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SCHOOL OF FOOD, BIOTECHNOLOGY AND DEVELOPMENT

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Study on biofilm-related microorganisms implicated in table olive fermentation

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Μελέτη βιοϋμενίων από μικροοργανισμούς που σχετίζονται με τη ζύμωση της επιτραπέζιας ελιάς

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Διδακτορική Διατριβή

Αθήνα, 2016

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Study on biofilm – related microorganisms implicated in table olive fermentation
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Στον πατέρα μου

Abstract

Fermentation is one of the oldest and most widespread methods for food preservation that appeared with the dawn of civilization. Table olives are among the post popular agricultural products of the Western world that are consumed as fermented. The fermentation process aims at the complete or partial removal of the phenolic compound oleuropein, which makes olive drupes too bitter for direct consumption upon harvest, and the development of a final product with enhanced sensory and preservation characteristics. Table olive fermentation has been attributed to the selective action of microorganisms originating from the olive's microbiota, mainly lactic acid bacteria (LAB) and yeasts. Until recently, the population dynamics of the microbial groups driving the fermentation process, mainly LAB and yeasts, was monitored in the cover brines whereas little attention was given on the analysis of the microbiota adhered on the surface of olive drupes forming mixed microbial communities known as biofilms. However, in the last years the focus has been shifted from the brine to olive drupes given that the food finally consumed is the olives whereas the brine is discarded. Hence, the main objective of the present thesis was to study the predisposition of selected starter cultures, mainly LAB and yeasts, to attach on the surface of the drupes and develop biofilms during fermentation, as well as the determination of their survival during the process.

Within this concept, selected microorganisms previously isolated from industrially fermented olive brines and reported for their functional properties (Argyri et al. 2013; Bonatsou et al. 2015) were first screened *in situ* for their ability to exploit the micro-architecture of olive surface for attachment and biofilm formation (Chapter 2). For this purpose, thermally processed (sterilized) table olives lacking the

indigenous microbiota and sterile brine solutions with physicochemical conditions (i.e., salt content, pH, acidity, fermentable substrates) resembling the industrial practice were used. The employed microorganisms were the LAB *Lactobacillus pentosus* B281 and the yeast *Pichia membranifaciens* M3A. Based on the obtained results, salt concentration in the brine highly affected the growth and dominance of the LAB strain while yeast growth was less affected. In most brining treatments, both strains were able to colonize the olive surface in high numbers ranging from 6.0–7.0 log CFU/g and 5.0–5.5 log CFU/g for *L. pentosus* B281 and *P. membranifaciens* M3A, respectively. Aggregates of the inoculated strains on the olive surface were further observed by Scanning Electron Microscopy (SEM) with microorganisms mostly located in stomal openings and discontinuations of the epidermis. The results showed the predisposition of the selected strains to adhere and form biofilms on olives and gave rise to further investigation on their potential use as starter cultures in fermentation conditions where they would have to compete with the interfering indigenous biota.

In this sense, *L. pentosus* B281 and *L. plantarum* B282 where used as starters during fermentation of Halkidiki green olives in low (8%) and high (10%) initial salt brines according to Spanish-style processing, while *L. pentosus* B281 and *P. membranifaciens* M3A were used as starters during fermentation of Conservolea natural black olives in 8% initial salt brine according to Greek-style processing (Chapters 3 and 4). The use of *L. pentosus* B281 resulted in a final product with appropriate physicochemical characteristics and good sensory properties in both processing styles. In the case of Spanish-style processing (Chapter 3), strain B281 dominated over strain B282 when they were co-inoculated in the brines regardless of salt concentration, while the latter LAB strain showed low imposition over the

indigenous LAB population during processing in high salt (10%) brines. In the case of Greek-style processing, the selected yeast starter could not be recovered from the olive surface at the end of fermentation, despite the high adherence on olives at the onset of the process (100%). However, the presence of the yeast starter resulted in proper fermentation and the final product was characterised by good sensory attributes and a milder acidic taste. In contrast, *L. pentosus* B282 either as single or combined culture with the yeast statrter could successfully colonize the surface of black olives presenting high recovery rate (100%) at the end of fermentation. Therefore, the use of a mixed starter culture (LAB/yeast) in the fermentation of black olives could result in the development of a final product that could be suitable for consumers who do not appreciate the sour taste of olives and prefer milder tastes.

In the present thesis, it was further shown that LAB and yeasts, the fermenting microbiota of table olives, can colonize and form biofilm communities on the surface of plastic vessels where fermentation takes place and most importantly they can be persistent in cleaning treatments (Chapter 5). The most frequently isolated and characterized yeasts belonged to *Candida* spp., followed by *Wickerhamomyces anomalus*, *Debaryomyces hansenii* and *Pichia guilliermondii* which are common members of the yeast fermenting microbiota of table olives. Regarding LAB species, *L. pentosus* was the most abundant species recovered from the biofilm. These results showed that biofilm development on the surface of fermentation vessels may serve as a natural means of brine inoculation with the necessary technological microbiota to support fermentation.

key words: olive fermentation, biofilm microbial community, *Lactobacillus pentosus, Lactobacillus planetarium,* starter cultures with functional properties

Περίληψη

Η ζύμωση είναι μία από τις παλαιότερες και πλέον διαδεδομένες μεθόδους για την επεξεργασία και συντήρηση των τροφίμων η οποία εμφανίστηκε από τις απαρχές της ανθρωπότητας. Οι επιτραπέζιες ελιές αποτελούν ένα από τα πιο σημαντικά τρόφιμα φυτικής προέλευσης στο Δυτικό κόσμο που καταναλώνονται ως ζυμωμένο προϊόν. Η διαδικασία της ζύμωσης έχει ως βασικό στόχο την μερική ή ολική απομάκρυνση της φαινολικής ουσίας ελευρωπαΐνη, η οποία ευθύνεται για την πικρή γεύση του νωπού ελαιοκάρπου καθιστώντας το μη εδώδιμο, και την ανάπτυξη ενός τελικού προϊόντος με βελτιωμένα οργανοληπτικά και φυσικοχημικά χαρακτηριστικά που θα εξασφαλίζουν την μικροβιολογική σταθερότητα του προϊόντος ακόμη και σε θερμοκρασία περιβάλλοντος, αλλά και την αποδοχή του από τον καταναλωτή. Η ζύμωση της επιτραπέζιας ελιάς οφείλεται στην επιλεκτική δράση της αυτόχθονης μικροχλωρίδας του καρπού, με την τελική επικράτηση των οξυγαλακτικών βακτηρίων και των ζυμών. Μέχρι πρόσφατα, η μελέτη της δυναμικής της μικροχλωρίδας κατά τη ζύμωση πραγματοποιείτο αποκλειστικά στην άλμη, χωρίς να λαμβάνεται υπόψη η δυνατότητα των μικροοργανισμών να προσκοληθούν στην επιφάνεια του καρπού και να σχηματίσουν μεικτές βιοκοινότητες γνωστές ως βιοϋμένια. Τα τελευταία χρόνια όμως το ενδιαφέρον της επιστημονικής κοινότητας έχει μετατοπιστεί από την άλμη στον καρπό της ελιάς, δεδομένου ότι το προϊόν που τελικά καταναλώνεται είναι ο καρπός ενώ η μητρική άλμη απορρίπτεται. Ως εκ τούτου, το αντικείμενο έρευνας της παρούσας διδακτορικής διατριβής ήταν η μελέτη της δημιουργίας βιοϋμενίων στην επιφάνεια του ελαιοκάρπου κατά τη ζύμωση με την εφαρμογή επιλεγμένων μικροοργανισμών που χρησιμοποιούνται ως καλλιέργειες

εκκίνησης, καθώς επίσης και ο προσδιορισμός της επιβίωσής τους κατά την επεξεργασία του καρπού.

Στο πλαίσιο αυτό, επιλεγμένοι μικροοργανισμοί οι οποίοι είχαν προηγουμένως απομονωθεί από άλμες ζύμωσης ελιάς σε βιομηχανική κλίμακα και μελετηθεί για τις προβιοτικές και τεχνολογικές τους ιδιότητες (Argyri et al. 2013; Bonatsou et al. 2015), εξετάστηκαν αρχικά για την *in situ* ικανότητά τους να προσκολλώνται στον ελαιόκαρπο και να δημιουργούν βιοϋμένιο (Κεφάλαιο 2). Για το σκοπό αυτό, χρησιμοποιήθηκαν θερμικά επεξεργασμένες (αποστειρωμένες) ελιές χωρίς την παρουσία αυτόχθονης μικροχλωρίδας και αποστειρωμένα διαλύματα άλμης με φυσικοχημικές συνθήκες (αλατότητα, pH, οξύτητα, ζυμώσιμα συστατικά) ανάλογες με αυτές που χρησιμοποιεί η μεταποιητική βιομηχανία της επιτραπέζιας ελιάς στη χώρα μας. Οι μικροοργανισμοί που επιλέχθηκαν ως καλλιέργειες εκκίνησης ήταν το οξυγαλακτικό βακτήριο *Lactobacillus pentosus* B281 και η ζύμη *Pichia membranifaciens* M3A. Τα αποτελέσματα έδειξαν ότι η αυξημένη συγκέντρωση άλατος στην άλμη (10%) επηρέασε σε μεγάλο βαθμό την ικανότητα αύξησης του οξυγαλακτικού βακτηρίου, σε αντίθεση με τη ζύμη της οποίας ο πληθυσμός παρουσίασε μικρές διακυμάνσεις. Στις περισσότερες συνθήκες που μελετήθηκαν οι δύο μικροοργανισμοί μπόρεσαν να αποικίσουν τον καρπό σε μεγάλους πληθυσμούς που κυμαίνονταν μεταξύ 6,0–7,0 log CFU/g και 5,0–5,5 log CFU/g για το βακτήριο *L. pentosus* B281 και τη ζύμη *P. membranifaciens* M3A, αντίστοιχα. Η παρουσία βιοϋμενίου επάνω στον καρπό επιβεβαιώθηκε περαιτέρω με παρατήρηση σε ηλεκτρονικό μικροσκόπιο σάρωσης, με τους μικροοργανισμούς να συγκεντρώνονται κυρίως στα στομάτια του καρπού και σε σημεία όπου υπήρχε ασυνέχεια της επιδερμίδας. Από τα εν λόγω αποτελέσματα προέκυψε ότι οι μικροοργανισμοί που

μελετήθηκαν έχουν την προδιάθεση προσκόλλησης και σχηματισμού βιοϋμενίου στην επιφάνεια του ελαιόκαρπου. Παράλληλα, οδήγησαν στην ανάγκη περαιτέρω μελέτης για τη χρήση τους ως καλλιέργειες εκκίνησης σε συνθήκες ζύμωσης διαφορετικών εμπορικών τύπων επιτραπέζιας ελιάς (πράσινες ελιές Ισπανικού τύπου, φυσικές μαύρες ελιές) όπου θα έπρεπε να ανταγωνιστούν την υπάρχουσα αυτόχθονη μικροχλωρίδα κατά την επεξεργασία.

Σε αυτή τη λογική, τα οξυγαλακτικά βακτήρια *L. pentosus* B281 και *L. plantarum* B282 χρησιμοποιήθηκαν στη συνέχεια ως εναρκτήριες καλλιέργειες κατά τη ζύμωση πράσινων ελιών ποικιλίας «Χαλκιδικής» σύμφωνα με την Ισπανική μέθοδο σε αρχική άλμη χαμηλής (8%) και υψηλής (10%) αλατοπεριεκτικότητας, ενώ το βακτήριο *L. pentosus* B281 και η ζύμη *P. membranifaciens* M3A χρησιμοποιήθηκαν ως εναρκτήριες καλλιέργειες κατά τη ζύμωση φυσικής μαύρης ελιάς ποικιλίας «Κονσερβολιά» σε αρχική άλμη 8%, σύμφωνα με την πρακτική που εφαρμόζεται από τη βιομηχανία στη χώρα μας (Κεφάλαια 3 και 4). Τα αποτελέσματα έδειξαν ότι η χρήση του βακτηρίου *L. pentosus* B281 οδήγησε στη δημιουργία τελικού προϊόντος με επιθυμητά φυσικοχημικά χαρακτηριστικά και καλές οργανοληπτικές ιδιότητες τόσο στην επεξεργασία ισπανικού τύπου όσο και στη ζύμωση της φυσικής μαύρης ελιάς. Στην περίπτωση της ζύμωσης ισπανικού τύπου (Κεφάλαιο 3), το βακτήριο *L. pentosus* B281 επικράτησε του *L. plantarum* B282 όταν ενοφθαλμίστηκαν στην άλμη ως συγκαλλιέργεια ανεξάρτητα από την αρχική αλατότητα της άλμης, ενώ το δεύτερο στέλεχος, όταν εμβολιάστηκε ως μονοκαλλιέργεια σε άλμη υψηλής αλατότητας (10%) επέδειξε χαμηλή ικανότητα επικράτησης έναντι της αυτόχθονης μικροχλωρίδας των οξυγαλακτικών βακτηρίων. Στην περίπτωση της ζύμωσης φυσικής μαύρης ελιάς, το επιλεγμένο στέλεχος της

ζύμης, παρά το υψηλό ποσοστό προσκόλλησης στον καρπό στο αρχικό στάδιο της επεξεργασίας (100%), δεν κατάφερε να επικρατήσει και να ανακτηθεί στο τέλος της ζύμωσης. Ωστόσο, η χρήση οδήγησε σε φυσιολογική ζύμωση με το τελικό προϊόν να χαρακτηρίζεται από καλά οργανοληπτικά χαρακτηριστικά και ήπια αίσθηση της οξύτητας. Αντίθετα, το βακτήριο *L. pentosus* B282, είτε ως μονοκαλλιέργεια είτε ως συγκαλλιέργεια με τη ζύμη, επικράτησε κατά την επεξεργασία και ανακτήθησε σε ποσοστό 100% στο τέλος της ζύμωσης. Επομένως, η εφαρμογή μικτής καλλιέργειας εκκίνησης (οξυγαλακτικού βακτηρίου/ζύμης) κατά τη ζύμωση του φυσικού μαύρου ελαιόκαρπου θα μπορούσε να συμβάλλει στη δημιουργία ενός τελικού προϊόντος με ήπια γεύση που απευθύνεται σε καταναλωτές που δεν εκτιμούν ιδιαίτερα την όξινη γεύση στην επιτραπέζια ελιά.

Στη συνέχεια, μελετήθηκε η δυνατότητα προσκόλλησης της αυτόχθονης μικροχλωρίδας και η δημιουργία βιοϋμενίου στην επιφάνεια της δεξαμενής ζύμωσης, καθώς επίσης και ο ποσοτικός και ποιοτικός χαρακτηρισμός των μικροοργανισμών του βιοϋμενίου σε διαφορετικές συνθήκες εξυγίανσης της δεξαμενής (Κεφάλαιο 5). Τα αποτελέσματα έδειξαν ότι οι μικροοργανισμοί που αποτελούν την επικρατούσα μικροχλωρίδα κατά τη ζύμωση, οξυγαλακτικά βακτήρια και ζύμες, έχουν την ικανότητα προσκόλλησης στην εσωτερική επιφάνεια του περιέκτη και δημιουργία βιοϋμενίων τα οποία επιβιώνουν έναντι των συνθηκών απολύμανσης. Κατά το χαρακτηρισμό του πληθυσμού των ζυμών βρέθηκε ότι τα πιο συχνά απαντώμενα είδη ήταν *Candida* spp. ακολουθούμενα από τα είδη *Wickerhamomyces anomalus*, *Debaryomyces hansenii* και *Pichia guilliermondii,* ενώ όσον αφορά στο χαρακτηρισμό των οξυγαλακτικών βακτηρίων, το βακτήριο *L. pentosus* ήταν το πιο συχνά ευρισκόμενο είδος. Τα αποτελέσματα δείχνουν ότι η δημιουργία βιοϋμενίων

στην επιφάνεια των δεξαμενών ζύμωσης μπορεί να λειτουργήσει ως ένας φυσικός τρόπος ενοφθαλμισμού της άλμης με μικροοργανισμούς που διαθέτουν τα κατάλληλα τεχνολογικά χαρακτηριστικά για να υποστηρίξουν τη ζύμωση.

Λέξεις κλειδιά: ζύμωση ελαιοκάρπου, μικροβιακή κοινότητα βιοϋμενίων, *Lactobacillus pentosus, Lactobacillus plantarum,* καλλιέργειες εκκίνησης με λειτουργικές ιδιότητες.

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Chapter **1**

Table olive fermentation

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Table olive fermentation

1.1. Introduction

The olive tree *Olea europaea* L. ssp. *europaea,* is among the oldest crops of the Mediterranean basin and one of the best biological indicators of the Mediterranean climate (Blondel et al. 2010). The exact geographic origins, the time and the reasons of its early exploitation are still open to controversies (Breton et al. 2009; Kaniewski et al. 2012; Besnard et al. 2015). The domestication of the olive tree was characterized by the selection and propagation of the most valuable trees and establishment of olive orchards (Breton et al. 2009). Based on archaeological remains, it is believed that the olive tree was first domesticated during the early Neolithic period in the Near East (Galili et al. 1989) and it was later introduced into the West of the Mediterranean Region via human migrations. The cultivation of the olive tree has been practiced from antiquity to modern times for its wood (utensils, furnishing, manufacturing, solid fuel) and for its fruits used in olive oil and table olive production (Riley 2002; Kaniewski et al. 2012). Especially the fruits of the olive tree are considered as one of the most extensively cultivated fruit crops in the world. The annual world consumption of table olives has steadily increased during the last two decades reaching approximately 2,398,300 tons from 2008/9 to 2013/14 crop seasons (IOC 2014). The world table olive production of 2014/15 crop season is estimated at 2,554,500 tones the majority of which comes from countries in the European Union (IOC 2014). Spain is the leader of producer countries followed by Greece and Italy while also Turkey, Egypt, Algeria, Argentina, Syria, Morocco, USA and Peru are major non European producer countries.

Olives are botanically classified as drupes and anatomically consist of three component tissues namely, the epicarp or skin, the mesocarp or flesh and the endocarp or pit which encloses one or, rarely, two seeds (Garrido-Fernández et al. 1997). The epicarp comprises a layer of epidermal cells rich in chloroplasts and is covered by a thin cuticle. The mesocarp, rich in protoplasm, surrounds the endocarp which progressively sclerifies during fruit development (Connor and Fereres 2005).

Fruit development, which starts approximately 30 days after fertilization and fruit set, is completed within 4 to 5 months and generally involves cell division, cell expansion and storage of metabolites. The primary stage of fruit development is characterized by intense cell division resulting in rapid growth of the endocarp with little mesocarp. During the middle stage, the drupes are covered by epicuticular wax layer, the mesocarp cells have developed vacuole cells while the endocarp has completely sclerified and its enlargement has ceased. Then a period of marked fruit growth follows due to expansion of pre-existing mesocarp cells. At the same time intense oil synthesis and accumulation in the mesocarp is observed which continues with a slower rate until the maturation/ripening phase. Upon maturation the color of the drupes changes from lime green to purple-black and the texture of the flesh becomes softer and easier to squash until some juice is released (Connor and Fereres 2005; Conde et al. 2008). Depending on the developed color of the fruit upon harvest they are categorized in (i) green olives, harvested at the early stage of maturity prior to coloring, having obtained the appropriate size, (ii) black olives, harvested at the full stage of maturity or slightly earlier, having attained deep violet black color, and (iii) turning color olives, harvested between the two stages presenting a wine – rose color (Garrido- Fernández et al. 1997; IOC 2004).

Nutrient components are present at the highest percent in the 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 (Wodner et al. 1988; Nergiz and Engez 2000; Marsilio et al. 2001a, b; Sakouhi et al. 2008; López-López et al. 2009; Lanza et al. 2013). Upon harvest the lipid content is dominated by oleic acid followed by palmitic acid, linoleic acid and stearic acid. Carbohydrates in olive drupes are represented mainly by soluble reducing sugars such as glucose and fructose and non reducing sugars such as mannitol. Their concentration is lower in comparison with any other drupes since they act as precursors for fatty acids synthesis during fruit growth (Wodner et al. 1988). The sugar content that remains serves further as carbon source for the development of desired microorganisms during table olive production (Garrido-Fernández et al. 1997). Complex sugars such as lignin, hemicellulose, cellulose and pectin are also distributed in the olive fruit. Lignin is present in the stone while the rest of the polymers are present in the mesocarp playing substantial role in the structural characteristics of olive flesh (Kailis and Harris 2007; Lanza et al. 2010). The protein content is low but of high quality due to the presence of essential amino acids for adults (threonine, valine, leucine, isoleucine, phenylalanine and lysine), and for children (arginine, histidine and tyrosine) (Lanza et al. 2010; Lanza et al. 2013). Phenolics are also present with oleuropein, a secoiridoid glucoside, being the representative phenolic compound and responsible for the bitter taste of the fruit. Its concentration decreases during ripening (Amiot et al. 1986) giving rise primarly to hydroxytyrosol and other simple phenolics (Bianchi 2003). Other simple phenolics present in the mature fruit are tyrosol, homovanillic alcohol, caffeic acid, coumaric acid, phloretic acid, vanillic acid (Boskou et al. 2006) contributing also to sensory and aromatic characteristics of the olive as well as impart pharmaceutical and physiological benefits (Tassou 1993; Kountouri et al. 2007; Omar 2010; Ghanbari et al. 2012).

1.2. Table Olive processing

1.2.1. Commercial preparations of table olives

Olive fruits when freshly picked are too bitter due to the high phenolic fraction they contain and thus unsuitable for direct consumption. In order to make them edible they need to undergo a series of processes involving complete or partial removal of the bitter content and eventually the development of a final product with enhanced sensory and preservation characteristics. The final product, "table olive", is defined by the International Olive Council as "the product prepared from the sound fruit of varieties of the cultivated olive trees (*Olea europea* L.) that are chosen for the 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; table olives are treated to remove their bitterness and preserved by natural fermentation, or by heat treatment, with or without the addition of preservatives; packed with or without covering liquid" (IOC 2004).

Depending on the method used for the debittering step table olives are broadly categorized in treated and untreated/natural olives. The difference lies in the use of lye/alkali treatment in the case of treated olives which results in the quick and complete removal of bitterness. Specifically, the drupes are immersed into a weak solution of sodium hydroxide which penetrates the fruit's epidermis and hydrolyzes the ester bond of the phenolic hydroxytyrosol with the rest of the oleuropein molecule (Brenes and de Castro 1998). After lye treatment the drupes are washed to remove the excess of alkali and further stored in brine, where complete or partial lactic acid fermentation takes place. The treatment of green olives in alkaline solution is

commonly applied in Spain, particularly in Seville. This type of processing, also known as "Spanish – style" method represents the first most important commercial preparation in the international table olive market (Garrido- Fernández et al. 1997; Sánchez Gómez et al. 2006) and is schematically illustrated in Figure 1.1.

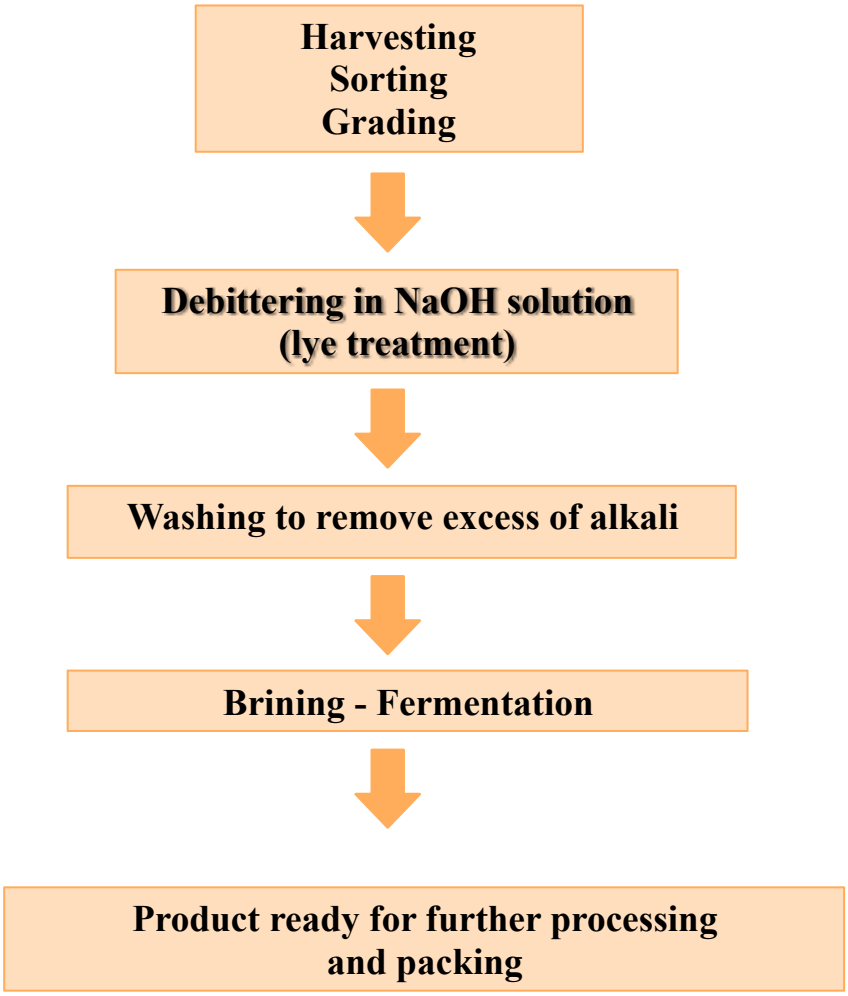

Figure 1.1. Flow diagram for the processing of Spanish-style green olives in brine.

Untreated olives on the other hand are naturally debittered without any prior alkali treatment. The most common practice to obtain natural olives is by directly placing the drupes in brine where the bitter phenolic compounds are diffused from the olive's mesocarp to the surrounding brine. In parallel, lactic acid fermentation develops

which may further assist the debittering process due to the reported ability of certain lactic acid bacteria (LAB) species to hydrolyze oleuropein (Ciafardini et al. 1994; Servili et al. 2006; Landete et al. 2008; Kaltsa et al. 2015). Green, black and turning colour olives can be prepared in this way. Natural green olives in brine are commercially known as "Siciliano style" olives (Colmargo et al. 2001). Natural black olives in brine are the main trade preparation in Greece, also known as "Greek style" in the international market, representing a widespread commercial preparation (Balatsouras 1990) (Fig. 1.2).

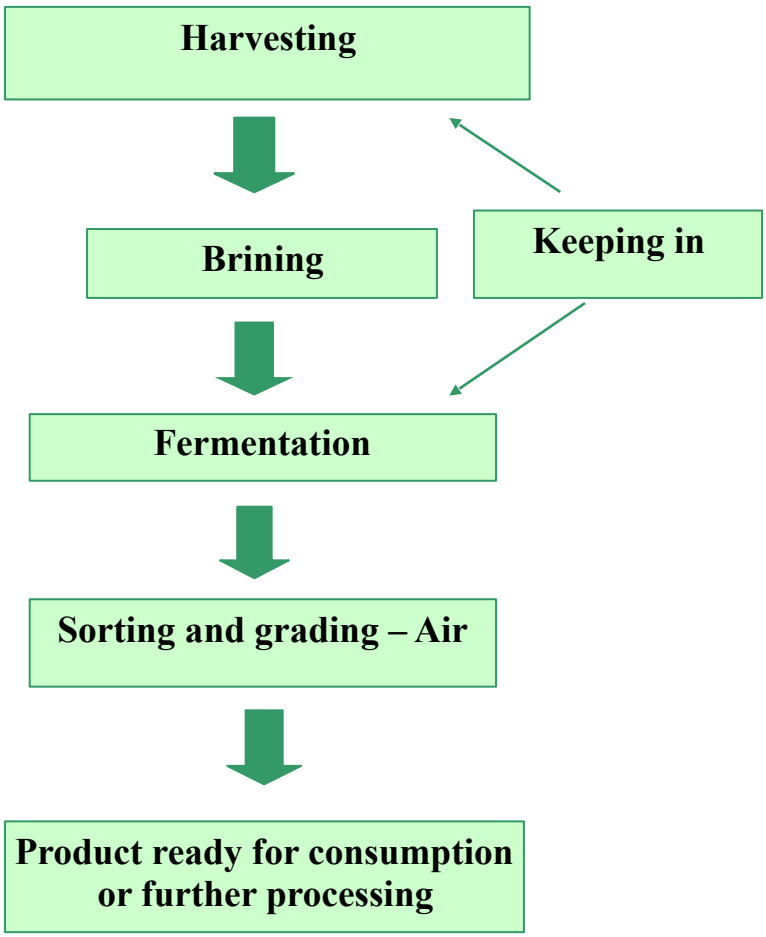

Figure 1.2. Flow diagram for the processing of Greek-style natural black olives in brine.

Another special type of untreated olives is obtained by placing the raw drupes between layers of coarse salt resulting in a product completely different from the other

preparations. This is mainly applied to fruits harvested at the stage of full ripeness and the final product is known as “naturally black dry salted olives” (Fig. 1.3). Due to high osmosis induced by the coarse salt the water content leaks out of the fruit while the salt is taken up by the olive (Panagou 2006). It has been hypothesized that along with the water content other solutes including oleuropein flow out resulting in the progressive debitterness of the fruit. Recently, it has been reported that olive debittering during the dry-salting process is due to enzymatic oxidation, in particular polyphenol oxidase (PPO) activity. The use of salt causes the rapture of the tissue thereby putting oleuropein component into contact with PPO (Ramírez et al. 2013).

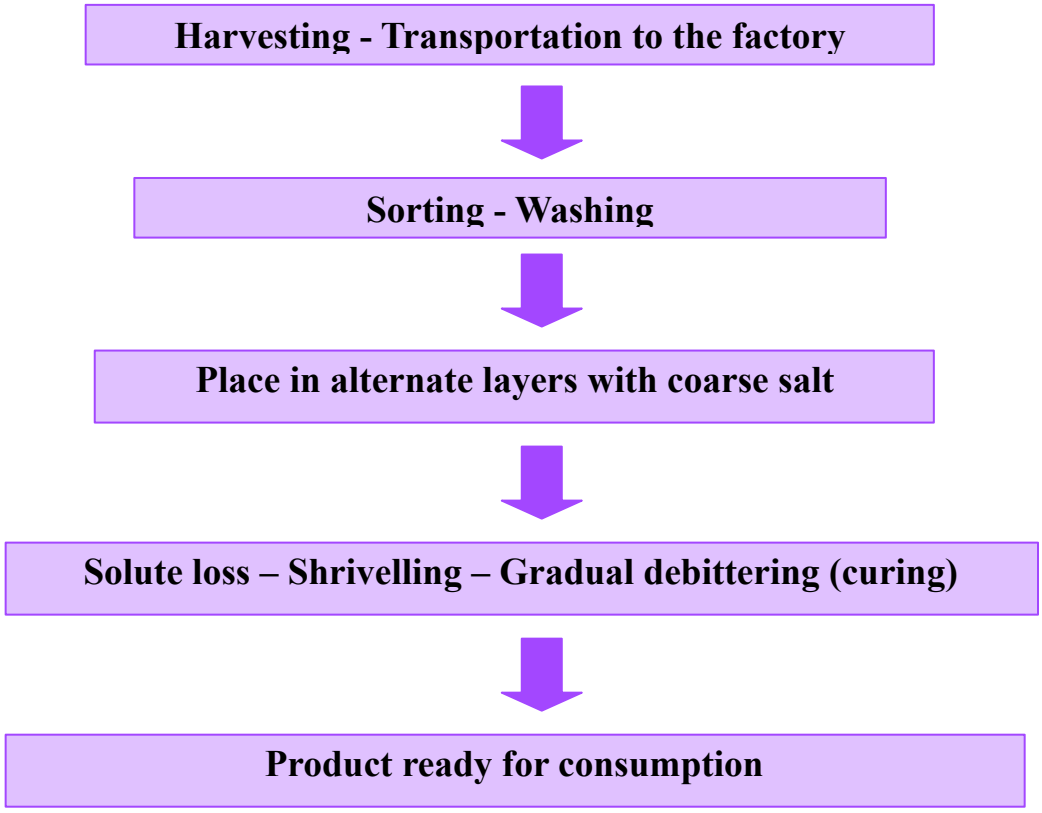

Figure 1.3. Flow diagram for the processing of natural black olives in dry salt.

Olives are finally shrivelled and characterized by a salty, bitter, and sweet taste. The physicochemical characteristics of the product are 4.5-5.5 pH, 0.75-0.85 water activity, 30-39g/100g moisture content, 35-39g/100g oil content, 2.0-2.5g/100g

reducing sugars and 10g/100g NaCl content of the flesh (Balatsouras 1995) Due to their high salt content, their share in the international market is relatively small and limited in the countries of the Mediterranean region (Panagou 2006).

Another commercially important elaboration of treated table olives is the black ripe olive processing technology which involves artificial darkening (oxidation) of the drupes in an alkaline solution (Fig. 1.4). This technology was initiated in California at the beginning of the 20th century, therefore olives processed in this way are also known as "Californian-style" olives. The fruits are subjected to three successive treatments with sodium hydroxide solution on three consecutive days, penetrating skin, 1-2 mm into the flesh, and to the pit, respectively. Between treatments, the drupes are immersed in water or dilute brine through which air is bubbled. Olives darken progressively, both in the flesh and on the surface and, once the colour is obtained, more water is added and aeration continues until the pH is around 7-8 units. Then, iron salts (ferrous gluconate or lactate) are added to stabilize black color. After 1 day at equilibrium, the product is canned and sterilized (Brenes-Balbuena et al. 1992, Marsilio et al. 2001a).

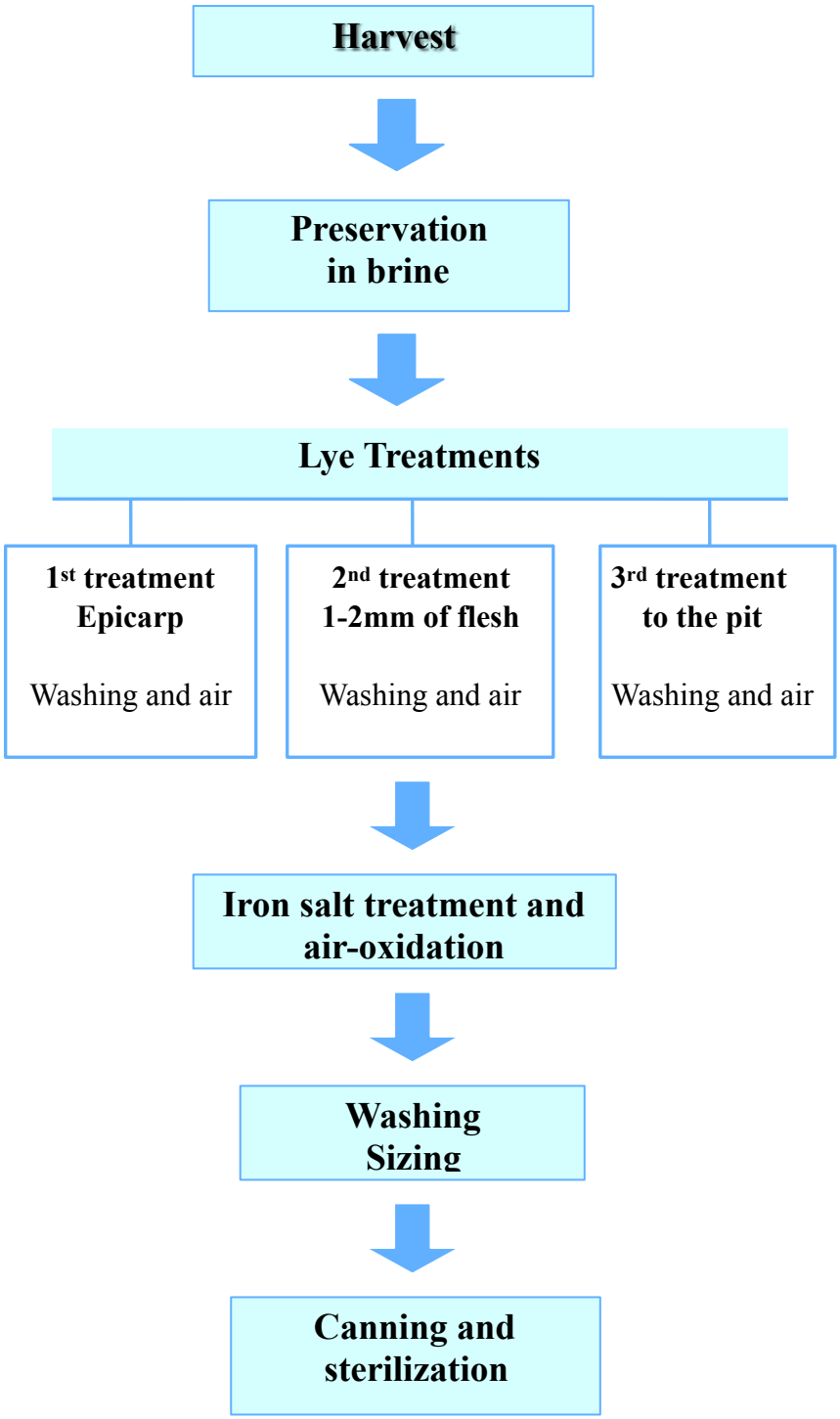

Figure 1.4. Flow diagram for the processing of Californian-style black ripe olives (adapted from Marsilio et al. 2001b).

The abovementioned processes reflect the fact that olive preparation may significantly vary depending on regional and national practices. All the different commercial preparations under which table olives are distributed in the market have

been previously elucidated in detail (Sánchez Gómez et al. 2006; Heperkan 2013) while the name of each one of them includes information on the ripeness stage of the harvested drupe (green, black or turning colour), the procedure used in the debittering step (treated or natural) and the preservation method (brining or dry salting) (Garrido-Fernández et al. 1997). In terms of international trade, the International Olive Council has adopted a unified qualitative standard applying to table olives wherein five commercial/trade preparations are established and described, namely (i) treated olives , (ii) natural olives, (iii) dehydrated and/or shrivelled olives, (iv) olives darkened by oxidation, and (v) specialities (IOC 2004). Based on this classification, treated and natural olives undergo a fermentation process the aspects of which will be further discussed.

1.2.2. Fermentation process

Fermentation is one of the oldest and most widespread methods of food preservation that appeared with the dawn of civilization. There are numerous examples of meat, milk, grain and vegetable derived foods and beverages consumed as fermented such as wine, beer, cheese, yogurt, kefir, sauerkraut, pickles, olives, sausages and many others. The action of microorganisms, mainly LAB and yeasts, results in the production of organic acids, alcohol and carbon dioxide through which preservation is achieved while also other produced metabolites enhance the sensory attributes of the product (Campbell-Platt 1994; Caplice and Fitzgerald 1999; Ross et al. 2002; Farnworth 2005; Bourdichon et al. 2012)

Table olives are of the most important fermented vegetables. The fermentation process starts spontaneously once the drupes, previously treated with alkali or not, are placed in brine. The whole process can be divided into three stages, namely (i) primary stage, lasting up to 15 days, (ii) middle stage, lasting up to 3 weeks, and (iii) last stage, until the end of fermentation (Tassou 1993; Balatsouras 1995; Garrido-Fernández et al. 1997).

During the primary fermentation stage, the brine is conditioned by nutrients diffused from the mesocarp serving as substrate for the growth of microorganisms coming from the olive's indigenous biota that is highly heterogeneous comprised by

Gram negative and Gram positive bacteria as well as yeasts and fungi (Tassou 1993; Balatsouras 1990). Species belonging to *Pseudomonas, Aeromonas, Flavobacterium* genera as well as enterobacteria form the group of Gram negatives are present during the initial stage of olive fermentation. Their presence has been associated with the formation of "gas pockets" resulting in softening of the fruit. These microorganisms while metabolizing sugars release carbon dioxide which is accumulated as pockets of gas either hypocuticularly or intramesocarpically (Lanza 2013). Macroscopically, hypocuticular gas appears as bubbles on the olive surface. Intramesocarpical gas results in the formation of gas fissures in the mesocarp and a narrow belt on the skin, a condition known as "fish eye" or "alambrado" (Sánchez Gómez et al. 2006; Lanza 2013). Other important Gram positive bacteria that may prevail during this stage and cause malodorous fermentation are *Clostridium* and *Bacillus* species. Butyric fermentation is caused by the action of butyric acid bacteria, such as *Clostridium butyricum, Clostridium beijerinckii, Clostridium multifermentans, Clostridium fallax.* Apart from the rancid butter smell they are also responsible for gas fissures formation in the olive's mesocarp (Gililland and Vaughn 1946). The precence of *Bacillus* species such as *Bacillus subtilis* and *Bacillus pumilus* has been associated with pectinolytic activity resulting in softening of the drupes (Nortje and Vaughn 1953). These microorganisms are present during the first days of fermentation and progressively decrease. By the end of the primary stage, LAB such as *Lactococcus, Pediococcus* and *Leuconostoc* prevail. Heterofermentative *Leuconostoc* and homofermentative *Pediococcus* cocci dominate the middle stage of the fermentation producing lactic acid which increases the acidity and lowers the pH in the brine. The developed acidity further favours the growth of lactobacilli. The dominance and fast growth of lactobacilli coupled with the production of lactic acid in high amounts characterize the last stage of the fermentation and guarantee the preservation properties of the final product. The process is considered complete once the fermentable substrates are exhausted. Meanwhile, the pH in the brine is maintained at around 4 (Hurtado et al. 2012). The lactobacilli group is represented mainly by *Lactobacillus plantarum* and *L. pentosus* and to a lesser extent by *L. paracasei, L. casei, L. brevis*. Non lactobacilli

species such as *Enterococcus faecium, Enterococcus casseliflavus* and *Leuconostoc mesenteroides* have also been reported (Hurtado et al. 2012).

Once the fermentation process is finished, the drupes are stored in the brine. Brines with properly fermented olives contain mainly lactic and acetic acids whereas the presence of succinic, citric, malic and tartaric acida has also been reported to lower concentrations (Bleve 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 "zapatera" spoilage. The first off odours to appear have been described as "cheesy" and as the spoilage progresses fecal odour develops. Mainly *Propionibacterium* species via propionic and acetic acid production (Plastourgos and Vaughn 1957) and *Clostridium* species (Kawatomari and Vaughn 1956) are the microorganisms associated with this condition. To control development of these bacteria, the pH should be kept <4.0 and the NaCl in the brine should be raised to >8% after the end of fermentation (Lanza 2013).

Apart from LAB, yeasts are also present throughout the process. *Candida, Pichia, Debaryomyces*, *Wickerhamomyces* and *Saccharomyces* are the main genera associated with table olive processing while their role can be either beneficial or detrimental (Arroyo-López et al. 2012b). For 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 (> 8 (w/v) % NaCl) the yeasts outnumber the LAB resulting in a final product with milder taste and less self-preservation characteristics due to lower acid production (Tassou et al. 2002; Hurtado et al. 2009; 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 (Vaughn et al. 1972). Some strains of *Rhodotorula minuta*, *W. anomalus* and *D. hansenii* have been reported to produce enzymes such as proteases, xylanases and pectinases causing softening of the fruits (Hernández et al. 2007; Bautista-Gallego et al. 2011b). Furthermore, their presence in packaged olives may cause clouding of the brines (Arroyo-López et al. 2005). On the beneficial side, yeasts may produce metabolites such as higher

alcohols, esters and other volatile compounds contributing in aroma and flavour development (Garrido-Fernández et al. 1997). Other technological properties such as degradation of polyphenols, production of killer toxins and enhancement of LAB growth have been reported (Psani and Kotzekidou 2006; Hernández et al. 2007; Aponte et al. 2010; Bautista-Gallego et al. 2011b; Silva et al. 2011; Bonatsou et al. 2015) while also the scientific interest in their probiotic potential and use as starter cultures is increasing (Psani and Kotzekidou 2006; Etienne-Mesmin et al. 2011; Silva 2011; Bevilacqua et al. 2013).

1.2.3. Control of the fermentation process

Olive fermentation is a spontaneous process influenced by factors such as pH, water activity, availability of nutrients, the level of salt concentration in the brine, phenol content (Spyropoulou et al. 2001; Tassou et al. 2002; Hurtado et al. 2009). During an uncontrolled fermentation development of spoilage microorganisms and deterioration of the product is likely to occur leading to economic losses for the table olive industry.

Constant adjustement of the salt concentration and acidification of the brine are the main industrial practices to control the process and avoid abnormal fermentation (Balatsouras 1990; Garrido–Fernández et al. 1997). During brining, the initial salt concentration is progressively taken up by the olive mesocarp and its level in the brine decreases until an equilibrium between fruit and brine is reached. Under low salt conditions, the survival of Gram negatives may be prolonged. In order to avoid spoilage the salt level should be progressively raised up to 8.5-9.5% in equilibrium. Acidification of the brine at pH 4.0-4.5 at the primary stage of fermentation is another common industrial practice in order to restrain the action of Gram negatives that are abundant during this stage. As a result, the duration of the primary stage decreases giving rise to the desired LAB growth. Lactic acid and acetic acid are widely used as acidifying agents for this purpose.

The use of starter cultures has also been proposed as a way to better control olive fermentation since it decreases the risk of spoilage and accelerates the course of fermentation and acidification of the brine (Montaño et al. 1993; Garrido-Fernández et al. 1997; Spyropoulou et al. 2001; Panagou and Tassou 2006; Corsetti et al. 2012).

Different cultures have been studied for different cultivars and preparation methods with focus mainly on *L. plantarum* and *L. pentosus* as the main species associated with fermented olives (Montaño et al. 1993; Panagou and Tassou 2006; Panagou et al. 2008; Peres et al. 2008; Sabatini et al. 2008; Hurtado et al. 2010; Perricone et al. 2010; Caballero-Guerrero et al. 2013; Pistarino et al. 2013; Randazzo et al. 2014; Rodríguez-Gómez et al. 2014). Their use has been employed as single or co-inoculated cultures and their effectiveness strongly depends on the type of cultivar and processing method (Panagou and Tassou 2006).

The selection of potential starter cultures is generally based on the following criteria: (i) fast and predominant growth, (ii) homofermentative metabolism, leading to lactic acid production and consequently to high acidification rate, (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 low temperatures, and (vi) ability to resist freezing or lyophilisation if required for commercial purposes (Hurtado et al. 2012). Apart from their use as protective cultures and their effectiveness in accelerating the fermentation, other functional properties such as bacteriocin production are desired. The bacteriocin producer strain *L. plantarum* LPCO10 was first isolated from green olive fermentation (Jiménez–Díaz et al. 1993) and reported to produce bacteriocins S and T with the former being active against *Propionibacterium* and *Clostridium* spoilage bacteria. Its later use as a starter culture in Spanish-style green olive fermentation (Ruiz-Barba et al. 1994) resulted in bacteriocin production in the brine and predomination over other *Lactobacillus* strains. Further on, the final concentration of lactic acid was higher in brines inoculated with *L. plantarum* LPCO10 strain than in uninoculated brines as well as brines inoculated with another non bacteriocin producing strain. Other bacteriocin producer strains have been studied and it seems that factors such as temperature, salt concentration in the brine and the presence of other bacteria affect bacteriocin production (Delgado et al. 2005, 2007; Ruiz-Barba et al. 2010; Hurtado et al. 2011). Recently, research on table olives has been shifted on the probiotic potential of starter cultures enhancing thus the significance of table olives from a traditional agricultural

product to a high added value functional food that provides new perspectives for the table olive sector (Peres et al. 2012).

1.3. Table olives as functional food

"Let food be thy medicine and medicine be thy food". This philosophy which was first adopted by Hippocrates, the father of medicine, was again embraced by the modern man in the 20th century. Modern nutrition emphasizes in a diet rich in vegetables, fruits, grains, legumes and low saturated fat, in order to reduce the risk of chronic diseases. Towards this "food as medicine" sense, there is an increasing interest in production and consumption of health promoting products. This has led the food industry to the development of a diverse range of food products with enhanced characteristics known as "functional foods" (Hasler 2002; Betoret et al. 2011).

Functional foods were first introduced as a concept in Japan in 1980s (Arai 1996) and today the Functional Food Science in Europe (FUFOSE) proposed a working definition 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." (EC 2010). With the aim of developing functional foods, probiotics have received increasing attention over the years. The term "probiotic" means "for life" and the concept is generally attributed to Nobel Prize winner Éli Metchnikoff who first suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff 1907). Today, probiotics can be defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001). *Lactobacillus* and *Bifidobacteria* are the main genera including bacterial strains with health promoting properties. Intake of probiotics contributes to the maintenance of the intestinal microbial balance of the host by inhibiting pathogens and lowers the risk of

gastrointestinal diseases. They are attributed with anticarcinogenic, antihypertensive and immunomodulatory properties while also alleviate certain intolerances, such as lactose intolerance and prevent food allergies (Salminen et al. 1998; Wollowski et al. 2001; Cross 2002; Commane et al. 2005; Tang et al. 2010). So far, the majority of probiotic foods found in the market are milk based products such as yogurts, cheeses and fermented milks (kefir). Due to the increasing number of individuals faced with lactose intolerance and/or milk protein allergy, the need for manufacturing non-dairy probiotic products arises (Gupta and Abu-Ghannam 2012).

In recent years, table olives have attracted the attention of the scientific community for their use as biological carriers of microorganisms with health promoting properties, transforming thus a traditional product into a novel functional food. The first attempt to incorporate probiotic microorganisms on table olives was published by Italian researchers (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 strains tested were able to adhere and colonize the olive surface and most of them exhibited high survival rates (above 10^6 CFU/g) after 90 days of storage. Furthermore, one of the strains, *L. paracasei* IMPC2.1, was recovered from four out of five human fecal samples from healthy volunteers after daily consumption of olives containing about 10^9 to 10^{10} viable cells. In another work (De Bellis et al. 2010), the same strain was used both as probiotic and as starter culture in the fermentation process of *cv* Bella di Cerignola green olives leading in a final product with functional appeal.

The main source of probiotic isolates is the human gastrointestinal tract meaning that the selected starter cultures are allochthonous to olives. The limitations of allochthonous starters have been previously mentioned (Di Cagno et al. 2013) while the exploitation and use of autochthonous strains with functional properties has been proposed (Vitali et al. 2012, Di Cagno et al. 2013). In this sense, a number of LAB originating from table olive environment has been screened for functional properties. Recently, a total of 144 LAB isolates obtained from naturally fermented

Aloreña green olives were studied for their potential as probiotic starter cultures (Abriouel et al. 2012). Fifteen *L. pentosus* strains and one *Lc. pseudomesenteroides* strain from this study exhibited antimicrobial properties and tolerance to low pH and high bile salt concentration. In another work, lactobacilli isolates from different spontaneous industrial green olive fermentations were selected by Italian and Spanish researchers (Bautista-Gallego et al. 2013a) by *in vitro* phenotypic tests related to probiotic potential. In the same line, four *L. pentosus* strains, previously isolated from diverse table olive processing and selected according to *in vitro* phenotypic tests related to probiotic potential, were used as starters to better control Spanish-style green olive fermentation (Rodríguez-Gómez et al. 2013). Moreover, the probiotic potential of LAB isolated from naturally fermented olives by *in vitro* probiotic tests has been also reported (Argyri et al. 2013). In this work, four strains of *L. pentosus*, three strains of *L. plantarum* and two strains of *L. paracasei* were found to possess desirable *in vitro* probiotic properties similar or superior to the reference probiotic strains *L. rhamnosus* GG and *L. casei* Shirota. Two of them, namely *L. pentosus* B281 and *L. plantarum* B282 were selected and used in the present thesis as starters during Spanish style fermentation of green olives (Blana et al. 2014) and heat shocked green olives (Argyri et al. 2014) in low and high salt brines. In both studies, the strain B281 was found to be more effective in terms of survival ability under the different salt levels in the brine while it also colonized the olive surface in higher populations than the strain B282. The first study is also part of the present thesis and more detailed discussion of the results can be found in the corresponding chapter (Chapter 3).

1.4. Biofilm development in table olive fermentation: Role and significance

Biofilm formation in food processing environments has been the focus of extensive scientific research, especially in the context of food hygiene, as many outbreaks have been associated with the presence of biofilms by foodborne

parthogens in food industries (Zottola and Sasahara 1994; Srey et al. 2013). On the other hand, biofilm formation by non pathogenic microorganisms may pose positive effects. Their role is vital for the health of ecosystems by decontaminating polluted sites and breaking down sewage (Hunter 2008). Biofilms by certain rhizobacteria species on plant roots have been found to induce plant growth and protect plants from phytopathogens (Bogino et al. 2013). The exploitation of industrial *Bacillus subtilis* and related species biofilms as biocontrol and bioremediation agents has been previously proposed (Morikawa 2006), while biofilms by bacteriocin producing LAB have also been reported to exhibit antilisterial activities (Guerrieri et al. 2009).

Biofilms are defined as functional consortia of microorganisms attached to a surface which are embedded in the extracellular polymeric substances (EPS) produced by the microorganisms (Monds and O'Toole 2009). It has long been established that microorganisms in natural environments tend to adhere to any solid surface, either biotic or abiotic, immersed in a liquid medium and assemble themselves in a complex multispecies consortium often embedded in a extracellular polymeric matrix (EPS) produced by the microorganisms (Sutherland 2001; Donlan 2002; Hall-Stoodley et al. 2004; Elhariry 2011). One of the prerequisites for biofilm development is a process known as "conditioning film" which involves the accumulation of nutrients at the solid surface immersed in the liquid medium (Donlan 2002). After film conditioning, microorganisms may adhere on the contact surface, gradually form microcolonies and finally assemble themselves in biofilms exhibiting high microbial diversity in terms of genera, species and strain level (Rickard et al. 2003; Burmølle et al. 2006).

In the case of table olives, the fermentation process can be described as a biofilm phenomenon. During the process, the brine solution is progressively enriched with fermentable sugars outflowing from the mesocarp. This may serve as a means of conditioning film on olive's epicarp and on the surface of the container where

fermentation takes place. Thus, in accordance to biofilm formation theory, the enriched brine supports the role of the liquid medium while the drupes and the fermentation container support the role of biotic and abiotic sites of adherence and biofilm development, respectively.

The first evidence supporting that olive drupes serve as a site of attachment and biofilm formation was published by Nychas et al. (2002). The authors, using Scanning Electron Microscope (SEM) observed aggregates of bacteria and yeasts in the stomal openings of naturally black *cv* Conservolea olives, with bacteria predominating in the intercellular spaces of the sub-stomal cells. More recently, Spanish researchers observed biofilm formation of LAB and yeast communities on the olive surface of Spanish-style green olives of Manzanilla (Arroyo-López et al. 2012a) and Gordal cultvivars (Domínguez-Manzano et al. 2012). In another study (Lavermicocca et al. 2005), the ability of different *Lactobacillus* and *Bifidobacterium* probiotic species to adhere and colonize the olive's epicarp was investigated. As observed by SEM all strains tested were able to adhere the olive surface retaining high populations during storage. One of them, *Lactobacillus paracasei* IMPC2.1, was selected and successfully employed during debittered green olives *cv* Bella di Cerignola fermentation (De Bellis et al. 2010) and later proposed as a suitable strain for the development of probiotic table olives (Sisto and Lavermicocca 2012).

The formation of biofilms on the abiotic surface of fermentation vessels, on the other hand, has received little attention by table olive research. To our knowledge, only two studies have been recently published focusing on this aspect. In the first study, the establishment of polymicrobial communities on glass slides that come into contact with the brine during Spanish style table olive fermentation was investigated (Domínguez- Manzano et al. 2012). The authors confirmed the ability of microorganisms to adhere and produce biofilms on the abiotic surfaces. In the second

study, which is part of the present thesis, the quantification and characterization of biofilm community formed on the surface of plastic containers used in the fermentation of green table olives at different sampling locations and different cleaning treatments of the vessel was investigated (Grounta et al. 2015). It was shown that multi-species biofilm communities were formed on the fermentation vessels exhibiting persistence during the applied cleaning treatments. Among LAB species, *L. pentosus* was the most abundant species recovered from the biofilm. The most frequently detected yeasts were characterized as *W. anomalus, D. hansenii* and *P. guilliermondii* which are common members of the yeast fermenting microbiota of table olives. More detailed discussion and presentation of the results can be found in the corresponding chapter (Chapter 5)

1.5. Scope and outline of the thesis

The scope of the present thesis was to study table olive fermentation as a biofilm phenomenon as a result from the action of microorganisms originating from the olive fermenting microbiota. The interest in this aspect was driven by recent findings in table olive research, indicating that the microorganisms isolated from olive indigenous microbiota, primarily LAB and yeasts, apart from their role in fermenting and finally transforming the harvested drupes into a product with enhanced sensory characteristics, may also exhibit a number of promising technological and functional properties. Until recently, the population dynamics of fermentation was routinely monitored in the cover brines whereas little attention was given on the analysis of the microbiota adhered on the surface of olive drupes forming biofilm communities that can transform table olives from a traditional agricultural product into a novel food with potential functional appeal.

Within this concept, selected microorganisms previously isolated from industrially fermented olive brines and reported for their functional potential, were first screened *in situ* for their ability to exploit the micro-architecture of olive surface for attachment and biofilm formation (Chapter 2). In this initial stage, thermally processed (sterilized) olives were used in the experiments to avoid the interfering indigenous microbiota that could hamper the activity of the inoculated cultures for attachment on olives. The obtained results were further extended in natural fermentation of both Spanish style green olives (Chapter 3) and Greek style black olives (Chapter 4), as both processing procedures constitute two major commericial preparations in the international market. In the case of Spanish style processing, the selected starter cultures were two LAB species, namely *Lactobacillus pentosus* and *Lactobacillus plantarum*, as these two species are the driving force of lactic acid fermentation of green olives. In contrast, for Greek style black olives, the selected microorganisms were a LAB species, *Lactobacillus pentosus*, and a yeast species, *Pichia membranifaciens*, commonly isolated from black olive fermentations. Further on, the potential of the fermenting microbiota of table olives to adhere and form biofilms on the surface of fermentation vessels was also elucidated (Chapter 5), together with the persistence of biofilm communities in various cleaning treatments.

Chapter **2**

Mono and dual species biofilm formation between *Lactobacillus pentosus* and *Pichia membranifaciens* on the surface of black olives under different sterile brine conditions

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Abstract

The purpose of this work was to investigate the *in situ* predisposition of *Lactobacillus pentosus* B281 grown in both monoculture and co-culture with the yeast *Pichia membranifaciens* M3A to adhere on the surface of black olives and develop single and mixed microbial communities under different sterile brine conditions. Black oxidized olives were submerged in brine solution and inoculated with an initial load of 5.0 log CFU/mL *L. pentosus* or with a combined culture of 5.0 log CFU/ml *L. pentosus* and *P.membranifaciens*. Two initial salt concentrations in the brines were investigated, namely 6 % and 10 %, corresponding to low and high salt brine, respectively. Brines were supplemented with (1) 0.2 % (v/v) lactic acid, (2) 0.5 % (w/v) glucose, and (3) both 0.2 % (v/v) lactic acid and 0.5 % (w/v) glucose. A brining treatment with no supplementation of glucose and lactic acid was also studied as a control treatment. Each brining condition was studied in duplicate at 20 °C for a period of 30 days. The population dynamics of the inoculated strains on the surface of olives were determined by plate count, whereas olive samples were observed at the end of storage under scanning electron microscopy (SEM). Results showed that, in the case of low salt brines, high population levels between 6.5 and 7.0 log CFU/g were reached on olive drupes for *L. pentosus* in both single and mixed culture inoculation regardless of brining treatment. However, in high salt brines no cells of the microorganism could be recovered from the control and glucose supplemented brines in the case of single culture inoculation as well as in acidified brines with/without glucose in mixed inoculations. The presence of biofilm on the surface of olives was

also confirmed by SEM. Aggregates of the lactic acid bacteria and the yeast could be observed located mostly in the stomatal apertures and on the epidermis.

2.1. Introduction

Lactic acid bacteria (LAB) include industrially important genera that are responsible for the fermentation of various foods, contributing to sensory characteristics and preservation of the final product (Leroy and De Vuyst 2004; Hurtado et al. 2012); health-promoting properties have also been attributed to many LAB strains (Vitali et al. 2012; Di Cagno et al. 2013). Table olives are a popular fermented food with worldwide acceptance that supports a fundamental food production sector in the Mediterranean region. The estimated world production of table olives for the crop season 2012/2013 was estimated at 2,315,000 tonnes with similar consumption volume (IOC 2013). The elaboration of this food has evolved throughout the years based on local practices and long tradition. However, from the industrial perspective, the International Olive Council (IOC) recognizes three main commercial preparations, namely (1) treated olives, (2) natural olives, and (3) olives darkened by oxidation (IOC 2004) for which elaboration processes have been well established (Garrido-Fernández et al. 1997; Sánchez Gómez et al. 2006). It is generally accepted that fermentation is a basic step in table olive processing, and is undertaken mainly by LAB in treated olives, and by a mixed community of LAB and yeasts in natural olives, thus inhibiting the growth and survival of other undesirable or hazardous microorganisms and enhancing the sensory attributes of the final product (Garrido-Fernández et al. 1997; Romeo 2012). The most representative species belong to the *Lactobacillus* genera, namely *L. pentosus*, *L. plantarum* and to a lesser extent *L.*

paraplantarum (Ercolini et al. 2006; Hurtado et al. 2008; Abriouel et al. 2012; Cocolin et al. 2013). The important contribution of yeasts in the process has been reviewed recently (Arroyo-López et al. 2008, 2012b; Bevilacqua et al. 2009, 2012).

Biofilms are defined as structured communities of microbial cells enclosed in a self-produced polymeric matrix adherent to abiotic or biotic surfaces (Steenackers et al. 2012). Microbial cells embedded in this matrix may communicate with each other and present a coordinated group behaviour mediated by a process known as quorum sensing (Coenye and Nelis 2010). In food environments biofilms are found to be highly complex communities exhibiting high microbial diversity at genus, species and strain level (Burmølle et al. 2006; Kawarai et al. 2007; Kubota et al. 2008). This is also the case with table olive fermentation, where diverse microbial groups compete in a dynamic process to define the physicochemical characteristics, microbiological stability and sensory properties of the final product. Until recently, the population dynamics of the microbial groups driving the fermentation process, mainly LAB and yeasts, was monitored in the cover brines whereas little attention was paid to the analysis of the microbiota adhering to the surface of olive drupes. However, in recent years the focus has shifted from the brine to olive drupes, taking into account that the food finally consumed is the olives whereas the brine is discarded. In this sense, aggregates of LAB and yeasts colonizing the surface of natural black olives were first described by Nychas et al. (2002) using scanning electron microscopy (SEM), while more recently similar observations describing mixed LAB-yeast communities on the epidermis of green olives have been reported by Spanish researchers (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012). In addition, Italian researchers (Lavermicocca et al. 2005; Sisto and Lavermicocca 2012) have investigated the

colonization and biofilm development on olive drupes inoculated with selected strains of lactobacilli and bifodobacteria in an attempt to use table olives as a carrier of probiotic microorganisms. Along the same lines, the changes in population dynamics of LAB and yeasts adhered on olive drupes during inoculated fermentation of green olives with selected starter cultures of LAB with probiotic potential have been investigated (De Bellis et al. 2010; Blana et al. 2014; Rodriguez-Gómez et al. 2013) but no attempt was made in these works to verify the presence of biofilms on olive drupes by SEM observation.

The purpose of the present study was to investigate the in situ predisposition of *L. pentosus* B281, grown in single culture and co-culture with the yeast *P. membranifaciens* M3A, to adhere and form biofilm on the surface of black olives under different brining treatments commonly employed by the Greek table olive industry. This LAB strain has been isolated previously from industrially fermented Greek table olives (Doulgeraki et al. 2013) and was investigated recently for its *in vitro* probiotic potential (Argyri et al. 2013). The selected yeast species is among the dominant microorganisms involved in the fermentation and storage of natural black olives (Nisiotou et al. 2010b; Doulgeraki et al. 2012) and it has been proposed by some authors to be employed in table olive preparations due to its desirable technological properties such as killer toxin production, which contributes to LAB maintenance and probiotic properties (Marquina et al. 1997; Silva et al. 2011).

2.2. Materials and methods

2.2.1. Microorganisms and preparation of inocula

The LAB *L. pentosus* (strain B281) in mono-culture and its co-culture with the yeast *P. membranifaciens* (strain M3A) were studied for their ability to colonize and form biofilm on the surface of black olive drupes. Both microorganisms belong to the culture collection of the Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, and have been isolated previously from the brines of black olive fermentations. LAB and yeast strains were stocked and maintained at −80 °C in de Man-Rogosa-Sharpe broth (MRS; Lab M, Heywood, UK) and YEPD (1 % yeast extract, 2 % peptone, 2 % glucose) broth, respectively, containing 20 % glycerol as a cryoprotective agent. The LAB strain was revived by adding 10 μL the stock culture to 10 mL MRS and incubating at 30 °C for 24 h.Working cultures were obtained by adding 100 μL revived culture to 10 mL MRS broth containing 6 % (w/v) NaCl and incubating at 30 °C for 24 h. The yeast strain was revived by adding 10 μL stock culture in 10 mL YEPD broth and incubating at 25 °C for 48 h with agitation. Working cultures were obtained by adding 100 μL revived culture in 10 mL YEPD broth and incubating at 25 °C for 24 h with agitation. LAB and yeast cells were centrifuged (5,000 g, 10 min, 4 °C), washed twice with sterile quarter strength Ringer's solution (Lab M) and resuspended in 10 mL sterile quarterstrength Ringer's solution resulting in a final concentration of ca. 9.0 log CFU/mL and 7.0 log CFU/mL for *L. pentosus* and *P. membranifaciens*, respectively, as assessed by plate counting.

2.2.2. Table olive samples and brining conditions

Commercial black oxidized table olives cv. Halkidiki were obtained from a table olive industry in a 5 kg can that was thermally processed (sterilized) by the manufacturer. The olives, harvested at the green stage of maturation, were subjected

initially to a series of dilute NaOH solutions for debittering. Between lye treatments they were suspended in water through which air was bubbled, resulting in progressive darkening of the drupes (oxidation process). Subsequently, they were treated with water to remove the residual alkali, placed in 3 % brine with ferrous gluconate to stabilize the black color and subjected to sterilization. This type of olives was selected as experimental material in order to exclude any interfering microorganisms and ensure that the formation of biofilm was due to the presence of the inoculated cultures only. Prior to opening, the external surface of the container was thoroughly sprayed with pure ethanol to avoid any incidence of contamination and left to dry inside a laminar flow cabinet. Subsequently, the container was opened under aseptic conditions and a sample (25 g) of olives was subjected to microbiological analysis to ensure absence of any contaminating microbiota. Olive samples (two olive drupes weighing ca. 12 g each) were placed in falcon tubes (50 mL capacity) and covered with 20 mL brine solution. Two different initial salt concentrations were prepared, namely 6 % and 10 % (w/v), to assess the potential of the selected microorganisms to colonize the drupes and form biofilms in both low and high salt brines. In addition, the brines were supplemented with 0.2 % (v/v) lactic acid (95 %, Sigma, St. Louis, MO) and/or 0.5 % (w/v) glucose. Overall, eight initial brine treatments were investigated, namely (1) 6 and 10 % salt brines without any addition of lactic acid and glucose (control treatment), (2) 6 and 10 % salt brines supplemented with 0.5 % (w/v) glucose, (3) 6 and 10 % salt brines acidified with 0.2 % (v/v) lactic acid, and (4) 6 and 10 % salt brines with 0.5 % (w/v) glucose and 0.2 % (v/v) lactic acid. All brine solutions were sterilized at 120 °C for 15 min. After 24 h of brining, in the case of mono-culture inoculation, the working culture of L. pentosus B281 was diluted

decimally and 200 μL of the second dilution was added to the brines to achieve an initial population of ca. 5.0 log CFU/mL in the brine. In the case of co-culture inoculation, 200 μL of the second decimal dilution of the working culture for *L. pentosus* B281 and 200 μL of the undiluted working culture for *P. membranifaciens* M3A were added to the brines to achieve an initial population of ca. 5.0 log CFU/mL for each microorganism. No attempt was made to inoculate the brines with *P. membranifaciens* M3A in mono-culture on the grounds that the table olive industry in Greece is oriented towards fermentation in which LAB prevail over yeasts, resulting in a process that is more lactic and less alcoholic. Thus, any attempt to develop a starter culture with commercial acceptability must include a LAB strain that will ensure the establishment of a lactic process attaining the proper values of pH and acidity to guarantee the stability of the product during storage and marketing. Overall, 22 falcon tubes were prepared per brining treatment and inoculation process and stored at 20 °C for a period of 30 days. On each sampling occasion two falcon tubes were withdrawn randomly and subjected to microbiological analysis.

2.2.3. Microbiological analysis

Olive drupes of each brining treatment were sampled at regular time intervals during the experiment for quantification of biofilm cells (11 sampling points per treatment). Olive drupes were removed carefully from the brine solution with sterile forceps and rinsed twice with 5 mL sterile Ringer's solution in order to remove loosely attached cells (Giaouris et al. 2005). The drupes were then depitted with sterile scalpel and forceps, transferred aseptically into a stomacher bag (Seward, London, UK) and diluted decimally with sterile Ringer's solution. The diluted sample

was homogenized in a stomacher device for 2 min (Lab Blender 400, Seward) at room temperature. Serial decimal dilutions in Ringer's solution were prepared and duplicate 1 or 0.1 mL from the appropriate dilution was poured or spread on agar medium. *L. pentosus* was enumerated on MRS medium (Lab M), supplemented with 0.05 % (w/v) cycloheximide (AppliChem, Darmstadt, Germany), overlaid with 10 mL molten medium and incubated at 30 °C for 48–72 h. *P. membranifaciens* was enumerated on Rose Bengal Chloramphenicol agar (RBC; Lab M) incubated at 25 °C for 48–72 h. Results were expressed as log values of colony forming units per gram (log CFU/g) of olives. Moreover, the pH in the brine was also measured routinely with a digital pH meter (model RL150, Russell, Boston, MA).

2.2.4. Scanning Electron Microscopy

At the end of storage, olive drupes were observed under a scanning electron microscope (SEM) for biofilm formation. Olive drupes were washed twice with phosphate-buffered saline (PBS) and then 3×3 mm^2 slices from the olive skin were obtained with a scalpel. Slices were fixed in 2.5 % glutaraldehyde in PBS overnight. The slices were washed five times in PBS for 5 min and then dehydrated in a series of increasing concentrations of ethanol (50 %, 70 %, 80 %, 90 %, 95% and 100%) for 5 min. Some of the slices were then dried in t-butyl alcohol (AppliChem, Darmstadt, Germany) or in critical point in a Polaron E3000 critical point dryer and finally coated with gold in a Denton Vacuum DV 502 coating unit. Samples were also treated with another protocol for SEM observation that included a freeze drying step. After fixation and dehydration, skin samples were freeze-dried in a Leybold- Hereaus GT-2 apparatus. After freeze-drying, the slices were treated with chloroform to remove the

lipids secreted from the olive skin, left to dry and finally coated with gold in a Denton Vacuum DV 502 coating unit. Images were taken with a Jeol JLM-6360 SEM (JEOL, Peabody, MA).

2.3. Results and Discussion

2.3.1. Population dynamics in mono species biofilm on olive drupes

The population changes of *L. pentosus* B281 biofilm cells under the different brining treatments assayed are illustrated in Figure 2.1. The concentration of salt in the brines strongly affected the attachment and growth of the microorganism. In all cases the brines were inoculated with ca. 5.0 log CFU/mL. However, the cells that were initially attached on the surface of olives were ca. 1–2 log cycles lower. Specifically, in low salt brines (6 % NaCl), the initially attached population (day 1) was 4.26 (±0.11) log CFU/g, while in high salt brines (10 % NaCl) it was 3.18 (±0.68) log CFU/g. At the same time, in brines supplemented with 0.5 % (w/v) glucose the initially attached population was 4.14 (±0.48) and 3.11(±0.19) log CFU/g for low and high salt brines, respectively. In the case of brines acidified with 0.2 % (v/v) lactic acid, the respective counts were 5.29 (±0.03) and 3.96 (±0.17) log CFU/g for low and high salt brines, respectively. Finally, the combination of glucose supplementation and acidification resulted in an initial attachment of the inoculated culture at 5.4 (±0.19) log CFU/g in low salt, and 4.21(±0.18) log CFU/g in high salt brines. In all treatments of low salt brines the microorganism was able to colonize the surface of olives in high numbers, exceeding 6.5 log CFU/g, that were maintained at this level throughout the storage period. However, in high salt brines, the potential of the inoculated LAB strain to colonize the surface of olives was affected by the brining treatment applied.

Specifically, in the control and glucose-supplemented brines, there was hardly any colonization of the inoculated culture on olives. In these treatments, the population of initially attached cells decreased rapidly until day 5 of storage and stayed close to the detection limit of the enumeration method (1 log CFU/g). On the contrary, in the acidified brines with/without glucose supplementation there was a reduction of the initially attached cells of ca. 2 log cycles within the first 5 days of storage, followed by a progressive increase until the end of storage, reaching population levels that were comparable with the low salt brine treatments. Our results are comparable with a previously published work (Lavermicocca et al. 2005) in which black oxidized olives were inoculated with probiotic LAB strains in mono-culture and

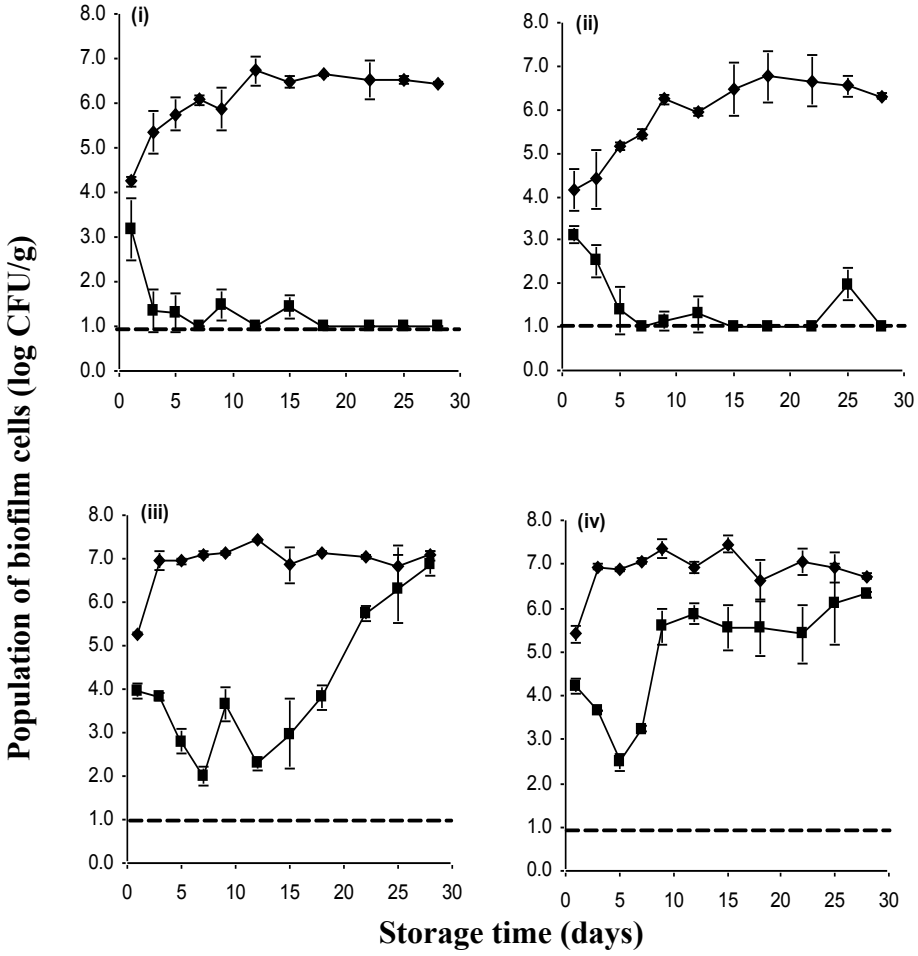

Figure 2.1. Changes in the population of *Lactobacillus pentosus* B281 in mono species biofilm development on olive drupes under 6% (◆) and 10% (■) salt brines; (i) control treatment, (ii) brines supplemented with 0.5% (w/v) glucose, (iii) brines acidified with 0.2% (v/v) lactic acid, and (iv) brines supplemented with 0.5% (w/v) glucose and 0.2% (v/v) lactic acid. Data points are average values of two replications per sampling point ± standard deviation. Dashed line indicates the detection limit of the enumeration method (1 log CFU/g).

their population dynamics were monitored in low salt brines (4 % NaCl) with pH ranging from 5.1 to 6.3 for a period of 3 months. The authors reported that the inoculated LAB strains were able to colonize the surface of olives in high populations (ca. 7.0 log CFU/g) that were maintained throughout the storage period. Salt concentration in the brines is an important factor in table olive preparations as it can influence the microbial association and the prevalence of different groups of microorganisms throughout the process. Depending on the cultivar, olives are fermented and stored in brines containing sodium chloride levels ranging from 4 % to 15 % (w/v) (Garrido-Fernández et al. 1997); however, concentrations maintained above 8 % are considered inhibitory for lactic acid bacteria (Tassou et al. 2002). In the present work, *L. pentosus* B281 was able to colonize the surface of olives in high populations in the case of low salt brines regardless of the additional treatment provided (acidification and/or sugar supplement). This is in line with a recent work (Bautista-Gallego et al. 2008) reporting that the minimum inhibitory concentration (MIC) of *L. pentosus* to salt was determined to be 82 g/L. However, as the tolerance of *L. pentosus* to salt is strain dependent, other researchers have reported higher salt levels up to 12 % (Hurtado et al. 2012; Romero-Gil et al. 2013).

The evolution of pH in the different brining treatments is shown in Figure 2.2. In the case of low salt brines the pH values were almost unchanged from a practical

point of view in the control treatment, ranging from 7.18 (±0.03) at the beginning of storage to 6.5 (±0.06) from day 7 onwards. Brine acidification with lactic acid resulted in an initial pH value of 5.5 that was maintained at this level until the end of storage. This trend in pH could be attributed to the fact that the culture was added to already processed table olives in which the fermentable substrates were limited and thus no fermentation could take place. As expected, the addition of glucose in the brines had a profound effect on pH reduction in the remaining treatments by 1.5 and 2 units for acidified/glucose and glucose supplemented brines, respectively. In the case of high salt brines, no changes in pH values could be observed throughout the 30- day storage period. This was expected for control and glucose supplemented brining treatments in which no survival of the selected LAB strain on olives could be observed. However, for the treatments containing lactic acid with/without the addition of glucose no changes in pH values could be monitored despite the fact that the inoculated culture was able to colonize the surface of olives in high populations. Lactic acid as an acidifying agent and glucose supplementation as a carbon source in the brine are often used by the Greek table olive industry to control lactic acid fermentation. Especially the addition of sugars in the brines as a mean of fast acid development has been reported previously in both green and black table olive processing (Balatsouras et al. 1983; Chorianopoulos et al. 2005; Perricone et al. 2010).

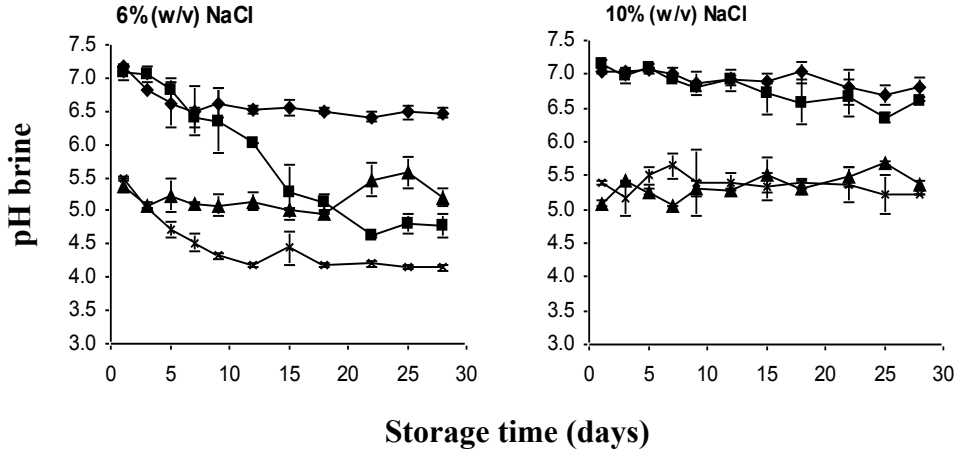

Figure 2.2. Evolution of pH in the brine during storage in the case of mono species biofilm development on olive drupes under different brining treatments: control treatment (◆), addition of 0.5% (w/v) glucose (■), acidification with 0.2% (v/v) lactic acid (▲), combination of 0.5% (w/v) glucose and 0.2% (v/v) lactic acid (×). Data points are average values of two replications per sampling point ± standard deviation.

2.3.2. Population dynamics in dual species biofilm on olive drupes

The attached population of *L. pentosus* B281 and *P. membranifaciens* M3A on olive drupes on day 1 varied between the different brining treatments (Fig. 2.3). Specifically, in low salt brines the initial attachment of both microorganisms was similar and ranged between 3.0 and 3.5 log CFU/g. However, the inoculated LAB species colonized the surface of olives more rapidly, reaching a population level that was maintained above 6.0 log units throughout storage. *P. membranifaciens* was also able to adhere on olives and form a mixed community with *L. pentosus*, but was recovered from olive skin in lower populations never exceeding 5.0 log CFU/ g during storage. It is notable that in brines supplemented with both lactic acid and

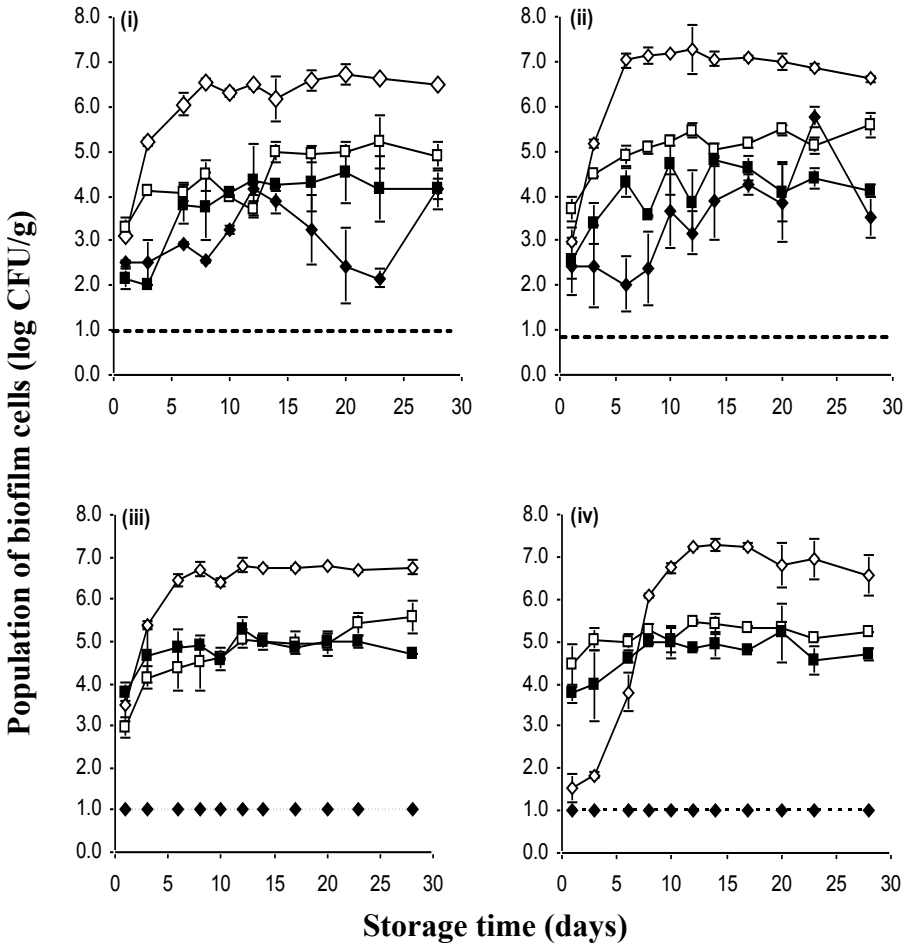

Figure 2.3. Changes in the population of *Lactobacillus pentosus* B281 (◆) and *Pichia membranifaciens* M3A (■) in dual species biofilm development on olive drupes under 6% (open symbols) and 10% (closed symbols) salt brines; (i) control treatment, (ii) brines supplemented with 0.5% (w/v) glucose, (iii) brines acidified with 0.2% (v/v) lactic acid, and (iv) brines supplemented with 0.5% (w/v) glucose and 0.2% (v/v) lactic acid. Data points are average values of two replications per sampling point ± standard deviation. Dashed line indicates the detection limit of the enumeration method (1 log CFU/g).

glucose, *L. pentosus* presented a different growth pattern compared with the other treatments. Under these conditions, the initial attachment of the bacteria was very low (ca. 1.5 log CFU/g) but the microorganism colonized the surface of olives very rapidly and became the dominant species, outgrowing the population of the yeast species that presented a similar growth profile as with the other brining treatments.

These findings are in accordance with a previous study (Nisiotou et al. 2010b) in which LAB prevailed over yeasts in brines with 6 % (w/v) NaCl, during fermentation of natural black Conservolea olives. Low salt brines favor the growth of LAB whereas high salt brines stimulate yeast growth (Tassou et al. 2002; Hurtado et al. 2009), which was also observed in our study since, in all high salt brining treatments, the yeast strain exhibited higher salt tolerance. It must be noted that in both high and low salt brining treatments the growth pattern of the yeast species on the surface of olives was similar. However, the colonization of *L. pentosus* B281 on olive skin exhibited a variable pattern. Specifically, in acidified brines with/without the addition of glucose, the inoculated LAB strain could not be recovered from olives, whereas in the remaining treatments it managed to colonize the olive skin in lower numbers compared to *P. membranifaciens* M3A. Presumably the combination of high salt and acidic conditions in the brines created an adverse environment for the survival of this LAB species. It should be noted that in high salt brines inoculated with *L. pentosus* B281 as a mono-culture, no survival of the LAB strain could be observed during storage (Fig. 2.1), but when the yeast strain was added to the brines (co-culture) then the bacterium could grow and colonize the olive skin in population numbers comparable to those of the yeast species (Fig. 2.3). This could be attributed to the enhancement of LAB growth in the presence of yeasts (Arroyo-López et al. 2012b). Indeed, it has been reported than certain table olive- related yeast species, including *P. membranifaciens*, have the potential to synthesize substances (e.g., B-complex vitamins, amino acids) that are essential for the growth of *Lactobacillus* species (Ruiz Barba and Jiménez-Díaz 1995; Viljoen 2006; Silva et al. 2011). In a recent work by Blana et al. (2014), the same LAB strain was employed as a starter culture in green

olive processing and its survival throughout fermentation was determined by molecular techniques. The results showed that the selected strain was able to compete successfully with indigenous microbiota and grow on olives to high population levels. However, no attempt was made in that work to define the predisposition of the strain to adhere to table olives or to visualize the resulting biofilm using electron microscopy.

The changes in pH in the different brining treatments for dual species colonization of table olives are illustrated in Figure 2.4. In both low and high salt brines, glucose supplementation resulted in a progressive decrease of the pH value by approximately 2.2 and 1 units, respectively. However, in low salt treatments, brine acidification with/without glucose addition did not result in any pH change. In the corresponding high salt brines a slight increase was observed by almost 1 unit due to the complete inhibition of lab growth and stimulation of yeast growth.

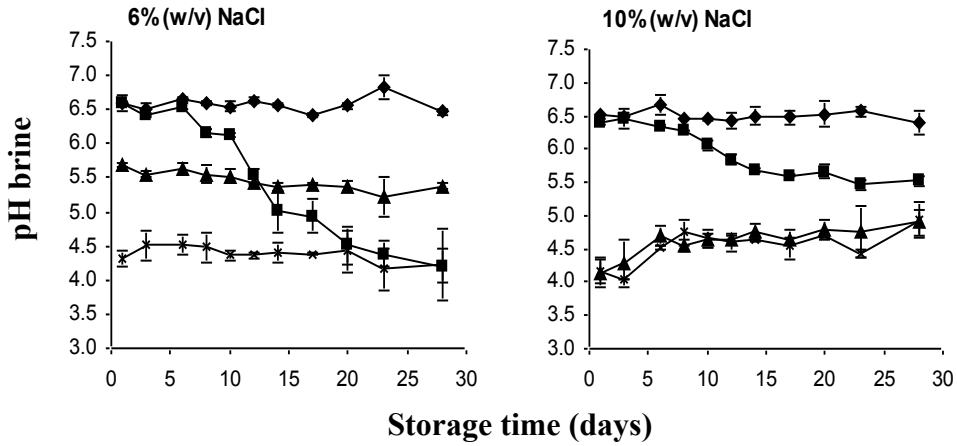

Figure 2.4. Evolution of pH in the brine during storage in the case of dual species biofilm development on olive drupes under different brining treatments: control treatment (◆), addition of 0.5% (w/v) glucose (■), acidification with 0.2% (v/v) lactic acid (▲), combination of 0.5% (w/v) glucose and 0.2% (v/v) lactic acid (×). Data points are average values of two replications per sampling point ± standard deviation.

2.3.3. In situ observation of mono and dual species biofilms by SEM

At the end of storage, representative olive samples were examined under SEM to observe biofilm formation on the olive epidermis. As mentioned above, the olive samples used in the present work were thermally processed (sterile) to ensure that the development of biofilm was due to the inoculated microorganisms only. This observation is in line with the microbiological analysis undertaken on olive drupes after the opening of the thermally treated can, where no cells could be enumerated. In general, in both mono and dual species biofilm the microorganisms adhered the olive surface and were found to be located mostly in stomatal openings and in epidermis discontinuations. No clear differences could be observed regarding brining treatment. In mono species biofilm, stomatal apertures were completely colonized by *L. pentosus* under all brining treatments, while spotty attached cells in groove areas were also observed. Judging from the SEM results, it was interesting but not surprising to find that different sample preparation treatments for SEM observation gave different images to describe the same phenomenon and this is due, in part, to non-standardized protocols for biofilm observation directly on plant tissues. Typical images of mono species biofilm by *L. pentosus* B281 in stomata on drupes in low salt brine supplemented with glucose and lactic acid are shown in Figure 2.5. It is clear that the bacterial cells appeared to be embedded in a biofilm matrix, with some cells trying to detach from the biofilm. Images of typical dual species biofilm are shown in Figures 2.6 and 2.7. Olive samples in these figures came from the same brining treatment, and sample drying was performed either by freeze drying (Fig. 2.6a, b), t-butyl alcohol (Figs. 2.6c), or critical point drying (Fig. 2.7). As observed, in freeze drying and t-butyl alcohol treatments, the microorganisms appear to be close together joined by a nondescriptive matrix and discrimination between them is rather difficult. These

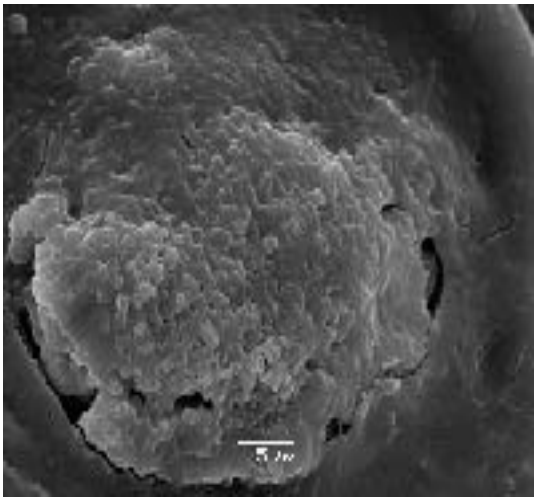

Figure 2.5. Micrograph of mono species biofilm on olive in low salt brines acidified with lactic acid and supplemented with glucose at the end of storage. Internalized biofilm cells of *L. pentosus* B281 are observed in a stomal opening in a freeze dried sample

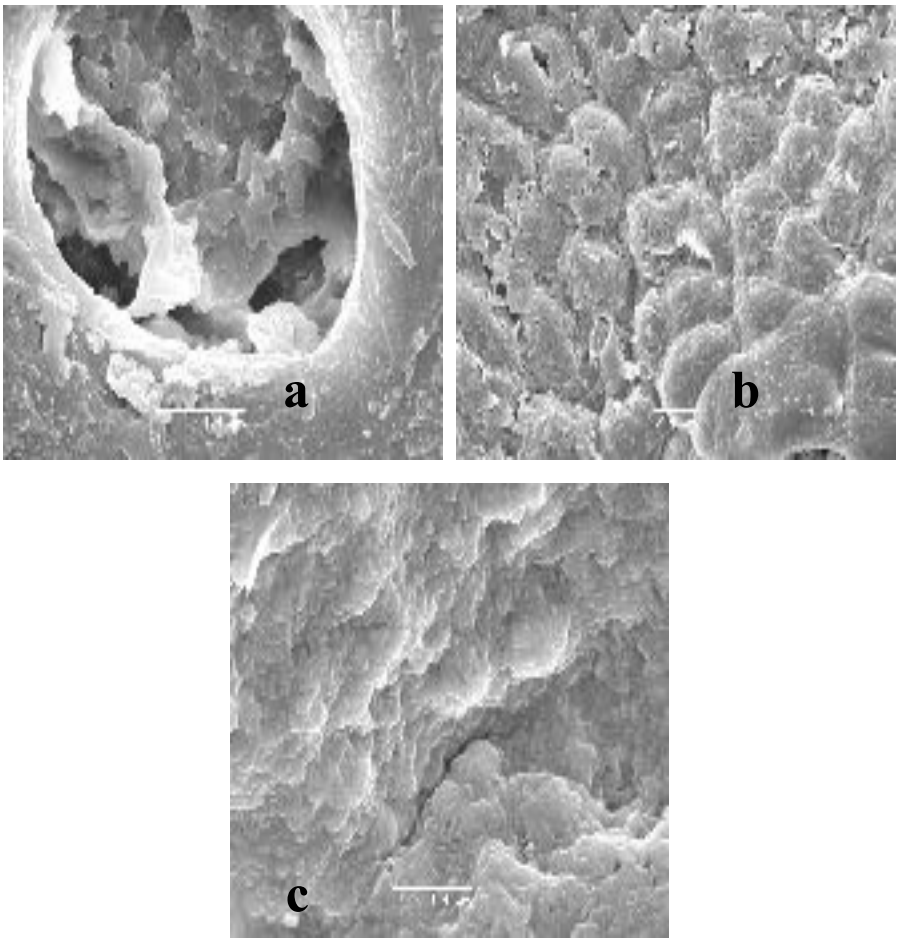

Figure 2.6. Dual species biofilm in a stomal opening (a) and grooves (b, c) at the end of storage in a freeze dried sample (a, b) and in a t-butyl dried sample (c).

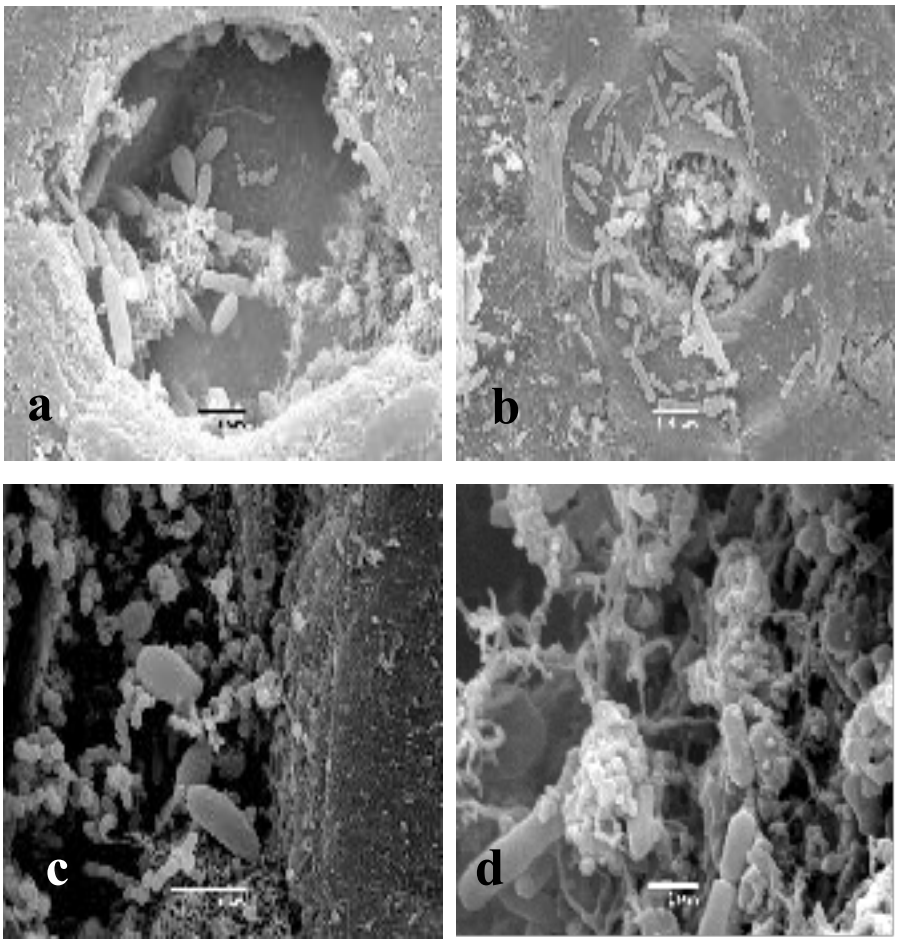

Figure 2.7. Dual species biofilm on olives in low salt brines supplemented with glucose and lactic acid. a, b Micrographs showing internalized lactic acid bacteria (LAB) and yeast cells in stomal apertures. c, d Micrographs showing LAB and yeast cells at greater depth inside stomata.

images are very similar with those obtained by previous researchers (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012), who studied the biofilm architecture on the skin surface of Spanish-style processed Gordal and Manzanilla green olives. On the other hand, LAB and yeast communities are clearly differentiated in the critical control drying treatment. Different types of secreted extracellular substances that are yet to be characterized were observed (Fig. 2.7). Images in Figures 2.7a and b are very like those obtained by Nychas et al. (2002), who first described biofilms

consisting of LAB and yeast aggregates in stomata on olive drupes during spontaneous fermentation of natural black Conservolea olives. Similar images were observed by Lavermicocca et al. (2005), who aimed to develop a probiotic product by inoculating ready-to-eat table olives with probiotic Lactobacilli and Bifidobacteria strains. Underneath the olive surface (ca. 5 μm), filaments were observed (Fig. 2.7a, c, d) between LAB and yeast cells, which could be regarded as an indication of dehydrated biofilm formed during the dehydrating and drying steps of sample preparation for SEM observation (Hassan 2008). Production and characterization of exopolysaccharides by LAB has been studied extensively in fermentation of dairy products as a means to modify the textural and functional properties of the final product (Cerning 1990; Ricciardi and Clementi 2000; Degeest et al. 2001; De Vuyst et al. 2001; Ercolini et al. 2003; Dabour et al. 2006; Hassan et al. 2007). In the case of table olive fermentation, only a few studies have demonstrated biofilm formation on olive surface either endophytically (in stomatal apertures) or in grooves on the epidermis (Nychas et al. 2002; Lavermicocca et al. 2005; Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012; Sisto and Lavermicocca 2012), the secreted matrix of which, however, remains uncharacterized and needs further investigation. Recent works on foodborne pathogens (Reyner et al. 2004; Kroupitski et al. 2009; Niemira and Cooke 2010; Elhariry 2011; Barak et al. 2011; Sirinutsomboon et al. 2011) focused on the attachment and biofilm formation on fresh produce and on microfabricated plant surface structures. It seems that cavities, such as stomata, grooves around the veins and discontinuations of the cuticle are the main sites where microorganisms preferentially adhere (Kroupitski et al. 2009; Barak et al. 2011) and form biofilms, which enables them to survive in the harsh phyllosphere and also gain

access to nutrients (Critzer and Doyle 2010). Therefore, it can be speculated that the stomatal apertures and cracks on the cuticle observed in the present work served as sites of attachment and colonization because there the microorganisms would be protected against the unfriendly brine environment and also gain access to nutrients. A recent study (Domínguez-Manzano et al. 2012) suggested that the attachment of microorganisms to the olive surface could be explained by the availability of nutrients at the olive/brine interface. In this sense, the microorganisms could detect a concentration gradient and attach to the surface, establishing mixed communities. Further to this, our observations indicate that substomatal cavities could be niches where nutrients are located, thus enabling the formation of biofilm not only on the surface of olives but also in areas inside the olive drupe where microorganisms could have access to a nutrient-rich environment.

2.4. Conclusion

The results of this work illustrate that *L. pentosus* B281 inoculated either alone or in co-culturewith *P. membranifaciens* M3A could colonize successfully the surface of black olives and develop mono and mixed LAB–yeast communities. The salt concentration in the brines affected the colonization of olive drupes as high salt levels were inhibitory or did not favour the dominance of the selected LAB strain, in contrast to the yeast species, which was found to be more resistant to high salt. The selection of black oxidized olives used to investigate the in situ predisposition of a microorganism for attachment in a biotic surface was successful despite the fact that the olives were already processed. In most brining treatments, the selected strains were able to colonize the olives in high numbers ranging from ca. 6.0–7.0 log CFU/g

and 5.0–5.5 log CFU/g for *L. pentosus* B281 and *P. membranifaciens* M3A, respectively. Since the colonization of table olives is a basic requirement for a microorganism to be considered as a potential starter culture, especially if the final product is to be characterized as probiotic, as in this case, or for other technological properties, this technique could serve as a screening method to assess potential cultures for their predisposition to adhere to the surface of olives and form biofilms. Future research will verify the results obtained for the predisposition of the selected culture to be embedded in biofilm structures in natural black olive fermentation where the selected starter culture will have to compete with the interfering indigenous microbiota of olives.

Chapter **3**

Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives

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Abstract

The performance of two strains of lactic acid bacteria (LAB), namely *Lactobacillus pentosus* B281 and *Lactobacillus plantarum* B282, previously isolated from industrially fermented table olives and screened in vitro for probiotic potential, was investigated as starter cultures in Spanish style fermentation of cv. Halkidiki green olives. Fermentation was undertaken at room temperature in two different initial salt concentrations (8% and 10%, w/v, NaCl) in the brines. The strains were inoculated as single and combined cultures and the dynamics of their population on the surface of olives was monitored for a period of 114 days. The survival of inoculated strains on olives was determined using Pulsed Field Gel Electrophoresis (PFGE). Both probiotic strains successfully colonized the olive surface at populations ranged from 6.0 to 7.0 log CFU/g throughout fermentation. PFGE analysis revealed that *L. pentosus* B281 presented higher colonization in both salt levels at the end of fermentation (81.2% and 93.3% in 8% and 10% NaCl brines, respectively). For *L. plantarum* B282 a high survival rate (83.3%) was observed in 8% NaCl brines, but in 10% NaCl the strain could not colonize the surface of olives. *L. pentosus* B281 also dominated over *L. plantarum* B282 in inoculated fermentations when the two strains were used as combined culture. The biochemical profile (pH, organic acids, volatile compounds) attained during fermentation and the sensory analysis of the final product indicated a typical lactic acid fermentation process of green olives

3.1. Introduction

Table olives are one of the major agricultural products that are consumed fermented. The primary purpose of table olive fermentation is to achieve a preservation effect and enhance the sensory attributes of the processed product (Sánchez Gómez et al. 2006). Diverse microbial groups are involved in olive fermentation determining the quality and sensory properties of the final product but it is generally accepted that LAB and yeasts are the most relevant microorganisms dominating the process (Arroyo-López et al. 2008; Hurtado et al. 2012). LAB influence fermentation in a variety of ways, the most important being the production of lactic acid from fermentable substrates resulting in pH decrease with a concurrent increase in acidity, ensuring thus the microbiological stability during storage even at ambient temperature for extended periods of time. Today, pure starter cultures of LAB are available in the market and used in several vegetable fermentations (Leroy and De Vuyst 2004; Di Cagno et al. 2013) but their use in table olive processing is still limited. For the selection of LAB as starter cultures in table olive processing certain technological properties must be assured including fast and predominant growth, antimicrobial activity, high acidification rate and fast consumption of fermentable substrates, utilization of non-digestible a-galactosidase sugars, tolerance to bile salts and acidic pH, screening of enzymes with biotechnological potential (Abriouel et al. 2012).

Probiotic food products are in general fermented foods containing an amount of viable and active microorganisms large enough to reach the intestine and exert an

equilibrating action on the intestinal microflora (FAO/WHO 2002). Intake of probiotics is considered to stimulate the growth of beneficial microorganisms, reduce the amount of pathogens and help boost the immune system, lowering thus the risk of gastro-intestinal diseases (Cross 2002; Reid 2008). Throughout the past two decades, probiotic health-promoting microorganisms have been increasingly included into commercial products in a response to the consumer demand for healthy foods that improve overall health, intestinal function, and digestion (Menrad 2003). Most of these microorganisms are LAB and, among them, lactobacilli represent one of the fundamental microbial groups which have been introduced in a wide range of food products. Until recently, probiotic foods have been restricted almost exclusively to dairy products (Saad et al. 2013). However, these foods cannot be consumed by certain groups of the population who suffer from lactose intolerance or need a diet based on non-milk derived products. Thus, in the last years, fermented foods of plant origin have been increasingly considered as vectors for incorporation of probiotic cultures following the well-established procedure of vegetable fermentation (Gupta and Abu-Ghannam 2012). In this way, fruits and vegetables already containing high levels of beneficial substances (e.g., antioxidants, vitamins, dietary fibres, minerals) can be reinforced with probiotic bacteria that can bring about additional health promoting features (Soccol et al. 2010; Peres et al. 2012). Recent research has focused on the exploitation of the microstructure of the olive surface as a carrier of probiotic strains of LAB confirming for the first time the suitability of the olive surface for this purpose (Lavermicocca et al. 2005; Saravanos et al. 2008; De Bellis et al. 2010; Arroyo-López et al. 2012a; Rodriguez-Gómez et al. 2013). A probiotic potential is expected to greatly enhance the already important nutritional value of table olives and

convey a favourable economic impact, especially knowing that such products originate in the less developed regions of the EU. However, depending on the geographical location, the cultivar, and the olive production process followed in each country, different LAB strains with diverse technological traits may prevail in the process and can be thus used as starter cultures. It is thus necessary to provide more evidence about the suitability of these strains to dominate the process and survive in high numbers in the final product.

The purpose of the current study was to assess the performance of two *Lactobacillus* strains, namely *L. pentosus* B281 and *L. plantarum* B282 as starter cultures in Spanish-style green olive fermentation in terms of microbiological and biochemical profiles attained during the process. Both strains have been previously isolated from naturally fermented olives (Doulgeraki et al. 2013) and selected for their *in vitro* probiotic potential (Argyri et al. 2013). The survival of the inoculated strains during the course of fermentation was determined using molecular techniques. In addition, two initial salt levels in the brines were used in an attempt to assess the starter performance at lower salt levels than the ordinary used by the table olive industry today in order to produce a low salt probiotic final product. Finally, the organoleptic attributes of fermented olives were assessed by a trained sensory panel to ensure the acceptability of the final product.

3.2. Materials and Methods

3.2.1. Olive cultivar and treatments

Green olives cv. Halkidiki were harvested in mid-September (season 2010-2011), subjected to quality control at the processor's installations to remove defective drupes and size graded to an average size of 111-120 fruits/kg. The olives were kindly provided by Konstantopoulos S.A., a table olive industry located in Northern Greece and processed in our laboratory according to the Spanish style method employed by the industry. A total amount of 110 kg of olives were initially subjected to a washing step with tap water to remove any impurities and subsequently immersed in a 1.9% (w/v) NaOH solution for 10-12 h at room temperature (20-22 ^{o}C) until the alkali penetrated approximately 2/3 of the flesh as measured from the epidermis to the pit. Debittering took place in the same vessels used for fermentation using 4.6 L of water and 87.4 g NaOH/vessel. A washing step was followed, replacing the NaOH solution with tap water. The process included two water changes at 4 and 8 h to remove the residual lye from the olive flesh.

3.2.2. Bacterial strains, preparation of inocula and inoculation

Two strains of LAB, namely *L. pentosus* B281 and *L. plantarum* B282, isolated previously from industrially fermented olives (Doulgeraki et al. 2013) and characterized for their *in vitro* probiotic potential (Argyri et al. 2013) were employed in the fermentations. Stock cultures were maintained in vials of treated beads in a cryoprotective fluid (Protect Bacterial Preservers, Lancashire, UK) at -80 ^{o}C until use. The cultures were revived by adding one bead of the frozen culture of each strain in 10 mL MRS broth medium supplemented with 4.5% (w/v) NaCl to allow adaptation of starter cultures to the saline environment of the brine and incubated at 30 ^{o}C for 24 h. Working cultures were prepared by adding 50 mL of each strain into 50 mL MRS

broth supplemented with 4.5% (w/v) NaCl and incubated at 30 °C for 24 h (ca. 9.0-9.5 log CFU/mL). Bacterial cells were centrifuged twice at 5.000 g for 15 min at 4 °C using a Heraeus Multifuge 1S-R centrifuge (Thermo Electron Corporation, Langenselbold, Germany), and the pellet was resuspended in 2.5 and 8.0 mL sterile ¼ Ringer's solution for *L. pentosus* B281 and *L. plantarum* B282, respectively to a final concentration of ca. 10.0 log CFU/mL. A 23-mL aliquot of the working culture was added in the fermentation vessels after 24 h of brining to achieve an initial population of ca. 7.0-8.0 log CFU/mL in the brine. In the case of combined cultures, equal volumes of each strain were mixed together and 23 mL of the resulting cocktail were added in the vessels. The cell concentration of the composite inocula in the brine was also ca. 7.0-8.0 log CFU/mL as in the case of monoculture.

3.2.3. Fermentation procedures

Fermentation was undertaken in 14 L total capacity screw capped plastic vessels containing 6.8 kg of olives and 4.6 L of freshly prepared brine (brine/olive ratio: 1.48/1). Two different initial salt concentrations were prepared namely, 8% and 10% (w/v) in order to evaluate the performance of the inoculated starter cultures at a lower salt level than those routinely employed by the Greek table olive industry today. At the onset of fermentation the brines were acidified with 0.1% (v/v) lactic acid (95%, Sigma) and 0.014% HCl following the industrial practice. Overall, eight initial fermentation processes were investigated namely, (i) spontaneous fermentation (control) in 8 and 10% salt brines, (ii) inoculated fermentation with *L. pentosus* B281 in 8 and 10% salt brines, (iii) inoculated fermentation with *L. plantarum* B282 in 8 and 10% salt brines, and (iv) combined inoculum of the two strains in 8 and 10% salt

brines. All treatments were performed in duplicate (i.e., two fermentation vessels per treatment). Fermentation took place at room temperature (ca. 20-22 ^{o}C) for a period of 114 days.

3.2.4. Microbiological analysis

Olive samples were analysed after 24 h of brining and at regular time intervals during the experiment (22 sampling points per treatment). Olives were recovered from the brine, the pit was aseptically removed with the aid of a sterile blade and olive flesh (25 g) was aseptically cut, added in 225 mL sterile ¼ Ringer's solution and homogenized in a stomacher (LabBlender, Seward Medical, London, UK) for 60 s at room temperature. The resulting suspension was serially diluted in the same diluent and 1 or 0.1 mL samples of the appropriate dilutions were mixed or spread on the following agar media: (i) de Man-Rogosa-Sharpe medium (MRS; 401728, Biolife, Milan, Italy) adjusted to pH 5.7 and supplemented with 0.05% (w/v) cycloheximide, overlaid with the same medium and incubated at 25 ^{o}C for 72 h; (ii) Rose Bengal Chloramphenicol agar (RBC; LAB 36 supplemented with selective supplement X009, LAB M, Bury, UK) for yeasts and moulds, incubated at 25 ^{o}C for 48 h; and (iii) Violet Red Bile Glucose agar (VRBGA; 402188, Biolife, Milan, Italy) for enterobacteria counts, incubated at 37 ^{o}C for 24 h. Results were expressed as log values of colony forming units per gram (log CFU/g) of olives.

3.2.5. Physicochemical analysis and sensory evaluation

During the period of fermentation physicochemical analyses were performed to monitor the changes in pH, organic acids and volatile compounds. The values of pH

were determined in both the brine and olive drupes using a digital pHmeter (Metrohm AG, Herisau, Switzerland). For olive drupes, the pH was measured in a sample (25 g) of olive flesh previously homogenized at room temperature in an Ultra-Turrax T25 Basic blender (IKA Werke, Staufen, Germany) in 50 mL of distilled water until obtaining a fluid slurry. Organic acids in the brine (lactic, acetic, formic, succinic, citric, malic, propionic) were determined by an ion exclusion chromatographic method with post-column pH-buffered electro-conductometric detection as described previously (Vergara et al. 2013). Finally, the major volatile compounds in the brine (ethanol, methanol, ethyl acetate, methyl acetate, propanol, 2-butanol) were monitored using the static headspace method described by Montaño et al. (1990) slightly modified (Vergara et al. 2013).

A sensory evaluation of olive samples was performed at the end of fermentation (114 days) by a taste panel consisted of ten persons according to the method of sensory analysis of table olives established by the International Olive Council (IOC 2011). The sensory attributes taken into account included the following descriptors: abnormal fermentation, salty, bitter, acid, hardness, fibrousness, and crispness. For each descriptor the median value of the score given by the members of the taste panel was calculated together with the robust standard deviation as defined by the method of sensory analysis of table olives. Sensory data were subjected to Kruskal Wallis non parametric analysis of variance (Quinn and Keough 2002) using Minitab release 14.1 (Minitab Ltd., Coventry, UK). The predetermined acceptable level of probability was 5% for all comparisons ($p < 0.05$).

3.2.6. Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from the highest dilution of MRS medium from the different sampling points. From each of the aforementioned samplings 20% of the colonies (i.e., 10-25 colonies) were randomly selected and purified (Harrigan 1998). Pure cultures were stored at -80 ^{o}C in MRS medium supplemented with 20% (v/v) glycerol. Before experimental use each isolate was subcultured twice, while the purity of the culture was always checked. A total of 305 isolates were picked at three different time intervals, namely onset of fermentation (day 1), middle (day 69) and at the end of fermentation process (day 114).

3.2.7. Characterization of the lactobacilli isolates

The survival of the selected probiotic strains during olive fermentation was determined using Pulsed Field Gel Electrophoresis (PFGE). Briefly, genomic DNA was prepared from all isolates according to Doulgeraki et al. (2010). The restriction enzyme ApaI (10U) (New England Biolabs, Ipswich, MA, USA) was applied according to the manufacturer's recommendation for 16 h. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris-Borate buffer on CHEF-DRII (Bio-Rad, Hercules, CA, USA) equipment with the following running parameters: 6 V/cm, 1 s initial switching time, 10 s final switching time, and 16 h of total run at 14 ^{o}C. Gels were then stained with ethidium bromide (0.5 mg/mL) in water for 1 h and distained for 2 h before being photographed using a GelDoc system. Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Bionumerics software, version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Isolates with profiles not similar to *L. pentosus* B281 and *L. plantarum* B282 were characterized using multiplex PCR analysis of the recA gene with species-specific primers for *L. pentosus*, *L. plantarum* and *L. paraplantarum*, following the protocol described by Torriani et al. (2001). Previously, DNA was extracted from 1 mL of an overnight culture of each isolate according to the procedure described by Doulgeraki et al. (2011).

3.2.8. Statistical analysis

Principal Components Analysis (PCA) was employed to investigate any underlying relationship among the different fermentation procedures based on the microbiological and biochemical profile obtained during the course of fermentation. The input matrix for the analysis consisted of the total area under the growth/decline curves of LAB, enterobacteria, and yeasts, as well as those corresponding to lactic and acetic acids, ethanol, methanol, ethyl acetate, methyl acetate, propanol, and 2-butanol. For the selection of the optimum number of PCs, factors with eigenvalues greater than 1.0 were retained. In addition the plot of the PCs enables the investigation of correlations between the variables of the input data set. To this end, the initial variables were projected into the subspace defined by the reduced number of principal components (first and second components) and correlated variables were identified. In the current approach, PCA analysis was implemented with XLStat software version 2006.06 (Addinsoft, Paris, France) using a varimax rotation.

3.3. Results

3.3.1. Microbiological changes during fermentation

The changes in the population dynamics of the enumerated microbial groups on olive fruits are illustrated in Figure 3.1. Generally, both starter cultures were well adapted in the fermentation environment as concluded by the high counts from the onset of fermentation that were maintained almost unchanged throughout the process. Specifically, the population of LAB in the inoculated fermentation with *L. pentosus* B281 was 6.5 log CFU/g on olive drupes at the beginning of fermentation (24 h), reaching a maximum at 7.8 log CFU/g within the first 14 days of fermentation, and then remained at levels exceeding 6.0 log units until the end of the process (Fig. 3.1b). The growth of LAB was not influenced by the salt concentration in the brine as exemplified by their growth curves that were similar during the whole fermentation process. A similar profile of LAB was observed in the process inoculated with a

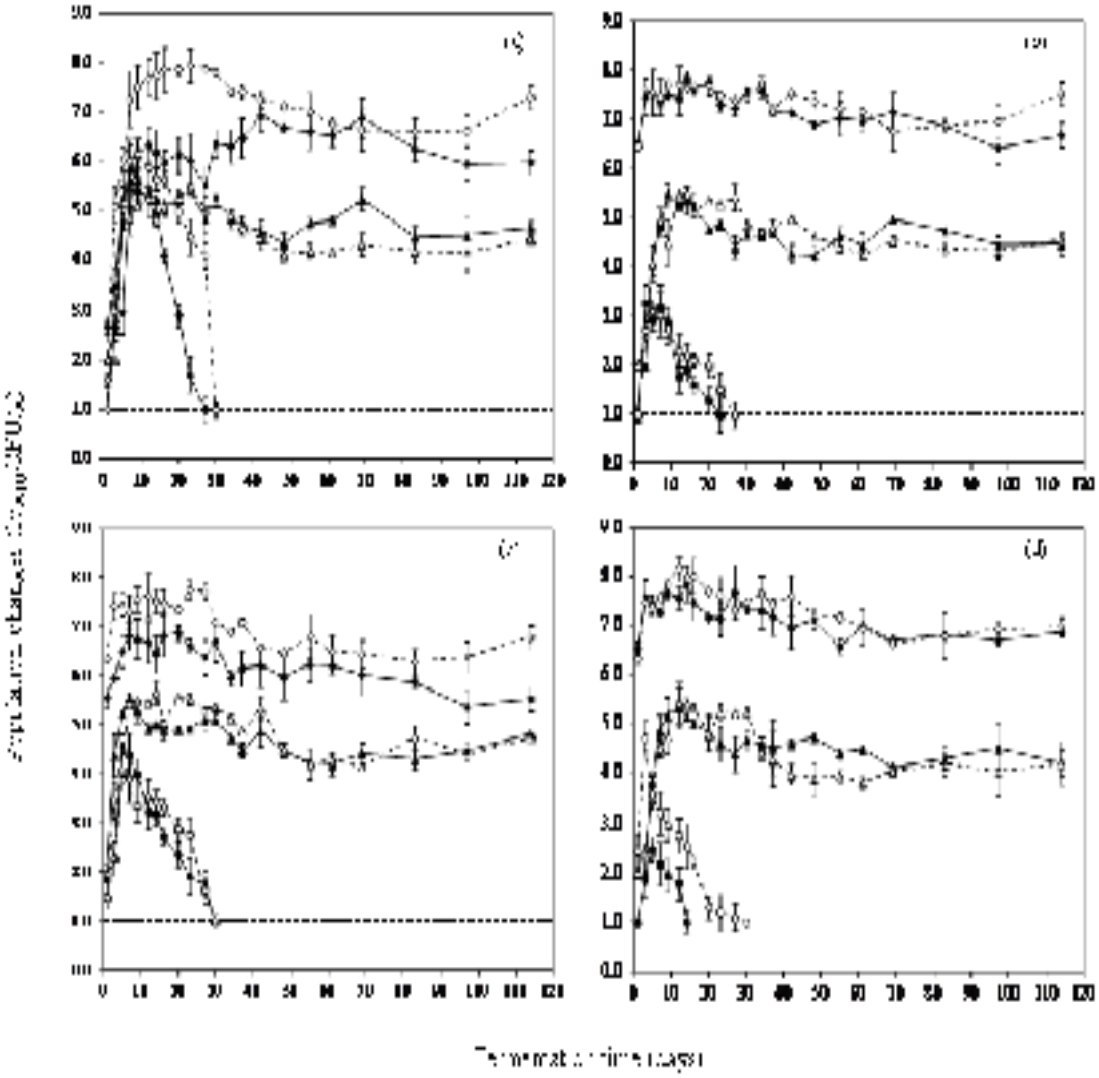

Figure 3.1. Changes in the population of LAB (◆, ◇), yeasts (▲, △) and enterobacteria (●, ○) on olive drupes during fermentation in 10% (solid line) and 8% (dotted line) initial salt concentration: (a) spontaneous process, (b) inoculated process with *L. pentosus* B281, (c) inoculated process with *L. plantarum* B282, (d) inoculated process with a co-culture of the two strains. Data points are average values of duplicate fermentations ± standard deviation. Horizontal dotted line indicates the detection limit of the method (1.0 $\log_{10}$ CFU/g).

combined culture of both strains, *L. pentosus* B281 and *L. plantarum* B282. Again, as in the case of previous fermentation, the concentration of salt did not influence the growth pattern of LAB (Fig. 3.1d). A different growth pattern was evident in the

inoculated process with *L. plantarum* B282 (Fig. 3.1c). In this case, there was a clear effect of salt concentration in the brines as the growth of LAB in high salt concentration was continuously lower by 0.6-0.8 log CFU/g on average compared with their growth at lower salt level, indicating a negative effect of high salt level on the growth of *L. plantarum* B282. In this treatment, *L. plantarum* B282 was inoculated at a somehow lower level (5.5 log CFU/g) on olive drupes at the beginning of fermentation in 10% salt brines and remained at a population below 6.0 log units at the end of the process. In the control treatment the evolution of LAB was rather typical for Spanish style processing, presenting a rapid increase within the first 12-14 days reaching 6.4 and 7.7 log CFU/g on olive fruits in 8% and 10% salt brines, respectively (Fig. 3.1a).

Yeasts coexisted with LAB but their population was generally lower by ca. 2.0-3.5 log CFU/g exhibiting a similar growth pattern in all fermentations (Fig. 3.1). Specifically, yeast growth showed a rapid increase within 12-16 days reaching a maximum at ca. 5.5 log CFU/g followed by a slight decline in their population that was maintained between 4.5 and 5.0 log CFU/g until the end of the experiment. Their population dynamics were not influenced by the salt level in the brines. Finally, Enterobacteriaceae showed a rapid increase within 7 days at populations ranging from 3.0 to 4.5 log CFU/g depending on fermentation process with the exception of control treatment where higher numbers (5.6 log CFU/g) were observed (Fig. 3.1). A sharp decline of this microbial group was evident thereafter, so that no viable counts could be detected after 30 days of fermentation.

3.3.2. Physicochemical changes during fermentation

The pH in olives was used to monitor the course of fermentation in the different processes (Fig. 3.2a and b). Specifically, in 10% salt brines the initial pH value in olives (8.3 ± 0.6) decreased rapidly within the first 20 days in all processes by about 3 pH units before reaching a plateau at about pH 5.0 (Fig. 3.2a). However, after day 83, the pH presented a slight decrease and reached a final value of 4.4 ± 0.1. It needs to be

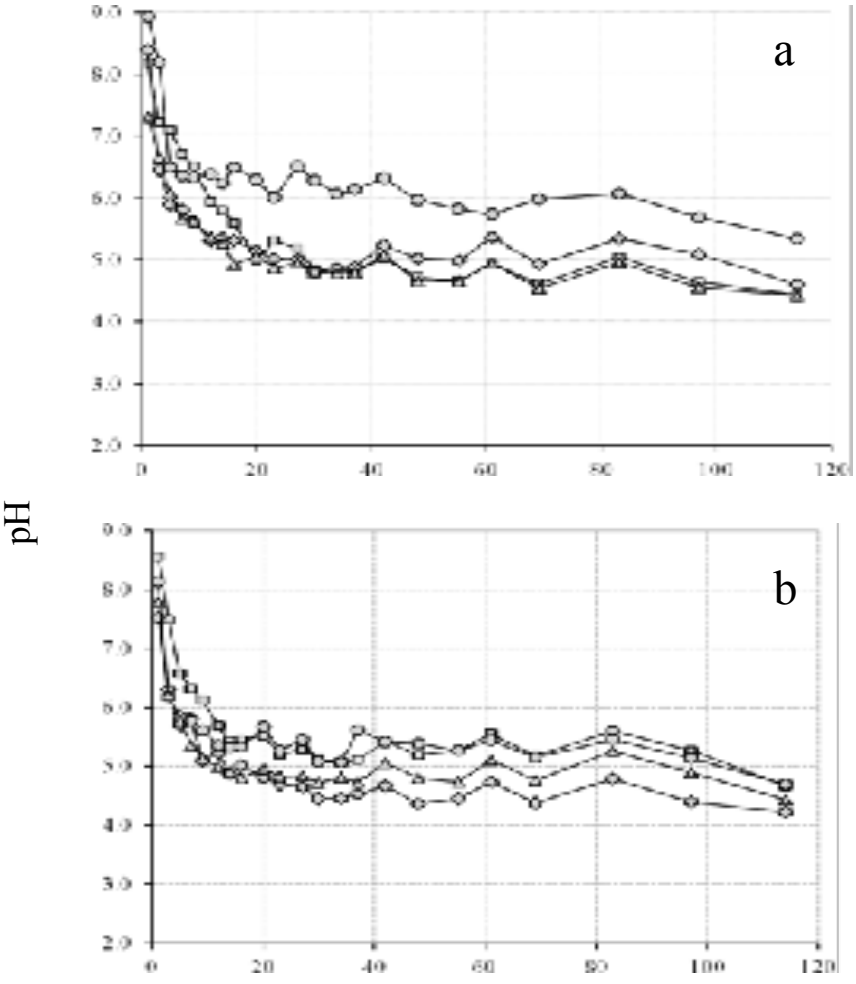

Figure 3.2. Changes in pH values in olive drupes during fermentation in 10% (a) and 8% (b) initial salt concentration: ( ) spontaneous process, (◇) inoculated process with *L. pentosus* B281, (○) inoculated process with *L. plantarum* B282, (△) inoculated process with a co-culture of the two strains. Data points are average values of duplicate fermentations. Error bars are not presented for clarity of the graph. Instead, pooled standard deviation (s_p) has been

calculated as follows. For 10% and 8% initial NaCl level: 0.3963/0.148, 0.194/0.215, 1.100/0.615, and 0.269/0.374 for spontaneous and inoculated processes with *L. pentosus* B281, *L. plantarum* B282, and co-culture of the two strains, respectively.

noted that the pH drop was faster in the inoculated process with *L. pentosus* B281 and in the co-culture of both strains *L. pentosus* B281 and *L. plantarum* B282. In contrast, high pH values (above 6) were recorded for the most part in the inoculated process with *L. plantarum* B282, where a pH value of 5.3 was attained at the end of fermentation. A similar pattern was observed in 8% salt brines with final pH values in the olives ranging from 4.2 to 4.7 (Fig. 3.2b). Inoculated processes with *L. pentosus* B281 and a coculture of the two strains presented lower pH values throughout fermentation compared with the *L. plantarum* B282 and control processes. Additionally, the changes of pH values in the brines reflected the trend of pH in olives, but they were lower by 0.5-0.6 units on average during the whole process (data not shown).

Organic acids analysis showed a marked increase of lactic acid after 5 days of fermentation in 8% salt brines inoculated with *L. plantarum* B282 and with the co-culture of the two strains at 50.6 and 57.7 mM, respectively. After this period the concentration of lactic acid was gradually reduced until the end of fermentation (day 114) reaching a value of 39 and 50 mM, respectively (Fig. 3.3a). In contrast, the concentration of lactic acid in the inoculated process with *L. pentosus* B281 increased gradually and reached a final value of 56 mM in the end of fermentation, presenting the highest lactic acid concentration compared with the other two treatments. It must be emphasized that in control treatment (uninoculated fermentation), lactic acid in the brine increased until day 20, but afterwards it started to decrease rapidly until day 55

where no lactic acid could be detected in the brine. In this treatment, propionic acid was detected in the brines from day 30 onwards reaching a value of 21.7 mM by the end of fermentation (data not shown). A similar profile for lactic acid was observed in 10% salt brines, with the highest rate of production being observed in the inoculated process with *L. pentosus* B281 and in the co-culture of the two strains (Fig. 3.3b). The other two treatments showed a hysteresis in lactic acid production but after 55 days of

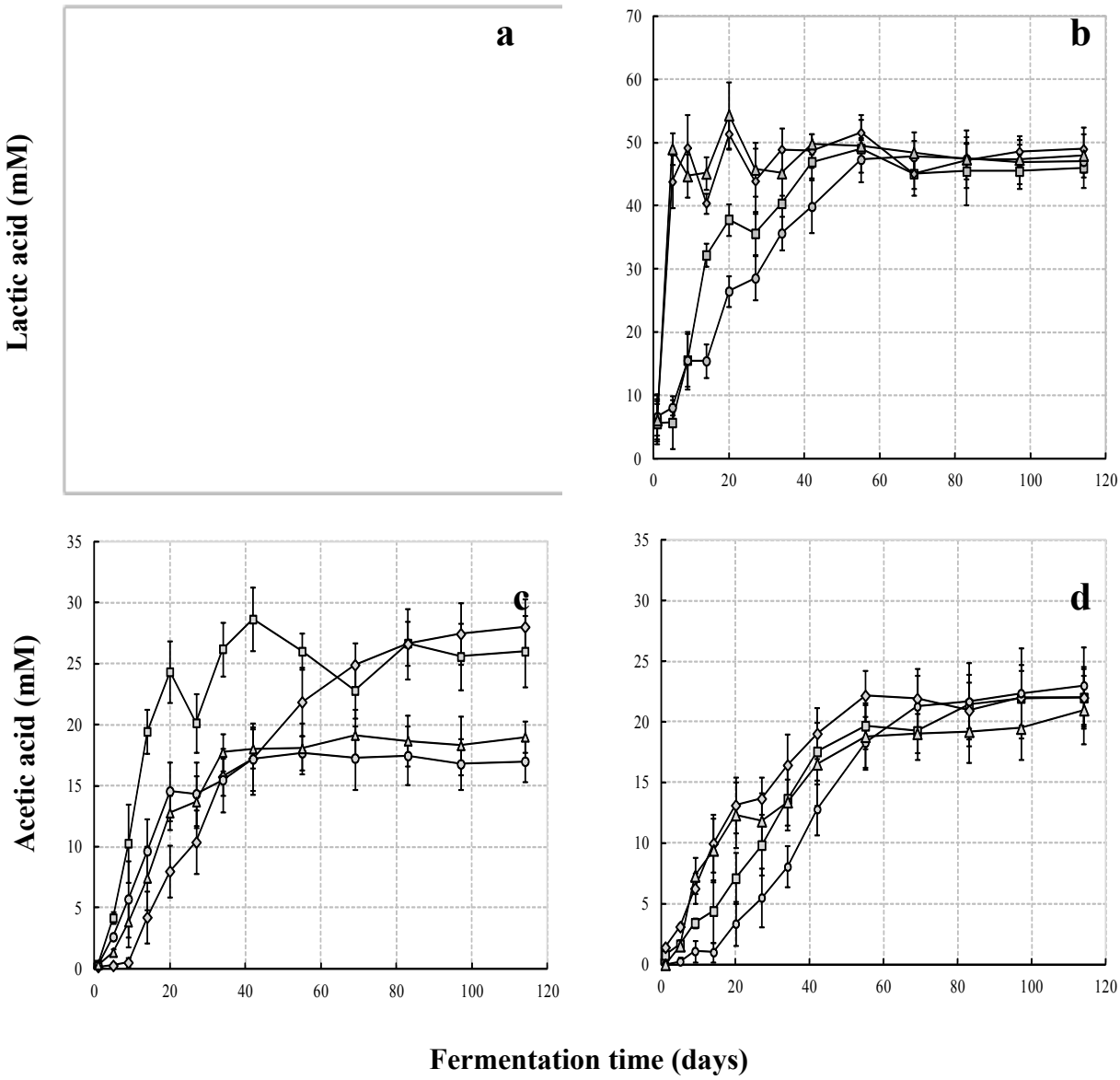

Figure 3.3. Changes in the concentration of lactic and acetic acid (mM) in the brines during fermentation in 8% (a, c) and 10% (b, d) initial salt concentration: ( ) spontaneous process,

(◇) inoculated process with *L. pentosus* B281, (○) inoculated process with *L. plantarum* B282, (△) inoculated process with a co-culture of the two strains. Data points are average values of duplicate fermentations ± standard deviation.

fermentation the concentration of this organic acid ranged between 46 and 48 mM in all processes. Acetic acid was also detected in the brines in concentrations not exceeding 30 mM and 25 mM in 8% and 10% salt brines, respectively (Fig. 3.3c and d). In low salt brines two different groups of procedures were differentiated with respect to acetic acid production, especially towards the end of fermentation, namely inoculated process with *L. pentosus* B281 and control treatment presenting the highest levels of acetic acid (26-28 mM), and inoculated process with *L. plantarum* B282 and co-culture of the two strains where lower concentration of acetic acid was attained (17-19 mM) (Fig. 3.3c). In 10% salt brines the pattern was fairly similar for all treatments (Fig. 3.3d) with final concentrations of acetic acid ranging from 21 to 23 mM.

The major volatile compounds identified in the brines were ethanol and methanol, whereas ethyl acetate, methyl acetate, propanol, and 2-butanol were detected in lower amounts (Tables 3.1 and 3.2). In 8% salt brines the highest concentration of ethanol was measured in the inoculated process with *L. pentosus* B281 (Table 3.1). Its level increased within the first 55 days of fermentation and then remained almost unchanged until the end of the process in concentration exceeding 1000 mg/L. In contrast, in 10% salt brines the highest concentration of this compound was detected in the inoculated process with *L. plantarum* B282 followed by the spontaneous fermentation (Table 3.2). Methanol was also detected in considerable amounts that were comparable with ethanol depending on fermentation process. The

highest amount of methanol was measured in low salt (8%) control fermentation in amounts reaching 1600 mg/L. Higher alcohols (2-butanol, propanol) were also detected in the brines. The former compound was detected in higher levels in control processes for both salt levels, ranging from ca. 20-24 mg/L, whereas for the inoculated fermentations it was maintained below ca. 10 mg/L and 6 mg/L in 8% and 10% salt brines, respectively. Propanol was detected at very low levels (below 7 mg/L) in 10% salt brines regardless of fermentation process, whereas in 8% salt the highest concentration was observed in the inoculated process with *L. pentosus* B281 and the co-culture of the two strains with *ca*. 17 mg/L and 14.4 mg/L, respectively. Finally, methyl and ethyl esters were present in the brines with the highest content detected in 8% salt brines inoculated with *L. pentosus* B281.

Table 3.1. Changes in the concentration (mg/L) of volatile compounds in 8% salt brines during fermentation of cv. Halkidiki green olives.

Process	Time (days)	Volatile compound (mg/L)					
		Ethanol	Methanol	2-Butanol	Propanol	Ethyl acetate	Methyl acetate
Control	1	47.8	76.2	-	-	-	-
	9	354.6	104.6	-	0.2	-	-
	20	236.4	724.6	3.3	1.5	0.1	0.1
	34	142.1	563.8	15.4	0.7	0.1	0.1
	55	2.7	723.0	14.0	0.3	0.1	0.1
	83	2.8	1690.3	23.7	0.4	-	0.1
	114	3.0	1603.9	24.0	0.4	-	0.1
L. pentosus B28	1	276.7	75.3	-	-	-	0.2
	9	646.4	243.5	-	0.4	0.5	0.3
	20	1691.1	387.6	-	1.3	2.0	0.6

1	34	1214.0	263.5	-	1.0	2.5	0.9
	55	1235.8	272.4	7.6	4.0	2.6	1.0
	83	1145.1	304.0	9.4	10.8	2.3	1.0
	114	1138.6	355.3	10.0	17.0	2.3	1.0
L. plantarum B282	1	44.6	49.3	-	-	-	-
	9	64.5	228.2	-	-	-	-
	20	70.4	90.4	-	-	-	-
	34	66.2	67.9	0.2	0.8	-	-
	55	67.9	52.4	1.8	1.9	0.1	0.1
	83	64.7	59.7	3.5	3.1	0.1	0.1
	114	70.1	75.8	4.0	4.7	0.1	0.1
Co-culture	1	71.6	85.0	-	-	-	-
	9	88.8	120.7	-	-	-	-
	20	95.1	86.4	0.6	1.9	-	-
	34	76.9	63.6	1.4	5.4	0.1	0.1
	55	95.5	74.4	2.3	8.4	0.1	0.1
	83	95.2	71.2	2.2	9.6	0.1	0.1
	114	119.1	90.9	2.9	14.4	0.1	0.1

Data points are average values of duplicate fermentations; standard deviation was less than 10% of the respective value.

Table 3.2. Changes in the concentration (mg/L) of volatile compounds in 10% salt brines during fermentation of cv. Halkidiki green olives.

Process	Time (days)	Volatile compound (mg/L)					
		Ethanol	Methanol	2-Butanol	Propanol	Ethyl acetate	Methyl acetate
Control	1	94.0	50.7	-	-	-	-
	9	549.0	106.9	-	0.3	-	-
	20	645.6	123.7	-	0.5	0.5	0.1
	34	686.9	159.6	-	0.5	0.7	0.4

	55	544.4	137.2	0.1	0.7	0.6	0.2
	83	547.6	135.9	16.0	1.9	0.7	0.4
	114	559.4	162.8	19.6	2.1	0.7	0.3
L. pentosus B281	1	53.5	50.9	-	-	-	-
	9	120.1	87.0	-	-	-	-
	20	74.0	105.4	-	-	-	0.1
	34	75.8	78.0	0.1	-	0.1	0.1
	55	109.4	98.3	4.9	2.3	0.1	0.1
	83	92.6	117.4	5.4	5.1	0.1	0.2
	114	102.3	107.0	5.9	6.9	0.1	0.2
L. plantarum B282	1	170.1	73.3	-	-	-	-
	9	1355.9	224.7	-	1.3	0.1	-
	20	1121.5	201.8	-	1.0	0.2	-
	34	962.0	189.6	-	0.8	0.9	0.1
	55	791.7	167.0	-	0.8	0.8	0.3
	83	655.2	162.6	1.2	1.5	0.9	0.4
	114	762.4	186.8	2.2	2.1	0.9	0.4
Co-culture	1	59.8	50.6	-	-	-	-
	9	76.9	73.0	-	-	-	-
	20	85.1	78.2	-	-	0.1	0.1
	34	73.1	79.9	0.3	0.6	0.1	0.1
	55	84.8	76.5	1.0	1.5	0.1	0.2
	83	91.3	94.8	2.0	2.2	0.1	0.2
	114	94.4	94.8	2.5	2.6	0.1	0.2

Data points are average values of duplicate fermentations; standard deviation was less than 10% of the respective value.

3.3.3. Sensory analysis

The results of the sensory evaluation of table olives at the end of fermentation (114 days) are presented in Table 3.3. The values for the perception of abnormal

fermentation in 8% salt brines were statistically significant (p=0.000) indicating that the taste panel detected an odour difference among the processes. Specifically, a higher score was given in the control treatment compared to inoculated processes, as the odour was not considered typical for green olive fermentation and the olives were

Table 3.3. Sensory profile (median ± robust standard deviation) of cv. Halkidiki green olives processed in different salt brines inoculated with *L. pentosus* B281, *L. plantarum* B282 and a co-culture of the two strains after 114 days of fermentation.

Sensory attribute	Fermentation process (8% salt brines)			
	Control	*L. pentosus* B281	*L. plantarum* B282	Co-culture (B281 and B282)
Abnormal fermentation	8.8 ± 1.2^{A}	2.5 ± 0.6^{B}	1.4 ± 0.4^{B}	1.7 ± 0.7^{B}
Salty	5.2 ± 0.5^{A}	7.5 ± 0.8^{A}	6.8 ± 1.2^{A}	5.5 ± 0.4^{A}
Bitter	7.5 ± 1.6^{A}	2.0 ± 1.6^{B}	1.7 ± 0.7^{B}	3.0 ± 0.7^{B}
Acid	3.6 ± 0.8^{A}	5.5 ± 1.1^{B}	3.9 ± 0.9^{A}	3.4 ± 0.3^{A}
Hardness	4.7 ± 1.1^{A}	5.8 ± 1.4^{A}	5.2 ± 1.2^{A}	4.9 ± 0.6^{A}
Fibrousness	2.9 ± 0.5^{A}	2.7 ± 1.4^{A}	3.2 ± 1.3^{A}	2.3 ± 1.5^{A}
Crunchiness	3. ± 0.9^{A}	3.4 ± 0.9^{A}	3.6 ± 0.6^{A}	3.2 ± 0.6^{A}
	Fermentation process (10% salt brines)			
Abnormal fermentation	1.7 ± 0.3^{A}	1.6 ± 0.2^{A}	1.5 ± 0.2^{A}	2.0 ± 0.4^{A}

Salty	8.0 ± 1.0[A]	8.3 ± 0.7[A]	7.5 ± 1.9[A]	6.5 ± 1.3[A]
Bitter	2.3 ± 0.9[A]	3.2 ± 0.6[A]	2.7 ± 0.6[A]	3.0 ± 0.7[A]
Acid	5.0 ± 0.9[A]	5.3 ± 1.0[A]	4.2 ± 1.2[A]	4.0 ± 0.3[A]
Hardness	4.4 ± 0.8[A]	4.0 ± 1.2[A]	4.2 ± 0.5[A]	3.9 ± 0.6[A]
Fibrousness	3.8 ± 1.5[A]	3.2 ± 1.2[A]	3.6 ± 1.0[A]	3.2 ± 0.8[A]
Crunchiness	3.2 ± 1.2[A]	3.3 ± 0.5[A]	2.5 ± 0.4[A]	3.2 ± 0.6[A]

Different letters within the same row indicate statistically significant difference at $p < 0.05$.

practically rejected by the panelists. The descriptors for hardness, fibrousness and crunchiness were not statistically significant (p = 0.087, 0.179, 0.215, respectively) and received low scores as the olives were subjected to the same treatment. The same holds for the perception of saltiness that did not differ among the different processes (p = 0.113) because the salt level at equilibrium was approximately the same since olives were immersed in the same brine solution. Moreover, the scores for acidity were statistically significant (p = 0.022) as the inoculated treatment with *L. pentosus* B281 received higher values by the taste panel compared to the other processes. Finally, bitterness received different scores by the taste panel with inoculated processes being statistically different from control fermentation (p = 0.004) that was perceived more bitter by the panelists. In the case of 10% salt brines no statistical differences among the sensory descriptors could be established between control and

inoculated treatments. As expected, olives received higher scores for saltiness as they were fermented in higher salt brines.

3.3.4. Survival of the inoculated LAB

To characterize the survival of the selected strains of LAB on olive fruits during the inoculated process, a total of 305 isolates were picked at the beginning (day 1), middle (day 69) and end of fermentation (114 days) and subjected to PFGE analysis. Results showed that at the onset of fermentation the inoculated strains of *L. pentosus* B282 and *L. plantarum* B282 were recovered 100% from the olive fruits in both salt concentrations (Table 3.4). During the course of fermentation it was found that *L. pentosus* B281 persisted in high numbers in both low and high salt brines until the end of the process, whereas *L. plantarum* B282 could not survive in high salt (10%) brines as it was not recovered at the final stage of fermentation. It needs to be noted that *L. pentosus* B281 was recovered in higher percentage (93.3%) in 10% brines compared to 81.2% in 8% salt brines indicating possibly a better adaptation of this strain to higher salt concentrations. This strain was also found to survive better in low salt (8%) brines as inferred by the high recovery numbers at the end of fermentation. Finally, in the case of inoculated fermentation with a co-culture of *L. pentosus* B281 and *L. plantarum* B282, only the former strain was able to be recovered at high numbers (>90%) from olive fruits at the end of fermentation in both salt concentrations.

Table 3.4. The survival rate of the inoculated strains on olive fruits during green olive fermentation according to PFGE analysis.

Inoculated strains	Fermentation time (days)	Brine 8% (w/v) NaCl Survival rate	Brine 10% (w/v) NaCl Survival rate
L. pentosus B281	1	100%	100%
	69	100%	100%
	114	81.25%	93.33%
L. plantarum B282	1	100%	100%
	69	100%	35.71%
	114	83.33%	0%
Co-culture (B281 and B282)	1	42.11% B281/ 57.89% B282	62.5% B281/ 37.5% B282
	69	80% B281/ 0% B282	90% B281/ 0% B282
	114	90% B281/ 0% B282	100% B281/ 0% B282

3.3.5. Multivariate data analysis of fermentation procedures

The results of the PCA analysis showed that there were three principal components (PCs) with eigenvalues higher than 1.00, accounting for 39.6%, 26.2%, and 19.6% of the total variance, respectively. This indicates that the initial eleven variables could be expressed as a linear combination of three PCs explaining 85.4% of the total variance. PC1 was mainly related to lactic acid, acetic acid, methanol, 2-butanol, and Enterobacteriaceae; PC2 was related to ethanol, methyl and ethyl acetate, and finally PC3 to LAB, yeasts and propanol (Table 3.5). The projection of the original variables on the plane of the two first PCs could clearly illustrate such

Table 3.5. Correlation of variables to the factors of the PCA analysis based on factor loadings.

Variable	Factor 1	Factor 2	Factor 3
LAB	0.046	0.003	**0.913**
Yeasts	0.038	0.230	**-0.749**
Enterobacteriaceae	**-0.743**	0.148	-0.321
Lactic acid	**0.936**	-0.228	0.106
Acetic acid	**-0.818**	0.267	0.252
Ethanol	0.348	**0.870**	-0.273
Methanol	**-0.890**	0.335	0.225
2-Butanol	**-0.897**	0.291	0.246
Propanol	0.514	0.217	**0.617**
Methyl Acetate	0.374	**0.910**	0.131
Ethyl Acetate	0.366	**0.925**	0.012

Values in bold within the same factor indicate the variable with the largest correlation

relationship as shown in the plot of loadings (Fig. 3.4a). The discrimination of the various fermentation processes can be visualized in the plot of scores (Fig. 3.4b) where three clusters could be clearly distinguished. The majority of fermentation processes at both salt levels was located near the centre of the axis and can thus be considered as very close to each other. However, there were two treatments that were clearly separated from the remaining, namely control fermentation and inoculated fermentation with *L. pentosus* B281 at 8% initial salt brines that were located in opposed quadrants to each other. Combining the information from the plot of loadings and scores, it can be inferred that the majority of fermentations were associated with lactic acid, whereas the inoculated process with *L. pentosus* B281 was related with volatile compounds, mainly ethanol, methyl acetate and ethyl acetate. The

spontaneous process in 8% salt brines was related to methanol, acetic acid, 2-butanol and Enterobacteriaceae. The variable for yeasts (RBC) is related to the spontaneous process and inoculated fermentation with *L. plantarum* B282 in 10% salt brines. Finally, the variable for LAB had a low correlation with the first two PCs to show a clear trend, but it seems to be positively correlated with lactic acid.

b

Figure 3.4. Plot of loadings (microbiological and biochemical profile) and scores (fermentation processes) formed by the first two principal components from the PCA analysis. (C_8): control fermentation in 8% salt brines; (C_10): control fermentation in 10% salt brines; (B281_8): inoculated fermentation with L. pentosus B281 in 8% salt brines; (B281_10): inoculated fermentation with L. pentosus B281 in 10% salt brines; (B282_8): inoculated fermentation with L. plantarum B282 in 8% salt brines; (B282_10): inoculated fermentation with L. plantarum B282 in 10% salt brines; (CO-8): inoculated fermentation with a co-culture of the two strains in 8% salt brines; (CO-10): inoculated fermentation with a co-culture of the two strains in 10% salt brines.

3.4. Discussion

In the last years considerable research effort has been undertaken on the elucidation of the technological properties of LAB that could transform a traditional fermented commodity into a functional food providing thus new perspectives for the table olive industry (Abriouel et al. 2012; Argyri et al. 2013; Bautista-Gallego et al.

2013a). The ability of LAB to colonize the surface of olives and form biofilms during fermentation provided new perspectives for the use of table olives as biological carrier of probiotic microorganisms (Sisto and Lavermicocca 2012). Today, the main source where commercial probiotic strains can be isolated is the human gastrointestinal tract and recently a clinical isolate of *L. paracasei* IMPC2.1 has been used as a probiotic culture in the fermentation of green and ripe olives with promising results (Lavermicocca et al. 2005; De Bellis et al. 2010; Sisto and Lavermicocca 2012) indicating that table olives could become a reliable vehicle for the transportation of probiotic bacteria into the human gastrointestinal tract. However, other ecosystems such as fruits and vegetables may harbour functional strains, as they possess intrinsic chemical and physical traits that mimic the conditions of the human gastrointestinal tract (Di Cagno et al. 2013). For this purpose, a large number of indigenous LAB isolates from fruits and vegetables, including table olives, has been screened for functional properties with promising results (Abriouel et al. 2012; Argyri et al. 2013; Vitali et al. 2012; Bautista-Gallego et al. 2013a). The LAB employed in this work as starter cultures have been reported as the two most dominant species in table olive fermentation (Hurtado et al. 2012). Both strains inoculated either as single or combined cultures were able to establish a vigorous lactic fermentation regardless of the initial salt concentration in the brines, with the exception of *L. plantarum* B282 that was slightly affected by the 10% salt brines. The inoculated strains were able to colonize the surface of olives at high populations ranging from 6.0 to 7.0 log CFU/g throughout the different processes. This is particularly important since a prerequisite to deliver the health benefits, probiotics need to contain an adequate amount of live bacteria (at least 10^6-10^7 CFU/g) (Oliveira et al. 2002; Boylston et al. 2004). An

important issue in inoculated olive fermentation is to monitor the survival of the specific inoculated strain during the process using molecular identification techniques, a fact that has received the attention of the scientific community only recently in similar studies (Panagou et al. 2008; Saravanos et al. 2008; De Bellis et al. 2010; Abriouel et al. 2012; Rodríguez-Gómez et al. 2013). In this work, identification of LAB isolates using PFGE analysis revealed several changes in the survival of the inoculated LAB strains. Specifically, *L. pentosus* B281 was able to survive at high rates in both salt brines in contrast to *L. plantarum* B282 that could not be recovered from 10% salt bines at the end of the process. It seems that *L. plantarum* B282 was sensitive to high salt concentration and was not able to impose over the native LAB microbiota on the surface of olives. Interestingly, the same LAB strain could not be recovered in the combined inoculum fermentation from the middle stage of the process (day 69) onwards, indicating a dominance of *L. pentosus* B281, making thus this strain a better selection for inoculated fermentation as it can survive at high rates in diverse salt concentrations and can also impose over the wild LAB strains on the surface of olives. These results illustrate the impact of different salt concentrations in the selection of the inoculated strains that will eventually dominate the process and at the same time demonstrate the potential to produce probiotic olives with less salt, an issue of paramount importance for the table olive industry today (Bautista-Gallego et al. 2013b).

The obtained results are in good agreement with a previous work undertaken in our laboratory with the same LAB strains and fermentation procedures on green olives that were subjected to a heat-shock treatment at 80 ^{o}C for 10 min prior to inoculation (Argyri et al. 2014). Both strains were able to colonize the olives,

complete a lactic fermentation and survive in high numbers at the end of the process. However, as the implementation of a heat-shock treatment is rather difficult to find acceptance on an industrial scale, the current work expands the existing data on the performance of the selected probiotic LAB strains in industrial fermentation procedures of green olives. In a recent work (Rodríguez-Gómez et al. 2013) Spanish researchers have reported on the imposition of four potential probiotic *L. pentosus* strains in the brines of green olive Spanish-style fermentation that have been previously isolated from the microbiota of table olives. They concluded that inoculated processes favoured the development of proper fermentation but the imposition of the selected strains in the cover brines was not assured. The new elements provided in this study compared to the existing literature are that (1) the LAB probiotic strains have been selected from the indigenous microbiota of table olives and they are not clinical isolates or from other food origin, (2) the imposition of the selected strains was determined on olive drupes and not in the cover brine in order to investigate the ability of the selected strains to colonize the surface of olives, and (3) fermentations were undertaken in two different initial salt concentrations including a lower salt level (8%) to ensure the development of a typical green olive fermentation with lower salt concentration.

The organic acids with a major presence in the brines were lactic acid as the main biochemical product during fermentation followed by acetic acid, the presence of which could be attributed to a shift from homo to hetero-fermentative metabolism of the inoculated LAB strains due to nutrient limitation, salt concentration (Nychas et al. 2002) as well as to yeast metabolism (Arroyo-López et al. 2008). It needs to be noted that organic acids were determined in fermentation brines and not directly in

olive flesh; however due to the rapid equilibrium between brine and olives in Spanish-style processing, the dynamics of organic acids in the brine could reflect quite accurately the changes occurring in olive flesh. The profile of organic acids was typical for table olive fermentation according to previously published works (De Castro et al. 2002; Panagou et al. 2008; Hurtado et al. 2010; Rodríguez-Gómez et al. 2013). Generally, inoculated processes presented higher lactic acid production rates depending on LAB strain and salt concentration in the brines, especially at the beginning of the process, but this difference was gradually diminished with time indicating that the beneficial effect of inoculation is more pronounced at the early stage of fermentation (Garrido-Fernández et al. 1997). In the case of the spontaneous process in 8% salt brines, a progressive decrease and finally absence of lactic acid was evident with a concurrent increase in propionic acid concentration. The presence of this organic acid that was detected only in 8% salt spontaneous fermentation process, affected the quality of the final fermented olives as itwas defined by the result of sensory evaluation. The taste panel detected a deviation from the typical green olive fermentation as inferred by the higher scores given to the sensory attribute 'abnormal fermentation', although they were not able to specify the type of deterioration detected. This irreversible deterioration that was observed in duplicate vessels of this treatment justifies the benefit of using starter cultures in green olive fermentation to achieve reproducible and high quality final products, especially when low salt fermentation brines are used. Ethanol and methanol were the major compounds identified in the brines according to GC analysis at varying concentrations depending on the inoculated LAB strain and salt concentration. The high level of ethanol observed in certain treatments could be attributed to the heterofermentative

activity of LAB as well as to yeast metabolism in the assayed condition (Garrido-Fernández et al. 1997). There was clearly an effect of salt concentration on the production of these volatile compounds that may result in the selection of the starter culture according to the salt level employed in fermentation. Thus, in low salt fermentation procedures *L. plantarum* B282 would have been a better option due to the lower amounts of ethanol and methanol produced in the brines. However in high salt fermentation brines *L. pentosus* B281 would be a better selection as a starter culture for the same reason. It is also noteworthy that a high concentration of 2-butanol was detected in 8% salt spontaneous fermentation, a treatment that was characterized as non typical in terms of flavour and odour by the taste panel. The presence of this volatile alcohol has been associated with off-odour development in the spontaneous fermentation of green olives providing a winey-off odour to the final product (Rodríguez-Gómez et al. 2013).

Multivariate data analysis has found extensive application in food processing including table olives (Bautista Gallego et al. 2011; Rodríguez-Gómez et al. 2012 a, b, 2013). PCA analysis was able to differentiate the fermentation profiles with respect to the different LAB strains and salt levels assayed. The spontaneous process in 8% salt level that was found not typical for green olive fermentation by the taste panel was clearly differentiated from the rest of the processes. Moreover, the majority of fermentations was close to each other and provided profiles that resembled the spontaneous process in 10% salt level. However, the inoculated process with *L. pentosus* B281 in 8% salt brine was clearly separated from the remaining treatments and associated with volatile esters which are major components of the aroma of fruits responsible for the pleasant flavour appreciated by consumers (Sabatini et al. 2009).

Therefore this strain could provide interesting perspectives to the table olive industry for the production of table olives with probiotic potential while maintaining the traditional character of green olive fermentation.

3.5. Conclusion

The results of the current investigation revealed promising perspectives for the application of the selected LAB strains as starter cultures in green olive fermentation to produce a high added value final product with probiotic potential. From the two strains, *L. pentosus* B281 was proved to be more effective as it was able to survive better in the different salt levels employed in fermentation and colonize the olive surface in higher populations compared to *L. plantarum* B282, dominating even in the case of mixed inoculation of the brine with the two strains. In contrast, the latter LAB strain could not be recovered from the olive surface in 10% salt brines at the end of fermentation indicating low imposition over the wild or contaminant LAB populations during the process and sensitivity to high salt levels. Consequently, *L. pentosus* B281 could become a promising candidate for the production of green olives with probiotic potential provided that these results could be confirmed at industrial scale, a process which is currently underway.

Chapter **4**

Biofilm formation on Conservolea natural black olives during single and combined inoculation with a functional *Lactobacillus pentosus* starter culture

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Abstract

Lactic acid bacteria (LAB) and yeasts are the associated microorganisms in table olive preparations and their assembly in biofilm community upon olive drupes has been reported together with the probiotic potential of selected strains, transforming thus a traditional product into a high added value functional food. The present study examines the ability of biofilm formation of *Lactobacillus pentosus* B281 and *Pichia membranifaciens* M3A, both coming from olive microbiota, used as starter cultures during Consvolea black olive fermentation. The probiotic potential of the former (Argyri et al. 2013) and its overall good performance during green olive fermentation cv. Halkidiki (Blana et al. 2014) has been previously shown while the later is among the dominating yeast species during black olive fermentation (Nisiotou et al. 2010b). Olives were directly brined in plastic fermenters (14Lt total capacity) with 8%(w/v) NaCl initial concentration. Three fermentation conditions in duplicates were employed namely, i) spontaneous fermentation, ii) inoculated fermentation with B281 and iii) co-inoculated fermentation with B281 and M3A. During fermentation the population dynamics of LAB and yeasts on drupes were determined by plate count routinely. The survival of the inoculated strains was confirmed by Pulsed Field Gel Electrophoresis whereas at the end of fermentation (153 days) biofilm formation was observed under Scanning Electron Microscope. In both cases of inoculated fermentations yeasts were between 3.5 to 4.5 log cfu/g whereas the population of lactic acid bacteria maintained between 5.5 to 6.5 log cfu/g. Lactic acid was the main organic acid found in the brine, as determined by High Performance Liquid Chromatography, recovered in high amounts in both inoculated fermentations followed by acetic and citric acids in varying concentrations depending on the

fermentation treatment. Gas chromatography indicated ethanol and methanol as the major volatiles in the brine while traces of propanol, acetaldehyde, ethylacetate and 2-butanol were detected. A sensory evaluation of the final product was also performed by a ten person taste panel by which no indications of abnormal fermentation or other disadvantages were perceived.

4.1. Introduction

It has long been established that microorganisms in natural environments tend to adhere to any solid surface immersed in a liquid medium and assemble in a complex multispecies consortium often embedded in a extracellular polymeric matrix (EPS) produced by the microorganisms known as biofilm (Hall-Stoodley et al. 2004; Wimpenny 2009). During table olive preparation the drupes are originally immersed in brine that is gradually enriched by nutrients coming from the olive mesocarp serving as substrate for microorganisms to initiate fermentation in order to obtain a microbiologically safe final product with enhanced sensory characteristics (Garrido-Fernández et al. 1997). The presence of aggregates of LAB and yeasts colonizing the surface of natural black olives was first decribed by Nychas et al. (2002) whereas biofilm development between LAB-yeasts species on Spanish-style green olives has been recently reported (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012). The most representative LAB species during table olive processing are *L. pentosus, L. plantarum* and to a lesser extent *L. paraplantarum* (Hurtado et al. 2012) whereas diverse yeast species belonging to the genera *Candida, Issatchenkia, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces* and *Wickerhamomyces* have been reported (Arroyo-López et al. 2008).

On the other hand, a number of studies focusing on the development of new functional probiotic foods have been carried out in the last years indicating that plant based products could be successfully employed as carriers of microorganisms with probiotic potential (Sun-Waterhouse 2011; Gupta and Abu-Ghannam 2012). Probiotics consumption is proposed to confer a number of beneficial health effects to the individual that consumes them such as inhibition of pathogens, alleviation of

allergic symptoms and in general boost the immune system (Isolauri 2001; Cross 2002). Towards this aim, Lavermicocca et al. (2005) used selected strains of lactobacilli and bifidobacteria as probiotic cultures in fermented olives with promising results in terms of adhesion and colonization on olive epidermis, survival rate and good recovery during storage. In another study (De Bellis et al. 2010), the strain *L. paracasei* IMPC2.1, isolated from human intestine, was used both as probiotic starter culture to better control the fermentation of Bella di Cerignola green olives resulting in a final product with functional appeal. Since the main source of probiotic isolates is the human gastrointestinal tract, the exploitation of fruits and vegetables, including table olives has been suggested as means of selection of autochthonous LAB strains with functional properties (Di Cagno 2013). In this sense, a number of LAB originating from the table olive environment has been screened for functional properties (Abriouel et al. 2012; Vitali et al. 2012; Argyri et al. 2013; Bautista-Gallego et al. 2013a) whereas their application as starters in table olive fermentations have indicated promising results (Bevilacqua et al. 2010; Abriouel et al. 2012; Argyri et al. 2013; Bautista-Gallego et al 2013a) and their application as starters in table olive fermentation has indicated promising results (Arroyo-López et al 2012a; Argyri et al. 2014; Blana et al. 2014; Rodríguez-Gómez et al. 2013, 2014). The exploitation of such species possessing functional and/or probiotic potential together with their ability to colonize and establish biofilms on olives could make this agricultural commodity a carrier of health promoting properties for consumers. However, the above works focused on green olive fermentation, whereas the applicability of multifunctional starter cultures in natural black olive fermentation remains mostly unexplored. In a recent work (Tufariello et al. 2015) selected yeast and LAB isolates

with technological and safety characteristics (β-glucosidase activity, absence of amino acids decarboxylation activity, presence of protease and lipase activity) were used as starter cultures in a sequential fermentation strategy of Italian and Greece black table olvie varieties with promising results in the control and standardization of the fermentation process, improvement of sensory characteristics and minimization of fermentation time compared with commercial products.

In the present study, the ability of multifunctional starter cultures namely, *L. pentosus* B281 and the yeast *P. membranifaciens* M3A was investigated during Greek-style processing of Conservolea natural black olives. The probiotic potential of the former microorganism has been previously elucidated (Argyri et al. 2013) whereas the selected yeast is part of the dominant yeast biota in black olive fermentation (Nisiotou et al. 2010b) and its technological properties have been recently reported (Bonatsou et al. 2015). The *in situ* predisposition of the selected microorganisms to colonize the surface of olives and form biofilm communities was recently reported using black oxidized olives as a model system (Grounta and Panagou 2014). However, as in this trade preparation of olives there is absence of indigenous microbiota, it was deemed necessary to extend the obtained results in natural black olive fermentation where the selected multifunctional cultures would have to compete with the interfering indigenous microbiota in order to colonize the olives and form biofilms.

4.2. Materials and Methods

4.2.1. Microorganisms used and preparation of inocula

The LAB *L. pentosus* B281 and the yeast *P. membranifaciens* M3A used in this study belong to the culture collection of the Laboratory of Microbiology and

Biotechnology of Foods, Agricultural University of Athens, and have been previously isolated from brines of fermented olives (Nisiotou et al. 2010b; Doulgeraki et al. 2013). The selected LAB strain exhibited *in vitro* probiotic potential including survival in low pH, resistance to bile salts, partial bile salt hydrolase activity, absence of *β*-haemolytic activity, adherence to *Caco-2* cells, antibiotic resistance to kanamycin, gentamycin and erythromycin (Argyri et al. 2013). Moreover, the selected yeast species presented resistance to salt, survival in simulated gastric digestion, *β*-glucosidase and esterase activity (Bonatsou et al. 2015). The LAB and yeast were maintained as stock cultures in de Man-Rogosa-Sharpe broth (MRS, Lab M, Heywood, UK) and YEPD (1 % yeast extract, 2 % peptone, 2 % glucose) broth respectively, supplemented with 20 % glycerol at -80^{o} C until use. The LAB strain was revived by adding 10 μL of the stock culture in 10 mL MRS broth and incubating at 30^{o} C for 24 h. Working cultures were obtained by adding 500 μL of the revived culture in 50 mL MRS broth containing 4.5 % (w/v) NaCl and incubating at 30^{o} C for 24 h. The yeast was revived by adding 10 μL of the stock culture in 10 mL YEPD broth and incubating at 25^{o} C for 48 h with agitation. Working cultures were obtained by adding 500 μL of the revived culture in 50 mL YEPD broth and incubating at 25^{o}C for 24 h with agitation. LAB and yeast cells were centrifuged at 5000 g for 10 min at 4^{o} C and the resulting pellet was resuspended in 50 mL ¼ Ringer's solution resulting in a final concentration of *ca.* 9.0 log CFU/mL and 7.0 log CFU/mL for *L. pentosus* and *P. membranifaciens*, respectively as assessed by plate counting.

4.2.2. Fermentation procedures and inoculation of the brine

The olives were of the Conservolea variety kindly supplied by Konstantopoulos S.A table olive industry. The drupes were harvested in mid-December, subjected to quality control at the processor's facilities to remove defective olives and transported to the laboratory within 24h. On arrival, the olives were transferred in 14 L plastic vessels containing 7.0 kg of olives and 5.0 L of freshly prepared 8% (w/v) NaCl solution. The brine was acidified at the onset of fermentation with 0.5 % (v/v) vinegar (*ca.* 6%, v/v, acetic acid). Inoculation was carried out after 24h of brining using a 50-mL aliquot of each working culture in order to achieve an inoculum level in the brine of 7.0 log CFU/mL and 5.0 log CFU/mL for the LAB and yeast starter, respectively. Overall, three fermentation treatments were investigated namely, (i) spontaneous fermentation (control), (ii) inoculated fermentation with *L. pentosus* B281 and (iii) inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A. During the period of fermentation the salt level was adjusted to 8% by periodical additions of coarse salt in the brine. All fermentations were performed in duplicate and the vessels were maintained at room temperature for a period of 153 days.

4.2.3. Microbiological analysis

Olive samples were analysed at regular time intervals (16 sampling points per treatment) to allow for the determination of the population dynamics on the surface of olives. It needs to be noted that the population dynamics in natural black olive fermentation of cv. Conservolea has been routinely monitored in the brine medium (Nychas et al. 2002; Tassou et al. 2002; Panagou et al. 2008; Bleve et al. 2015; Tufariello et al. 2015). However, in this work the focus was given on the disposition of the selected starters to colonize the olive surface and form biofilms and thus it was

deemed necessary to monitor the changes in the microbial population directly on the olive drupes. For this reason, three olives (ca. 20-22 g total weight) were randomly sampled from each fermentation vessel and rinsed twice with 10 mL sterile Ringer's solution to remove the loosely attached cells (Giaouris et al. 2013) and then depitted with sterile scalpel and forceps under aseptic conditions. Subsequently, the drupes were transferred into a stomacher bag, decimally diluted with sterile quarter-strength Ringer's solution and homogenized in a stomacher device for 2 min (Lab Blender 400, Seward Medical, London, UK) at room temperature. Serial decimal dilutions were prepared in Ringer's solution and 1 or 0.1 mL from the appropriate dilution were poured or spread on the following agar media: (i) de Man-Rogosa-Sharpe agar (MRS; Lab M, Lancashire, UK) for the enumeration of LAB, supplemented with 0.05% (w/v) cycloheximide (AppliChem, Darmstadt, Germany), overlaid with 10 mL of molten medium and incubated at 30 ^{o}C for 48-72 h, (ii) Violet Red Bile Glucose Agar (VRBGA; Biolife, Italy) for the enumeration of enterobacteria, overlaid with 10 mL of molten medium and incubated at 37 ^{o}C for 18-24 h, and (iii) Rose Bengal Chloramphenicol Agar (RBC; Lab M, Lancashire, UK) for the enumeration of yeasts, incubated at 25 ^{o}C for 72 h. Results were expressed as log values of colony forming units per gram of olives (log CFU/g).

4.2.4. Physicochemical analysis and sensory evaluation

Brine samples were analysed at the same time intervals as for microbiological analysis for the determination of pH, titratable acidity, and organic acids. Specifically, the changes in pH values were measured by a digital pHmeter (model RL150, Russel Inc. Boston, MA). Titratable acidity was determined according to Garrido-Fernández

et al. (1997) and organic acids in the brine were analysed by HPLC as described elsewhere (Blana et al. 2014). Changes of volatile compounds (methanol, ethanol, 2-butanol, ethyl acetate, propanol, acetaldehyde, dimethylsulfide) in the brine were analysed by Gas Chromatography following the static headspace technique (Montaño et al. 1990) slightly modified as detailed elsewhere (Villegas Vergara et al. 2013). Sensory attributes of the final product were evaluated by a ten person taste panel according to the method of sensory analysis of table olives established by the International Olive Council (IOC 2011). Negative (abnormal fermentation, other defects), gustatory (bitter, salty, acid) and kinaesthetic (hardness, fibrousness, crispness) sensations were evaluated and results were expressed as the median score provided by the members of the panel together with the robust standard deviation. Sensory data were subjected to analysis of variance using Statgraphics Centurion XV (Statpoint, Inc., Warrenton, Virginia) to define significant differences among the sensory descriptors for the three fermentation procedures ($p < 0.05$).

4.2.5. Determining the survival of the probiotic strain

The survival of *L. pentosus* B281 during fermentation was determined by Pulsed Field Gel Electrophoresis (PFGE). A total of 100 lactobacilli isolates were picked at three different time points from inoculated fermentations, namely early (day 1), middle (day 72) and late stage (day 153) of the process. Isolates were picked from the highest dilution of MRS agar medium and pure cultures were stored at -80 ^{o}C in MRS broth supplemented with 20% (v/v) glycerol. Genomic DNAwas extracted from the isolates according to Doulgeraki et al. (2010). The restriction enzyme ApaI (10U) (New England Biolabs, Ipswich, MA, USA) was applied according to the

manufacturer's recommendation for 16 h. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris-Borate buffer on CHEF e DGII (Bio e Rad, Hercules, CA, USA) equipment with the following parameters: 6 V/cm, 1 s initial switching time, 10 s final switching time and 16 h of total run at 14 ^{o}C. The obtained gels were stained in water with ethidium bromide (0.5 mg/mL) for 1 h, distained in water for 2 h and finally photographed using a GelDoc system.

The survival of *P. membranifaciens* M3Awas determined by RFLP analysis. A total of 53 yeast isolates were picked from the RBC medium at the same time points as for *L. pentosus* B281. Pure cultures of yeast isolates were stored at -80 ^{o}C in YPD broth (1% yeast extract, 2% peptone and 2% dextrose) supplemented with 20% (v/v) glycerol until use. Genomic DNA was extracted from pure cultures and the 5.8S-ITS rDNA region amplified by PCR (Nisiotou et al. 2010b). PCR products were separately restricted by 10 units of restriction endonoucleases HinfI, HaeIII and HhaI (New England Biolabs, Ipswich, MA, USA) and the restriction fragments were separated by gel electrophoresis in 3% (w/v) agarose, stained with ethidium bromide and photographed. The obtained restriction profiles were compared with the profile of the inoculated yeast *P. membranifaciens* M3A as reference strain.

4.2.6. SEM observation

At the end of the process, olive drupes from each fermentation procedure were observed under Scanning Electron Microscope (SEM) for the presence of biofilm. The drupes were washed twice with Phosphate Buffered Saline (PBS) and 3×3mm2 slices were cut from the olive epidermis and fixed in a 2.5% (w/v) glutaraldehyde solution overnight. Subsequently, slices were washed five times in PBS for 5 min and

then dehydrated in an increasing concentration series of ethanol (50%, 70%, 80%, 90%, 95% and 100%) for 5 min. Finally, the slices were dried in critical point in a Polaron E3000 critical point dryer, and coated with gold in a Denton Vacuum DV 502 coating unit. Images were taken with a Jeol JLM-6360 scanning electron microscope (JEOL, USA, Inc. Peabody, MA).

4.2.7. Exploratory data analysis

The heterogeneous data collected throughout the different sampling times in the experiment comprising microbiological (counts of LAB and yeasts) and physicochemical data (pH, acidity, organic acids, volatile compounds) were subjected to multivariate statistical analysis to investigate any discriminating potential of the processes based on their overall profile. A supervised classification technique, namely Discriminant Function Analysis (DFA) was chosen in this work, in which for a given number of groups (i.e., fermentation processes) corresponding to the dependent variable Y, the method searches for a linear arrangement in the hyperspace constructed on the independent variable X (i.e., microbiological and physicochemical data) that produces the largest mean difference between analysed groups, thereby maximizing between-class distance while minimizing within-class distance (Gromski et al. 2015). DFA analysis was performed with Statistica version 8.0 (Statsoft Inc., Tulsa, OK, USA).

4.3. Results and Discussion

4.3.1. Microbiological changes during fermentation

The population dynamics of LAB, yeasts and *Enterobacteriaceae* on olive drupes during fermentation are presented in Figure 4.1. In the spontaneous fermentation (Fig.4.1a) the counts of *Enterobacteriaceae* and LAB were below the enumeration limit (<1.0 log CFU/g) resulting in a process dominated by yeasts. In the inoculated treatment with *L. pentosus* B281 (Fig. 4.1b), the initial population of *Enterobacteriaceae* was 3.64 (±1.36) log CFU/g and rapidly decreased below the enumeration limit within 16 days, whereas the population of LAB was initially enumerated at 5.74 (±0.17) log CFU/g and progressively increased maintaining an

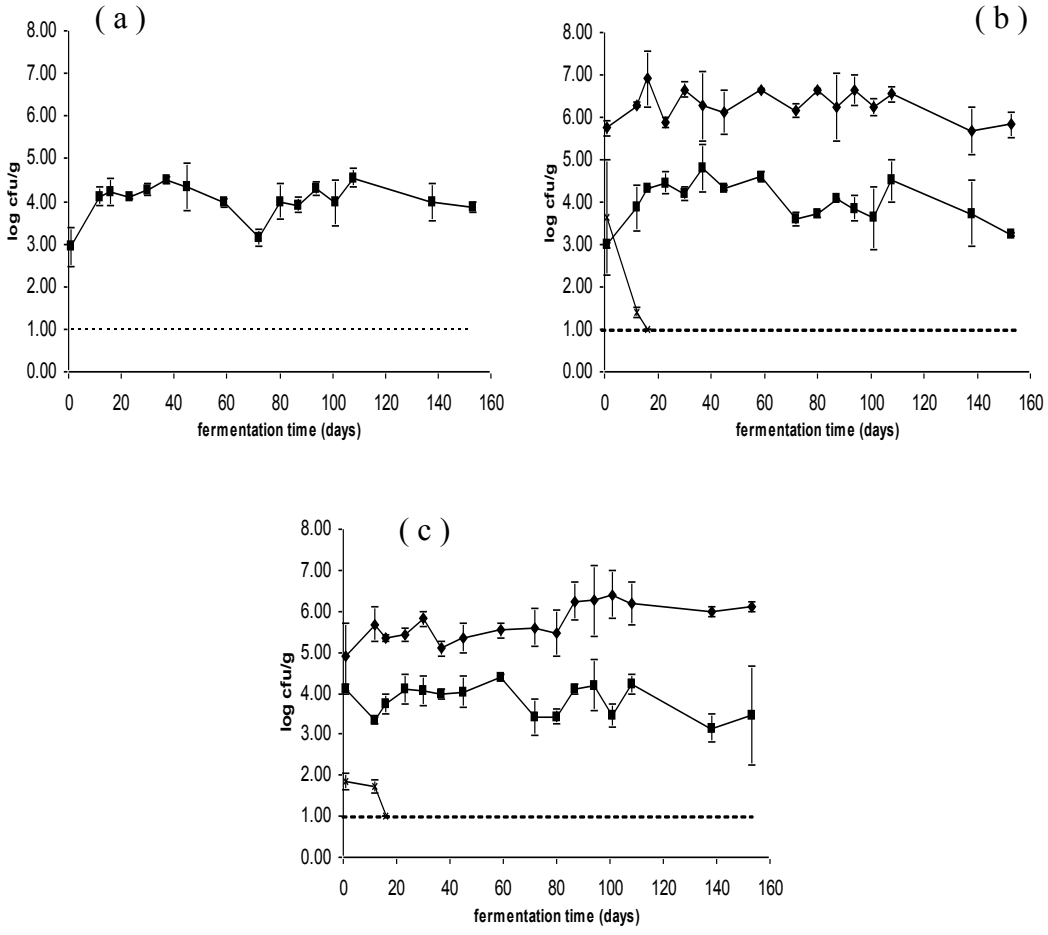


Figure 4.1. Microbial changes of LAB (A), yeasts (-) and enterobacteriaceae (×) on olive drupes during (a) spontaneous fermentation, (b) inoculated fermentation with *L. pentosus* B281 and (c) inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A. Data points are average values of duplicate fermentations ± standard deviation. Dashed line indicates the enumeration limit of the method (1.0 log CFU/g).

average value of 6.2 log CFU/g until the end of the process. A similar trend was observed in the case of combined inoculation with *L. pentosus* B281 and *P. membranifaciens* M3A, where *Enterobacteriaceae* were initially counted at 1.85 (±0.21) log CFU/g and decreased rapidly below the enumeration limit within 16 days (Fig. 4.1c). In the same process, the recovered LAB population on olive drupes was enumerated at 4.88 (±0.83) log CFU/g at the onset of fermentation and maintained at around 6.0 log CFU/g towards the end of fermentation. In both inoculated treatments yeasts co-existed with LAB but in lower numbers ranging between 3.5 and 4.5 log CFU/g.

The absence of LAB in the spontaneous process is in good agreement with a previous study in which naturally black olives of Conservolea variety were processed in different salt levels and temperature conditions (Tassou et al. 2002). The authors reported that the highest salt level used in the brine (8%, w/v) resulted in retarded LAB growth leaving the salt tolerant yeasts almost unaffected dominating the process. Delayed growth of LAB was also reported in a recent work (Bleve et al. 2015) in which black olives of the Conservolea and Kalamata varieties were subjected to Greek-style processing in 8% NaCl for a period of 180 days. The authors reported that LAB could not be enumerated in brine samples until the 105th day of fermentation in both varieties. LAB growth in both inoculated treatments could be attributed to the initial high inoculum of *L. pentosus* B281 and also to the fact that prior to inoculation, the strain was cultured in a medium supplemented with 4.5% (w/v) NaCl allowing adaptation to the saline environment of the brine (De Castro et al. 2002). In comparison to our findings, *L. pentosus* B281 has been previously employed as mono-

culture and in combination with a *L. plantarum* strain as starter culture in Spanish-style fermentation of cv. Halkidiki green olives using 8 and 10% (w/v) initial salt concentration in the brine (Blana et al. 2014). Results showed that *L. pentosus* B281 was able to dominate the process and adhere on the surface of olives in higher populations (6.0-7.5 log CFU/g) either in single or combined inoculation presenting better adaptation to higher salt concentrations. Furthermore, as mentioned in the introduction of the present work, *L. pentosus* B281 has been previously investigated for its *in situ* predisposition to adhere on the surface of black oxidized olives cv Halkidiki under different sterile brine conditions in single or coinoculation with the same yeast employed in the present study (Grounta and Panagou 2014). In most brining treatments, the selected strains were able to colonize the olives in high numbers ranging from ca. 6.0-7.0 log CFU/g and 5.0-5.5 log CFU/g for *L. pentosus* B281 and *P. membranifaciens* M3A, respectively.

4.3.2. Physicochemical changes during fermentation

The initial pH values in the brine ranged from 4.2 to 4.7 due to the acidification step with vinegar (Fig. 4.2). The inoculated process with *L. pentosus* B281 with/ without the presence of *P. membranifaciens* M3A resulted in a pH decrease within the first 12 days to 3.87 (±0.03) and 4.01 (±0.15), respectively, followed by a plateau thereafter until the end of fermentation where the pH was maintained at 3.9-4.0 (Fig. 4.2a). The profile of titratable acidity was similar in inoculated processes presenting a gradual increase until the end of fermentation reaching a final value of 0.48 g of lactic acid/100 mL of brine. On the other hand, the dominance of yeasts during the spontaneous fermentation was reflected by the high pH values in the brine ranging

between 4.6 and 4.8 and the low values of titratable acidity that were constantly below 0.2 g of lactic acid/ 100 mL of brine (Fig. 4.2b). This product does not meet the specifications of the trade standard applying to table olives of the IOC (IOC 2004) where for natural fermentations the maximum limit of pH should be 4.3 and the minimum acidity 0.3 g of lactic acid/ 100 mL of brine. Organic acids with major presence in the brines (lactic, acetic, citric) are presented in Figure 4.3. Lactic acid was detected in higher concentrations in the inoculated brines with progressively increasing levels within the first month of the process. However, from day 37 to 94, the concentration of lactic acid was higher in the brines inoculated with *L. pentosus* B281 alone, but from this point onwards no differences could be observed between the inoculated processes reaching a final concentration of 80 mM (Fig. 4.3a). In the case of spontaneous fermentation no lactic acid production could be detected due to the restriction of LAB growth. The amount of acetic acid at the onset of fermentation was ca. 12.3 (±1.8) mM on average in all fermentation procedures, due to the initial acidification of the brines with vinegar (Fig. 4.3b), presenting a progressive increase until the end of fermentation. Higher concentrations of acetic acid were measured in inoculated fermentation with *L. pentosus* B281 with/without the presence of *P. membranifaciens* M3A, amounting to 56.4 (±1.8) mM and 58.5 (±1.8) mM, respectively.

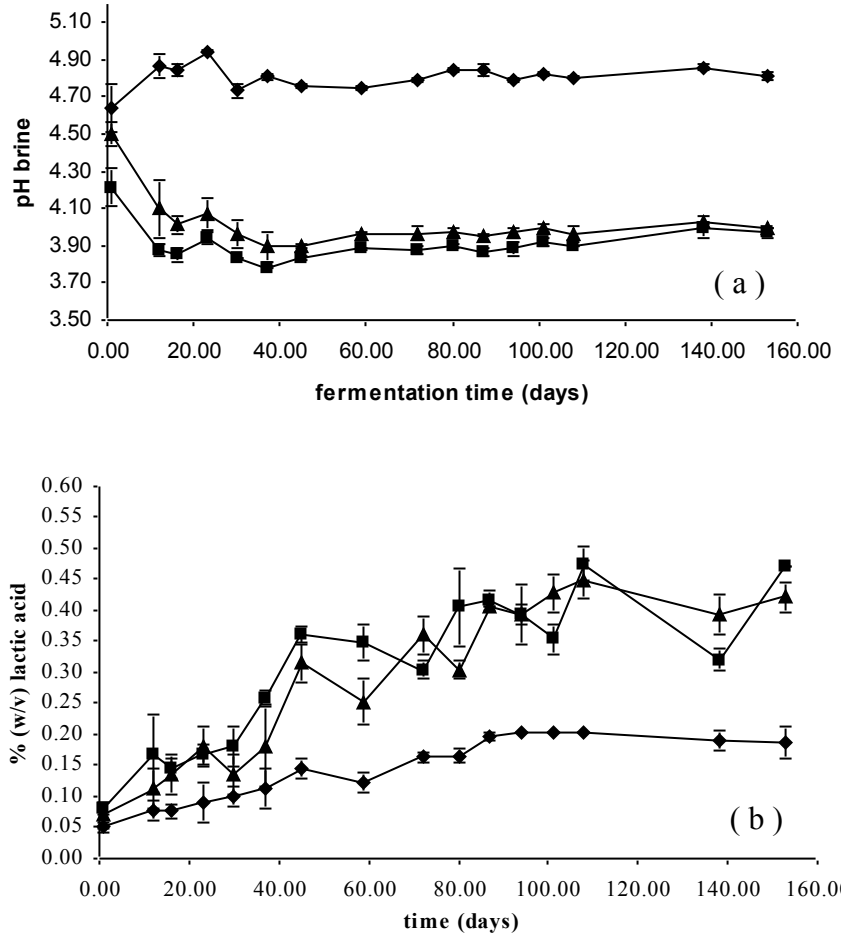
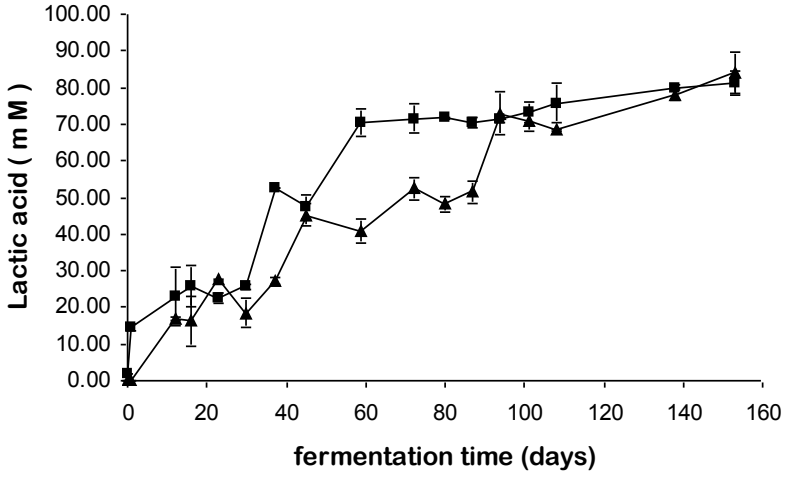


Figure 4.2. Changes in pH (a) and titratable acidity in the brines (b) during spontaneous fermentation (♦), inoculated fermentation with *L. pentosus* B281 (■) and inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A (▲). Data points are average values of duplicate fermentations ± standard deviation.



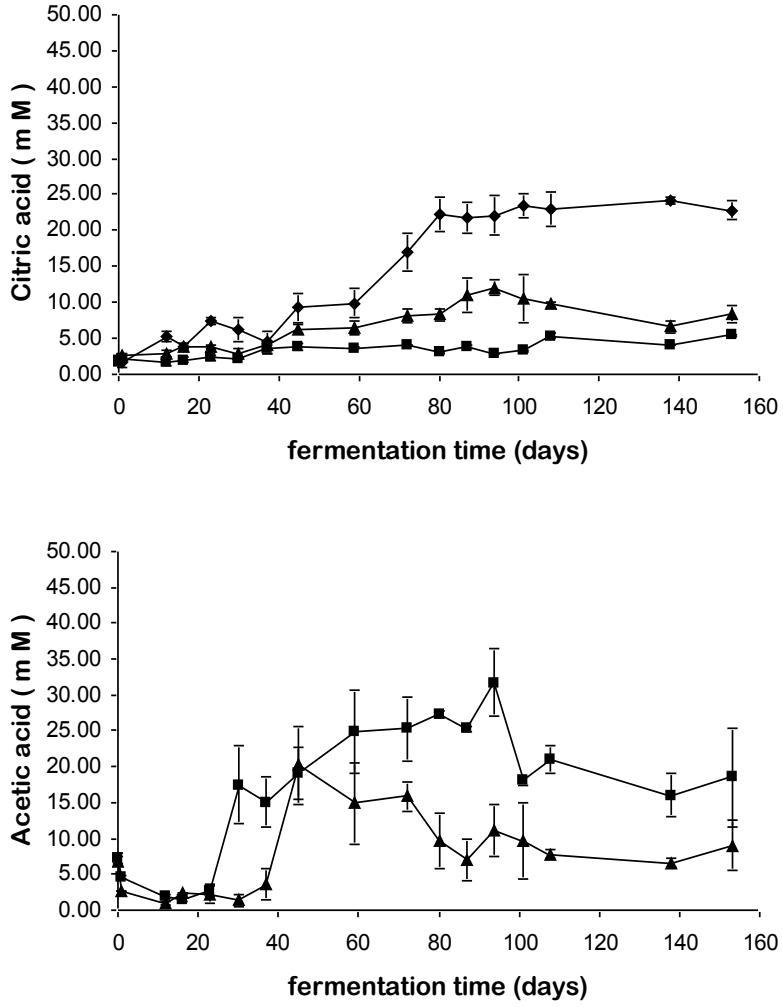


Figure 4.3. Changes in the concentration of lactic, citric and acetic acid (mM) during spontaneous fermentation (♦), inoculated fermentation with *L. pentosus* B281 (■) and inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A (▲). Data points are average values of duplicate fermentations ± standard deviation.

It has been reported that the presence of this metabolite may be due to a shift from homo to hetero-fermentative metabolism of the inoculated LAB species due to environmental stress (Bobillo and Marshall 1991,1992) as well as to yeast metabolism (Arroyo-López et al. 2008). Citric acid was most abundant in spontaneous fermentation reaching a concentration at ca. 22 mM from day 80 onwards (Fig. 4.3c). It was detected in lower amounts in dual species inoculated fermentation, while very low levels of this compound were also observed in the brines inoculated with *L.*

pentosus B281 that never exceeded 5 mM. In line with our results, enhanced acetic acid production as outcome of citric acid metabolism has been reported in brines inoculated with *L. plantarum* and *L. pentosus* during Conservolea green and black olive fermentation (Panagou et al. 2008; Panagou and Tassou 2006).

The volatile compounds identified in the brine are presented in Table 4.1 among which ethanol was the most abundant followed by methanol, whereas propanol, 2-butanol, ethyl acetate, acetaldehyde and dimethyl sulfide were detected in lower amounts. A statistically significant ethanol production ($p < 0.05$) was observed between spontaneous and inoculated-driven processes, due to the dominance of yeasts, giving final concentrations exceeding 3000 mg/L at the late stage of the spontaneous process. In the brines inoculated with *L. pentosus* B281, a progressive increase was observed reaching the highest peak of 1350.1 mg/L at day 80 but decreased thereafter until the end of the process. In the coinoculated process ethanol production was even lower giving a peak of 872.85 mg/L at day 59 and later on decreasing at 747.03 mg/ L. High ethanol concentration was also reported in the fermentation of Conservolea black olives after sequential inoculation, initially with *Debaryomyces hansenii* A15-44 followed by *L. plantarum* A135-5 after 63 days of fermentation (Tufariello et al. 2015). The authors reported that the ethanol content was similar in starter driven and in spontaneous fermentations due to the dominance of yeasts throughout the process. However, in this work the inoculation of the LAB starter from the onset of fermentation either alone or in combination with the yeast starter resulted in lower ethanol levels due to the dominance of *L. pentosus* B281 on the olives from the early stage of fermentation (Fig. 4.1). Ethanol is the precursor of ethyl esters with ethyl acetate (ethereal fruity notes) being among the most important

ones; however, its presence is generally undesirable at high concentrations exceeding 200 mg/L (Roza et al. 2003). In the present study, its presence was detected in all types of fermentation treatments but in low concentrations ranging between 0.69 and 3.39 mg/L without presenting a statistical difference ($p = 0.091$) among the processes. It needs to be noted that in the work of Tufariello et al. (2015) esters presented an increasing concentration during the course of fermentation and they were associated with the late stage of fermentation (90 days). However, this observation was not confirmed in our work where the highest concentration of ethyl acetate was detected in the spontaneous and co-inoculated process at 3.39 mg/L and 3.16 mg/L after 30 and 59 days, respectively. Methanol being the second major metabolite detected in the brines exhibited a similar trend between the different treatments (no statistical differences, $p = 0.885$). Specifically, it was initially detected at 53.27 mg/L for spontaneous fermentation, 57.43 mg/L for inoculation with *L. pentosus* B281 and 68.75 mg/L for inoculation with *L. pentosus* B281 and *P. membranifaciens* M3A which progressively increased giving final values of 365.07, 371.47, and 333.41 mg/L, respectively. Methanol production during naturally black olive fermentation has been previously reported (Panagou et al. 2008) as well as in green olive fermentation (Montaño et al. 1992; Panagou and Tassou 2006; Blana et al. 2014) and its presence has been associated with pectinolytic activity by enzymes excreted by yeasts (Silva et al. 1994). Higher alcohols (2-butanol, propanol) were also detected in the brines. The former compound was either sporadically detected in the case of inoculated fermentations or not detected at all (spontaneous fermentation). The presence of this volatile alcohol has been reported in green olive fermentation providing a winey off-odour to the final product (Rodríguez-Gómez et al. 2013). Propanol was also

identified in low amounts (<7.0 mg/L) regardless of fermentation treatment (no statistical difference, p = 0.077). This alcohol has been previously detected in inoculated and uninoculated Kalamata and Moresca table olives (Sabatini et al. 2008), in Nocellara del Belice green olives (Sabatini and Marsilio 2009), in Conservolea black olives (Panagou et al. 2011) and Halkidiki green olives (Blana et al. 2014) but in higher concentrations. Furthermore, a statistical difference (p = 0.002) in the changes of acetaldehyde between spontaneous and starter-driven fermentations was observed. Thereby, at the end of the process the final value of this compound amounted to 9.33 mg/L while lower amounts were detected in the inoculated treatments. This metabolite is regarded as an important sensory carbonyl compound originating mainly from yeast metabolism (Osborne et al. 2000) and together with ethanol is considered to contribute to the secondary odour of fermented olives (Fleming et al. 1969). Finally, dimethyl sulfide (DMS), a sulphur compound, was detected in low amounts not exceeding 1.5 mg/L without presenting significant differences among the different processes (p = 0.860). DMS has been reported to originate from the degradation of sulphur-containing amino acids due to microbial activity and is described as having sulphurous, cabbage-like odour (Casaburi et al. 2015). A similar compound, methyl sulfide, has been reported from the green olives processed by the Spanish method (Fleming et al. 1969).

Table 4.1. Changes of volatile compounds in the brines expressed as mg/L during during cv. Conservolea black olive fermentation

Fermentation Process	Fermentation time (days)	Volatile compound (mg/L)

		Ethanol	Methanol	2 - Butanol	Propanol	Ethyl acetate	Acetaldheyde	Dimethylsulfide
Spontaneous fermentation	0	283.83±62.83	53.27±13.46	-	-	0.69±0.02	1.65± 0.15	0.59±0.01
	16	1086.69±203.89	125.12±24.57	-	4.44±0.08	3.16±0.06	1.07± 0.19	0.62±0.01
	30	1538.35±332.75	174.46±44.74	-	4.78±0.02	3.39±0.26	1.25±0.50	0.64±0.01
	59	1999.28±272.52	220.21±20.09	-	5.45±0.38	1.48±0.19	2.53±0.11	0.67±0.06
	80	3016.4± 85.38	250.31±44.46	-	5.58±0.59	1.59±0.79	5.77±0.04	0.76±0.03
	108	3487.32±70.71	323.77±24.27	-	6.39±0.08	1.28±0.19	3.26±0.74	0.83±0.14
	153	3293.2±317.12	365.07±46.88	-	6.09±0.10	1.00±0.14	9.33±0.06	1.2±0.28
L. pentosus B281	0	311.52±30.35	57.43±5.24	-	-	0.69±0.05	0.73±0.08	0.58±0.02
	16	591.31±67.33	128.79±23.14	-	3.97±0.23	0.93±0.10	1.33±0.24	0.65±0.02
	30	653.89±48.90	133.56±2.98	0.62±0.02	4.12±0.27	1.00±0.12	1.19±0.16	0.61±0.04
	59	1279.52±198.45	278.82±2.45	-	4.01±0.34	1.81±0.14	2.04±0.12	0.84±0.09
	80	1350.1±170.88	269.84±16.36	0.51±0.10	4.30±0.16	1.54±0.34	3.28±0.29	0.85±0.001
	108	1095.76±76.96	300.81±38.26	-	4.38±0.06	1.77±0.32	2.01±0.04	0.97±0.05
	153	786.87±23.41	371.47±20.55	0.69±0.07	5.57±0.45	1.77±0.27	1.59±0.26	1.46±0.12
L. pentosus B281 and	0	375.04±14.16	68.75±6.14	-	-	0.71±0.01	1.11±0.09	-
P. membranifaciens M3A	16	360.39±84.48	116.57±3.05	-	4.02±0.12	1.19±0.71	1.15±0.15	0.65±002
	30	358.02±63.45	112.72±17.98	-	3.94±0.15	1.89±0.94	0.86±0.46	0.63±0.03
	59	872.85±296.92	190.69±34.19	0.83±0.09	4.20±0.26	3.16±1.52	1.16±0.10	0.73±0.04
	80	600.88±201.72	272.49±39.16	0.73±0.05	5.48±0.23	1.41±0.44	2.19±0.19	0.78±0.14
	108	791.99±79.22	319.14±14.59	1.11±0.12	5,37±1.73	1.22±0.13	1.71±0.25	0.89±0.01
	153	747.03±11.94	333.41±28.19	1.78±0.16	6.56±1.78	1.24±0.05	1.71±0.94	1.19±0.07

4.3.3. Survival of the inoculated strains

RFLP analysis revealed that at the early stage (day 1) the recovered yeast biota was dominated (100%) by *P. membranifaciens* M3A, but after 72 days its survival was reduced to 40%, whereas at the end of fermentation the inoculated yeast was not recovered at all (Table 4.2). The inability of the selected yeast starter to dominate the process could be attributed to competition for growth substrates with the inoculated LAB culture as well as the indigenous yeast biota of olives (Viljoen 2006). At the same time points, the survival of *L. pentosus* B281 in the inoculated and co-inoculated process was monitored by PFGE. Results showed that *L. pentosus* B281 was recovered at 100% in all fermentation stages and in both inoculation treatments, indicating the efficacy of this strain to dominate the autochthonous LAB microbiota, adhere on the surface of olives and maintain its viability throughout fermentation. The specific strain has recently exhibited high survival potential in inoculated green olives cv Halkidiki (Argyri et al. 2014; Blana et al. 2014) with surviving rates up to 100%. It is noteworthy that the imposition of a mixed starter culture of LAB and yeast has not been extensively studied in table olive fermentation. The first report on this issue was recently published (Hurtado et al. 2010) considering a co-inoculated fermentation in Arbequina naturally green olives using, among other treatments, a strain of *L. pentosus* 5E3A18 in combination with *Candida diddensiae*. Results showed that the LAB starter dominated in the brines over the indigenous LAB population in rates exceeding 80% and the presence of *C. diddensiae* resulted in better microbial profile of the inoculated LAB starter as yeasts could be considered as adjunct starters to improve LAB development (Tsapatsaris and Kotzekidou 2004; Segovia Bravo et al. 2007; Arroyo-López et al. 2008). Furthermore, four other *L. pentosus* strains with

probiotic potential were employed as multifunctional starter cultures in Spanish-style table olive fermentation (Rodríguez-Gómez et al. 2013) and it was reported that the selected strains resulted in a better control of the fermentation process but their imposition in the brine was not assured. In a further work, the same researchers (Rodríguez-Gómez et al. 2014) employed two multifunctional starters of *L. pentosus* at pilot plant scale fermentation of green olives and reported that the selected LAB were able to prevail over the indigenous population of lactobacilli and adhere on the surface of olives in high populations. Overall, the obtained results illustrated that in inoculated processes the selected LAB strain was able to dominate the process and colonize the surface of olives in high populations from the early stage of fermentation enhancing thus the probiotic potential of natural black olives.

Table 4.2. Survival of *Lactobacillus pentosus* B281 and *Pichia membranifaciens* M3A during single and co-inoculated fermentation of Conservolea natural black olives.

Inoculated process	**Fermentation time (d)**	***L. pentosus* B281**		***P. membranifaciens* M3A**	
		No of isolates	**Survival rate**	**No of isolates**	**Survival rate**
L. pentosus B281	1	15	15/15 (100%)	-	-
	72	23	23/23 (100%)	-	-
	138	24	24/24 (100%)	-	-
	1	10	10/10 (100%)	22	22/22 (100%)

L. pentosus B281 & *P. membranifaciens* M3A	72	17	17/17 (100%)	19	8/19 (42%)
	138	17	17/17 (100%)	12	0/12 (0%)

4.3.4. Biofilm development on olive drupes

Dominance of yeasts in the spontaneous process was confirmed by SEM (Fig. 4.4a) with yeasts appearing in stomatal openings whereas a mixed association of LAB and yeasts embedded in EPS matrix in stomata was observed in inoculated processes (Fig. 4.4b). It needs to be noted that apart from the microbial aggregations in stomatal apertures, no adherent microorganisms could be observed in the remaining area of olive epidermis. This observation can be confirmed by a previous work on natural black olives (Nychas et al. 2002) where mixed communities of yeasts and LAB were located in stomata and sub-stomal cavities. The spatial distribution of the same microorganisms on black oxidized olives was reported recently (Grounta and Panagou 2014) indicating a different location of the adherent cells not only in the stomatal apertures but also on the surface of olives. This difference could be attributed to the fact that natural black olives are not subjected to lye treatment prior to brining and thus the olive cuticle remains intact and may act as a barrier for the release of nutrients disabling thus the microorganisms to ‘sense’ a concentration gradient and migrate toward the olive skin (Domínguez-Manzano et al. 2012).

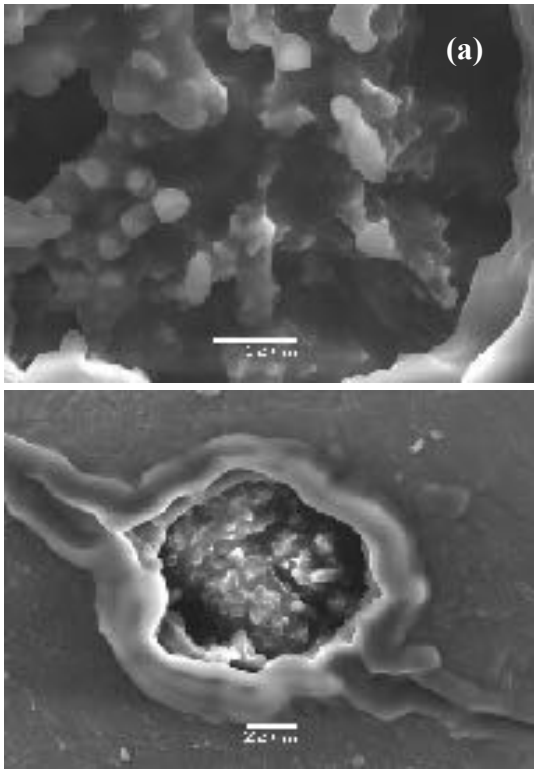


Figure 4.4. SEM images of biofilm development in the case of (a) spontaneous fermentation, and (b) inoculated fermentation with L. pentosus B281 at the end of the fermentation process

4.3.5. Sensory analysis

No off odours indicating abnormal fermentation (i.e., butyric, putrid fermentation, or zapateria) were detected by the panelists in any of the processes (Table 4.3). Other defects were not detected but five out of ten panelists noticed a distinct odour in olives from the spontaneous fermentation described as "wine odour" or "ethanol odour" due to the dominance of yeasts in this process resulting in higher amounts of ethanol as confirmed by volatile compounds analysis. For the gustatory sensations of saltiness and bitterness no statistically significant differences could be established among the different fermentations. Concerning acidity, olives from the inoculated process with *L. pentosus* B281 were given higher scores, whereas olives from the spontaneous and co-inoculated processes with *P. membranifaciens* M3A

developed a milder taste receiving lower values of acidity. No significant differences were also observed between the different processes concerning the kinaesthetic sensations of hardness, fibrousness and crunchiness as all of them received similar scores.

Table 4.3. Sensory attributes (median ± standard deviation) of cv. Conservolea black olives at the end of the spontaneous and inoculated fermentation processes.

Sensory attribute	Fermentation process		
	Spontaneous	*L. pentosus* B281	*L. pentosus* B281 & *P. membranifaciens* M3A
Abnormal fermentation	1.00 ± 0.16 [a]	1.00 ± 0.67 [a]	1.5 ± 0.78 [a]
Other defects	1.25 ± 2.82 [a]	1.00 ± 0.09 [a]	1.00 ± 0.2 [a]
Salty	6.15 ± 1.19 [a]	7.55 ± 2.01 [a]	6.6 ± 1.42 [a]
Bitter	4.1 ± 1.63 [a]	4.05 ± 1.38 [a]	4.15 ± 1.89 [a]
Acid	3.7 ± 1.02 [a]	5.00 ± 1.53 [b]	3.75 ± 1.61 [a]
Hardness	3.2 ± 1.53 [a]	3.85 ± 1.7 [a]	3.2 ± 0.99 [a]
Fibrousness	1.7 ± 1.09 [a]	1.6 ± 1.73 [a]	1.55 ± 0.93 [a]
Crunchiness	2.75 ± 1.38 [a]	3.2 ± 0.72 [a]	2.95 ± 0.98 [a]

*Median values with different letters in the same row are statistically significant ($p < 0.05$).

4.3.6. Exploratory data analysis of fermentation profiles

The DFA plot showed a clear separation between control and inoculated processes (Fig. 4.5). Fermentation with *L. pentosus* B281 presented negative scopes in DF1 and positive scores in DF2, whereas co-inoculated fermentation with the LAB starter and *P. membranifaciens* M3A had negative scores in both DFs. It is evident that DF1 discriminated mostly between spontaneous and inoculated-driven processes, whereas DF2 distinguished mostly between LAB and LAB/yeast inoculated fermentations. In order to assess the discriminating capacity of the variables the Wilks' Lambda and F-tests were performed (Table 4.4). Small values of Wilks'

Lambda and high values of F-test indicate high discriminating capacity of the variable. Results showed that the most discriminating variables were the population of LAB, citric acid, volatile alcohols (ethanol, methanol, propanol), and dimethyl sulfide. The coefficients of the discriminant functions (DF) in raw and standardized form are provided in Table 4.5. The raw coefficients could be used to classify new observations among the three fermentations whereas the standardized coefficients are customarily used for interpretation as they pertain to the standardized variables and therefore refer to comparable scales. Thus, DF1 is weighted most heavily to the population dynamics of LAB, pH, acidity, lactic acid, methanol, and propanol, whereas DF2 is associated with yeasts, acetic and citric acids, ethanol, ethyl acetate, and dimethyl sulfide. In addition, DF1 accounted for 97.7% of the explained variance and hence almost all discriminatory power was explained by this function. Multivariate data analysis has found extensive application in food science lately to extract useful information from multiple and in most cases heterogeneous variables monitored during processing. In the case of table olives, DFA has been successfully employed in the characterization of olives according to their nutrient composition

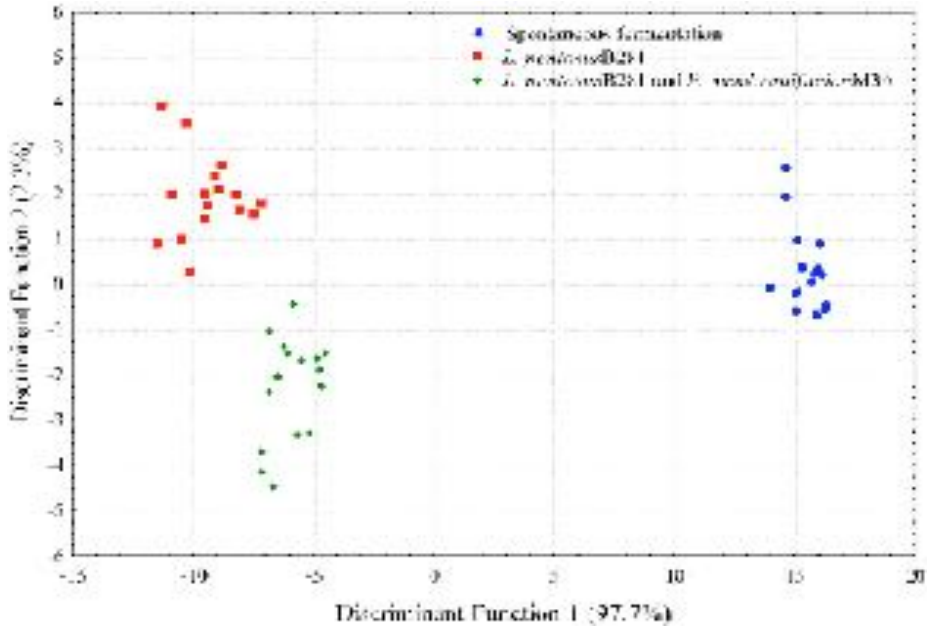


Figure 4.5. DFA plot showing the discrimination between the different fermentation procedures based on microbiological and physicochemical variables measured throughout processing.

Table 4.4. Wilks' Lambda and F-tests of group means for the fermentation processes as derived by DFA analysis.

Variable	Wilks' Lambda	F	p-level
LAB	0.0051	27.78	0.000
Citric acid	0.0041	19.57	0.000
Dimethyl sulfide	0.0023	4.78	0.015
Ethanol	0.0025	6.12	0.005
Methanol	0.0022	3.94	0.029
Propanol	0.0022	3.66	0.037
Yeasts	0.0022	3.23	0.052
Acetaldehyde	0.0021	3.09	0.059
Acidity	0.0021	2.77	0.077
Ethyl acetate	0.0020	1.66	0.204
pH	0.0019	1.44	0.251
Lactic acid	0.0018	0.24	0.781
Acetic acid	0.0018	0.09	0.915

Values in bold are statistically significant ($p < 0.05$).

Table 4.5. Raw and standardized coefficients for the discriminant variables of the DFA analysis.

Variable	Raw coefficients		Standardized coefficients	
	DF_1	DF_2	DF_1	DF_2
Constant	-1.879	-4.922	-	-
LAB	-3.385	-0.125	**-1.140**	-0.042
Yeasts	-0.773	1.736	-0.342	**0.769**
pH	4.351	-1.055	**0.462**	-0.112
Acidity	12.07	-8.878	**1.301**	-0.956
Lactic acid	0.017	0.003	**0.355**	0.075
Acetic acid	-0.006	0.016	-0.049	**0.121**
Citric acid	0.190	-0.532	1.016	**-2.845**
Ethanol	0.002	0.003	0.094	**1.754**
Methanol	-0.023	0.011	**-2.176**	0.982
Propanol	0.609	-0.553	**0.877**	-0.796
Ethyl acetate	-0.062	-0.661	-0.047	**-0.499**
Acetaldehyde	-0.547	0.814	-0.553	**0.822**
Dimethyl sulfide	-1.412	6.352	-0.317	**1.426**

Values in bold indicate major contribution to discriminant factors

(López-López et al. 2007, 2008), the classification of olives from different cultivars (Pinheiro and Esteves Da Silva 2005), and the differentiation of different fermentation procedures of green Conservolea olives processed by the Spanish method (Villegas Vergara et al. 2013).

4.4. Conclusion

The obtained results illustrated that the use of *L. pentosus* B281 resulted in a final product with appropriate physicochemical characteristics and well appreciated sensory properties. Also, its ability to survive in high numbers on olive drupes forming biofilm together with its in vitro probiotic potential makes it a good candidate for the production of natural black olives with functional characteristics. In contrast, *P. membranifaciens* M3A could not be recovered from the olive surface at the end of fermentation despite the high adherence on olives at the onset of the process. However, the presence of the yeast starter, even if not recovered at the end of the process, resulted in a proper fermentation process rendering a final product with good sensory attributes and a milder acid gustatory sensation. Hence, this product could be suitable for consumers who do not appreciate the acid taste of natural black fermented olives and prefer milder tastes. Overall, the use of multifunctional starter cultures in natural black olive fermentation provides interesting perspectives in the transformation of table olives from a traditional agricultural commodity to a high added value functional product. Our findings are further supported by Italian researchers (Tufariello et al. 2015) who proposed a new fermentation process for natural black olives based on a sequential inoculation strategy of selected yeast and LAB starters with specific technological properties in order to standardize the process. Moreover, as the role of yeasts in black olive fermentation is continuously elucidated, further research is needed to unravel the technological/probiotic potential of this microbial group in order to produce functional black olives.

Chapter **5**

Quantification and characterization of microbial biofilm community attached on the surface of fermentation vessels used in green table olive processing

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Abstract

The aim of the present study was the quantification of biofilm formed on the surface of plastic vessels used in Spanish-style green olive fermentation and the characterization of the biofilm community by means of molecular fingerprinting. Fermentation vessels previously used in green olive processing were subjected to sampling at three different locations, two on the side and one on the bottom of the vessel. Prior to sampling, two cleaning treatments were applied to the containers, including (a) washing with hot tap water (60 °C) and household detergent (treatment A) and (b) washing with hot tap water, household detergent and bleach (treatment B). Population (expressed as log CFU/cm^2) of total viable counts (TVC), lactic acid bacteria (LAB) and yeasts were enumerated by standard plating. Bulk cells (whole colonies) from agar plates were isolated for further characterization by PCR-DGGE. Results showed that regardless of the cleaning treatment no significant differences were observed between the different sampling locations in the vessel. The initial microbial population before cleaning ranged between 3.0-4.5 log CFU/cm^2 for LAB and 4.0-4.6 log CFU/cm^2 for yeasts. Cleaning treatments exhibited the highest effect on LAB that were recovered at 1.5 log CFU/cm^2 after treatment A and 0.2 log CFU/ cm^2 after treatment B, whereas yeasts were recovered at approximately 1.9 log CFU/ cm^2 even after treatment B. High diversity of yeasts was observed between the different treatments and sampling spots. The most abundant species recovered belonged to *Candida* genus, while *Wickerhamomyces anomalus, Debaryomyces hansenii* and *Pichia guilliermondii* were frequently detected. Among LAB,

Lactobacillus pentosus was the most abundant species present on the abiotic surface of the vessels.

5.1. Introduction

Table olives are one of the most important groups of vegetables marketed and consumed as fermented. A proper fermentation procedure results in a microbiologically safe final product with enhanced sensory attributes. This is achieved primarily by the growth of LAB that metabolize fermentable sugars diffused from the olive mesocarp into the brine and produce lactic acid which decreases the pH in the brine, resulting thus in the inhibition of undesirable microorganisms and the extended preservation of the end product even at ambient temperature (Corsetti et al. 2012; Heperkan 2013). Development of some yeast species is also desirable as they contribute in the production of aroma compounds and maintenance or even stimulation of LAB populations (Viljoen 2006; Arroyo-López et al. 2008, 2012b). The population dynamics of these diverse microbial groups throughout the fermentation process were, until recently, monitored in the cover brines. However, it has been shown that LAB and yeast communities colonize the surface of the naturally black and Spanish style table olives drupe forming mixed species aggregates characterized as biofilms (Nychas et al. 2002; Domínguez-Manzano et al. 2012; Grounta et al. 2016).

Biofilm formation in food processing environments has been the focus of extensive scientific research, especially in the context of food hygiene, as many

outbreaks have been associated with the presence of biofilms in food industries (Srey et al. 2013). Biofilms are defined as functional consortia of microorganisms attached to a surface which are embedded in the extracellular polymeric substances (EPS) produced by the microorganisms (Monds and O'Tool 2009). It has been realized that biofilm formation is a natural phenomenon which occurs whenever there are microorganisms and surfaces, either biotic or abiotic, surrounded by a high or a low level of nutrients (Elhariry 2011; Giaouris et al. 2014). In these environments, the accumulation of food nutrients at the solid/liquid interface on food surfaces leads to a higher concentration of nutrients compared to the fluid phase which as a process is known as "conditioning film" (Donlan 2002). After film conditioning, microorganisms may adhere on the food contact surface, gradually form microcolonies and finally assemble themselves in biofilms exhibiting high microbial diversity in terms of genera, species and strain level (Borucki et al. 2003; Burmølle et al. 2006). A similar phenomenon takes place during table olive fermentation as the outflow of nutrients from the mesocarp into the brine medium may serve as a means of conditioning film on both the olive epidermis and the surface of the container where fermentation takes place and thus form sites of adherence and biofilm development. The presence of biofilm on the surface of olive drupes has been extensively studied by various research groups in the last years, since the olive surface may serve as a carrier of beneficial microorganisms to the consumer, transforming thus table olives from a traditional agricultural commodity to a high-added functional food (Lavermicocca et al. 2005; Arroyo-López et al. 2012a; Rodríguez Gómez et al. 2013; Blana et al. 2014). So far, the formation of biofilms on the abiotic surface of fermentation vessels has received little attention by the table olive research

community. Recently, Spanish researchers (Domínguez-Manzano et al. 2012) investigated the establishment of polymicrobial communities on abiotic (glass slides) surfaces that come into contact with the brine during Spanish style table olive fermentation and confirmed the ability of microorganisms to adhere and produce biofilms. The presence of this microbial community on the abiotic surfaces, composed primarily of members of the technological microbiota may have an impact on olive fermentation. Nowadays, it is well established that the natural microbiota of olives is greatly reduced during lye treatment and the effectiveness of the process depends mainly on the number of LAB introduced during the subsequent washing and brining operations (Garrido-Fernández et al. 1997). However, biofilm formation is a stepwise and dynamic process consisting of several stages, among which dispersal is the last step in the biofilm cycle, allowing thus cells to revert into their planktonic form (Srey et al. 2013). In this sense, the biofilm formed on the abiotic surfaces of the fermentation vessels may contribute to the process with microorganisms of the technological microbiota, namely LAB and yeasts, through the mechanism of biofilm detachment into the brine.

The objective of the present study was to investigate the biofilm community formed on the surface of plastic containers used in the fermentation of green table olives at different sampling locations and different cleaning treatments of the vessel, as well as to characterize the microbiota assembling this community by molecular fingerprinting. To our knowledge, this is the first report on the characterization of microbial biofilms on the surface of fermentation vessels and the results of this work could provide further insights in the fermentation process of green olives.

5.2. Materials and methods

5.2.1. Sampling from the vessels and microbiological analysis

The fermentation vessels employed in this work were screw-capped plastic vessels (14 L total capacity) in which spontaneous Spanish style fermentation of cv. Halkidiki green olives was undertaken as described elsewhere (Blana et al. 2014). At the end of fermentation olives and brine were discarded, the vessels were washed with pressurized tap water, left to dry and subsequently two sequential cleaning treatments were applied namely, (a) cleaning with hot tap water (60 ˚C) and household detergent (treatment A) and (b) cleaning with hot tap water, household detergent and bleach (treatment B). Before and after each cleaning treatment, the vessels were subjected to sampling at three different locations on the inside of the container, namely, (i) on the upper spot (near the opening of the vessel), (ii) on the middle spot (halfway up the side), and (iii) on the bottom of the vessel. Sampling was implemented using a fixed template of 56 cm^2 (7 cm wide x 8 cm long) for the upper and middle spots, whereas for the bottom of the vessel a fixed area of 77 cm^2, (corresponding to the half bottom of the vessel) was sampled. The sampling areas were thoroughly scrubbed with sterile gauze which was submerged in sterile urine collectors containing 30 mL Phosphate Buffered Saline (PBS). The gauze was left for about 20 min to allow suspension of cells in the PBS solution and microbiological analysis was carried out from this suspension. The resulting PBS suspension was serially diluted in Ringer's solution and duplicate 1 or 0.1 mL from the appropriate dilution were poured or spread on agar media. TVC were enumerated on Tryptone Soy Agar (TSA; LabM, Lancashire, UK) incubated at 25 ^oC for 48 h. Yeasts were enumerated on Rose Bengal Chloramphenicol agar (RBC; LabM, Lancashire, UK) incubated at 25 ^oC for 48-72 h. LAB were enumerated on de Man-Rogosa-Sharpe agar (MRS; LabM, Lancashire, UK) supplemented with 0.05 % (w/v) cycloheximide (AppliChem, Darmstadt, Germany), overlaid with 10 mL of molten medium, incubated at 30 ^oC for 48-72 h. Results were expressed as log values of colony forming units per cm^2 of surface area (log CFU/cm^2). Following cleaning treatments, the vessels were left to dry and 60 days later, the vessels were subjected in the same cleaning treatments and sampling

was performed in the same sampling spots to investigate any potential changes in the biofilm communities during storage of the vessels. Duplicate vessels were analyzed for each treatment denoted herewith as vessel a and b, respectively.

5.2.2. Preparation of bulk cells and DNA extraction

Genomic DNA was extracted from bulk cells on MRS, RBC and TSA plates for each cleaning treatment and sampling spot. The whole cultivable community from each agar plate was diluted in an appropriate volume of Ringer's solution and 1 mL of the bulk was stored in a cryoprotect vial at -80 ^{o}C for further use. DNA was extracted using the protocol described by Doulgeraki et al. (2012). One millilitre of bulk cells was centrifuged at 9,000 g for 5 min at 4 ^{o}C, washed with Tris-EDTA buffer (100 mM Tris, 10 mM EDTA) and finally resuspended in 0.5 mL buffer solution (1 M sorbitol, 0.1 M EDTA, pH 7.4) containing 25 mg/ml lysozyme. After incubation at 37 ^{o}C for 2 h, the samples were centrifuged at 17,000 g for 10 min at 4 ^{o}C and the pellet was resuspended in 0.5 mL buffer solution (50 mM Tris - HCl, 20 mM EDTA, pH 7.4). Subsequently, 50 µl of 10 % SDS were added and then the samples were incubated at 65 ^{o}C for 30 min. After incubation, 0.2 mL potassium acetate 5 M was added to the samples which were left on ice for 30 min and then centrifuged at 17,000 g for 20 min at 4 ^{o}C. The supernatant was precipitated with 1 mL ice cold isopropanol. After precipitation, the samples were centrifuged at 17,000 g for 10 min at 4 ^{o}C and the resulting pellet was resuspended in 0.5 mL ice cold 70 % ethanol. The samples were then centrifuged at 17,000 g for 10 min at 4 ^{o}C and the resulting pellet was left to dry. Finally, the dried pellet was resuspended in 50 µL sterile double distilled H_2O and stored at -80 ^{o}C until further use.

5.2.3. PCR-DGGE analysis

Genomic DNA from bulk cells on TSA and MRS plates was amplified by PCR targeting the variable V6 – V8 region of the 16S rRNA as described by Ercolini et al.

(2006). Genomic DNA from bulk cells on RBC and TSA plates was amplified by PCR targeting the 5.8S – ITS rDNA region according to Nisiotou et al. (2010b). PCR products were confirmed by gel electrophoresis in 1.0% (w/v) agarose gel followed by ethidium bromide staining. Subsequently, positive PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) using a DCode apparatus (BioRad) according to Ercolini et al. (2006) with a gradient from 22 to 55% and 20 to 60 % for bacteria and yeasts, respectively. Electrophoresis was performed at 200V for 4 h (with an initial 10 min at 50V) at 60 ^{o}C in 1 × TAE buffer. The gels were then stained with ethidium bromide for 5 min, rinsed for 20 min in distilled water and photographed using a Gel- Doc system (Biorad, Hercules, CA, USA).

The obtained DGGE profiles were compared with those of bacteria and yeast strains belonging to the Food Microbiology Culture Collection (FMCC) of the Agricultural University of Athens, previously isolated from environments associated with table olive fermentations which were used as reference strains. For bacteria characterization, *L. pentosus* B281 and *L. plantarum* B282 were used as reference strains (Blana et al. 2014). For yeast characterization, *Pichia guilliermondii* Y16, *Pichia kluyveri* Y17, *Wickerhamomyces anomalus* Y18, *Candida silvae* Y19, *Saccharomyces cerevisiae* Y22, *Debaryomyces hansenii* Y57, and *Pichia membranifaciens* Y67 were used as reference strains (Nisiotou et al. 2010b). DNA bands of interest, not matching the reference strains' profile, were excised from DGGE gels, reamplified by PCR and sequenced. Sequence similarities were searched for by Basic Local Alignment Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database.

5.2.4. Multiplex PCR for the recA gene

Bulk cells collected from TSA and MRS subjected to multiplex PCR amplification for further differentiation of *L. plantarum* group species as described elsewhere (Torriani et al. 2001). Briefly, primers pentF (59-CAG TGG CGC GGT

TGA TAT C-39), pREV (59-TCG GGA TTA CCA AAC ATC AC-39), paraF (59-GTC ACA GGC ATT ACG AAA AC-39) and planF (59-CCG TTT ATG CGG AAC ACC TA-39) were used to amplify the *rec*A gene giving PCR products of about 107, 218 and 318 bp for *L. paraplantarum*, *L. pentosus* and *L. plantarum* species, respectively.

5.2.5. Scanning Electron Microscopy

Three sections (5×5 mm^2) cut from the vessels, corresponding to the three different sampling spots after the second cleaning treatment at 60 days, were fixed in 2.5 % glutaraldehyde in PBS overnight. The sections were washed five times in PBS for 5 min and then dehydrated in a series of increasing concentrations of ethanol (50 %, 70 %, 80 %, 90 %, 95 % and 100 %) for 5 min. They were dried in critical point in a Polaron E3000 critical point dryer, coated with gold in a Denton Vacuum DV 502 coating unit, and finally observed under a Jeol JLM-6360 scanning electron microscope (JEOL, USA, Inc. Peabody, MA).

5.2.6. Statistical analysis

The microbiological data were subjected to analysis of variance (ANOVA) taking as factors the location of the sampling point (upper and middle spot on the side and bottom of the vessel) and the sampling treatment (before or after cleaning). Statistically significant differences between the microbial counts were assessed using Tukey's Studentized Range (HSD) test at a significance level of $p<0.05$. Statistical analysis was performed using the JMP ver.8 package (SAS Institute Inc., Cary NC, USA).

5.3. Results and discussion

5.3.1. Quantification of biofilm community before and after cleaning treatments

The population of each microbial group enumerated before and after the cleaning treatments from the different sampling spots of the vessels is illustrated in

Figure 5.1. TVC population before the cleaning treatments was 5.28 (± 0.88), 5.23 (± 0.14) and 4.77 (± 0.34) log CFU/cm^2 for the three sampling points (i.e., upper and middle spot on the side and the bottom of the vessel), respectively. The corresponding yeast population was 4.12 (± 0.46), 4.58 (± 0.12) and 4.59 (± 0.05) log CFU/cm^2 for the same locations, while those of LAB were 4.54 (± 0.91), 4.15 (± 0.27) and 3.00 (± 1.08) log CFU/cm^2. It is characteristic that no significant difference was observed in the enumerated population of all microbial groups between the three sampling points (upper, middle, and bottom) in the fermentation vessels. The effect of the cleaning treatments was more pronounced on LAB population (Fig. 5.1c) resulting in up to 3.0 log units reduction, while TVC numbers (Fig. 5.1a) and yeasts (Fig. 5.1b) declined by approximately 2.0 log units. It needs to be noted that all groups of microorganisms were recovered after each treatment at statistically significant lower populations (p<0.05), but they were not completely eliminated even after treatment B. For this reason, right after the cleaning procedures, the plastic vessels were left to dry and 60 days later microbiological analysis was performed again in the same sampling locations to quantify the reformed biofilm community. At that time, TVC population was 3.26 (± 0.51), 3.89 (± 0.08) and 3.89 (± 0.05) log CFU/cm^2 for the upper, middle spot on the side and the bottom of the vessel, respectively. The higher tolerance of yeasts over LAB under dehydrated conditions was evident since the former microbial group was recovered at considerable levels, ranging between 2.76 and 3.94 log CFU/ cm^2 while no population of LAB was recovered after 60 days.

The presence of biofilms on the surface of fermentation vessels was also verified by scanning electron microscopy (SEM) images (Fig. 5.2). Typical SEM images provide evidence that microorganisms aggregate and colonize the surface of vessels while being embedded in a biofilm matrix with some cells trying to detach from the biofilm (Fig. 5.2a). Mixed biofilm community between LAB and yeasts has been also verified by SEM analysis on the surface of glass slides immersed in the brine during green olive fermentation (Domínguez-Manzano et al. 2012). The authors reported that at the onset of fermentation microorganisms appeared to be individually attached on the slides, forming mixed communities with the progress of fermentation. However, at the end of the process (80 days) only a few mixed species colonies could

be observed from the biofilm that appeared to be disrupted. It is worth noting that the development of biofilm communities on the surface of table olive fermentation vessels has not been reported so far by other researchers. However, in a similar study the characteristics of the microbial biofilm on wooden vats used in the production of PDO Salers cheese were reported under different cheese processing conditions (Didienne et al. 2012). In this study biofilm samplings were undertaken from two different locations on the inside of the vat (bottom and side) and revealed a great diversity of LAB, yeasts and moulds, and Gram negative bacteria. The authors indicated differences in the spatial distribution of the biofilm microbiota, namely LAB and *Pseudomonas* spp., with the bottom of the vats presenting higher counts compared to the inner side. However, this observation is not in line with the findings of this work where no statistically significant difference in the enumerated microorganisms could be established between the different sampling locations in the fermentation vessels. The authors of the previous study (Didienne et al. 2012) attributed this difference to the volume of the vats indicating that the higher the volume the more marked the difference between the bottom and the sides. This could also be the case in our research where the small volume of the fermentation vessels could not allow differentiation in the counts of the microorganisms between the different sampling locations.

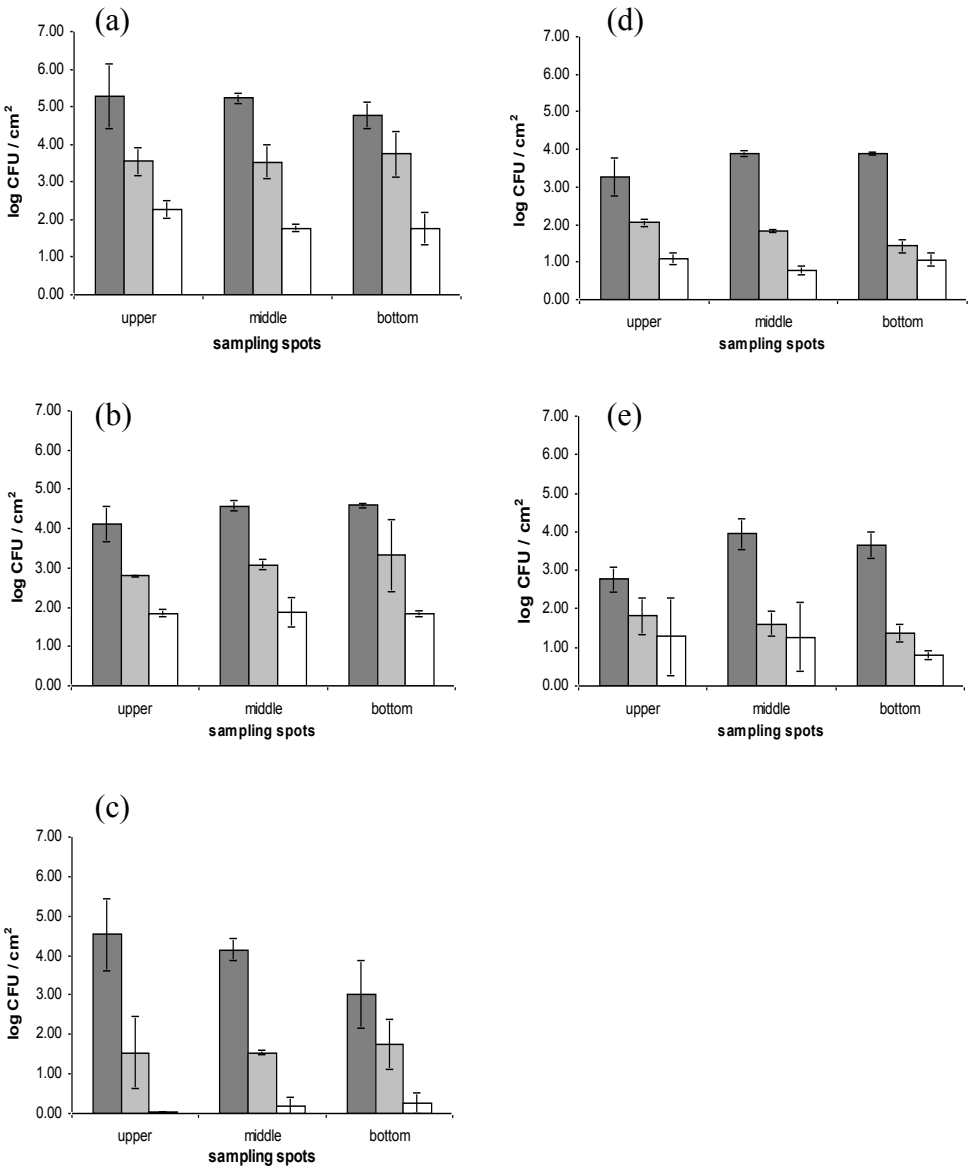

Figure 5.1. Recovered population (log CFU/cm²) of TVC (a, d), yeasts (b, e) and LAB (c) from each sampling spot in the fermentation vessels after each cleaning treatment (no treatment: dark grey bars; cleaning with hot water and detergent: light grey bars; cleaning with hot water, household detergent and bleach: white bars) at the end of spontaneous Spanish style fermentation (t=0) (a–c) and 60 days later (t=60) (d-e). Data points are average values ± standard deviation of two replications

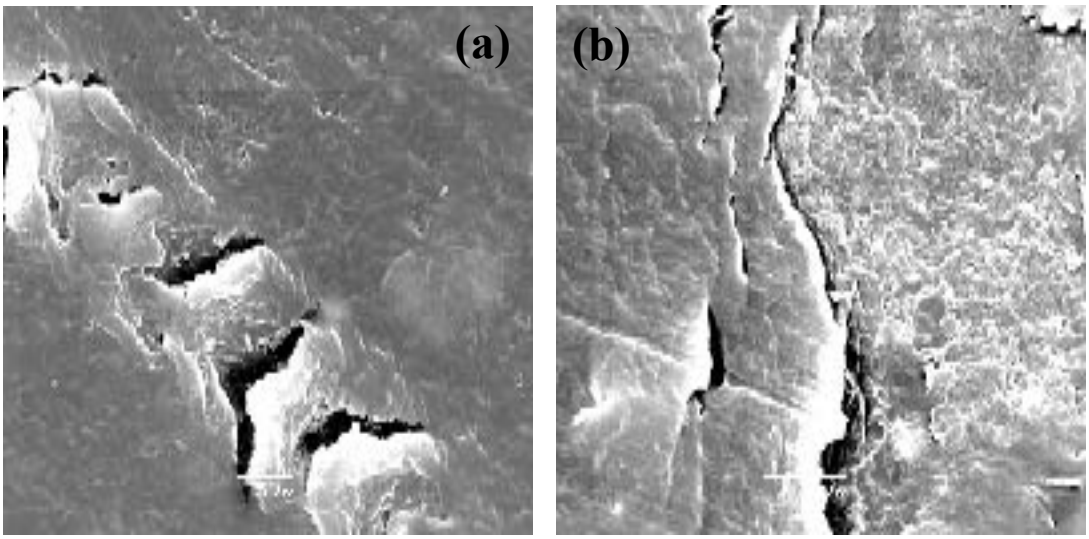

Figure 5.2. Scanning Electron Microscopy (SEM) microphotographs of the fermentation vessels from the middle (a) and upper (b) sampling spots after treatment B after 60 days.

It was also reported that yeasts and LAB were retained on wooden vats despite washing and cleaning with hot water at 60 °C, a fact that was also observed in our work with the persistence of yeast cells on the surface of fermentation vessels, even after the most severe cleaning treatment with hot water, household detergent and bleach (Fig. 5.1b,e). The authors concluded that the presence of the biofilm microbial community on the surface of wooden vats could be considered as an indigenous starter culture ensuring rapid inoculation of milk during cheese processing without using an external starter culture, a fact that needs further research for verification in the case of table olives.

5.3.2. Characterization of biofilm community before and after cleaning treatments

A total of 90 bulk samples collected from bulk cells on TSA, RBC and MRS medium for each sampling point were subjected to PCR-DGGE analysis to characterize the microbiota comprising the biofilm community on the surface of each fermentation vessel after the end of fermentation. The DGGE profile from samples recovered from MRS and TSA medium was compared with that of *L. pentosus* B281 and *L. plantarum* B282 which were used as reference strains (Blana et al. 2014). All

MRS samples shared the same profile with the reference strains and it was therefore concluded that the developed LAB community in the vessels belonged to the *L. plantarum* group species (Fig. 5.3a). In fact, it has been recognized that the identity of 16S rRNA sequences is not necessarily enough to guarantee species characterization (Fox et al. 1992). Thus further characterization was achieved by multiplex PCR assay targeting the *rec*A gene (Torriani et al. 2001). The results of this assay, illustrated in Fig. 5.3b, indicated that the recovered LAB population comprised of *L. pentosus*, which is one of the most dominant species in both green and black table olive fermentations (Hurtado et al. 2012; Lucena Padrós et al. 2014; Tofalo et al. 2014).

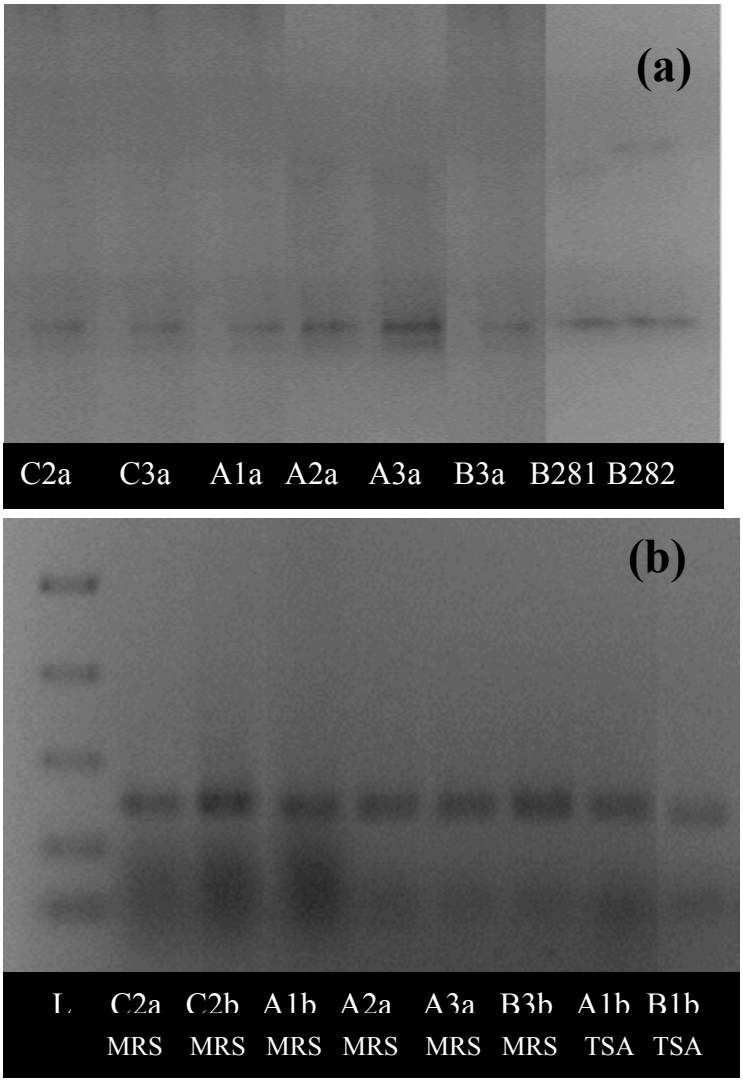

Figure 5.3.a. PCR-DGGE profile of representative samples as recovered from MRS medium, before the cleaning treatments (A), after treatment with hot tap water and household detergent (B), hot tap water, household detergent and bleach (C), at the upper spot (1), middle spot (2) and bottom (3) of replicate vessel a, compared with the profile of *L. pentosus* B281 and *L. plantarum* B282.**b.** Multiplex PCR assay using specific primers targeting the *recA* gene, for representative samples from MRS and TSA medium before cleaning treatments (A), after cleaning with hot tap water and household detergent (B), after cleaning

with hot tap water, household detergent and bleach (C), at the upper spot (1), middle spot (2) and bottom (3) of replicate vessel a and b. Lane 1: DNA ladder with resulting bands at 50, 150, 300, 500 and 700bp.

The functional properties of *L. pentosus* strains as well as other lactobacillis species have been studied for their use as starter cultures in table olive preparations by several authors. Ruiz-Barba et al. (1994) have shown that plantaricin production by *L. plantarum* LPCO10, now classified as *L. pentosus*, was essential for the control of lactic acid fermentation in Spanish-style green olives. In another study, *L. pentosus* LP RJL2 and LP RJL3, strains with plantaricin producing property and high amounts of exopolysaccharides ability respectively, were successfully used as paired starter culture in Spanish-style green olive fermentation (Ruiz-Barba and Jiménez-Díaz, 2012). The *in vitro* probiotic potential of different lactobacilli strains, all previously isolated from naturally fermented olives of Greek cultivars has been recently shown (Argyri et al. 2013). The authors of the study concluded that four strains of *L. pentosus,* three strains of *L. plantarum* and two strains of *L. paracasei* were found to possess desirable *in vitro* probiotic properties while also two of them namely, *L. pentosus* B281 and *L. plantarum* B282 have been successfully used as starter culture in green olives *cv.* Halkidiki and proposed as candidates for production of green olives with probiotic potential (Argyri et al. 2014; Blana et al. 2014). In the same sense, four strains of *L. pentosus,* previously screened and selected for their probiotic potential (Bautista – Gallego et al. 2013a), were used in Spanish-style fermentation leading to a proper lactic acid fermentation process with strain *L. pentosus* TOMC LAB2 giving the best results of imposition in the cover brines (Rodríguez-Gómez et al. 2013). The same strain together with strain *L. pentosus* TOMC LAB4 have also been reported for their ability to adhere to Manzanilla olive's epidermis rendering them promising candidates for the production of functional table olives (Rodríguez-Gómez et al. 2014).

DGGE profiles obtained from samples recovered from TSA and RBC media indicated a high diversity of yeast biota between the different cleaning treatments and sampling spots. These profiles revealed a high diversity of additional DNA bands not matching the reference strains' profile. Figure 5.4 shows a DGGE gel from which

unknown DNA bands with high abundance were isolated, coded as 1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M and 9M and sequenced. They were further subjected to BLAST search in the National Centre for Biotechnology Information (NCBI) data base. It has to be noted that, despite the success of PCR-DGGE to provide a rapid survey of the mixed community, the technique has several acknowledged drawbacks that have been reviewed in the past (Ercolini 2004).

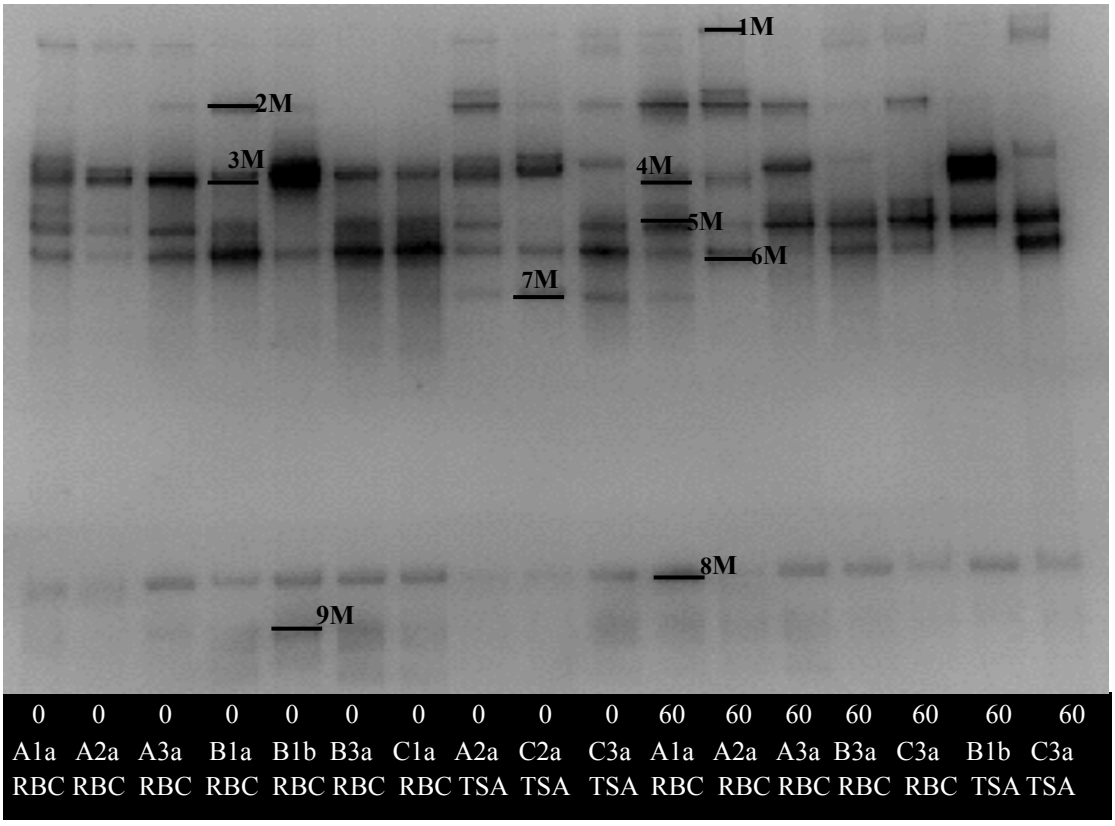

Figure 5.4. Representative DGGE gel profile for yeasts from TSA and RBC medium before the cleaning treatments (A), after cleaning with hot tap water and household detergent (B), and after cleaning with hot tap water, household detergent and bleach (C) at the upper spot (1), middle spot (2) and bottom (3) of replicate vessels a and b, at the end of Spanish style green olive fermentation (t = 0) and in the case of reformed biofilms in empty vessels (t = 60 days).

In the present study, the main drawback could be attributed to the use of small fragment size of the PCR products amplification of 300–400 bp that may not contain enough information for a precise taxonomic classification (Ovreas 2000; Nocker et al. 2007). The yeast succession at the end of fermentation, as well as in the reformed biofilms in the dried vessels after 60 days are summarized in Tables 5.1 and 5.2,

respectively, based on the comparison with the reference strains and the isolated additional DNA bands.

Table 5.1. Succession of yeast biota recovered from TSA and RBC medium for the different sampling spots and cleaning conditions after the end of Spanish style green olive fermentation (t=0).

Sampling spot	**Before cleaning treatments**	**Treatment A**	**Treatment B**
Upper spot a*	Bands 1M, 2M, 3M, 5M, 6M, 8M	Bands 1M, 2M, 3M, 5M, 6M, 8M, 9M	Bands 3M, 6M, 8M, 9M
Upper spot b	Bands 3M, 5M	*W. anomalus* *D. hansenii* *P. guilliermondii* Bands 1M, 2M, 3M, 5M, 6M, 8M, 9M	*W. anomalus* *D. hansenii* *P. guilliermondii*
Middle spot a	Bands 1M, 2M, 3M, 5M, 6M, 8M	*W. anomalus* *D. hansenii* *P. guilliermondii* Bands 2M, 5M, 6M	*P. guilliermondii* Bands 2M,3M, 6M, 7M, 8M
Middle spot b	Bands 3M, 5M	Bands 3M, 5M, 6M	*W. anomalus* *D. hansenii*
Bottom a	Bands 1M, 2M, 3M, 5M, 6M, 8M, 9M	Bands 3M, 6M, 8M	*W. anomalus* Bands 1M, 2M, 3M, 5M, 6M, 7M 8M
Bottom b	Bands 3M, 5M	Bands 3M, 5M	*P. guilliermondii* Band 3M

* a, b indicate replicate fermentation vessels.

Among the tested reference strains, *Wickerhamomyces anomalus* and *Pichia guilliermondii* like species were detected after the cleaning treatments on the different sampling spots, followed by *Debaryomyces hansenii* (Table 5.1). In the dried vessels (t=60) however none of the reference strains was detected (Table 5.2).

Table 5.2. Succession of yeast biota recovered from TSA and RBC medium for the different sampling spots and cleaning conditions in the case of reformed biofilms in empty fermentation vessels after 60 days from the initial sampling.

Sampling spot	Before cleaning treatments	Treatment A	Treatment B
Upper spot a	Bands 1M, 2M, 5M, 6M, 8M	Bands 1M, 2M 5M, 6M, 9M	Band 9M
Upper spot b	Bands3M, 5M, 6M	Bands 1M, 3M, 5M, 6M, 8M	Band 3M
Middle spot a	Bands 1M, 2M, 5M, 6M, 8M	Band 9M	Band 9M
Middle spot b	Band 5M, 3M	Bands 3M, 5M, 6M	Band 3M
Bottom a	Bands 2M, 3M, 5M, 8M	Bands 1M, 5M, 6M, 8M	Bands 1M, 2M, 5M, 6M, 8M
Bottom b	Band 3M, 5M, 6M	Band 3M, 5M	Band 3M

* a, b indicate replicate fermentation vessels

Among the isolated bands, before the cleaning treatments, 3M and 5M were recovered in all samples with 3M persisting even after treatment B on most sampling spots and 5M being detected only on the bottom of vessel a for the same treatment. The remaining of the detected biota, although less frequent than 3M and 5M before the cleaning treatments, persisted at the different sampling spots after treatment B. Among them, it was observed that the bands 1M and 2M were recovered almost exclusively from vessel a, with the exception of the upper spot in vessel b after treatment A. Furthermore, 7M was unique for vessel a, found on the middle spot and bottom after treatment B (Table 5.1) which however was not detected again in the dried vessel (Table 5.2). After 60 days, 5M was again abundant in all samples before the cleaning treatments but detected only in the bottom of vessel b after treatment B, while 3M was recovered exclusively from vessel b and under all cleaning conditions.

Bands 1M and 2M were again detected exclusively from vessel a with the exception of the upper sampling spot in vessel b after treatment A, where 1M was detected (Table 5.2). In both sampling occasions (for 0 and 60 days), 8M and 9M were detected mainly from vessel a with the former persisting on the bottom and the latter on the upper and middle spots of vessel a after treatment B. The closest relative species to the isolated DNA bands along with the percentage of identity and accession numbers as resulted from the BLAST search in the NCBI database are presented in Table 5.3, indicating that species belonging to *Candida, Zygoascus, Wickerhamomyces* and *Debaryomyces* genera were closely related to the bands. Specifically, bands 1M and 2M were found to be close to species like *Z. steatolyticus* (AY447032, AY447015, AY447007) and *Z. hellenicus* (AY447014, AY447004). To our knowledge, it is the first time that *Zygoascus* species is reported in Greek table olives, although the presence of *Z. hellenicus* has been previously reported in French black olive fermentation (Coton et al. 2006). The band 3M which was one of the most prevailing bands in most samples was close to *C. boidinii* species (KJ794107, FJ196745, FJ914946, GQ855213, EU293427, AY791700), while 5M shared a high percentage of identity with *P. mexicana* (FM180550, EU809452, DQ778990 EU326118), *Y. mexicana* (AB365477, KC006440), *Candida* sp. (JQ247716, AY640215, JN204249), *C. friedrichii* (FR819701, FR772348, FN667839), *C. olivae* (GU327624) and *C. tenuis* (FR690080) species.

Table 5.3. Closest relative yeast species to the isolated DNA bands from DGGE analysis as resulted from BLAST analysis.

Band	Closest relative species	% identity	GenBank Accession Numbers
1M	*Z. meyerae*	93%	HM450997
	Z. steatolyticus	93%	AY447032
	Uncultured yeast isolate DGGE band	92%	JQ864401

2M	*Z. meyerae*	96%	HM450997
	Z. steatolyticus	96%	AY447032, AY447015, AY447007
	Z. hellenicus	96%	AY447014, AY447004
	Zygoascus sp.	96%	EU708990
3M	*C. boidinii*	92%	KJ794107, FJ196745, FJ914946, GQ855213, EU293427, AY791700
	Uncultured fungus clone	92%	HQ143201
	Uncultured fungus isolate DGGE band	87%	JX041503
5M	*Candida* sp.	99%	JQ247716, AY640215, JN204249
	C. olivae	99%	GU327624
	C. tenuis	99%	FR690080
	P. mexicana	99%	FM180550, EU809452, DQ778990 EU326118
	Y. mexicana	99%	AB365477, KC006440
	C. friedrichii	99%	FR819701, FR772348, FN667839
6M	*Candida* sp.	99%	FJ794977, EU605795, EU622124
	C. olivae	98%	GU327624
	C. tenuis	98%	FR690080
	C. tumulicola	97%	AB365456
7M	*Wickerhamomyces sp.*	94%	AB774379, AB774400
	W. anomalus	94%	KF959845, JX188244, HF952838, KC515371
	D. hansenii	94%	KC515371, KJ095639, HF934036, JQ912667
	Uncultured fungus isolate DGGE band	94%	JX041503
8M	*C. olivae*	95%	GU327624
9M	Uncultured saccharomycete isolate DGGE gel band	91%	DQ028950
	Uncultured fungus isolate DGGE band	91%	JX041503

D. hansenii	91%	FJ475230, KJ095639, HF934036, JQ912667

The latter two species also neighbored with 6M while the band 8M was by 95% identified as *C. olivae* (GU327624). Several *Candida* species have been reported in the literature as part of the yeast population associated with table olive procedures. *C. boidinii* has been previously reported during the preservation stage of black olives (Arroyo-López et al. 2006) and consists one of the representative species in French black olives (Coton et al. 2006). *C. olivae* has been previously identified as a new species associated with Greek style black olive fermentation (Nisiotou et al 2010a), while *C. tenuis* has been also isolated from directly brined olives in Portugal (Garrido-Fernández et al. 1997). The band 7M, unique for vessel a, seems to belong to either *Wickerhamomyces* or *Debaryomyces* genera. *W. anomalus* has been commonly isolated from both black (Coton et al. 2006; Nisiotou et al. 2010b) and green table olives (Hurtado et al. 2008; Bautista-Gallego et al. 2011b; Muccilli et al. 2011) while *D. hansenii* has been previously recovered from olive brines as well (Marquina et al. 1992; Kotzekidou 1997; Nisiotou et al. 2010b). *Wickerhamomyces* and *Debaryomyces* are important genera since they contain species with negative and positive role in table olive fermentation (Arroyo-López et al. 2012b). Some strains of *W. anomalus* and *D. hansenii* have been related with softening of olive drupes by the production of enzymes such as xylanases, proteases and pectinases (Hernández et al. 2007) and olive bloater spoilage during processing (Faid et al. 1994). On the other hand, strains of *W. anomalus* have been attributed with antioxidant properties due to catalase activity, biodegradation of phytate complexes due to phytase activity as well as the ability of phenol degradation due to β-glucosidase activity (Bautista-Gallego et al. 2011b; Psani and Kotzekidou 2006). *D. hansenii* has been found to enhance LAB growth when inoculated 48h prior to inoculation with *L. plantarum* in black olive juice (Tsapatsaris and Kotzekidou 2004) while also diverse strains of this species possess a probiotic potential by tolerating high bile salts concentrations and low pH values (Psani and Kotzekidou 2006), surviving the gastrointestinal tract and adhering

to intestinal Caco-2 cell line (Moslehi-Jenabian et al. 2010). Recently, the multifunctional features of 12 yeast strains, all isolated from the yeast biota in Greek natural black table olives, were studied for application as starters in natural black table olive processing (Bonatsou et al. 2015). Among them, *P. guilliermondii* Y16, *W. anomalus* Y18 and *D. hansenii* Y57, which were used as reference strains in the present study, were found to be the most promising strains in terms of stress conditions (salt concentration, gastric and pancreatic digestion) and other cellular and extracellular enzymatic activities such as esterase, β-glucosidase and phytase activity. The authors of the study proposed the use of *W. anomalus* Y18 for technological applications and the use of *P. guilliermondii* Y16 as potential probiotic agent.

5.4. Conclusions

In this study, it was showed that the technological microbiota, namely LAB and yeasts, employed in green olive fermentation can colonize the abiotic surface of fermentation vessels and form biofilm communities that are persistent in cleaning treatments applied on the vessels during the operational procedures of green olive processing. In terms of species biodiversity, the most frequently detected yeasts belonged to *Candida* genus followed by *W. anomalus, D. hansenii* and *P. guilliermondii* which are common members of the yeast fermenting microbiota of table olives. Concerning LAB identification, it was found that *L. pentosus* was the most abundant species recovered from the biofilm, confirming the importance of this species in table olive fermentation. *Zygoascus* like species however are reported for the first time in Greek table olives. Given the dynamic process of biofilm formation, development, maturation and dispersion, the presence of this multispecies microbial community could have a beneficial impact on green olive processing, since biofilm detachment from the surface of the vessels into the brine could contribute to a certain extent in brine inoculation with the necessary technological microbiota to support fermentation.

Epilogue

The results of the present thesis indicate that the ability of biofilm formation by olive originated microorganisms with functional properties can transform table olives from a traditional product to a high added product with functional appeal. In both Spanish-style and Greek-style procedures that were studied, the microorganisms employed as starter cultures resulted in accelerated fermentation process and the final product obtained good physicochemical characteristics and enhanced sensory properties in comparison with the spontaneous (control) process. The selected LAB strains were recovered in high numbers throughout the process with *L. pentosus* B281 exhibiting higher ability to dominate at the end of fermentation providing a final product with functional appeal. Further, development of polymicrobial biofilm communities on the surface of fermentation vessels could have a beneficial impact on olive processing, since biofilm detachment from the surface of the vessels into the brine could contribute to the natural inoculation of the brine with the necessary technological microbiota to support fermentation.

Based on these findings, further research should be undertaken to elucidate the use of table olives as a carrier of beneficial microorganism to the consumer. Research on the probiotic properties of LABs originating from olives is still recent hence novel candidates remain to be further studied. Since the beneficial role of yeast species in table olive processing has been mentioned in the literature, identification of novel yeast species with probiotic properties is proposed as well. In a first and simple approach, the use of invertabrate model hosts could serve as low cost *in vivo* tools to screen for health-promoting properties. A number of health-promoting properties that could be studied include antagonism with pathogens, enhanced phagocytosis after infection with various pathogens, expression of immunorelative genes of the host and production of antimicrobial peptides due to LAB intake whereas such properties can be further investigated for yeasts as well. In a next step, *in vivo* experiments using mammalian models and clinical trials are proposed to verify the probiotic character of the studied candidates. In addition, the characterisation of the EPS matrix produced during biofilm formation and its role in table olive production remains to be elucidated. Such characterisation could render table olives, apart from a rich pool of beneficial microorganisms, a rich source of prebiotics as well.

Industrial application

From the industrial perspective, the results of the present thesis have great significance and can be further exploited as follows:

- The inoculation of the brines with LAB as starter cultures during olive processing is strongly advised at industrial scale, as their use results in an accelerated lactic acid fermentation process with high final values of acidity and low pH that ensure the safety of the fermented product during storage.
- The use of high initial salt concentration in the brine should be avoided as it may have detrimental effect on LAB growth. Specifically, in the case of natural black olive fermentation, initial salt concentration below 8 % (w/v) is proposed in order to avoid the dominance of yeasts and the development of alcoholic fermentation rendering a final product with high pH and low acidity and hence with less shelf-preservation characteristics. Moreover, the combination of LAB starter cultures in low salt brines is further suggested as this approach guarrantees the safety of the final product and at the same time meets the trend of modern nutrition which demands consumtion of food products with low salt content.
- The fermenting microbiota of table olives, namely LAB and yeasts, can colonize and form biofilm communities on the surface of plastic vessels where fermentation takes place and may serve as a natural means of brine inoculation with the appropriate technological microbiota to support fermentation.
- The potential of selected LAB and yeasts starter cultures with specific technological and functional properties to attach on the surface of olives forming mixed microbial communities (biofilms) may turn table olives into a high added value food providing new perspectives for the table olive industry.

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