. Based on these evidences, in the present study, 117 LAB strains, previously isolated from naturally fermented green cracked Cypriot olives, were evaluated to a proposed pre-selection protocol, applying conditions that mimic the natural conditions taking place during the process. As a preliminary step, a formulation reproducing the main physicochemical constraints usually found into the fermenting brines of Cypriot olives was settled. All studied isolates were firstly assayed for their ability to grow in a SMB and secondly for both acidification and oleuropein degradation capacities. The composition of the SMB was

formulated based on the results of previous study (Chapter 2), in order to be used as a reliable and reproducible substrate for the *in vitro* technological assay. In all experiments the incubation temperature was applied at 22ºC ± 2, which represents the average value occurring at industrial-scale controlled conditions in Cypriot olive processing, during the whole fermentation process. Results showed that 51 out of 117 studied strains satisfying the above parameters and they were further studied, indicating that they could not only become potentially dominant strains throughout fermentation, but also exhibited both high acidifying and high oleuropein degradation capacities. These are two of the most crucial technological traits for a successful olive fermentation (Bonatsou et al., 2017).

Enzymatic profile is an important factor for the selection of starter culture, because its contribution can either favor or not the quality of fermentation process, producing several metabolites and secondary compounds; thus they could lead to both positive and negative traits, affecting the organoleptic characteristic of the final product (Heperkan, 2013). In this study, a pool of enzymatic assays was tested to the selected strains, using the API-Zym system. Results demonstrated a plethora of interesting profiles for the majority of the strains. More deeply, all *Lc. mesenteroides* showed a positive reaction for the esterase and esterase lipase enzymes, while few strains from the other species (1 *L. plantarum* and 3 out of 5 *L. coryniformis*) were weakly reacted. Those lipolytic enzymes contribute to olive's flavor, as they catabolize the free fatty acids, resulting in the formation of desirable aromatic compounds such as ethanol, glycerol, higher alcohols, esters, and other volatile compounds (Bevilacqua et al., 2012; Rodríguez-Gómez et al., 2012). Other interesting enzymes are the α and β-glucosidase. Among them, the former is an undesirable trait, as it has been reported that the

production of such compounds is highly connected with for type 2 diabetes mellitus (Nurhayati et al., 2017). Thus, the absence of this enzyme from our isolates is considered a positive feature. Regarding β-glucosıdase, it has a significant impact on flavor due to the production of secondary metabolites. However, its ability to hydrolyze phenolic compounds, particularly oleuropein, producing glucosyl derivates, hydroxytyrosol and elenolic acid, is its most important function (Pino et al., 2019). As a consequence, the fact that all *L. plantarum*, *L. pentosus* and the majority of *L. coryniformis* strains showed the ability to produce β-glucosidase, is a promising feature, concerning the selection of a functional starter, for table olives debittering in a more biological approach (Arroyo-López et al., 2012). The presence of this enzyme has been previously reported for *Lactobacillus plantarum* group (Ghabbour et al., 2011; Iorizzo et al., 2016). However, Abriouel et al. (2012) reported that β-glucosidase was found more frequently in *Lc. mesenteroides* strains, which is not in agreement with the present study. Furthermore, the finding that *Lc. mesenteroides* possess a-galactosidases, which is not synthesized by human organism, is a beneficial trait for LAB associated with fermented foods, since it has been related with the prevention of intestinal disorders, due to its ability to hydrolyze galactose oligosaccharides which are responsible for such situations (Chen et al., 2014). As for β-galactosidase, where all Lactobacilli exhibited strong reaction, it has been previously noted its contribution in improving lactose tolerance in the gut, making the presence of this enzyme pivotal, with regards to probiotic potential (Colombo et al., 2018). Continuously, alkaline phosphatase is responsible for the release of inorganic phosphorus, which is useful for cell membranes (Młodzińska and Zboińska, 2016). Concerning the present results, it can be assumed that only strains belonging to *Lc. mesenteroides* could produce alkaline phosphatase, whereas most of the assayed strains, showed a positive reaction for acid phosphatase.

These findings are in line with previous studies (Iorizzo et al., 2016; Pessoa et al., 2017), where *Lactobacilli* presented acid phosphatase activity and only a few strains belonging to *Lc. mesenteroides* showed alkaline phosphatase activity. Furthermore, all strains shown a positive reaction to the enzyme Naphthol-AS-BI-phosphohydrolase. Both enzymes (acid phosphatase and naphthol-AS-BI-phosphohydrolase) participate in phytic acid degradation, constituting desirable characteristics of starter cultures for the production of several fermented vegetables (Kostinek et al., 2007). Those enzymes are absent from the human gastrointestinal tract. Thus the degradation of the insoluble complexes, created by the ability of phytic acid to form chelates with several inorganic compounds, could be succeeded by microorganism's enzymes action (Bonatsou et al., 2018a). Moreover, although degradation of amino acids plays a significant role in the olive's flavor, the absence of proteolytic enzymes could also be a positive characteristic as well, because in some cases protein degradation may generate off-flavors and toxic compounds such as biogenic amines (Iorizzo et al., 2016). According to the literature, it has been not clear yet, whether this trait should be considered as a desirable trait (Songré-Ouattara et al., 2008a). Nevertheless, arylamidases are of great important proteolytic enzymes for the liberation of amino acids and the development of desirable flavor. These enzymes have also been shown to play a role in the debittering aspect (Herreros et al., 2003). In this regard, the finding that all *Lactobacilli* exhibited leucine and valine arylamidase activity, while all of them plus 3 *Lc. mesenteroides* have been shown to have cystine arylamidase activity, could be consider as a covetable characteristic. Concerning the activities of enzymes correlated with carbohydrate catabolism, α-mannosidase and α-fucosidase were not detected. Additionally, negative activity for trypsin, α-chymotrypsin, and the undesirable β-glucuronidase activity was

observed, indicating the safeness of the studied strains. The latter is in agreement with the literature (Ji Jang and Kim 2015; Colombo et al. 2018).

Adhesion ability to epithelial cells is one of the main commonly encountered criteria for the selection of potentially probiotic strains (Monteagudo-Mera et al., 2019). It has been shown that those strains able to adhere to cell monolayers, are also exhibit auto-aggregation and hydrophobicity characteristics and thus both traits have been proposed as a preliminary screening to find out potentially adherent isolates (Porru et al., 2018). Most of the LAB strains investigated in our study showed high percentage of auto-aggregation which was increasing for 24 h. On the other hand, hydrophobicity had a strongly scattered distribution, with some isolates showing values above 30%, while most of them showed hydrophobicity below 10%. The differentiation among the strains could be explained due to some strains present slower elimination kinetics from the digestive tract than others after cessation of their administration (Martins et al., 2009). Nevertheless, these values are much higher than those found for other Lactobacilli isolates (Bautista-Gallego et al., 2013; Ramos et al., 2013; Teles Santos et al., 2016). However, Pérez et al., (1998), supported that the limit of hydrophobicity for a strain, in order to be able to adhere to the epithelial cell, should be at least 85%, although it has been reported that sometimes there is no clear correlation between those two variables (Binetti et al., 2013; Perricone et al., 2014). Thus, this hypothesis should be construed with reservation, because the adhesion to model cell lines does not necessarily mean a real adhesion, because human cell lines of tumor origin cannot always simulate cells deriving from normal tissues, especially regarding surface components that interact with bacteria (Bautista-Gallego et al., 2013; Zoumpopoulou et al., 2018). Nevertheless, it would be interesting to perform additional tests to these

strains to better understand the mechanisms involved, such as in vivo testing using animal models, in order to ascertain the benefits observed in vitro.

Concerning bacteriocin production, no inhibition at all was observed, as the pathogens were grown in the presence of near-neutral supernatants (pH 6.5). Thus, inhibition effects cannot be explained by bacteriocin action and are most probably due to the production of organic acids; mainly lactic; resulting to the reduction of pH, a fact which is in line with the literature (Li et al., 2017). Nevertheless, the fact that the acid cell-free extract produced by the tested strains cause antimicrobial activity like bacteriocin, could be also considered as a desirable trait; with regards to beneficial health effects; which is in accordance with previous reports (Maragkoudakis et al., 2006; Ji et al., 2015; Li et al., 2017; Zoumpopoulou et al., 2018). LAB able to produce bacteriocin like inhibitory substances (BLIS) are of great importance to food fermentation industry, playing the role of natural preservatives, since they are able inhibit the growth of many potential spoilage and pathogenic bacteria. However, a more deep study is required, even in molecular level, in order to be clarified this topic. Finally, as it has been proven that most of our strains produced BLIS against at least one indicator strain, it is expected that human intestinal flora could be benefited, improving protection against occurrences of diarrhea, food poisoning, and enteric infection, caused by such undesirable microorganisms.

Free radicals, which are growing during oxidative stress, are highly unstable molecules that interact with other molecules in human body, provoking damage to DNA, enzymes and cellular membranes, while they are linked to aging acceleration and the development of many diseases, like cancer and heart diseases (Zoumpopoulou et al., 2018). Antioxidant substances donate electrons or hydrogen atoms to the free

radical to create a complex. DPPH has been used widely as a radical to estimate the capacity of a potential antioxidant. It accepts electrons or hydrogen atoms from antioxidant substances, commuting them into inconvertible stable molecules, to prevent detriment activity, described above (Kedare and Singh, 2011). Results from the present study indicating that the majority of *L. pentosus*, *L. plantarum* and *L. coryniformis* presented high scavenging DPPH radical. As a result, it could be mentioned that Lactobacilli isolated from Cypriot table olives could be considered as a very promising source of strains exhibiting high antioxidant capacity. This is in consistence with the literature, where it has been reported the high ability of Lactobacilli to scavenge free radicals (Chen et al., 2014; Xing et al., 2015). It is worth noting that 8 strains (MITLAB 2, 14, 15, 55, 58, 59, 60, 63) had equal capacity to the probiotic reference strains. Noteworthy the satisfying antioxidant activity exhibited by other 3 strains, as well (MITLAB 25, 35, 45).

Regardless all the above cited characteristics, the most important feature for a strain in order to be characterized as potential probiotic, is its own survival to the unfavorable environmental conditions, existing in human stomach. More specifically it should be resistant to gastric and pancreatic juices, both of which are simulating the conditions of the path they have to go through, in order to reach the intestine in sufficient quantity and confer health benefits to the host (FAO/WHO, 2002). Because of the high acidic conditions in this environment, where the pH is around 2.0, it is essential to select probiotic strains with high tolerance to such situations. In this study, survival during transit through the gastrointestinal tract was extremely satisfied, while quite a lot of strains were able to reach higher and equal survival rates to the well-known probiotics *L. rhamnosus* and *L. acidophilus*, respectively. It is crucial to be

mentioned that 18 *L. pentosus* strains and 1 *L. plantarum* had even higher survival rate than the second probiotic control. However, it has been reported that the specific behavior of strains to each of those 2 independent assays might lead to misleading results (Bautista-Gallego et al., 2013). Consequently, overall survival should be more plausible, compare to the findings obtained with the application of separate assays; but in line with the findings in gastrointestinal and pancreatic digestions, survival of the above cited isolates was still higher and/or equal than the reference strains, adding further evidence for the strong probiotic profile of the strains from our study.

BSH activity was not found in any of the isolates. However, opinions for this characteristic are deflected. Although the ability of strains to hydrolyze bile salts is usually considered among the main criteria for potential probiotic strain; due to its connection with the reduction of cholesterol (Bonatsou et al., 2017); it has been also reported as possibly harmful to the human host and thus it is not yet completely clear if it could be considered as a desirable feature or not for the selection of a potential probiotic (Zoumpopoulou et al., 2018).

The PCA was useful in showing the relationships among isolates. It has clearly differentiated some *L. pentosus* strains (MITLAB 14, 15, 25, 45) based on their interesting probiotic profiles and satisfying enzymatic activity. Notable that these strains exhibited quite equal profile to *L. acidophilus* used, a well-known probiotic strain. Other strains including 1 *L. plantarum* (MITLAB 2), 1 *L. pentosus* (MITLAB 24) and 2 *L. coryniformis* (MITLAB 61, 62) also far apart from the rest, having close relation to *L. rhamnosus* probiotic control, could have, in principle, limited application as potential probiotics because their differences were linked mainly to characteristics related to high SPD, DPPH scavenging and proteolytic activity. Finally, the enzymatic

profile of *Lc. mesenteroides*, especially in the case of strong reacted lipolytic enzymes, should be paid attention, because such enzymes (esterase, esterase-lipase) are related with oleuropein degradation leading to olives debittering. Thus, it is suggested their potential use as adjunct cultures in table olives fermentation.

Last but not least, the safety profile of isolates has been verified, since neither hemolytic activity nor biogenic amines genes exhibited by any of them. The latter molecules are necessary for several critical functions in humans. However high concentrations can cause unwanted physiological effects to human health; their amount is closely related to both amino acid compositions of fruits and bacteria metabolism (Bevilacqua et al., 2015). Thus, all strains should be characterized as Generally Recognized as Safe (GRAS), as their safety parameters are in line to the criteria established by EFSA (European Food Safety Authority: QPS status, Qualified Presumption of Safety).

4.5. Conclusions

This study provides the multifunctional properties; with regards to technological and probiotic potential; of novel LAB strains isolated from Cypriot green cracked table olives, in order to be used as starter and/or adjunct cultures, that could play a crucial role in the improvement not only to the fermentation process, but also to human health. Primary, a pool of assays has been proposed, in order to establish a pre-selection protocol, for the selection of the most appropriate strains.

According to the present work, 5 *L. pentosus* (MITLAB 14, 15, 24, 25, 45), 2 *L. coryniformis* (MITLAB 61, 62) and 1 *L. plantarum* (MITLAB 2) showed promising

probiotic and differentiated technological characteristics, as was proven by PCA, which were similar or even superior to the reference probiotic strains. However, it would be an omission not to mention the interesting enzymatic profile exhibited by most of *Lc. mesenteroides* strains, making them good candidates for possible use as adjunct cultures. Furthermore, the lack of any potentially non-safe isolates, raises optimism about their application in fermented foods. As a consequence, those strains could be considered as good candidates for further investigation with regards to *in vivo* studies (animal models), to elucidate both their probable health benefits as well as their contribution to fermentation improvement. Their use by the industry, is expected to enhance the production of novel functional Cypriot or other table olives with high quality in combination with a plethora of probiotic features.

Chapter 5: Study of technological and probiotic properties of yeasts isolated from Cypriot table olives

ABSTRACT

In the present study, the technological and potential probiotic features of yeasts, previously isolated from naturally fermented Cypriot green cracked table olives, were investigated, aiming to produce novel starter and/or adjunct cultures for the production of functional olives. A total of 72 isolates were screened in a proposed pre-selection protocol, including tolerance to synthetic model brines, oleuropein degradation and lactic acid assimilation and the selected strains (7) were molecularly identified and screened for further attributes. Results shown that during the simulated gastric and pancreatic digestions, 4 isolates were able to reach equal survival rates to the well-known probiotic *L. acidophilus* LA-5. All of them showed high auto-aggregation and hydrophobicity, while 2 *S. cerevisiae* strains (Y42, Y45) exhibited higher DPPH scavenging activity than the control. None isolate showed bile salt hydrolase activities, while they exhibited antimicrobial activity perhaps to all indicators. Interesting enzymatic profile was observed for most of them, especially regarding to lipolytic enzymes, arylamidases, β-glucosidase, α-galactosidase, Naphthol-AS-BI-phosphohydrolase and phosphatases. Finally, the obtained results led to the selection of 3 strains, all belonging to *S. cerevisiae*; with multifunctional properties, raising their potential use as starter cultures (single or combined), in Cypriot olives processing.

5.1. Introduction

Yeasts are single-celled eukaryotic microbes, belonging to the kingdom of Fungi. Nowadays, more than 1500 species are identified, corresponding to 1% of all described fungal species (Arroyo-López et al., 2008). Both their survival and growth are ensured by consuming starches and sugars. Plenty of yeasts are commercially available and can provide an inexpensive source for biochemical and biotechnological applications.

While LAB have been extensively studied regarding their multifunctional properties (Bevilacqua et al., 2010; Argyri et al., 2013; Bautista-Gallego et al., 2013; Randazzo et al., 2017; Pavli et al., 2019), yeasts were considered for years to be harmful in the succession of table olives fermentation (Abriouel et al., 2012). It has been reported a plethora of risks caused by yeasts, such as reduction the shelf life (Tofalo et al., 2012), as well as production of bad odors and flavors, clouding of brines, softening of fruits and assimilation of lactic acid (Arroyo-López et al., 2012a). Although many of these aspects have been revised in the last twenty years, they should always be strictly under consideration. Several studies have upgraded the role of yeasts, underlying their vital role principally to quality and flavor of the final product (Silva et al., 2011; Arroyo-López et al., 2012b; Bevilacqua et al., 2012; Bonatsou et al., 2015, 2018a; Oliveira et al., 2017; Tufariello et al., 2019). Nowadays, it is generally accepted that many yeast species are closely related with the production of compounds with important organoleptic attributes, which are caused by their enzymatic and other activities, such as lipase, esterase, β-glucosidase, catalase, production of killer factors etc. (Bonatsou et al., 2018a). Noteworthy is their contribution during fermentation to the enhancement of LAB population, due to the production of several compounds (vitamins, purines),

which are essential for LAB growth (Arroyo-López et al., 2012b). Furthermore, it has been noted their crucial probiotic potential (Oliveira et al., 2017; Bonatsou et al., 2018a; Porru et al., 2018). The latter has increased the interest in finding yeast strains with potential significant effects on several aspects to human health, such as prevention and treatment of intestinal diseases (Oliveira et al., 2017), bioavailability of minerals through the hydrolysis of phytate (Ragon et al., 2008), anti-inflammatory effects (Mumy et al., 2008), detoxification of mycotoxins and antioxidant capacity (Menezes et al., 2019; Sampaolesi et al., 2019).

The aforenoted aspects are of great importance to food industry due to their potential benefits. Therefore, the selection of the most appropriate yeast strains to be used as starters, in combination with LAB or not, is a promising future target which may improve the added value of the final product (Arroyo-López et al., 2012a; Bonatsou et al., 2017). It is crucial to mention that the use of yeasts to lead the fermentation process of different table olive types has been evaluated in the last years at experimental, pilot and industrial scale with promising results (De Angelis et al., 2015; Tufariello et al., 2015, 2019; Benítez-Cabello et al., 2019; Chytiri et al., 2020). These works support the application of yeasts inoculation to table olive fermentations and enhance the need for more detailed and specific studies to determine their multifunctional features for the selection of the most promising strains.

For the above cited reasons, the aim of the present work was to evaluate both the technological and the potential probiotic properties of yeast strains, previously isolated from Cypriot naturally fermented green table olives, during semi-industrial scale process. Strains exhibiting interesting multifunctional profile, could be potentially used as novel starter or adjunct cultures by the table olive industry.

5.2. Materials and methods

5.2.1 Yeasts strains and pre-selection protocol

A total of 72 yeasts strains were investigated in this study (Table 5.1). They have been previously isolated from the spontaneous fermentation of Cypriot green cracked table olives (Chapter 2) at intervals. All isolates were deposited in the culture collection of the Laboratory of Agricultural Microbiology and Biotechnology of Cyprus University of Technology.

A specific protocol for the selection of yeast strains was applied (Bleve et al., 2015). All strains were initially tested for their tolerance to a synthetic model brine (SMB), which was formulated based on the results obtained by previous chapter (Chapter 2), in order to be represented; as much as possible; the physicochemical attitudes occurred during the first period of Cypriot olives fermentation (ca. 30 days). More specifically, the SMBs (20 ml) contained 10% (w/v) NaCl, 0.4 mg/ml hydroxytyrosol, 4 mg/ml oleuropein, 50 mM citric acid, 50 mM mallic acid, 8 mM tartaric acid and 40 mM glucose. Overnight cells (18 h) were harvested (10.000 x g, 5 min, 4^{o}C), washed twice with NaCl (0.85% w/v) and re-suspended in 1.5 mL of a sterile saline solution (10% w/v) to obtain a final concentration of about 7 log CFU/mL (confirmed by plate counting on PDA). These suspensions were used to inoculate the SMBs and incubated at 20^{o}C for 15 days. Enumeration of yeast population was applied by plate counting on PDA. Results were expressed as survival score [3 high survival (>6 log CFU/ml), 2 medium survival (4.5-6 log CFU/ml, 1 low survival (2-4.49 log CFU/ml and 0 no survival (<2 log CFU/ml), as they emerged by means (log cfu/ml) and standard deviations of three replicates. Only the strains able to exhibit score 3 or 2 after 15 days of incubation were further analyzed.

Continuously, the selected strains were further investigated in a second pre-selection step, constituting their ability to degrade oleuropein in SMBs containing 10% w/v NaCl, 4mg/ml oleuropein and 40 mM glucose. Yeast cells from fresh (18 h) cultures were harvested (10,000 x g, 5 min, 4 C), washed twice with NaCl (0.85% w/v) and re-suspended again in 1.5 mL of a sterile saline solution (10% w/v) to obtain a final concentration of about 7 log CFU/mL (confirmed by plate counting), following by inoculation to the SMBs and incubated at 20^{o}C for 7 days. Oleuropein degradation was determined by High Pressure Liquid Chromatography (HPLC) analysis, as reported by (Tataridou and Kotzekidou, 2015b). A non-inoculated SMB was used as negative control. Results were expressed as percentage (%), as emerged by means (mg/ml) and standard deviations of three replicates. Only the strains degrade at least 50% of oleuropein content after 7 days, were further studied.

The promoting strains were studied for their potential assimilation of lactic acid. More specifically, SMBs, representing the conditions occurring in the final stage of fermentation process (where lactic acid has been produced by LAB) (based on the results of Chapter 2), contained 10% (w/v) NaCl, 0.5 mg/ml hydroxytyrosol, 50 mM acetic acid, 8 mM tartaric acid, 150 mM lactic acid and 40 mM glucose, were formulated and the potential reduction of lactic acid was determined by HPLC (Papadelli et al., 2015). A non-inoculated brine was used as negative control. Results were expressed as percentage (%) of lactic acid assimilation, as emerged by means (mM) and standard deviation of three replicates. Only the strains which could not assimilate lactic acid more than 10%, were proceed for further screening.

5.2.2 Identification of the selected strains

Overnight (18h) cells grown in Y.E broths, were used for total genomic DNA extraction and purification. DNA from each strain was obtained by adding 20 µl lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 65˚C for 24 h. The cell lysate was spin by short centrifugation and diluted with 180 µl buffer (10 mM Tris-HCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000 x g was performed to remove cell debris. Supernatants were stored checked for their purity (260/280, 260/230) using Nanodrop and stored at -20˚C. Continuously, DNA of the selected strains were procced to the amplification of ITS1-ITS2 region of 5.8s rRNA gene, according to Fernandes et al., (2019) using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC3′), in a final volume of 25 µl containing 3 mM $MgCl_2$, 0.2 µM of each primer, 1 U Taq polymerase (KAPA Taq PCR kit, KAPA Biosystems, United States), 0.2 mM dNTP's (Invitrogen) and 5 ng/µl of template DNA. Amplification was carried by an initial denaturation at 95°C for 5min, following by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, and extension at 72°C for 2min, plus a final extension step at 72°C for 7min. The PCR products were subjected to electrophoresis in 1% agarose gels, using 1X TAE (100 V for 50 min). Thereafter PCR products were sent for sequencing to Macrogen (Seoul, Korea). The sequences were aligned in GenBank using the BLAST search program (http://www.ncbi.nlm.nih.gov/blast/).

5.2.3 Enzymatic and probiotic assays

Both enzymatic and probiotic features (auto-aggregation, hydrophobicity, antimicrobial activity, BSH, antioxidant capacity, SGD, SPD, overall survival) were applied as described in the previous chapter (Chapter 4, *paragraphs 4.2.3, 4.2.4*), with the modification that yeast strains were grown to PDA medium.

5.2.4 Statistical analysis

Statistical analysis of the quantitative characteristics (auto-aggregation, hydrophobicity, DPPH, antimicrobial activity and in vitro simulated gastric, pancreatic and overall digestions), was carried out by analysis of variance (One-Way Anova), being the means compared by the LSD test ($p<0.05$), using SPSS v.20 software.

5.3. Results

5.3.1. Pre-selection assay

The results of the pre-selection protocol are summarized in Table 5.1. It was indicated that out of 72 yeast isolates, 47 were able to survive in SMBs, having a survival score more than 2. However, the number of selected strains was further reduced, in the next pre-selection assays. Oleuropein degradation, after incubation at 20^{o}C for 7 days, was low (<50%) for further 22 strains. From the 25 remaining strains, 7 were able to assimilate lactic acid less than 10%. Thus, 7 (Y1, Y36, Y40, Y42, Y45, Y46 and Y52) strains were selected as the most promising and studied for further characteristics.

Table 5. 1. The 72 studied strains and results of the pre-selection protocol. Survival to SMBs, acidification activity and oleuropein degradation capacity of the 72 yeast strains. Data are means values ± standard deviation of three replicates. Gaps indicating that strain did not satisfied the previous pre-selection assay. Bolt face indicates the selected isolates.

Strain	Model Brine Tolerance	Oleuropein degradation loss (%)	Lactic acid assimilation (%)
Y1	**3**	**70±6.61**	**5.2±1.3**
Y2	1		
Y3	1		
Y4	1		
Y5	1		
Y6	1		
Y7	1		
Y8	1		
Y9	1		
Y10	1		
Y11	1		
Y12	3	30±6.61	
Y13	3	24.1±15.2	
Y14	3	38.3±7.63	
Y15	3	24.1±12.3	
Y16	3	19.1±12.3	
Y17	1		
Y18	1		
Y19	1		
Y20	1		
Y21	3	74.1±10.1	37.5±4.7
Y22	2	62.5±6.6	46.9±3.3
Y23	2	65±9.01	49.8±8.6
Y24	1		
Y25	3	83.3±10.1	33.4±7.4

Y26	3	80±9.01	56.6±11.8
Y27	3	80.8±8.0	57.8±6.5
Y28	1		
Y29	1		
Y30	1		
Y31	1		
Y32	1		
Y33	1		
Y34	1		
Y35	3	60.8±8.7	63.1±4.2
Y36	**3**	**79.1±6.2**	**1.1±0.5**
Y37	3	20±13.9	
Y38	2	13.3±8.7	
Y39	2	19.1±8.0	
Y40	**2**	**75±7.5**	**6.3±2.2**
Y41	3	76.6±5.2	70.5±4.3
Y42	**3**	**61.6±11.2**	**4.6±2.7**
Y43	3	37.5±13.9	
Y44	3	26.6±8.7	
Y45	**3**	**65.83±10.1**	**5.6±1.1**
Y46	**3**	**60.8±8.7**	**5.8±2.3**
Y47	3	18.3±7.6	
Y48	3	29.1±15.2	
Y49	3	20.8±12.5	
Y50	3	29.1±17.7	
Y51	3	20.8±10.1	
Y52	**3**	**81.6±8.7**	**5.3±1.4**
Y53	3	70.4±13.5	83.2±2.3
Y54	3	70±10	73.8±2.8
Y55	3	65±9.0	64.7±4.3
Y56	1		
Y57	1		
Y58	1		
Y59	3	17.5±9.0	

Y60	3	27.5±6.6	
Y61	2	24.1±8.7	
Y62	3	15±7.5	
Y63	3	62±11.3	37.4±4.5
Y64	3	64.8±11	57.8±5.1
Y65	3	70±12.5	31.8±4.8
Y66	3	77.5±5	72.4±4.9
Y67	3	66.6±10.1	56.7±16.1
Y68	2	60±6.6	56.6±5.9
Y69	2	66.6±8.7	59.1±5.1
Y70	3	36.6±8.7	
Y71	3	16.6±14.2	
Y72	3	18.3±9.4	

5.3.2. Identification of selected strains

According to molecular identification, among the selected strains, they were included 1 *P. membranifaciens*, 1 *D. prosopidis*, 3 *S. cerevisiae*, *1 P. manchurica* and 1 *P. kluyveri* (Table 5.2).

Table 5. 2. Molecular identification of the selected yeast strains.

Strain	Species ID	Identity (%)
Y1	*Pichia membranifaciens*	99
Y36	*Debaryomyces prosopidis*	99
Y40	*Saccharomyces cerevisiae*	100
Y42	*Saccharomyces cerevisiae*	99
Y45	*Saccharomyces cerevisiae*	99
Y46	*Pichia manchurica*	98
Y52	*Pichia kluyveri*	99

5.3.3. Enzymatic profile

The enzymatic activity of yeast strains is presented in Table 5.3. Generally, all strains exhibited interesting enzymatic profile. More specifically, they exhibited strong and weak leucine and valine arylamidase activities, respectively. However, only *S. cerevisiae* strains (Y40, Y42, Y45) showed positive reaction to cystine arylamidase, while *D. prosopidis* (Y36) were weakly reacted. Concerning α-galactosidase, 5 strains (Y1, Y40, Y42, Y45 Y52) were weakly reacted, while all of them except from Y1 exhibited weak (Y40, Y42, Y45, Y46) or strong reaction (Y36, Y52) to α-glucosidase activity. Notably that all of them were positive to β-glucosidase and acid phosphatase enzymes, while only *P. membranifaciens* (Y1) was positive to alkaline phosphatase. It is noteworthy that all strains, exhibited positive reaction for the enzymes esterase, esterase lipase and lipase, while 2 strains (Y46, Y52) were weakly reacted to those enzymes. Continuously, all strains were strongly positive to Naphthol-AS-BIphosphohydrolase, while only *D. prosopidis* (Y36) was reacted with N-Acetyl-*β*-glucosaminidase. Finally, negative reactions were observed for the proteolytic enzymes trypsin, α-chymotrypsin, as well as α-mannosidase, α-fucosidase, β-galactosidase and the unwanted β-glucorinidase.

Table 5. 3. Enzymatic profile of the selected yeast strains. 0: No reaction, 1: Weak reaction, 2: Positive reaction.

Strain	β-Glucuronidase	Alkaline phosphatase	Esterase(C4)	Esterase lipase (C8)	Lipase (C14)	Cystine arylamidase	Trypsin	α-Chymotrypsin	α-Galactosidase	α-Mannosidase	α-Fucosidase	Leucine arylamidase	Valine arylamidase	Naphthol-AS-BIphosphohydrolase	β-Galactosidase	α-Glucosidase	β-Glucosidase	N-Acetyl-βglucosaminidase	Acid phosphatase
Y1	0	2	2	2	2	0	0	0	1	0	0	2	1	2	0	0	2	0	2
Y36	0	0	2	2	2	1	0	0	0	0	0	2	1	2	0	2	2	2	2
Y40	0	0	2	2	2	2	0	0	1	0	0	2	1	2	0	1	2	0	2
Y42	0	0	2	2	2	2	0	0	1	0	0	2	1	2	0	1	2	0	2
Y45	0	0	2	2	2	2	0	0	1	0	0	2	1	2	0	1	2	0	2
Y46	0	0	1	1	1	0	0	0	0	0	0	2	1	2	0	1	2	0	2
Y52	0	0	1	1	1	0	0	0	1	0	0	2	1	2	0	2	2	0	2

5.3.4. Probiotic properties

The auto-aggregation capacity of yeasts is shown in Fig. 5.1. Results indicated that auto-aggregation started within the first 4 h of incubation, while all strains exhibited strong capacity after 24h. Notably that 4 of them (3 *S.cerevisiae* and 1 *D. prosopidis*) had even stronger ability than the probiotic control. More specifically, *S. cerevisiae* strains (Y40, Y42, Y45) showed the highest phenotype capacity (ca. 96%) after 24h of incubation, without significant differences between them, following by *D. prosopidis* (Y36), the capacity of which was close to 80.5 ± 2.1. The auto-aggregation activity of the remaining strains (Y1, Y46, Y52) was significant lower, even from the control strain, as well.

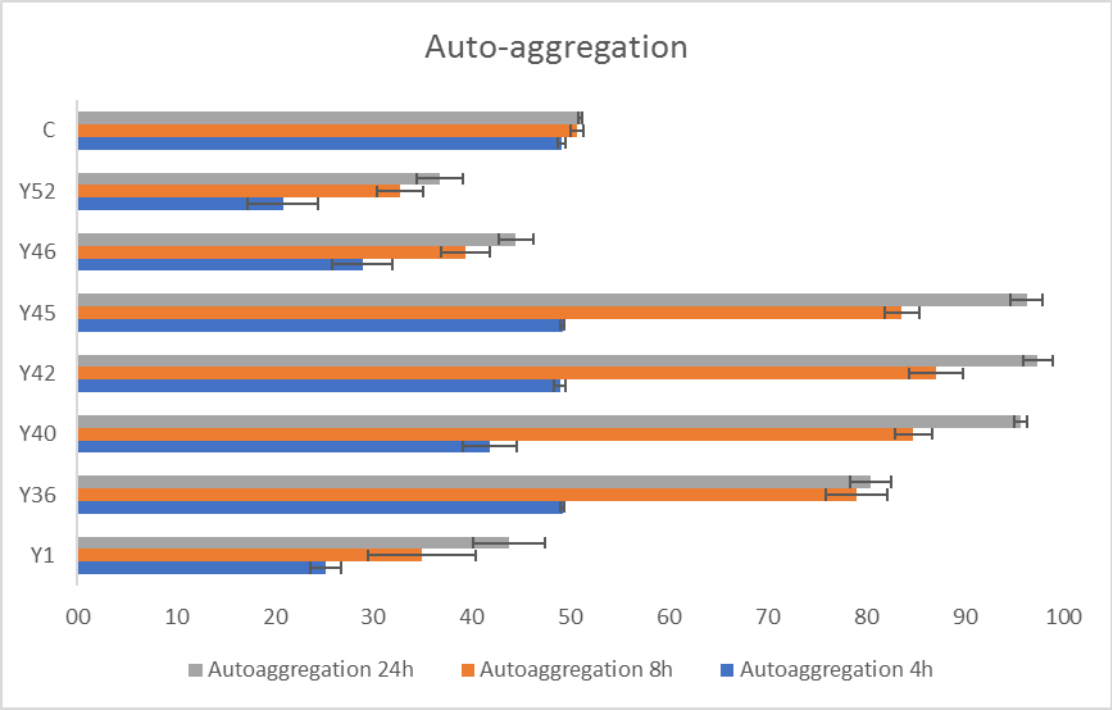


Fig. 5. 1. Autoaggregation capacity (%) of the selected strains assayed after 2, 4, and 24 h. Data expressed as means values ± standard deviation of three replicates.

Furthermore, most of strains were strongly hydrophobic, reaching values between 45% and 95% (Fig. 5.2). The highest levels exhibited by *S. cerevisiae* strains (Y40, Y42, Y45), showing values higher than 95%. No significant differences were recorded between those strains. Continuously, *P. membranifaciens* (Y1) and *D. prosopidis* (Y36), showed strong hydrophobicity as well, reaching levels close to 82%, without significant differences each other. Finally, the lowest values observed for Y46 and Y52 (45.9% and 49.2%, respectively). However, it is crucial to be mentioned that all strains exhibited higher hydrophobic levels than the probiotic control (36.05% ±1.2).

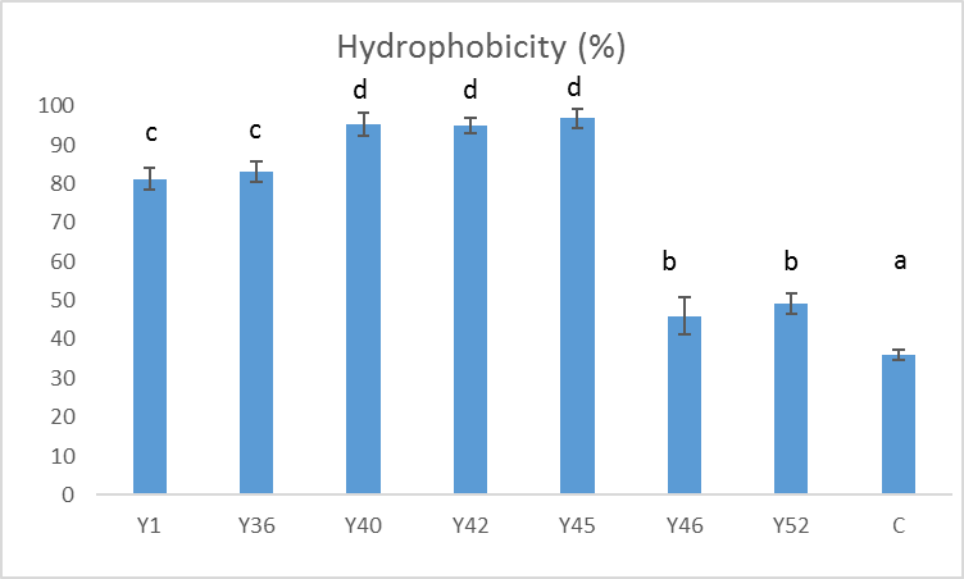


Fig. 5. 2. Hydrophobicity (%) of the selected strains. Data expressed as means values ± standard deviation of three replicates. Means not sharing common superscript statistically differ ($p<0.05$).

Concerning antimicrobial activity, all strains displayed inhibitory capacity against all indicator strains (Table 5.4), with limited exceptions. For example, *S. aureus* were able to grow in the presence of Y36 and Y52. The latter was unable to inhibit *E. coli* and *Ps. aeruginosa*, as well. The higher inhibition was observed from Y1 and Y36,

against all indicator strains (5-6 mm), except from *S. aureus*, where the inhibition was more limited (2 mm). However, it is noteworthy that the highest inhibition of *S. aureus* was observed by *S. cerevisiae* strains (7mm).

Table 5. 4. Inhibition zone (mm) of the selected strains against food pathogens.

Strain	*L. monocytogenes* *NCTC* 1994	*L. monocytogenes* NCTC 33411	*S. aureus* NCTC 6571	*E. coli* ATCC35150	*P. aeruginosa* NCTC 10662
Y1	6	5	2	6	6
Y36	6	6	0	6	7
Y40	2	2	7	2	2
Y42	2	2	7	2	2
Y45	5	3	7	2	2
Y46	5	4	2	2	2
Y52	1	1	0	0	0
C	3	4	2	3	3

As for DPPH free radical scavenging activity (Fig. 5.3), strains shown low capacity, while the antioxidant activity of Y52 did not even determined. Y46 shown the lowest activity (2.3% ± 0.6), following by Y1 (4.1% ± 1.1) and Y36 (8.2% ± 1.7). Significant differences were observed between those isolates. However, *S. cerevisiae* strains shown satisfied activity. Specifically, Y40 recorded medium antioxidant capacity (25.5% ± 4.3), while Y42 and Y45 exhibited the highest capacities (ca. 52% without significant differences), which was equal to the probiotic control (50.4% ± 1.2).

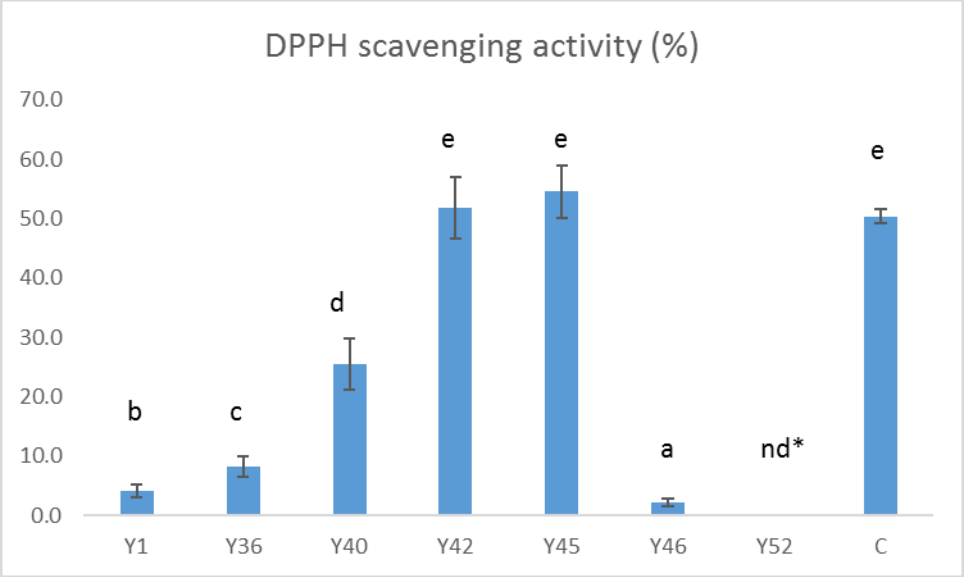


Fig. 5. 3. DPPH radical scavenging activity (%) of the selected strains. Data expressed as means values ± standard deviation of three replicates. Means not sharing common superscript statistically differ ($p<0.05$). *nd: not determined.

Four strains (Y36, Y40, Y42, Y45) exhibited high SGD rates, between 70% and 76% (Table 5.5). These strains showed equal rates to the control (72.7%), However, the remaining isolates (Y1, Y46, Y52), shown low survival rates, which was less than 30%, in any case.

Additional trend was observed during SPD. The partial survival rate for the pancreatic digestion was generally between 56% and 94%, while most of strains (Y40, Y42, Y45, Y46) exhibited rate higher than the probiotic control (73.49% ± 4.26). The highest survival was observed for the 3 *S. cerevisiae* strains, having values more than 90%, while the lowest survival observed in Y52 (no survival).

Concerning overall survival, the rates were generally high, showing that most of the strains had percentages between 46-78%. However, 3 *S. cerevisiae* strains, as

well as *D. prosopidis* (Y36) showed higher rates than the probiotic control. No significant differences were observed between *S. cerevisiae* strains, the survival of which was close to 78%, while the lowest rates were observed for Y52 (no survival), following by Y1 and Y46 (20.83 and 21.64, respectively).

Table 5. 5. Gastric, pancreatic and overall survival (%) of the selected strains, during simulating digestion. Data are means values ± standard deviation of three replicates.

Strain	SGD	SPD	Overall
Y1	21.9±0.09	64.3±17.1	20.8±0.04
Y36	71.7±1.5	56.3±8.2	46.5±12.8
Y40	75.1±8.6	93.3±2.6	77.6±7.7
Y42	70.5±2.2	93.9±3.3	77.5±3.3
Y45	75.7±5.9	93.9±1.1	79.9±4.7
Y46	26.4±0.4	81.2±0.3	21.6±0.09
Y52	17±3.7	ns*	ns
C	72.7±1.1	73.4±4.2	39.2±5

Regarding BSH, none of the strains shown bile salt hydrolase activity.

5.4. Discussion

Table olive is an important fermented product, and it is globally recognized as the future functional food (Bonatsou et al., 2017). On this point, one of the main aims of the food industry is to modernize olives production. The establishment of starter culture is the number one innovation challenge, while the need for isolation and characterization of novel strains from different olive cultivars, is always in the

foreground (Perpetuini et al., 2020). In the last two decades, yeasts have been recognized among the essential microorganisms in the production of table olives, as they contribute to the characteristics of the final product, producing desirable metabolites and other compounds. Many yeast species have been proposed to be used as starter cultures in olives fermentation, as it has been deeply approved by their interesting biochemical profile by many researchers (Silva et al., 2011; Bevilacqua et al., 2012; Bonatsou et al., 2015). Furthermore, their probiotic view has also been noted (Oliveira et al., 2017; Bonatsou et al., 2018b; Porru et al., 2018). In this regard, it is important to be mentioned that table olives have been recognized, as a perfect carrier for probiotic strains, due to their microstructure, being able to guarantee their survival through digestion (Pino et al., 2019). Thus, the use of potential probiotic strains isolated from olives has many chances to manifest their health benefits to consumers. However, the selection criteria of the most appropriate yeast strains should include specific attributes, as yeasts may cause serious problems during the fermentation process resulting in process distortion and deficient final products (Hernández et al., 2007). Based on those pieces of evidence, in the present study, 72 yeast strains, previously isolated from naturally fermented green cracked Cypriot olives, were evaluated to a proposed pre-selection protocol, applying conditions that mimic the natural conditions taking place during the process. All strains were tested for their ability to grow in a SMB as well as oleuropein degradation capacity and lactic acid assimilation. SMB was formulated based on the results of Chapter 2, to be used as a reliable and reproducible substrate for the in vitro technological assay. In all experiments, the incubation temperature was applied at 22^{o}C ± 2, which represents the average value occurring at industrial-scale controlled conditions in Cypriot olive industries, during the whole fermentation process. Results indicated that 7 of the tested strains satisfied the above

parameters, and further studied; indicating that they could not only become potentially dominant strains throughout fermentation, but also contribute to olives debittering, as well as to safety process with minimal lactic acid assimilation. It must be mentioned that the absence of the latter trait is very desirable (Bonatsou et al., 2017), especially in the case of the potential use of multi starter culture, including LAB and yeast strains.

Microbial enzymatic activity is one of the main factors regarding the selection of an appropriate starter culture (Heperkan, 2013). In this study, a pool of enzymatic assays was applied to the selected strains, using the API-Zym kit. Results indicated an interesting enzymatic profile for the 7 selected yeast strains. Specifically, all strains exhibited strong and weak leucine and valine arylamidase activities, respectively, while only *S. cerevisiae* strains (Y40, Y42, Y45) showed a positive reaction to cystine arylamidase. Those proteolytic enzymes are responsible for the liberation of amino acids and, thereby the improvement of flavor. However, despite their crucial role, it has also been noted that the absence of those proteolytic enzymes could also be a positive characteristic. Extensive protein degradation may generate undesirable compounds (Iorizzo et al., 2016). As a consequence, it has not been clear yet, if this trait should be considered as desirable (Songré-Ouattara et al., 2008b). Furthermore, one of the most important enzymes a potential starter should exhibit is β-glucosidase. This enzyme is closely linked with olives debittering, via its ability to hydrolyze phenolic compounds, mainly oleuropein. By this biochemical pathway, other simpler compounds (oleuropein derivatives) are generated, such as oleuropein aglycon and hydroxytyrosol. The latter is of great importance for human health, as it is linked with strong antioxidant and antimicrobial capacities (Anagnostopoulos et al., 2020). Thus, the observation that all selected strains were able to produce β-glucosidase activity, is a very desirable

characteristic, regarding the selection of a starter culture, that can debitter olives in a biological process, rather than chemical. This in addition may also provide health benefits to consumers (Bevilacqua et al., 2012; Bonatsou et al., 2018). According to literature, plenty of yeast species, isolated from both Spanish and Greek style process, have been reported to exhibit this enzymatic activity, which is in good line with the present work (Perricone et al., 2014; Bonatsou et al., 2015; Porru et al., 2018). As for α-glucosidase, this is an enzyme resulting in the release of α-glucose by hydrolyzing several carbohydrate molecules, releasing a-glucose (Bonatsou et al., 2018a). This is an undesirable trait, as it has been reported that the production of such compounds is highly connected with type 2 diabetes mellitus (Nurhayati et al., 2017) and thus, the observation that all of the studied strains, except Y1, shown weak or positive reaction, should be taken under consideration. Furthermore, the finding that 5/7 strains (Y1, Y40, Y42, Y45, Y52) possess a-galactosidase, which is not synthesized by the human organism, is a very beneficial trait, since it has been related with the prevention of flatulence, diarrhea, indigestion and abdominal pain, due to its ability to hydrolyze galactose oligosaccharides which are responsible for such situations (Chen et al., 2014). However, the observation that none of the studied strains shown β-galactosidase activity, could be consider as a disadvantage, since this enzyme contributes to the improvement of lactose tolerance in the gut, making the presence of this enzyme pivotal, concerning probiotic potential (Colombo et al., 2018). Nevertheless, the latter is in line with a previous study (Bonatsou et al., 2018a). Acid phosphatase, which is involved in phytic acid degradation, was found in all studied strains. Phytase activity is a desirable feature of starter cultures for fermentation processing of several products (Iorizzo et al., 2016). Continuously, alkaline phosphatase is a hydrolytic enzyme responsible for the de-phosphorylation of various types of molecules (nucleotides,

proteins and alkaloids). As a result, inorganic phosphorus is released and becomes available, expediting its transfer into the cell membranes (Młodzińska and Zboińska, 2016). Concerning the results of the present study, it can be assumed that only strain Y1 belonging to *Pichia membranifaciens* could produce this enzyme, whereas as previously mentioned, all of them showed a positive reaction for acid phosphatase. The latter is in good agreement with previous studies (Bonatsou et al., 2018a). Furthermore, all tested strains shown positive (Y1, Y36, Y40, Y42, Y45) or weak (Y46, Y52) reaction to the enzymes esterase (C4), esterase lipase (C8) and lipase (C14). Those lipolytic enzymes contribute to olive's flavor, as they catabolize the free fatty acids, resulting in the formation of desirable aromatic compounds such as ethanol, higher alcohols, esters, etc. (Bevilacqua et al., 2012; Rodríguez-Gómez et al., 2012). The observation that all strains were positively reacted to Naphthol-AS-BIphosphohydrolase, is very desirable trait, as this enzyme involved in phytic acid degradation, while is absent from human gastrointestinal tract and thus, the degradation of the insoluble complexes, created by the ability of phytic acid to form chelates with several inorganic compounds, could be succeeded by microorganism's enzymes action (Bonatsou et al., 2018a). Finally, negative activity for the undesirable β-glucuronidase was observed, indicating the safeness of the studied yeast isolates.

One of the most important features, a potential probiotic starter should exhibit, is its ability to adhere to epithelial cells (Monteagudo-Mera et al., 2019). According to literature, it has been proved the strong relation between adhesion ability with both strain's auto-aggregation and hydrophobicity. As a consequence, both traits have been proposed as a fundamental screening, to find out potentially adherent isolates (Porru et al., 2018). In the present study, most of the selected strains showed a high percentage

of auto-aggregation, which was increasing for 24 h. It is noteworthy that *S. cerevisiae* strains (Y40, Y42, Y45), as well as *Debaryomyces prosopidis* (Y36), showed significant higher auto-aggregation capacity than the probiotic control. Additionally, all strains exhibited higher hydrophobicity than the control, while all 3 *S. cerevisiae* strains (Y40, Y42, Y45) showed the higher capacities (no differences within them). The strong yeast capacities of both auto-aggregation and hydrophobicity are in line with previous works (Ogunremi et al., 2015; Oliveira et al., 2017). Both abilities are crucial prerequisite for the colonization of probiotic strain in gastrointestinal tract, preventing their elimination by the peristaltic movements and the existing microbes (Syal and Vohra, 2013). Nevertheless, it would be interesting to perform additional tests to these strains to better understand the mechanisms involved, such as in vivo testing using animal models, in order to verify the in vitro findings.

Regarding antimicrobial activity, all strains displayed antibacterial activity against perhaps all indicator strains. It must be mentioned that *S. cerevisiae* strains shown the lower inhibition ability to all pathogens, except to *S. aureus* where the inhibition zone was the highest (7mm). *Pichia membranifaciens* (Y1) and *Debaryomyces prosopidis* (Y36) displayed the highest inhibition to all pathogens. This is in agreement with previous works (Marquina et al., 2002; Santos and Marquina, 2004), although Oliveira et al., (2017) did not find any inhibition by *Pichia* and *Debaryomyces* spp.

Free radicals, are very dangerous molecules for human health, provoking a series of diseases (Zoumpopoulou et al., 2018). Thus, potential antioxidant capacity of microorganisms, is of great importance. It is believed that the antioxidant activity of yeast is mainly due to the high content of (1,3)-β-D-glucan and other β-glucans found

in the cell wall (Jaehrig et al., 2007). Results from the present study indicating that only 2 strains belonging to *S. cerevisiae* (Y42, Y45), shown higher antioxidant capacity (ca. 56%) than the control, while Y1, Y46 and Y52 shown limited or none capacity. However, it must be mentioned that the values obtained from the 3 *S. cerevisiae* strains are much higher than the findings of previous works (Gil-Rodríguez et al., 2015; Oliveira et al., 2017), using a similar method to the present work. Thus, it could be mentioned that *S. cerevisiae* isolated from Cypriot table olives could be considered as a very promising source of strains exhibiting high antioxidant capacity.

Due to the acidic conditions in the environment of human stomach, it is crucial to select strains with high tolerance to such situations. In this study, the majority of the studied strains (Y36, Y40, Y42, Y45) were able to reach equal and higher SGD and SPD, respectively survival rates to the well-known probiotic *L. acidophilus*. Additionally, overall survival of the abovementioned strains was higher and/or equal than the reference strains, enhancing the hypothesis of the strong probiotic profile of our yeast strains. These strains exhibited satisfied high survival rates, suggesting good adaptability to these environmental conditions. Similar results were also reported by previous works (Bonatsou et al., 2015, 2018a; Oliveira et al., 2017; Porru et al., 2018), reporting a considerable resistance of table olives related yeasts to simulated gastric, pancreatic and overall digestions.

BSH activity was not found in any of yeast strains. However, it is not clear yet if BSH is a desirable trait, as there have been reported both beneficial and negative traits for human health (Zoumpopoulou et al., 2018).

5.5. Conclusions

This study aimed to select the most promising yeast strains in order to be used as starter or adjunct cultures in Cypriot olives fermentation. 3 *S. cerevisiae* strains (Y40, Y42, Y45) showed the most promising characteristics, which were similar or even superior to the reference probiotic strains. However, it is noteworthy the high antimicrobial capacity exhibited by Y1 and Y36, as well as their satisfactory antioxidant activity, making them good candidates as adjunct cultures. Finally, before their application at industrial scale, further *in vivo* studies are suggested (e.g animal models, clinical assays), in order to confirm the findings from the present study.

Chapter 6: The role of Enterococci in Cypriot table olives fermented with high sodium content

A slightly modified version of this chapter has been published

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ABSTRACT

The aim of this work was to study the microbiological profile, as well as the technological characteristics and the potential probiotic properties of the predominant microflora, isolated from Cypriot green table olives, fermented with high sodium content. Results indicated that the dominant group was LAB. From a collection of 92 isolates from spontaneously fermenting green olives, 64 Gram positive isolates were firstly identified to genus level using biochemical tests, and secondly to species level using multiplex species-specific PCR amplifications of the *sodA* gene. Moreover, each strain was tested for potential technological and probiotics properties, as well as for safety characteristics, using biochemical and molecular methods, in order to be used as starter cultures. Finally, to discriminate the most promising isolates on the base of their technological and probiotics properties, Principal Component Analysis was used. Results indicated that all isolates were identified as *Enteroccocus faecium*, having interesting properties, while pathogenicity determinants were absent. Principal component analysis showed that some isolates had a combination of the tested parameters. These findings demonstrate that Enteroccoci from Cypriot table olives should be considered as a new source of potential starter cultures for fermented products, having possibly promising technological and probiotic attributes.

6.1. Introduction

In Chapter 1 it was noted that except from the main type of olives processing in Cyprus (directly brined, NaCl 10% w/v, citric acid), there is another remarkable type of fermentation, where olives are directly brining with an increased salt content (on average 15%), which is kept stable with weekly addition, in combination with an organic acid (mainly citric acid). By this way, the time for olives to become edible is shortened, due to the fact that the penetration of excessive sodium chloride and acid into the pulp of the fruit, causes a breakdown of the tissue and, consequently, the endogenous hydrolase enzymes act on the oleuropein molecule (Ramirez et al., 2016). Olives are ready for consumption in about 1 month. A part of Cypriot market demands a freshly debitter product with the following characteristics: green bright color, crunchy texture and taste characteristics of freshly cracked green olives. Freshly cracked green olives are traditionally served after removal of excessive salt, by washing them and adding olive oil, coriander seeds, etc.

Among LAB, the most frequent predominant species of table olives are *L. plantarum* and *L. pentosus*. However, in extreme brine conditions (such as high salinity and acidity at the beginning of fermentation), Lactobacilli may be unable to grow and survive. In this case, other tolerant species may predominate during and until the end of the process. It is well known that Enterococci are such microorganisms (De Castro et al., 2002).

Enterococci are Gram-positive, non-spore forming, catalase negative, oxidase negative cocci and they occur singly, in pairs, or in chains. From a taxonomic point of view, the genus Enterococcus has been reviewed several times (Fisher and Phillips, 2009). The genus includes more than 20 species, with *Enterococcus faecium* and

Enterococcus faecalis being the most prevalent species in fermented foods (Hancock et al., 2014). It has been shown that those species produce bile salt hydrolases, presenting potential probiotic properties related to a reduction of serum cholesterol levels by promoting a higher excretion of deconjugated bile salts (Franz et al., 2011). Due to their medium and/or high acid and salt tolerance, Enterococci may be used in food fermentations as starter cultures, being responsible for the formation of unique flavors, as well as the production of enterocins (Gomes et al., 2010). Moreover, Enterococci seem to be involved in table olive fermentation. In previous studies, it has been reported the presence of those LAB group in Spanish-style green olives (De Castro et al., 2002; Ben Omar et al., 2004). More specifically, in a previous work (Randazzo et al., 2004), four strains of Enterococci belonging to the species *E. faecium*, *E. casseliflavus*, and *E. hirae* were isolated from naturally fermented green olives collected from different areas of Sicily region. Furthermore, it has been proposed the use of Enterococci as starter cultures for olive fermentation, because they play a crucial role at the initial stage of the process, due to their tolerance to high pH and salinity (Randazzo et al., 2004; Gonza et al., 2014). However, there are many concerns about this species group, due to some indicates for undesirable characteristics they may exhibit, such as pathogenicity or antibiotic resistance (Ben Omar et al., 2004). Thus, this latter aspect should be deeply investigated before further application.

The aim of this work was a) to study the microbiological profile of fermented green Cypriot olives, processing by the method described above and b) to study the technological and safety characteristics, as well as the potential probiotic properties of its predominant microflora in order to discover new potential starter cultures for food fermentation industry.

6.2. Materials and Methods

6.2.1. Samples collection

Nine samples of Cyprus variety of green cracked olives in round containers of 100L brine (<13% w/v, added once a week), supplemented with citric acid (0.6% w/v) fermented for a period of 1 month (average temperature 25°C), were collected from the company named "King of olives" in Agglisides area, in Cyprus. Samples were transported to the laboratory and placed on sterile plastic containers where they were stored in darkness at room temperature for analysis within 24h.

6.2.2. Enumeration of microorganisms

The samples were evaluated for their Total Viable Count (TVC), *Enterobacteriaceae*, LAB, yeasts, Coliforms, *Micrococcaceae*, Gram-negative cocci and salt tolerant using the standard pour and spread plate methods after serial dilutions in saline water (0.85% w/v) (Table 6.1). More specifically, 10 g of table olives (flesh tissue) were transferred aseptically to stomacher bags with 90 ml saline solution (0.85% w/v NaCl) and homogenized for 2 min using a Stomacher at 220 rpm speed for 2 minutes (Bug Mixer, Interscience). Volumes of 0.1 ml or 1 ml (spread and pour plate, respectively) of serial dilutions in saline solution, were placed in petri dishes for enumeration of the microorganisms described. All samples were analyzed in triplicates.

Table 6. 1 Microbiological media used for microflora enumeration.

Growth media	Microorganisms	Method	Incubation Conditions

Plate Count Agar (PCA) (Merck, Darmstadt, Germany)	Total Viable Count	Pour plate	30ºC/72h
Man-Rogosa-Sharpe agar (MRS) (Oxoid, Basingstoke, UK) + natamycin 4%	Lactic Acid Bacteria	Pour plate/Overlay	30ºC/5d
M17 (Oxoid, Basingstoke, UK)	Lactic Acid Bacteria	Pour plate	37ºC/72h
Sabouraud Agar (Oxoid, Basingstoke, UK)	Yeast and Molds	Spread plate	25ºC/3d
Violet Red Bile Glycose Agar (VRBGA) (BD, Sparks, MD)	Enterobacteriacae	Pour plate/Overlay	37ºC/24h
Violet Red Bile Lactose Agar (VRBL) (Oxoid, Basingstoke, UK)	Coliforms	Pour plate/Overlay	30ºC/24h
Baird Parker egg yolk tellurite (BPM) (Oxoid, Basingstoke, UK)	Micrococcaceae	Spread plate	37ºC/48h
Nutrient Agar Crystal Violet (NACV) (Oxoid, Basingstoke, UK)	Gram$^-$ cocci	Spread plate	21ºC/48h
Mannitol Salt Agar (MSA) (Oxoid, Basingstoke, UK)	Salt resistant	Spread plate	30ºC/48h

6.2.3. Isolation of LAB

Representative colonies according to their different morphological characteristics (size, shape, color etc.) were retrieved from MRS agar Petri dishes with colony counts at the range of 30-300. Purity of the isolates was checked by streaking twice on MRS agar, followed by microscopic examination. Finally, stock cultures of purified isolates were stored at -80ºC in 20% (v/v) glycerol/MRS broth.

6.2.4. Physiological and molecular characterization of LAB strains

The purified isolates were examined for Gram staining and catalase (Shinozaki-Kuwahara et al., 2016). Gram-positive, catalase negative cocci were presumptively identified as LAB. Further classification was applied according to the biochemical criteria, such as growth at various temperatures (10ºC, 15ºC, and 45ºC), salt tolerance (2, 4, 6.5, 8% w/v NaCl), production of CO_2 from glucose as unique source of carbon and pH (9.6) (Estifanos, 2014).

The identification of the isolates at species level was achieved by applying molecular techniques by multiplex PCR amplifications of the sod*A* gene (Jackson et al., 2004). Three PCR master mixes consisting of different primer sets for each species were prepared (Table 6.2); Group 1 was *E. durans, E. faecalis, E. faecium*; group 2 was *E. casseliflavus, E. gallinarum*; group 3 was *E. hirae*. PCR reactions contained 2.5µl template DNA (10ng/µl), 1x PCR reaction buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 16 µM of each primer, 2.5U of Kappa Hot Start DNA polymerase (KAPPA Biosystems), and distilled water was added to a final volume of 22.5µl. Following an initial denaturation at 95°C for 4 min, products were amplified by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for Group1, 57°C for Group 2and 60°C for Group 3 for 1 min, and elongation at 72°C for 1 min. Finally, amplification was followed by a final extension at 72°C for 7 min. Eight µl of PCR product was electrophorized on a 1.2% agarose gel, 1x TAE (Tris-acetate-EDTA), stained with SYBR™ Safe DNA Gel Stain (Invitrogen) and visualized under UV light. A 100 bp DNA ladder (Nippon Genetics) was used as marker.

Table 6. 2. PCR primers, products, and reference strains.

Strain	Primer	Sequence (5'–3')	Product size (bp)
*E. durans*ATCC19432	DU1	CCTACTGATATTAAGACAGCG	295
	DU2	TAATCCTAAGATAGGTGTTTG	

*E. faecalis*ATCC19433	FL1	ACTTATGTGACTAACTTAACC	360
	FL2	TAATGGTGAATCTTGGTTTGG	
*E. faecium*ATCC19434	FM1	GAAAAAACAATAGAAGAATTAT	215
	FM2	TGCTTTTTTGAATTCTTCTTTA	
*E. casseliflavus*ATCC25788	CA1	TCCTGAATTAGGTGAAAAAAC	288
	CA2	GCTAGTTTACCGTCTTTAACG	
*E. gallinarum*ATCC49673	GA1	TTACTTGCTGATTTTGATTCG	173
	GA2	TGAATTCTTCTTTGAAATCAG	
*E. hirae*ATCC 8043	HI1	CTTTCTGATATGGATGCTGTC	187
	HI2	TAAATTCTTCCTTAAATGTTG	

6.2.5. Technological characteristics

6.2.5.1. Acidification activity

Acidification activity of the isolates was tested as described previously (Fuka et al., 2017). In particular, tubes containing 10 ml of sterile skimmed milk (RSM 10% w/v; Oxoid) were inoculated with fresh overnight cultures (1% v/v) and incubated at 37ºC. The pH value was determined after incubation for 0, 6 and 24 h using a pH meter (Hanna instruments). The analysis was carried out in triplicate. A non-inoculated skim milk was used as negative control. The acidification rate was calculated as $\Delta pH = pH_{(0h)}$-pH (6h or 24 h respectively).

6.2.5.2. Proteolytic activity

For the determination of proteolytic activity, 2μl of fresh overnight cultures (10^8 cfu/ml) were spotted on the surface of a skim milk agar (10% w/v skim milk, 2% w/v agar, Oxoid), and were incubated at 37ºC from 2 to 4 days (Franciosi et al., 2009). The results were considered as positive when a clear zone was formed around the colonies. The analysis was performed in triplicate.

6.2.5.3. Lipolytic activity

For the determination of lipolytic activity, a loopful of fresh culture (24 h) was placed on tributyrin agar (Oxoid) and was incubated at 37ºC for 4 days (Dinçer and

Kıvanç, 2018). The results were considered as positive when a clear zone appeared around the colonies. The analysis was repeated three times.

6.2.5.4. Exopolysaccharide production (EPS)

EPS production was evaluated with ruthenium red staining method in ruthenium milk agar (0.5% yeast extract, 10% skim milk powder, 1% sucrose, 1.6% agar, 0.08 g/L ruthenium red) (Imène et al., 2017). The coagulated cultures were considered as EPS positive when a white loop was formed after incubation at 37°C for 24h. The analysis was conducted in triplicate.

6.2.5.5. β-Glucosidase activity

β-Glucosidase activity was determined using X-Gluc- 5-bromo-4-chloro-3-indolyl-*β*-D-glucopyranoside (Sigma) as substrate. The test was conducted by spreading 0.2 ml of a N,N-dimethyl formamide solution to a final concentration of 0.3% (wt/vol) X-Gluc on MRS agar. The plates were left to dry for 3h in darkness, and were incubated at 37°C for 48 h after the inoculation (2µl) (Ghabbour et al., 2011). Isolates producing *β*-glucosidase were recognized when the colony was colored blue. The analysis was repeated for three times.

6.2.5.6. Catabolism of citric acid

The determination of catabolism of citric acid was performed by growth in Simmons Citrate Agar (Oxoid), which contains citric acid as the only carbon source. Freshly prepared cultures were inoculated by spread to the medium and the plates were incubated at 37°C for 7 days. Results were considered positive when colonies turned

the color of the substrate from green to blue, otherwise the test was considered as negative. The analysis was performed in triplicates.

6.2.6. Pathogenicity

6.2.6.1. Hemolytic activity

Freshly prepared cultures were inoculated by spread on Columbia Agar Base (Oxoid), supplemented with 5% v/v horse blood and incubated at 37ºC for 24 h. Hemolytic activity was characterized "α" when a green zone was formed round the colonies, "β" when clear zones were created around colonies, and "γ" when no zones were appeared around colonies (Fuka et al., 2017). The analysis was repeated in triplicates.

6.2.6.2. DNAse production

Screening for DNAse production was held according to (Ribeiro et al., 2014) . The tested strains were grown at 37ºC in MRS broth overnight. A loopful of freshly prepared cultures was spotted on DNAse agar (Oxoid) and a few drops of 1N HCl were added onto the colony, after incubation for 24 h at 37ºC. Production of DNAse was indicated when a clear zone appeared around the colonies. The analysis was repeated three times.

6.2.6.3. Genotyping analysis for testing virulence activity

All the selected strains were checked for the presence of genes encoding virulence, antibiotic resistance and amino acid decarboxylase activity (Chajęcka-Wierzchowska et al., 2017) (Table 6.3). DNA was extracted using Bacterial DNA kit

(Macherey-Nagel) according to the manufacturer's instructions, following by amplifications of genes described in Table 3 using specific primers. PCR reactions contained 1 µl template DNA (10ng), 1xPCR reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 16 µM of each primer, 1U of Kappa Taq DNA polymerase (KAPPA Biosystems), and distilled water was added to a final volume of 20 µl. Following an initial denaturation at 95°C for 4 min, products were amplified by 30 cycles of denaturation at 95°C for 30 s, annealing of each primer for 30 s, and elongation at 72°C for 1 min. Finally, amplification was followed by a final extension at 72°C for 7 min. Eight µl of PCR product was electrophorized on a 1.2% agarose gel, 1x TAE (Tris-acetate-EDTA), stained with SYBR™ Safe DNA Gel Stain (Invitrogen) and visualized under UV light. A 100 bp DNA ladder (Nippon Genetics) was used as marker. The DNA of the reference strain *Enterococcus faecium* ATCC 29212 was used as positive control in the corresponding PCR reactions.

Table 6. 3. List of primers used for the amplification of pathogenicity related genes.

Target gene	Primer Sequence	Annealing temperature (ºC)	Fragment size (bp)	Reference
Aggregation substance (*asa1*)	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	50	375	(Vankerckhoven et al., 2004)
Adhesion of collagen protein (*ace*)	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	48	1008	(Martín-Platero et al., 2009)
Cytolysin (*cylA*)	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	52	688	(Vankerckhoven et al., 2004)
Endocartidis antigen (*efaA*)	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	57	688	(Martín-Platero et al., 2009)
Enterococcal surface protein (*esp*)	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG	50	510	(Vankerckhoven et al., 2004)
Gelatinase (*gelE*)	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	47	213	(Vankerckhoven et al., 2004)

Hyluronidase (*hyl*)	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	53	276	(Vankerckhoven et al., 2004)
Vancomycin Resistance (*vanA*)	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	52	377	(Martín-Platero et al., 2009)
Vancomycin Resistance (*vanB*)	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC	60	529	(Martín-Platero et al., 2009)
Histidine decarboxylase (*hdc1*)	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	46	367	(De Las Rivas et al., 2005)
Histidine decarboxylase (*hdc2*)	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	50	534	(De Las Rivas et al., 2005)
Tyrosine decarboxylase (*tdc*)	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	55	924	(De Las Rivas et al., 2005)
Ornithinedecarboxylase (*odc*)	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	54	1446	(De Las Rivas et al., 2005)

6.2.7. Screening for probiotics characteristics

6.2.7.1. Resistance to low pH

The survival of cultures on MRS broth buffered at four different pH values (6, 4, 3 and 2) was studied. The isolates were inoculated (1% v/v) in MRS broth with 4 different pH values described above and incubated at 37ºC for 48h. Furthermore, 1ml of sample was taken at 0, 1 and 3h of incubation and following appropriate dilutions, was inoculated into MRS agar. Finally, after the incubation at 37ºC for 48h the forming colonies were counted. The survival ratio of the 1st and the 3rd hour was calculated using the log of the surviving cells (cfu/ml) in 1 and 3 hours, divided by the log of the starting cells (cfu/ml) at 0 h (Ilavenil et al., 2015). The analysis was repeated three individual times.

6.2.7.2. Resistance to bile salts

Resistance to MRS broth containing 0.3% of bile acids (Sigma-Aldrich) was tested as follows. Firstly, the samples (1% v/v) of 0 and 3 hours were received from a water bath (37ºC), following by appropriate dilutions and finally inoculated into MRS agar. Colony counting took place after 48 h of incubation at 37ºC. The survival ratio was calculated using the log of the surviving cells (cfu/ml) in 3 hours divided by the log of the original cells (cfu/ml) at 0h (Song et al., 2015). The analysis was repeated for three individual times.

6.2.8. Statistical analysis

The results were expressed as the mean and standard deviation with triplicate determinations, followed by frequencies distribution. Significant differences for microbiological and technological analyses between samples were determined using the statistical software MINITAB 12.0 by the method of one-way analysis of variance (ANOVA).

Principal component analysis (PCA) was used to discriminate all isolates on the base of their technological and probiotics properties, while the discriminating variables were proteolytic activity, lipolytic activity, acidification activity, survival to pH 2 at 1h, survival to pH 3 at 1h, survival to pH 3 at 2h, survival to bile salts (0.3%). Factors with eigenvalues higher than 1.00 were retained according to the Kaiser criterion (Song et al., 2015). In the case of not numeric values (proteolytic and lypolitic activity), before the use of PCA, it was used the method of Optimal Scaling, so our factorial points of the objects obtain new, complex, quantitative variables, which are linearly independent. PCA was performed using the statistical software SPSS v.20.

6.3. Results and Discussion

6.3.1. Microbial enumeration

The results of total aerobic bacteria, *Enterobacteriaceae*, LAB on MRS and M17 agar, yeasts and molds, coliforms, *Micrococcaceae*, Gram negative cocci of the fermented green cracked Cypriot table olives are summarized in Table 6.4. As shown in Table 6.4, LAB were the predominant microorganisms, having an average value of 8.04± 0.04 log_{10}cfu/g. However, yeasts and molds counts were counted at lower levels (3.48±0.06log_{10}cfu/g). In accordance with our results, in other study it was found that LAB from Spanish style fermented "Bella di Cerignola" table olives were the predominant microorganisms, having a population of 6.74± 0.16 log_{10}cfu/g, at the end of fermentation process, while yeasts population was more limited (Campaniello et al., 2005). Another study reports that in natural processing of olives the predominant organisms are yeasts contrary to lactic acid bacteria dominate the fermentation of Spanish style treated olives (Aponte et al., 2010). It is worthwhile to mention that in the present study, the population of *Enterobacteriaceae* and coliforms was found at quite high levels (4.66±0.02 log_{10}cfu/g and 3.83±0.01 log_{10}cfu/g, respectively), despite the low pH values (3.89 ± 0.01). *Enterobacteriaceae* can be found at the beginning of the fermentation and are quickly inhibited by pH decrease due to LAB activity which produce lactic acid as well as bacteriocins responsible of the safety of fermented products (Panagou et al., 2003). A possible justification for these high numbers of *Enterobacteriaceae* could be the short period of time (30 days) of fermentation. Reports from other studies show that *Enterobacteriaceae* disappear in 30-40 days (Panagou et al., 2003). On this point, it must be mentioned that this way of olives processing, directly brining with an increased salt content (more than 15%), in combination with an

organic acid, is disputed whether it should be considered as fermentation, while microbiological testing are necessary to reduce concerns regarding risk of safety. Further, it would be interesting to monitor this alternative process, during different time points, to better understand the microbial interactions. Finally, a sensory evaluation of the final product, as well as its compare with naturally fermented olives, would be of great interest.

Table 6. 4. Mean values (log10cfu/g; x±SD) of the counts of different microbial groups, as well as pH values of Cypriot green cracked table olives. Mean values of three individual plate counts.

Medium	**Log cfu ml^{-1}**
TAC[a]	9.18±0.07
Yeasts and Molds	3.48±0.06
LAB on MRS agar	8.04±0.04
Coliforms	4.66±0.02
Enterobacteriaceae	3.83±0.01
Micrococacceae	n.d.*
LAB on M17 agar	4.86±0.06
Salt Resistant Bacteria	7.81±0.08
Gram -ve	5.37±0.03
pH	3.89±0.01

* nd: not detected; [a]: Total Aerobic Counts

6.3.2. Isolation and identification of LAB

Ninety-two colonies were randomly picked from plates containing 30- 300 colonies. Sixty four Gram positive isolates were considered as LAB due to the fact that, they were cocci and catalase negative, and thus it seems that they belong to the genus of enterococci, according to the phenotypic identification (growth to all NaCl

concentrations, pH and temperatures, negative to heterofermentation). Molecular identification via multiplex species-specific PCR showed that all 64 isolates were *Enterococcus faecium*.

Enterococci were isolated from Spanish-style green olive fermentations (De Castro et al., 2002). Isolates have been identified, characterized and utilized with *L. pentosus* as starter cultures for Spanish-style green olive fermentation. In other study (Ben Omar et al., 2004), three strains of Enterococci were isolated from olives, belonged to the species *E. faecium*. Furthermore, it has been isolated a total number of 52 LAB from naturally fermented green olives collected from different areas of Sicily (Randazzo et al., 2004). Even though the majority of these strains belonged to the genus of Lactobacillus, some of them were identified as *Enterococcus* spp., as well.

6.3.3. Technological properties

6.3.3.1. Acidification activity

A good mesophilic fast acid producing starter culture will reduce the pH of the milk at 5.3 after 6h of incubation at 37^{o}C (Aspri et al., 2017). In the present study, none of isolated strains reduced the milk pH to <6.0 after 6 h of incubation (Fig. 6.1, Table 6.5). However, after 24h of incubation, 9 out of 64 isolates reduced milk pH levels to>4.60, 34 of 64 from 5.10 to 4.60, while 21 of them kept the pH value to<5.10. Thus, those isolates could be considered as slow or medium acidifiers (Sarantinopoulos et al., 2001), and could be classified in three main groups: (i) those showing a high acidifying capacity, with a pH decrease of 2 pH units (14.1%) and more, (ii) those with an intermediate acidifying activity showing a pH decrease ranging from 1.5 to 2.0 pH units (53.1% of the isolates), and (iii) those with low acidifying capacity (32.8% of the

isolates) showing a decrease in pH value less than 1.5 pH units (Fig. 6.1, Table 6.5). It is known that the rate of acid production is a crucial factor when it comes to select a proper starter culture (Bonatsou et al., 2017). Besides that, pH prevents the growth of undesirable microorganisms such as spoilage and pathogenic bacteria, is also responsible for the organoleptic properties of the final product. The high capacity acidifiers (14.1%) identified, could be used as starter cultures for the production of fermented table olives because they could reduce the pH value at low levels during fermentation. According to literature, considerable work has been done on acid production of Enterococcus species. In general, enterococci show low or medium acidifying ability, a fact that agrees with the present study (Ribeiro et al., 2014). Particularly, in another work, it has been reported that the pH value of milk did not fall below 5.5 after 24h inoculated with *Enterococcus faecium* (Morea et al., 1999). Some other studies on enterococci confirmed the poor acidifying activity of these microorganisms in milk, giving a small percentage of strains showing a pH below 5.0 - 5.2 after 16-24 h of incubation at 37^{o}C (Morea et al., 1999; Suzzi et al., 2000; Sarantinopoulos et al., 2001). It has been also shown that *E. faecalis* has higher acidification activity than *E. faecium* in general (Suzzi et al., 2000).

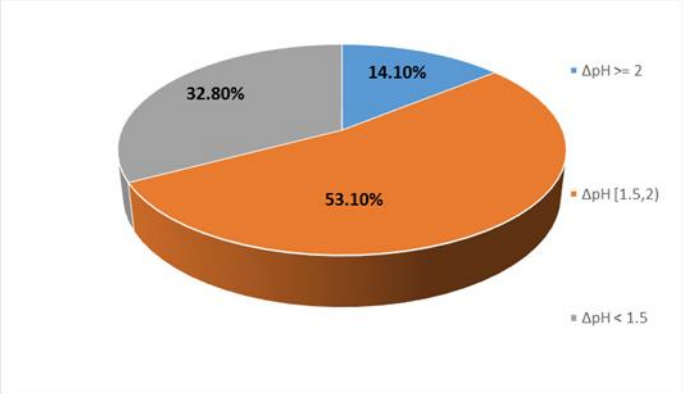

Fig. 6. 1. Percentage of number of isolates categorized in three acidification activity groups (low ΔpH<1.5, medium ΔpH {1.5,2) and high ΔpH>=2) after 24h incubation at 37°C in 10% skim milk.

Table 6. 5.. Isolates of Enterococcus spp showing positive or negative proteolytic activity, positive or negative lipolytic activity and acidification activity of high medium or low level [low ΔpH<1.5, medium ΔpH {1.5,2) and high ΔpH>=2 after 24h incubation at 37°C in 10% skim milk].

	Proteolytic activity (Positives)	Proteolytic activity (Negatives)	Lipolytic activity (Positives)	Lipolytic activity (Negatives)	Acidification activity (High)	Acidification activity (Medium)	Acidification activity (Low)
Isolates (*Enterococcus*)	7, 11 - 14, 16 - 18, 27 - 37	1 - 6, 8 - 10, 15, 19 - 26, 38-64	11 - 13, 16 - 18,32,36,42,53,54,62,63	1 - 10,14, 15,19 - 31,33, 34, 35, 37 – 41, 43 -52, 55 – 61, 64	3,23,26,27,28 ,42,47,52,59	1,2,4 - 10,13,14,18,21, 22,24,25,29 - 33,35,37,39,40, 41,43,50,51,55, 56,57,60 - 64	11,12,15,16,17,19,20,34,36 ,38,44,45,46, 48,49,53,54,58

6.3.3.2. Proteolytic activity

Proteolysis is one of the main industrial phenomena that contribute to the development of the organoleptic characteristics of a fermented product. The proteolytic activity of the isolates was recorded by the presence of a clear halo (positive result) on 10 % Skim Milk agar. Nineteen out of 64 strains (29.7%) showed proteolytic activity. According to the literature, it has been reported that between 7 strains of Enterococcus, only one was able to degrade casein, being positive for proteolytic activity (Ribeiro et al., 2014). Our isolates have less proteolytic activity (Fig. 6.2a, Table 6.5) in comparison with other Enterococcus strains that have been characterized in other studies. In particular, according to other studies, enterococci were characterized having weak proteinase activity (Suzzi et al., 2000). The same conclusion was drawn in another systematic study (Sarantinopoulos et al., 2001), in which 129 *E. faecium, E. faecalis*, and *E. durans* strains were screened for their technological characteristics. It was found that all strains showed low extracellular proteolytic activity, with the *E. faecalis* strains

being more active. Generally, there are few data regarding the proteolytic system of enterococci in comparison with other LAB species. The proteolytic activity is low in Enterococcus strains, except of *E. faecalis* strains (De Castro et al., 2002). It is worthwhile to mention, that in the present study there is no correlation between proteolytic and acidification activities, because the fact that only 2 strains were positive to proteolytic activity, were able to reduce pH value to more than 2 units, as well. This finding agrees with those of a study performed by other researchers (Xanthopoulos and Tunail, 2001), suggesting that no clear relationship was observed between proteolytic and acidification activities. However, according to other report, it has been observed that the majority of the acidifying strains also had proteolytic activity (Suzzi et al., 2000). Nevertheless, more and deeper physiological studies are needed in order to investigate whether or not the proteolytic ability of enterococci is one of its characteristics and how is regulated.

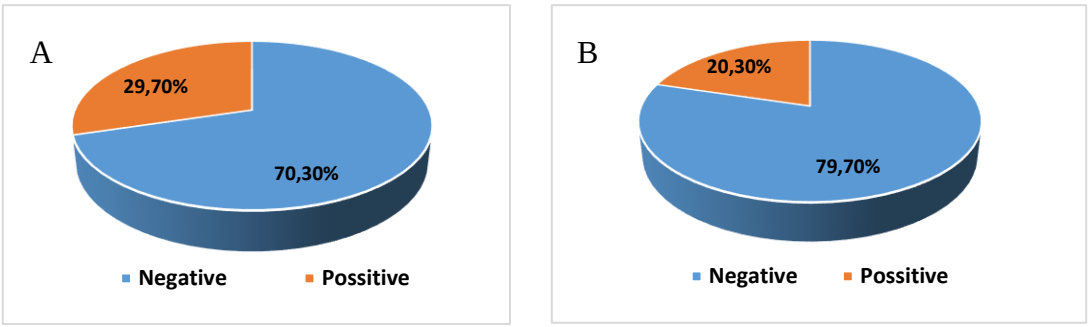

Fig. 6. 2. Percentage of number of: (A) proteolytic positive isolates and (B) lipolytic positive isolates.

6.3.3.3. Lipolytic activity

The lipolytic activity is a desirable characteristic of a strain to be used as a starter culture in order to highlight certain organoleptic characteristics of the final product. Limited reports exist on lipolytic activity of enterococci, especially those isolated from

table olives. Regarding the results of this study, it seems that enterococci have generally low lipolytic activity when using tributyrin agar. Namely, only 20.31% (13 out of 64) of strains were positive to lipolytic activity (Fig. 6.2b, Table 6.5). Additionally, there are some studies showing low or no lipolytic activity of Enterococcus species (Morea et al., 1999; Sarantinopoulos et al., 2001). Moreover, in another work, it has been showed that the existence of lipolytic activity was only confirmed for two out of 7 Enterococcus isolates (Ribeiro et al., 2014). However, there is a study reported that among 129 enterococci, the majority of them (90%) hydrolyzed all tributyrin substrates (Sarantinopoulos et al., 2001). Furthermore, in the same work, it has been concluded that *E. faecalis* strains were the most lipolytic, followed by the *E. faecium* and *E. durans*. All studies lead to the conclusion that lipolytic activity of enterococci, is strain depended as well as related to the type of the examined food.

6.3.3.4. Exopolysaccharide production (EPS)

According to our results about EPS production, all isolates were not able to produce EPS, since they were showing pink to red colonies. EPS is a protection barrier against lethal influence of the environment (desiccation, phagocytosis, phage attack, osmotic stress, antibiotics or toxic compounds) and the major component of bacterial biofilm, enhancing the colonization of probiotic bacteria in cell-host interactions in the gastrointestinal tract (Kanmani et al., 2013). This is a beneficial trait for probiotics in their endeavor to colonize the gut. However, according to two other studies 26 out of 72 tested strains were able to produce EPS and 7 out of 25 strains, respectively (Suzzi et al., 2000; Song et al., 2015). However, more studies are required in order to learn more about the EPS production from Enterococcus spp.

6.3.3.5. β-Glucosidase activity

None of the isolates gave positive result (blue colony), so they are not able to produce the β-glucosidase enzyme. The enzyme of β-glucosidase is closely related to the oleuropein hydrolysis and the debittering process in table olives so it is a desirable characteristic for a potential starter culture (Charoenprasert and Mitchell, 2012).

6.3.3.6. Catabolism of citric acid

Citrate metabolism by LAB is essential in a wide range of fermented foods and beverages, since it serves as a precursor for the formation of plenty other compounds contributing to the final organoleptic characteristics. None of the isolates could catabolize the citric acid as unique source of carbon. The metabolism of citric acid has been extensively studied and it is well documented in several Lactococcus, Lactobacillus, and Leuconostoc species (Laëtitia et al., 2014). In contrast, only a few data deals with citrate metabolism by Enterococcus strains are available. According to them, strains of *E. faecium* have less ability to utilize citrate as unique source of carbon (Sarantinopoulos et al., 2001).

6.3.4. Pathogenicity

Pathogenicity tests (DNase production, hemolytic activity) were negative for all strains, suggesting their safety as starter cultures but the study of virulence factors by molecular methods in Enterococcus spp. coming from foods, is necessary due to the risk of genetic transfer since these genes are usually located in conjugative plasmids (Chajęcka-Wierzchowska et al., 2017). The presence of virulence factors in enterococci

can greatly contribute to enhance the severity of hospital infections. The isolates were tested for the presence of genes encoding potential virulence factors and biogenic amines. Five (7.8%) of our isolates were positive for *gelE* gene. However, in previous studies, it has been found that strains having this gene, did not produce gelatinase (Vankerckhoven et al., 2004; González et al., 2010), because the fact that the *fsr* operon could be damaged, lost, or suffer deletions due to physiological stresses from laboratory storage. Hemolysin production increases the severity of enterococcal infections, and the presence of hemolysin/cytolysin genes is considered a risk pathogenicity factor. Regarding the *cylA* gene, 2 (3.1%) isolates gave a positive result, but according to phenotypical tests, only γ-haemolysis was observed. As a result, *cylA* has been considered as a "silent gene" and its gene expression can be influenced by the environmental factors and conditions used for phenotypic tests (Aspri et al., 2017). The genes *esp*, *efaA*, and *ace* have been involved in the colonization and adhesion at biotic and non-biotic surfaces, and the host immune system evasion. The genes *esp*, *efaA* and *ace* were not detected in none of the isolates. However other authors found high incidence of *esp*, *asa1* and *efaA* genes in *E. faecalis* (Creti et al., 2004). Moreover, none of the isolates gave a positive result for *hyl*, which is related to the production of hyaluronidase facilitating the spread of toxins and bacteria throughout the host tissue by causing tissue damage. Similarly, aggregation substance (*asa1*) was not detected in our isolates. This gene is a sex pheromone plasmid-encoded surface protein, which promotes the conjugative transfer of sex pheromone plasmids by formation of mating aggregates between donor and recipient cells. Finally, none of the isolates was positive for *vanA* and *vanB* genes. This is in agreement with the results from previous studies (Franz et al., 2001; Aspri et al., 2017), reporting that *vanA* and *vanB* genes have not been found frequently in enterococci isolates from food sources. However, in other

work it has been reported that three strains (50%) of *Enterococcus faecalis* were positive to *vanA* gene (Ribeiro et al., 2014). Regarding the presence of several-amino decarboxylase genes, histidine (*hdc1* and *hdc2*), ornithine (*odc*) and tyrosine (*tdc*) decarboxylase, no amplification occurred for either *hdc1* and *hdc2* or *odc*. However, the *tdc* gene was present in 11 (17.1%) isolates, which is in accordance with the literature reported that despite the fact that tyramine production is a common characteristic of enterococcal isolates, it is considered to be a negative trait for their possible use in foodstuffs (Klein, 2003; Ribeiro et al., 2014; Song et al., 2015).

The safety profile of Enterococci isolated from Cypriot green table olive, revealed that all of them were negative to the most clinically relevant antibiotics such as *vanA* and *vanB* genes. The van gene is transferable, making this antibiotic resistance the most important safety factor to be evaluated in food-grade Enterococci (Klein, 2003). It is crucial to be mentioned that the lack of the determinants of infectivity and antibiotic resistance in our strains raises optimism about their further application in fermented foods. It must be mentioned that, the presence of some virulence genes such as *gelE*, which was found in some of our isolates, cannot be considered as a negative trait, since Enterococci are a part of the spontaneous microbiota of table olives and these genes have been also found in commercial Enterococci starter cultures with a long history of safe use (Rosado et al., 2015).

6.3.5. Screening for probiotic potential

Nowadays, probiotic potential is one of the main factors in the case of choosing a starter culture. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of probiotic bacteria in the small intestine of the host. Results indicated that all

tested isolates were resistant to bile salts since the majority of them were grown successfully in MRS broth supplemented with 0.3% bile salts representing the physiological concentration of human bile (Table 6.6). Moreover, the isolates exhibited high tolerance to acidic conditions, surviving in pH 4.0, 5.0 and 6.0 (100% survival; data not shown), in pH 2 (after 1h of incubation), as well as in pH 3.0 (after 1 and 3 h of incubation), having high survival rates. In addition, after 3h of incubation at pH 2.0, none of the isolates survived (data not shown). However, all of isolates could be characterized as potential probiotics. According to other study among seven selected strains, three strains of *E. faecium* survived at pH 3.0 after 3 h (over 85%), while the other four strains showed lower survival rates (Strompfová et al., 2004). The results indicated that all tested strains could possibly survive through the human's stomach and might possess the ability to reach the intestinal environment in which they may effectively work. In the same work all tested strains survived in the presence of 1% bile salts over the rate 85%. Based on these results, *E. faecium* strains have the prerequisites to survive in the gastrointestinal tract. Finally, other researchers revealed that *Enterococcus* spp. strains from different sources could have the ability to reach the intestinal lumen and stay alive in that environment (Martin et al., 2005; Song et al., 2015). However, further in vitro studies are needed with these strains in order to establish their probiotic potential.

Table 6. 6. Percentage of isolates for survival at various rates to low pH and bile salts.

Survival rate	pH2 (1h)	pH3 (1h)	pH3 (3h)	Bile salts 0.3%
>70%	89%	57.8%	82.9%	10.9%
{70%, 80%)	11%	20.3%	9.3%	20.3%
{80%, 90%)	0%	12.5%	7.8%	34.4%
<=90%	0%	9.4%	0%	34.4%

Table 6. 7. Isolates of Enterococcus spp. surviving at different rates for various pH and exposure times or bile salts.

Survival rate	pH2 (1h)	pH3 (1h)	pH3 (3h)	Bile salts 0.3%
>70%	2, 5 - 14, 17, 18, 20, 21, 22, 24 -64	7,8,9,10,12,13,14,17,18 ,21,24,25,26,29 -39,41 -46,50,53,57,58,62,63, 64	5,7,8,9,10,11,12,13,14, 17,18,20,21,22,24-51,53,54,55,56,57,58,59,61,62,63,64	5,7,8,17,36,37,59
{70%, 80%)	1,3,4,15,16,19 ,23	5,11,27,28,40,48,49,51, 54,55,56,59,61	6,16,19,23,52,60	10,18,23,24,28,29,35,43,45,46,52,55,58
{80%, 90%)	-	6,16,19,20,22,47,52,60	1,2,3,4,15,	9,14,15,16,20,21,22,27, 30,31,39,40,41,42,50,51,53,54,56,57,60,61
<=90%	-	1,2,3,4,15,23	-	1,2,3,4,6,11,12,13,19,25,26,32,33,34,38,44,47, 48,49,62,63,64

6.3.6. Multivariate analysis of phenotypic characteristics related to probiotic potential

According to PCA, three eigenvalues had value higher than 1. The study of the contribution of variables to factors (Table 6.8) showed that Factor 1 (43,019% of variance) was related to 5 variables (acidification activity, survival to bile salts, survival to $pH2_{1h}$, survival to $pH3_{1h}$, survival to $pH3_{3h}$), Factor 2 (18,515% of variance) was related to 1 variable (lipolytic activity), and Factor 3 (13,093% of variance) to 1 variable (proteolytic activity) (Fig 6.3a). A projection of the variables on the plane formed by the first two factors (Fig. 6.3b) shows a clear relationship between the variables described above. However, they are quite scattered among them, but they can be distinguished mainly by 3 groups in which are close to each other. The first group is placed on the negative part of the two factors and involves isolates that do not combine proteolysis and lipolysis (or absence of both), having low resistance to pH but quite good resistance to bile salts (up to 75%), as well. Also, the majority of them are characterized as medium acidifiers. In the second group (negative of first factor and positive of second one), they are isolates with a greater resistance to pH and bile salts

than the first group, and proteolytic and lypolitic activity are combined (both presence and absence). However, in this group microorganisms are characterized as slow and/or medium acidifiers. In the third group, which is placed on the positive side of factor 1 and negative of factor 2, there are isolates with high survival to pH (more than 75% in pH3 after 3 hours) and bile salts (up to 90%). Furthermore, the majority of them are high acidifiers, but they do not have proteolytic and lypolytic activity. Apart from three main groups described above, there are also a few isolates separated from the rest. For example, isolates 11 and 12 have medium resistance to pH but high resistance to bile salts (93% and 100% respectively). They are also a few low acidifiers, but they have both proteolytic and lipolytic activity. Furthermore, another group of 4 isolates (1, 2, 3, 4) was characterized by high values of acidification, while they have high resistance to pH (average 74% to pH2 and 84% to pH3 after 3hours) and bile salts (100%, 97% 100%, and 94%, respectively). None of these isolates have proteolytic and lipolytic activity. Isolate 16 which is depicted a bit away from the others (on positive side of factor 1 and negative of factor 2) presents intermediate values for all the parameters. Based on those results, we conclude to 7 isolates (1, 2, 3, 4, 11, 12, 16) having possibly promising technological, probiotic attributes and safeness, as described above.

Table 6. 8. Contribution of variables (proteolytic activity, lipolytic activity, acidification activity, survival to pH2 for 1h, survival to pH3 for 1h, survival to pH3 for 3h and survival to bile salts) to the factors in the PCA based on correlations.

Variable	Factor 1	Factor 2	Factor 3
Proteolytic activity	<0.01	<0.01	0,715
Lipolytic activity	<0.01	0.661	0,261
Acidification activity	0.179	0,362	<0.01
Survival to $pH2_{1h}$	0.533	0.011	0,251
Survival to $pH3_{1h}$	0.557	<0.01	0,122
Survival to $pH3_{3h}$	0.545	<0.01	0,124
Survival to bile salts	0.080	<0.01	<0.01

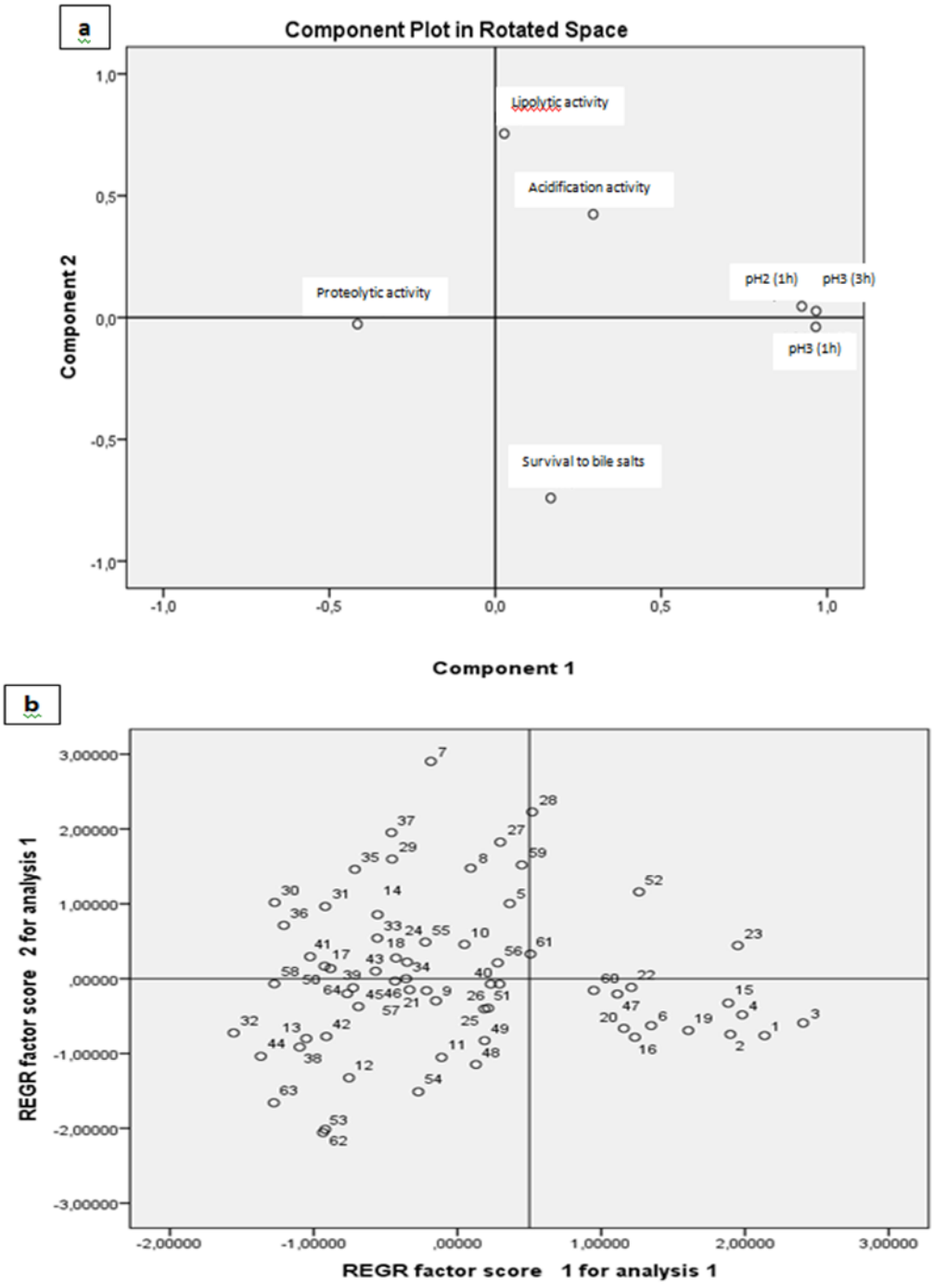

Fig. 6. 3. Projection of the variables (a) cases (b) isolates onto the plane formed by the first two factors.

6.4. Conclusions

Enterococci could become the predominant group during highly brining content of table olives. It seems that some strains could find possible applications in food

industry, due to their interesting technological and probiotic properties, such as their resistance to low pH and to bile salts. However, a deep screening regarding pathogenicity is mandatory prior any application. Finally, more research is required in those essential characteristics and scientists should give more attention in the future in the genus as it becomes apparent that is a dominant group of microorganisms in fermented food products.

Chapter 7: Epilogue

Table olives is more than a fermented product. It is the most widely consumed fermented food in the Mediterranean countries, with a high nutritional and economic contribution to consumers and producer countries, respectively. However, despite its crucial impact, its processing is still inconstant, leading to the production of an unstable final product, ranging from year to year, depending on regional practices, climate conditions etc. The latter could be easily shown by the results of the present thesis. Specifically, it was highlighted the different microbiological profile as well as the different predominant microflora of Cypriot olives, fermenting by the 2 main processes applied in Cyprus **(Chapter 2** & **Chapter 6).** Consequently, the standardization of fermentation process is a must, in order to produce a stable final product, without risk concerns.

While industry demands new methods to improve the processing of table olives, most innovations are still at a research stage. Thus, it is clear that we stand among the old and the next generation processing method of table olives, aiming to produce stable and reproductive products, worldwide. The most significant advances in table olive processing are represented by using microorganisms with positive technological traits. Nowadays, several starter cultures are being available in the market to enhance fermentation performances. Although there in an increasing interest in their application, they are still not commonly used. This is because it is uncertain whether the desirable control of the process can be achieved, since the commercial strains have not been optimized for this particular fermentation. On this point, according to the results of the present thesis, the use of the commercial starter *L. plantarum* to drive both Cypriot **(Chapter 2)** and Picual **(Chapter 3)** olives processing, gave very promising findings, raising the optimism for starter culture application by the Cypriot industry. In particular,

the starter culture dominates the indigenous microflora, leading to the fermentation control by higher brine's acidification and faster degradation of oleuropein, indicating the shortened of fermentation completion. Moreover, the reduction of sodium content (from 10 to 7%), which is one of the main challenges of Cypriot and global industry, resulted in a successful lactic fermentation. The final products fulfilled microbiological criteria and exhibited more appreciated sensorial characteristics. In addition, the production of table olives with low salt content is healthier and more suitable for consumers at risk of hypertension, opening a new era for table olives industry. The effect of table olives inoculum for the production of stable quality final product is not dependent on the alternating indigenous microflora. Overall, the findings of the present thesis enhance the contribution of starter culture, raising in parallel the possibility to produce table olives with reduced salt content. Further, the use of the commercial starter to ferment other table olives cultivated in Cyprus (i.e *Kalamon*, *Conservoeli*a, *Chalkidikis*) is strongly recommended.

Taking into account that table olives are a suitable matrix to convey probiotics, while autochthonous starters could be more easily adapted and drive the fermentation process, the study of multifunctional characteristics of indigenous microflora, to select the most appropriate strains to be used as starters, is of great importance both to industry and consumer demand for a more added value functional product. This thesis provided the multifunctional properties, with regards to technological and probiotic potential, of novel LAB **(Chapter 4 & 6)** and yeast **(Chapter 5)** strains isolated from Cypriot green cracked table olives. Primary, a pool of assays has been proposed for the first time, in order to establish a pre-selection protocol, for the selection of the most appropriate strains. A series of strains showed promising probiotic and technological

characteristics, as was proven by PCA, which were similar or even superior to the reference probiotic strains. As the next step, further investigation is proposed for those strains, such as experiments using animal models and experimental application at industrial-scale fermentation, to exemplify both their potential health benefits as well as their contribution to fermentation improvement. Finally, a meta-analysis after clinical trial is also recommended, using volunteers who will consume the product, in order to verify if the probiotic starter appears in their faeces, certifying the health benefits that could provide to their intestinal.

Methodologies, such as water potential and electrical conductivity, used for the first-time during olives processing, provided strong indications that they could be used as markers for the monitoring of fermentation progress. On this point, further studies and development of kinetic models are required to establish a validated protocol.

Finally, NGS analysis fill in the limitations and knowledge gaps of culture-dependent methodologies. The microbiota during table olives fermentation that could not imprint with classical molecular methods, can now be highlighted. This indicates the need of such kind of works, to improve our knowledge regarding microbiota formation during table olives fermentation.

Summarizing, the results of the present thesis have great significance both to industry and scientific aspects and can be further explored as follows:

- The brine inoculation with appropriate starter cultures is strongly recommended at industrial scale, resulting in an accelerated fermentation, ensuring stability, added value, functionality and safety of the final product.
- The common use of high amount of sodium or any other alternative salt-based in the brine can be reduced with the aid of an appropriate starter, avoiding any

risk concern. Specifically, in the case of Cypriot and Picual olive fermentation, the reduction of salt concentration close to 7 % seems feasible, meeting both guidelines advices and the trend of modern nutrition for the consumption of food products with low salt content. Experimental attempts to reduce salt to even lower levels (~5%) are also proposed.

- The potential use of the selected LAB and yeasts strains may turn Cypriot table olives into a novel functional food with high added value, providing new perspectives for the table olive industry.
- The use of both water potential and electrical conductivity as fermentation markers, could be useful tools for the industry, to easily determine the fermentation course.
- The use of NGS analysis could be the 'key' to better understand the interactions between microbial communities and biochemical attributes, occurring during fermentation process. The combination of microbiome with the formation of volatile compounds would be of great interest. This method could be also used in the near future for the determination of microbiome-based authenticity of table olives (microbial terroir), leading to the potential certification of geographical indication.

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Thesis deliverables

Research articles & reviews in Peer-Reviewed journals

- **Anagnostopoulos D.A.,** Kamilari E. and Tsaltas D. (2020). Evolution of Bacterial Communities, Physicochemical Changes and Sensorial Attributes of Natural Whole and Cracked Picual Table Olives During Spontaneous and Inoculated Fermentation. Front. Microbiol. 11:1128. https://doi.org/10.3389/fmicb.2020.01128
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- **Anagnostopoulos D.A,** Kamilari E., Papademas P. & Tsaltas D. (2018). Identification of authenticity and enhacement of the competitiveness of local traditional products of the Agri-Food sector. 5th ISEKI_Food 2018, July 2018, Stuttgart, Germany.
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Abstracts in National Conference Proceedings

❖ **Anagnostopoulos D.A.,** Kamilari E., Antoniades A. & Tsaltas D. (2019). Bacterial communities of whole and cracked Picula table olives during spontaneous and inoculated fermentation; a metagenomic approach. 13ο Συνέδρειο Χημείας Ελλάδας-Κύπρου. Θεματική ενότητα 'ΑΓΡΟΤΑΥΤΟΤΗΤΑ', Νοέμβριος 2019, Λευκωσία, Κύπρος.

❖ Kamilari E., **Anagnostopoulos D.A.,** Antoniades A., Kamilaris A., Papademas P. & Tsaltas D. (2019). Cyprus Halloumi bacterial communities' characterization through metagenomic sequencing. 13ο Συνέδρειο Χημείας Ελλάδας-Κύπρου. Θεματική ενότητα 'ΑΓΡΟΤΑΥΤΟΤΗΤΑ', Νοέμβριος 2019, Λευκωσία, Κύπρος.

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