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PhD Thesis

"*IN VITRO* PROBIOTIC ATTRIBUTES OF LACTIC ACID BACTERIA AND THEIR SPOILAGE POTENTIAL IN MEAT PRODUCTS"

"ΠΡΟΒΙΟΤΙΚΑ ΧΑΡΑΚΤΗΡΙΣΤΙΚΑ ΟΞΥΓΑΛΑΚΤΙΚΩΝ ΒΑΚΤΗΡΙΩΝ *IN VITRO* ΚΑΙ ΜΕΛΕΤΗ ΤΟΥ ΔΥΝΑΜΙΚΟΥ ΑΛΛΟΙΩΣΗΣ ΣΕ ΠΡΟΙΟΝΤΑ ΚΡΕΑΤΟΣ"

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"ΠΡΟΒΙΟΤΙΚΑ ΧΑΡΑΚΤΗΡΙΣΤΙΚΑ ΟΞΥΓΑΛΑΚΤΙΚΩΝ ΒΑΚΤΗΡΙΩΝ IN VITRO ΚΑΙ ΜΕΛΕΤΗ ΤΟΥ ΔΥΝΑΜΙΚΟΥ ΑΛΛΟΙΩΣΗΣ ΣΕ ΠΡΟΙΟΝΤΑ ΚΡΕΑΤΟΣ"

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ΠΕΡΙΛΗΨΗ

Τη σημερινή εποχή το καταναλωτικό ενδιαφέρον και οι τάσεις στην αγορά των τροφίμων έχουν μεταβληθεί σημαντικά. Τρόφιμα που μπορεί να είναι ωφέλιμα για την υγεία και βοηθούν στην ευζωία εμφανίζουν αυξημένη ζήτηση και για το λόγο αυτό οι βιομηχανίες τροφίμων καθώς και η έρευνα επικεντρώνονται προς αυτή την κατεύθυνση. Μεγάλη μερίδα της αγοράς των λειτουργικών προϊόντων, κατέχουν τα προβιοτικά προϊόντα και ο συγκεκριμένος τομέας φαίνεται να επεκτείνεται. Τα γαλακτοκομικά προϊόντα έχουν εκτενώς μελετηθεί ως πηγές προβιοτικών καλλιεργειών και ως προϊόντα-φορείς των προβιοτικών, όμως άλλες πηγές πέρα των γαλακτοκομικών, θεωρούνται πολύ σημαντικές έτσι ώστε να αυξηθούν οι κατηγορίες των τροφίμων στις οποίες τα προβιοτικά είναι διαθέσιμα σε όλους τους καταναλωτές. Στο πλαίσιο αυτό, η παρούσα μελέτη εστιάζει στο: α) να μελετηθεί το προβιοτικό δυναμικό οξυγαλακτικών βακτηρίων που έχουν απομονωθεί από παραδοσιακά ζυμούμενα ελληνικά προϊόντα, β) να χρησιμοποιηθούν επιλεγμένα οξυγαλακτικά βακτήρια σαν εκκινητήριες συγκαλλιέργειες ή πρόσθετες καλλιέργειες σε ζυμούμενα προϊόντα κρέατος, γ) να ερευνηθεί η δυνατότητα χρήσης εναλλακτικών μεθόδων όπως αυτή των εδώδιμων μεμβρανών, για τη μεταφορά των προβιοτικών καλλιεργειών σε προϊόντα κρέατος θερμικής επεξεργασίας και δ) να αξιολογηθεί η τεχνική της φασματοσκοπίας υπερύθρου με μετασχηματισμό Fourier σαν εργαλείο για την πρόβλεψη της ποιότητας των ανωτέρω προϊόντων κρέατος.

Σε πρώτο στάδιο οξυγαλακτικά βακτήρια, απομονωμένα απο παραδοσιακά ζυμούμενα ελληνικά γαλακτοκομικά και ζωϊκά προϊόντα, ταυτοποιήθηκαν μοριακά και μελετήθηκε το προβιοτικό τους δυναμικό *in vitro*. Δεκαεννέα (19) απο τα 47 στελέχη οξυγαλακτικών επιλέχθηκαν ως τα καλύτερα με τη βοήθεια μιας σειράς δοκιμών. Επιπρόσθετα, άλλα στελέχη οξυγαλακτικών, που απομονώθηκαν από ελιές, μελετήθηκαν επίσης ως προς τα προβιοτικά τους χαρακτηριστικά, με

17 απο αυτά να εμφανίζουν τις καλύτερες ιδιότητες. Τα ίδια στελέχη μελετήθηκαν στη συνέχεια ως προς την ικανότητα παραγωγής γ-αμινοβουτυρικού οξέος καθώς και αυτεπαγωγέων-2 (μορίων-σημάτων επικοινωνίας), οπού κανένα απο τα στελέχη δεν εμφανίστηκε θετικό ως προς την παραγωγή των συγκεκριμένων μορίων. Τέσσερα (4) απο αυτά τα στελέχη εξετάστηκαν περαιτέρω για την ικανότητα τους να παράγουν αυτεπαγωγείς-2, υπό συνθήκες καταπόνησης, παρόμοιες με αυτές που απαντώνται σε ζυμούμενα τρόφιμα και στο πεπτικό σύστημα του ανθρώπου. Παράλληλα, πραγματοποιήθηκε μελέτη της έκφρασης του γονιδίου *luxS* (υπεύθυνου για την παραγωγή αυτεπαγωγέων τύπου-2) υπο αυτές τις συνθήκες καταπόνησης με τη χρήση PCR σε πραγματικό χρόνο. Παρατηρήθηκε σημαντική διακύμανση στα επίπεδα έκφρασης του γονιδίου μεταξύ των διαφορετικών συνθηκών καταπονήσεων και μεταξύ των διαφορετικών στελεχών, αν και γενικά τα επίπεδα θεωρήθηκαν χαμηλά.

Σε δεύτερο στάδιο, επιλεγμένα στελέχη οξυγαλακτικών που εμφάνισαν προβιοτικό δυναμικό χρησιμοποιήθηκαν σαν πρόσθετες καλλιέργειες για την παραγωγή ζυμούμενων αλλαντικών (σαλάμι αέρος). Μικροβιολογικές, φυσικοχημικές (pH και a_w) και οργανοληπτικές αναλύσεις πραγματοποιήθηκαν κατά τη διάρκεια της ζύμωσης, ωρίμανσης και συντήρησης των σαλαμιών στους 4 και 12 °C. Η παρουσία του προβιοτικού στελέχους μέχρι και το τέλος της διάρκειας ζωής του προϊόντος επιβεβαιώθηκε με την τεχνική της ηλεκτροφόρησης αγαρόζης σε εναλασσόμενο πεδίο. Τα αποτελέσματα ήταν πολύ ικανοποιητικά, αφού το προβιοτικό στέλεχος διατηρήθηκε σε υψηλά επίπεδα (>6 log CFU/g), η καλλιέργεια φάνηκε να προσδίδει τα επιθυμητά τεχνολογικά χαρακτηριστικά και τα νέα προϊόντα είχαν αποδεκτά οργανοληπτικά χαρακτηριστικά.

Σε επόμενο στάδιο ερευνήθηκε η αποτελεσματικότητα της εφαρμογής των εδώδιμων μεμβρανών αλγινικού νατρίου ως φορείς των προβιοτικών καλλιεργειών σε συνδυασμό με την τεχνική της

Υπερυψηλής Υδροστατικής Πίεσης (ΥΥΠ) σε φέτες ζαμπόν. Τα προβιοτικά οξυγαλακτικά βακτήρια ενσωματώθηκαν στο διάλυμα αλγινικού νατρίου. Οι φέτες ζαμπόν (με ή χωρίς προηγούμενη μεταχείριση με ΥΥΠ:500MPa για 2 min) συσκευάστηκαν σε κενό, σε επαφή με τις εδώδιμες μεμβράνες και στη συνέχεια συντηρήθηκαν στους 4, 8 και 12 °C, για 66, 47 και 40 ημέρες αντίστοιχα. Πραγματοποιήθηκαν μικροβιολογικές και φυσικοχημικές (pH, a_w) αναλύσεις καθώς επίσης και μέτρηση του χρώματος. Επιπλέον πραγματοποιήθηκε οργανοληπτική αξιολόγηση, ενώ η παρουσία και το ποσοστό επιβίωσης του κάθε προβιοτικού στελέχους κατά τη συντήρηση επιβεβαιώθηκε με ηλεκτροφόρηση πηκτής αγαρόζης σε εναλασσόμενο πεδίο. Σε φέτες ζαμπόν χωρίς τη μεταχείριση με ΥΥΠ, τα επίπεδα των προβιοτικών βακτηρίων ήταν της ταξης των 6 log CFU/g κατά τη συντήρηση σε όλες τις θερμοκρασίες. Παρόμοια αποτελέσματα έδωσαν οι φέτες ζαμπόν που υποβλήθηκαν σε μεταχείριση με ΥΥΠ, αλλά με διαφορετικές τιμές pH μεταξύ των δυο περιπτώσεων. Τα ζαμπόν στα οποία είχαν προστεθεί προβιοτικές καλλιέργειες παρουσίασαν μια πιο όξινη γεύση και άρωμα σε σύγκριση με το μάρτυρα, παρόλο που αυτά τα χαρακτηριστικά μειώθηκαν αρκετά στην περίπτωση του ζαμπόν με ΥΥΠ. Γενικά τα αποτελέσματα θεωρήθηκαν ενθαρρυντικά, αφού τα προβιοτικά βακτήρια κατάφεραν να περάσουν επιτυχώς απο την εδώδιμη μεμβράνη στο ζαμπόν, ανεξάρτητα απο τη μεταχείριση με ΥΥΠ. Συγχρόνως, παρόμοια μελέτη πραγματοποιήθηκε εστιάζοντας στην αντιμικροβιακή δράση των εδώδιμων μεμβρανών αλγινικού νατρίου εμπλουτισμένων με αιθέριο έλαιο ρίγανης, ενάντια στο παθογόνο *Listeria monocytogenes* κατά την παρουσία του στο ίδιο προϊόν με ή χωρίς μεταχείριση με ΥΥΠ. Πραγματοποιήθηκαν οι ίδιες αναλύσεις με αυτές που αναφέρθηκαν στην προηγούμενη μελέτη, ενω η απουσία/παρουσία και το ποσοστό επιβίωσης του κάθε στελέχους *Listeria monocytogenes* κατά τη διάρκεια της συντήρησης πραγματοποιήθηκε με την ηλεκτροφόρηση αγαρόζης σε εναλασσόμενο πεδίο. Σύμφωνα με τα αποτελέσματα η ΥΥΠ είχε επίδραση στη *Listeria*, ανεξάρτητα απο τη θερμοκρασία συντήρησης. Η προσθήκη αιθερίου

ελαίου ρίγανης στις εδώδιμες μεμβράνες προκάλεσε μείωση ~1.5 log στον πληθυσμό της *Listeria* στους 8 και 12 °C, στο τέλος της συντήρησης και περίπου 2.5 log μείωση στους 4 °C. Ο συνδυασμός ΥΥΠ και εδώδιμων μεμβρανών εμπλουτισμένων με αιθέριο έλαιο ρίγανης στο ζαμπόν θεωρήθηκε η πιο αποτελεσματική μεταχείριση προκαλώντας σημαντική μείωση ή ακόμη και απουσία του παθογόνου, επηρεάζοντας ταυτόχρονα τους υπόλοιπους βακτηριακούς πληθυσμούς.

Σε τελικό στάδιο, αξιολογήθηκε το δυναμικό της φασματοσκοπίας Υπερύθρου με μετασχηματισμό Fourier (FTIR) σαν εργαλείο για τον προσδιορισμό των ποιοτικών χαρακτηριστικών των προϊόντων κρέατος εμπλουτισμένων με προβιοτικές καλλιέργειες. Τα προϊόντα που μελετήθηκαν ήταν αυτά που προέκυψαν από τις προηγούμενες μελέτες όπως τα ζυμούμενα αλλαντικά, οι φέτες ζαμπόν (χωρίς περαιτέρω μεταχείριση-μάρτυρας), οι φέτες ζαμπόν με εδώδιμες μεμβράνες προβιοτικών και εδώδιμες μεμβράνες με αιθέριο έλαιο ρίγανης, με ή χωρίς μεταχείριση με ΥΥΠ. Μοντέλα Μερικών Ελαχίστων Τετραγώνων (PLS) αναπτύχθηκαν με στόχο τη συσχέτιση των φασματικών δεδομένων απο την ανάλυση με FTIR με την αλλοίωση κατά τη συντήρηση στις διάφορες θερμοκρασίες ανάλογα με το εξεταζόμενο προϊόν. Ο σκοπός της μοντελοποίησης ήταν ο διαχωρισμός των δειγμάτων με βάση την αντίστοιχη ποιοτική τους κατηγορία, καθώς επίσης και να προβλεφθεί ο μικροβιακός πληθυσμός απευθείας από τα φασματικά δεδομένα. Τα αποτελέσματα έδειξαν ότι η επεξεργασία των προϊόντων επηρέασε την ακρίβεια της κατηγοριοποίησης των δειγμάτων σε ποιοτικές κλάσεις. Η αξιολόγηση της ακρίβειας των μοντέλων PLS ως προς τον υπολογισμό των μικροβιακών πληθυσμών βασίστηκε σε γραφήματα και στατιστικούς δείκτες (B_f, A_f, RMSE, % PE). Τα δεδομένα που καταγράφηκαν για πρώτη φορά έδειξαν ότι ενω η τεχνική της φασματοσκοπίας υπερύθρου έχει τη δυνατότητα να χρησιμοποιηθεί για την ταχεία αξιολόγηση των προϊόντων,

πρόσθετες επεξεργασίες όπως η ζύμωση, τα πρόσθετα και η ΥΥΠ, μπορούν να επηρεάσουν την αποτελεσματικότητα της τεχνικής.

Επιστημονική περιοχή: Μικροβιολογία Τροφίμων

Λέξεις κλειδιά: προβιοτικά τεστ *in vitro*, *lux*S, Αυτεπαγωγέας-2, γ-αμινοβουτυρικό οξύ, προϊόντα κρέατος, εδώδιμες μεμβράνες με προβιοτικά, αντιμικροβιακές εδώδιμες μεμβράνες, Υπερυψηλή Υδροστατική Πίεση (ΥΥΠ), Φασματοσκοπία Υπερύθρου με μετασχηματισμό Fourier (FTIR).

ABSTRACT

Nowadays, consumer's interest and trends on buying food products have changed drastically. Food products that can be beneficial for human health and support wellbeing, are particularly on demand and a lot of attention is being paid from both industry and research. A big portion of the functional food market, belongs to probiotic food products and this sector will be expanding. Dairy products have been well-studied as probiotic sources and probiotic carriers with excellent results, however, alternative vehicles are also important in order to increase the food categories containing probiotics, hence making them available to everyone. In this context, this study focuses on (a) exploring traditionally fermented Greek products for the presence of lactic acid bacteria with probiotic potential (b) use these lactic acid bacteria as co-starters or adjunct cultures in fermented meat products (c) investigating the potential of alternative probiotic carriers, such as edible films in thermally-processed meat products (d) assessing Fourier-Transform Infrared Spectroscopy as a tool to predict the quality status of the above meat products.

As a first step, lactic acid bacteria isolated from traditional greek fermented meat and dairy products were molecularly characterised and their probiotic potential was examined *in vitro*. Nineteen (19) out of 47 strains were selected as good probiotic candidates after a series of tests. Additionally, other LAB strains previously isolated from olives were also studied for probiotic attributes, with 17 of them showing good potential. These LAB strains were also studied for their ability to produce γ-aminobutyric acid and AI-2 signal molecules, with none of them being a producer of either compounds. Four of these strains were further investigated for their ability to produce AI-2, under stress conditions related to those encountered in foods and human gastrointestinal tract. In parallel, the gene expression of *lux*S (responsible gene for AI-2 production) under these stress conditions was monitored with Real-Time PCR. The results

revealed a great variability on the expression levels amongst strains and the different stress factors, however, the levels were considered as generally low.

At a second stage, selected lactic acid bacteria with probiotic potential were used as adjunct cultures for the preparation of fermented sausages. Microbiological, physicochemical (pH and a_w) and sensory analyses were performed during fermentation, ripening and storage of the sausages at 4 and 12 °C. The presence of the probiotic strain was confirmed with PFGE till the end of the shelf-life. The results were very promising, since the adjunct cultures remained in high levels (> 6 log CFU/g), the potentially probiotic adjunct culture was found to possess desirable technological characteristics and the sensorial attributes of the new products were highly accepted.

At a later stage, the efficacy of Na-alginate edible films as vehicles for delivering probiotic bacteria to sliced ham in combination with High Pressure Processing (HPP) was investigated. LAB strains with probiotic potential were incorporated in Na-alginate forming solution. Ham slices (pretreated or not with HPP: 500MPa for 2 min) were packed under vacuum in contact with the films and were then stored at 4, 8 and 12 °C for 66, 47 and 40 days, respectively. Microbiological and physicochemical (pH, a_w) analysis was performed in parallel with color measurements. Sensory characteristics were assessed, while the presence and the relative abundance of each probiotic strain during storage was evaluated using PFGE. In ham slices without HPP treatment, probiotic bacteria were enumerated above 6 log CFU/g during storage at all temperatures. Similar results were obtained in cases of HPP treated samples, but pH measurements showed differences between the two treatments. Sensory evaluation revealed that probiotic samples had a more acidic taste and odor/smell than the control ones, however these characteristics were markedly compromised in samples treated with HPP. Generally, the results seemed promising since probiotic bacteria were successfully delivered in the products by edible

films regardless of the HPP treatment. Simultaneously, a similar study was performed focusing on the antimicrobial activity of Na-alginate edible films supplemented with oregano essential oil on the pathogen *Listeria monocytogenes* when present in the same product with or without a HPP pre-treatment. The same analyses were performed as the previous study, while the presence/absence and the relative abundance of each *L. monocytogenes* strain during storage, was monitored using PFGE. Results showed that HPP had an effect on *Listeria*, regardless the storage temperature. The oregano essential oil incorporation in the films, caused ~1.5 log reduction in *Listeria* levels at 8 and 12 °C at the end of storage period, and almost 2.5 log reduction at 4 °C. The combined application of HPP and oregano essential oil edible films on the ham slices was the most effective treatment, causing a significant reduction or even absence of the pathogen, whilst having an effect on the other bacterial populations.

At a final stage, the potential of FTIR spectroscopy as a tool to determine the quality of meat products supplemented with probiotics was assessed. The products examined were the ones produced previously, such as dry-fermented sausages, ham slices without any treatment (control case), ham slices with probiotic edible films and ham slices with edible films supplemented with probiotic cultures or oregano essential oil with or without HPP treatment. A series of Partial Least Squares (PLS) models were developed to correlate spectral data from FTIR analysis with spoilage during storage at different temperatures, according to the product studied. The scope of the modelling approach was to discriminate the products in their respective quality class and additionally to predict the microbial population directly from spectral data. The results obtained demonstrated that the processing of the samples affected the performance of classification in the sensory classes. The performance of PLS regression models on providing quantitative estimations of microbial counts were based on graphical plots and statistical indices (B_f, A_f, RMSE, % PE). The results demonstrated for the first time that although FTIR has a potential to be used for the

rapid assessment of many foods, additional processes such as fermentation, additives or HPP can affect its performance.

Scientific field: Food Microbiology

Related keywords: probiotic tests *in vitro*, *lux*S, Autoinducer-2, γ-aminobutyric acid, meat products, probiotic edible films, antimicrobial edible films, High Pressure Processing (HPP), Fourier Transform Infrared Spectroscopy (FTIR)

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Without you none of this would indeed be possible.

F. Pavli, 2020

“Not everything that can be counted counts,
and not everything that counts can be counted.”

Albert Einstein

List of Abbreviations

LAB - Lactic Acid Bacteria

AI-2 - Autoinducers-2

PFGE - Pulsed Field Gel Electrophoresis

HPP - High Pressure Processing

PLS - Partial Least Squares

FTIR - Fourier Transform Infrared Spectroscopy

FAO - Food and Agricultural Organization

WHO - World Health Organization

IBS - Irritable Bowel Syndrome

PCR - Polymerase Chain Reaction

GIT - Gastrointestinal Tract

GABA - γ-Aminobutyric Acid

GAD - Glutamate Decarboxylase

QS - Quorum Sensing

AHL - Acylated Homoserine Lactones

AI-3 - Autoinducers-3

AIP - Autoinducing Peptides

MC - Methylcellulose

HPMC - Hydroxypropylmethylcellulose

EO - Essential Oils

GRAS - Generally Recognized as Safe

SNV - Standard Normal Variate

RMSE - Root Means Square Error

PBS - Phosphate Buffer Saline

OEOF - Oregano Essential Oil-Free

OEOS - Oregano Essential Oil-Supplemented

A_f - Accuracy Factor

B_f - Bias factor

PE - Prediction Error

APE - Acceptable Prediction Error

APZ - Acceptable Prediction Zone

LC - Latent Components

PCA - Principal Component Analysis

HCA - Hierarchical Cluster Analysis

ANN - Artificial Neural Networks

SR - Survival Rate

CNC - Coagulase-Negative cocci

RTE - Ready-To-Eat

TVC - Total Viable Counts

PF - Probiotic-Free

PS - Probiotic-Supplemented

MIC - Minimal Inhibitory Concentration

BSH - Bile Salt Hydrolase

ANOVA - Analysis of Variance

CFS - Cell-Free Supernatant

OD - Optical Density

TABLE OF CONTENTS

CHAPTER 1

Introduction/Literature Review/Objectives

1.1. Introduction and Literature Review

1.1.1. Modern needs towards a healthy lifestyle

Trends in modern diet and lifestyle and consequently, in food production have an impact on consumer's health status, as well as on the environment. The patterns related to food consumption and nutrition still vary significantly between USA, Europe and Asia and are linked to financial, social and food security issues. Specifically, around Europe nowadays, differences in food consumption are apparent, especially when comparing South and North European countries. It is generally noted that today's consumers are more than ever aware and cautious regarding food safety and the connection between food and health-related issues or wellbeing. Obesity, osteoporosis, gastrointestinal dysfunctions, cardiovascular diseases, allergies and cancer are only some examples of health-related issues of modern times, in which diet has a great impact on.

Although modern consumers are aware of the link between eating and health, still the main criterion when buying food is the convenience. Lately, food manufacturers have developed products falling in the category of the so called "functional foods", which include products that can contribute positively in human health. Traditional foods in particularly, are deemed to promote wellbeing and therefore, the public interest regarding their nutritional and health impact is on increase, together with their demand (Cencic & Chingwaru, 2010). Functional foods are different from the conventional ones, due to their demonstrated benefits on human health. Functional food products have become a part of the food market with increasing market share. However, many products-not only from this category-still fail to maintain their position in the market soon after their launch. It is important to consider the factors that can influence the consumer's decisions whether to purchase a product or not. According to consumer studies, the

willingness to purchase functional foods can be compromised by the sensory and non-sensory features of the product, the price, the country of origin or the brand, the benefits attributed to the product and the advertisement provided (Plasek and Temesi, 2019).

1.1.2. Assessment of probiotic potential

The widely accepted definition of probiotics according to FAO/WHO (2002) is "live microorganisms that when administered in adequate amounts, confer a health benefit on the host". As mentioned previously, foods containing probiotics fall in the category of functional foods and together with prebiotics represent the largest segment of the functional market, worldwide. Some of the health benefits that have been attributed to probiotics include the maintenance of a healthy gut microbiota, enhancing protection against gastrointestinal disorders, the treatment of antibiotic-associated diarrhea, promoting the body's natural defense mechanisms, alleviating lactose-intolerance symptoms, relief from constipation and IBS-related symptoms amongst others. A variety of bacteria, mainly bifidobacteria and lactobacilli have been assessed for their probiotic potential and have been applied as added cultures in several food products. However, one of the biggest challenges are the criteria used to verify the performance of the tested bacteria. For probiotics to confer a health benefit to the host, several established criteria should be fulfilled, thus still *in vitro* studies cannot predict adequately the *in vivo* performance. Consequently, a proper assessment of a potentially probiotic strain is of utmost importance for a future implementation as adjunct culture in food products. Having said that, *in vitro* tests that are routinely used as indicators to evaluate the probiotic performance, will be discussed in the introduction of this thesis as well as difficulties and challenges in their application. It has to be noted that any health benefits or claims related to certain bacteria will not be examined, since it is out of the scope of the thesis. For a probiotic food to be linked with

certain health benefits and be authorised, clinical studies are mandatory and have to be performed before introducing the probiotic food to the market. Before the clinical studies, *in vitro* tests as well as animal studies are required, even to immune-compromised animals, to further verify the probiotic safety (Pineiro and Stanton, 2007).

Before assessing the benefits that a potential probiotic can confer to the host, several other criteria should be taken into consideration and these include the *in vitro* assessment of the performance under conditions similar to those encountered on the GI tract, but also the technological contribution of such cultures in the selected food matrix. Having said that, a multistep approach is required for obtaining a solid image. At first a basic strain characterization and taxonomy is needed to gather information about the strain's identity, followed by *in vitro* tests and finally by *in vivo* assays. Strain identity is important to relate a strain to a specific health effect as well as to enable precise investigations and epidemiological studies (FAO/WHO, 2002; Pradhan, 2020). Currently, the molecular techniques used for strain identification include PCR, 16S rRNA sequencing and DNA fingerprinting techniques such as ribotyping and PFGE. Additionally, strain typing methods such as Randomly Amplified Polymorphic DNA, Amplified Ribosomal DNA Restriction Analysis, rep-PCR, PFGE and Multi Locus Sequencing have been used so far. Nowadays, also the application of whole-genome sequencing of probiotic isolates is becoming popular.

In general, probiotic strains should be safe for human consumption. Most of the probiotic strains belong to *Lactobacillus* and *Bifidobacterium* or *Streptococcus* species, that have a long history or safe use and are GRAS (Giraffa, 2012). Despite the fact that the safety of these bacteria is taken for granted, a few reports do exist in the literature, associating LAB with pathological conditions such as bacteraemia, and therefore, the safety of these bacteria is to be determined

individually and from zero point. Some of the safety aspects that are taken into consideration include the origin of the strain, the absence of haemolytic activity and the absence of acquired antibiotic resistance. Haemolysis is a serious issue, causing anemia and edema in the host. Alpha (α) haemolysis, is caused by hydrogen peroxide produced by the bacterium, oxidizing haemoglobin to green methaemoglobin, which gives the green colour to the bacterial colony when blood is present. Beta (β) haemolysis is the complete lysis of the red cells, which is evident in media in the presence of blood and gives a transparent/yellow colour. In some studies α-haemolysis in LAB isolates has been reported, while β-haemolysis is quite rare (Pradhan, 2020). Regarding the origin of probiotics, a variety of sources has been reported including human, animal or food sources. Although strains isolated from humans are deemed to be better in human applications, many food-associated LAB are gaining interest, due to the excellent technological attributes and their good adaptation in food matrices. Regardless of the origin of the isolates certain tests are required to verify the absence of β-haemolytic activity or the absence of enzymes that have been associated with health problems, such as β-glucosidase, β-glucuronidase or N-acetyl-β-glucosaminidase (Delgado et al. 2007; Dabek et al. 2008).

Another very important safety aspect is the antibiotic resistance of these cultures. It has been reported in the literature that LAB (especially enterococci) are involved in the transfer of resistance traits through plasmids over species or genus area, a fact that raises concerns with regard to their safety (Giraffa, 2014). Obviously, the presence of such resistant bacteria is abundant in humans or animals treated with antibiotics on a regular basis. In the case where antibiotic resistance exists in some bacteria, but is not transferrable, is not of a major concern. In such cases the resistance to a particular antibiotic is intrinsic to bacterial species or genus and enhances survival in environments where certain antibiotics are present. Therefore, as long as

such resistance is chromosomically encoded and not horizontally transferred the risk is kept to a minimum. On the contrary, acquired resistance to antibiotics might be horizontally spread and can pose a risk due to a possible transfer of this resistance *in vivo*. Although antibiotic resistance varies significantly among LAB species, it is generally reported that LAB show resistance to vancomycin or kanamycin, gentamycin or streptomycin and this resistance is considered mainly intrinsic. Differently, LAB are sensitive to penicillin and β-lactamases, chloramphenicol and tetracyclines (Pradhan, 2020). For this purpose, the Panel of Additives and Products or Substances used in Animal Feed (FEEDAP) has proposed "microbiological breakpoints" for some LAB, with the latest report published in 2012 (EFSA, 2012).

In vitro tests applied as a first step to assess the probiotic potential of strains can provide a lot of information on the performance of the isolate under gastrointestinal conditions. Some of the tests that are routinely used include resistance to low pH and bile acid, transit tolerance to simulated GIT juices, adherence to cell-lines, antimicrobial activity against pathogens, bile salt hydrolase activity and many others. Although it has been reported that dead cells of probiotics have the ability to provide health benefits, it is still mandatory that probiotics reach the gastrointestinal tract when still alive. The survival of probiotics under the conditions encountered in the human GIT is very important in order to assess their performance especially, in the case that probiotics are administered as free-cells and non-encapsulated. After ingestion, bacterial cells have to face the very acidic conditions of the stomach. Although the pH of the stomach is not stable and varies from 1-2 when is empty to up to 4.5 when is full of food, still for the *in vitro* resistance, very low pHs are examined. Furthermore, the *in vitro* tests for acid resistance are performed in buffers with adjusted pH and in the absence of any nutrients, which is improbable and leads to the selection of the very resistant strains only. On the contrary, the bacterial cultures

used for these tests usually involve cells derived from the stationary phase and not the exponential; a fact that is unrealistic since the bacterial cells in the foods have encountered several stresses due to food processing that makes them more susceptible to acid. Having said that, it is evident that the ways of studying the acid resistance of the potential probiotic candidates differs significantly from the real case scenario and might provide invalid results.

Another approach on assessing the probiotic potential *in vitro* is by using artificial gastric and pancreatic juices. The advantage of these tests is the addition of enzymes which are normally present in GIT. These tests are considered to be more accurate on mimicking the human GIT conditions, however the use of bovine or porcine bile in these assays, might have a different impact on the cells compared to human bile. Yet again, these assays are performed in artificial juices without nutrients, which does not represent the conditions encountered in the GIT. Nowadays, the development of GIT simulators can provide more accurate data regarding probiotic survival in GIT (Minekus et al. 1999). A good example of a GIT simulator is the SHIME, in which the data obtained had a good correlation with data obtained from studies *in vivo*. The main disadvantage of using these simulators is the initial capital cost and also the cost of maintenance and operation of the equipment as well as the ability to evaluate a particular probiotic candidate rather than performing a screening of many isolates.

Another important attribute of probiotics is their potential antimicrobial activity. Probiotic secretion of antimicrobials and/or the competition on pathogen's growth or colonization are considered desirable. Antimicrobial compounds produced by probiotic cells include organic acids or bacteriocins, while the most common tests for antimicrobial activity are the well diffusion assay and the agar-spot test. Additionally, it is desirable for probiotics to compete with pathogens while binding on the surface of the GIT. *In vitro* test that is widely used to assess this particular

ability is the co-aggregation test, which refers to the aggregation between probiotic and pathogenic cells (Collado, Meriluoto and Salminen, 2007). The tests regarding antimicrobial activity, can only indicate certain characteristics of a probiotic, but are not representative, since the produced antimicrobial compounds *in vitro* might not be produced under *in vivo* conditions in the host and the opposite. The conditions encountered in the human GIT, the vast range of different bacteria present in the host's microbiome and the relationships between them can alter the production of these compounds by probiotics.

For a probiotic to be able to exert health benefits to the host, efficient colonization is required. *In vitro* tests that gather information regarding colonization are usually adherence tests on epithelial cells. Intestinal epithelial cell lines offer a good representation of the GIT tissues and in several studies human epithelial cell lines such as HT-29 and CaCO-2 have been used. A possible disadvantage of these *in vitro* tests for colonization is the low reproducibility observed between different laboratories resulting from using different variants of cell lines however they can offer a rough indication of the actual colonization in the host (Papadimitriou et al. 2015). Other *in vitro* tests aiming to give an indication of the colonizing properties of probiotics, include the hydrophobicity and auto-aggregation tests. The auto-aggregation ability of individual cultures might correlate well with the actual *in vivo* adherence, while the hydrophobicity test remains controversial, due to the non-solid conclusions given by the results of this test (Papadimitriou et al. 2015).

As a general note, even though *in vitro* tests are useful to screen probiotic candidates, they exhibit variable effectiveness and they are performed differently by workers. This makes comparisons difficult and therefore the reproducibility of the methods difficult. Although *in vivo* assays are more appropriate for a precise screening, they are very limited due to costs and ethical

issues. Consequently, *in vitro* screening will probably always be the first step for the discovery of new probiotics (Papadimitriou et al. 2015). In this study, after the molecular characterization and identification of LAB that originated from traditionally fermented products, the aforementioned *in vitro* tests were performed to assess the probiotic potential of the isolated LAB strains (Chapter 2).

1.1.3. GABA production as a desirable probiotic feature

GABA is a non-protein amino acid that can be found everywhere in the nature from microorganisms to animals and plants. L-glutamate and GABA are known for their neuro-transmitting role in the central nervous system. GABA has been reported to induce hypotension, and having diuretic and tranquilizing effects (Li and Cao, 2010). Probiotic strains have been reported to modulate mood and stress responses in humans as well as to reduce anxiety and relieve from depression symptoms. Modifications in glutamate and GABA circuits in the central nervous system have been linked with anxiety and depressive disorders. Due to the fact that GABA has a potential as bioactive component in foods, the development of functional foods containing GABA is of industrial interest. Many studies have investigated the potential of certain probiotics for GABA production serving as bacterial cell-factories. Several LAB strains of the species *Lb. plantarum*, *Lb. paracasei*, *Lb. brevis*, *Lb. buchneri* and *Lactococcus lactis* can synthesize glutamate, while GABA is synthesized by decarboxylation of glutamate by the glutamate decarboxylase (GAD). Generally, GAD is present in both Gram-positive and Gram-negative bacteria and is linked to maintaining the pH homeostasis and the production of ATP molecules. *Lactobacillus*, *Lactococcus* and *Streptococcus* species have been studied for their ability to synthesize GABA (Siragusa et al. 2007; Li and Cao, 2010), although this ability is strain-related (Mazzoli and Pessione, 2016). It has to be noted that LAB with high GAD activity

can have a potential as probiotics through the exploitation of health promoting properties due to GABA. Many GABA producing strains have been isolated from cheeses (Nomura et al. 1998; Siragusa et al. 2007), however a further screening for isolation should be expanded to other sources so that GABA-producing LAB from different origins and matrices will offer wider applications and higher flexibility as starter cultures (Li and Cao, 2010). Considering the above, a part of the the current study was focused on examining the ability of LAB strains to produce GABA, which would be an additional probiotic trait of the selected strains (Chapter 2).

1.1.4. Quorum sensing in probiotics

Communication between bacterial cells is believed to occur based on quorum sensing (QS) signal molecules. These signal molecules include acylated homoserine lactones (AHLs), Autoinducer-2 (AI-2), Autoinducer-3 (AI-3) and Autoinducing Peptides (AIPs). QS as per definition is involved in the regulation of specific physiological functions in bacteria. With QS the bacterial cells achieve adaptation due to controlled gene expression initiated by the detection of specific signal compounds. For the case of probiotic bacteria, which mainly involve LAB (Gram-positive), two QS systems could be examined. The first system involves small AIPs and the second is the LuxS-mediated system producing AI-2, which is a universal signal-molecule and can be found in both Gram-positive and Gram-negative species and is used for both inter and intra- species communication (Federle and Bassler, 2003; Moslehi-Jenabian, Gori and Jespersen, 2009). AI-2 is a furanosyl borate diester, which is controlled by the *luxS* gene and was first detected in *V. harveyi* (Bassler et al. 1993). Many functional roles have been attributed to this molecule such as adhesion enhancement, increased adaptation in environmental conditions, biofilm formation, luminescence and others. For the case of probiotics, although the data are still

scarce, *lux*S homologues have been found in specific lactobacilli (Yeo et al. 2015). Relevant studies dealing with the detection of QS molecules in LAB are presented in Table 1.

As mentioned before, the probiotic potential of different bacterial strains varies a lot amongst the same bacterial species. Since one of the main requirements for the selection of probiotic bacteria is the effective transit through the GIT, where the bacterial cells are exposed to extreme environmental conditions, it would be important to identify those mechanisms involved in such resistance (Moslehi-Jenabian, Gori and Jespersen, 2009). In complex environments such as the human intestine, QS systems might play an important role in bacterial interaction (Kaper and Sperandio, 2005). Although the production of QS molecules has been detected in many bacterial species, including LAB, it can only give an indication of the actual scenario, since in most experiments single-strain effects are examined, while in actual fact such an environment is not realistic and is far away from natural conditions (Park et al. 2014). AI-2 and AIPs are considered as two different communication systems, however, it has been found that the presence of AI-2 is linked to bacteriocin production in co-culture systems (Chanos and Mygind, 2016). Such an observation reveals the great complexity and interactions taking place in natural niches. In a general note, QS has been involved in food fermentations and food environments with certain compounds to be associated with spoilage due to LAB, *Pseudomonas* spp. and *Enterobacteriaceae* proliferation (Nychas, Marshall and Sofos, 2007). However, data are still limited and more research is required to better understand the significance of such compounds. As a first step in the study of QS, phenotypic assays have been developed and used widely, followed by genomic and transcriptomic analyses. However, given the number of extracellular metabolites, proteomic approaches are utilized lately, since they provide a more “complete” image for proteins expressed by the genome and give more details regarding the behavior of

bacteria during QS phenomena (Di Cagno, De Angelis, Calasso, Gobbetti, 2011). Part of the current thesis aimed to investigate the production of AI-2 compounds of the selected LAB strains with probiotic potential, under standard growth conditions using the AI-2 bioassay. At a second stage, a genomic and transcriptomic approach was employed to study the expression of the gene involved in AI-2 production (*lux*S) under conditions typically encountered in fermented foods (presence of NaCl or changes in temperature) and in the human GIT (acidic conditions, presence of bile) (Chapter 3).

Table 1. Studies dealing with the detection of QS molecules in LAB and probiotics.

Bacterial strain	QS System	Reference
Lb. plantarum	AIP	Brurberg, Nes and Eijsink, 1997; Maldonado et al. 2004
Lb. salivarius	AIP	Flynn et al. 2002
Lb. sake	AIP	Brurberg, Nes and Eijsink, 1997
Streptococcus mutans	AI-2	Wen and Burne, 2004; Sztajer et al. 2008
Lb. rhamnosus GG	AI-2	Lebeer et al. 2007; Moslehi-Jenabian, Gori and Jespersen, 2009; Yeo et al. 2015
Lb. acidophilus NCFM	AI-2	Moslehi-Jenabian, Gori and Jespersen, 2009
Bifidobacterium breve	AI-2	Christiaen et al. 2014
Bifidobacterium longum NCC2705	AI-2	Sun et al. 2014
Lb. plantarum NC8	AIP	Ruiz-Barba et al. 2010
Lb. sakei NR28	AI-2	Park et al. 2014
Lb. plantarum DC400	AIP	Calasso et al. 2013
Lb. sanfranciscensis DPPMA174	AIP	Calasso et al. 2013
Lb. plantarum WCFS1	AIP	Sturme et al. 2007; Sturme et al. 2005
Lb. acidophilus La-5	AI-2	Medellin-Pena and Griffiths, 2009
Lb. plantarum NR74	AI-2	Yeo et al. 2015
Lb. plantarum C11	AIP	Diep, Havarstein and Nes, 1995
Lb. plantarum	AI-2	Park et al. 2016
Lb. brevis	AI-2	Park et al. 2016

1.1.5. Optimising probiotic performance and challenges in the use of probiotics by the industry

The viability of probiotics can be compromised during the manufacturing stages and shelf-life, putting barriers in their industrial application. Therefore, several aspects need to be analysed to ensure suitability of probiotics present in specific food products. Potential probiotic strains should be able to survive well under food processing and biological stresses, such as extreme temperature, pH, osmotic or oxidative stress. When probiotics are used as adjunct cultures in fermented products the possible antimicrobial effect of the starter culture against the added probiotic should be evaluated, too. Furthermore, the probiotic cultures should have no adverse effect on the sensory characteristics of the product, e.g. causing extreme acidification during shelf-life, compromising the aroma and taste.

One of the most important challenges related to probiotics is the enhancement of stress responses before the bacteria are required to survive the extreme conditions encountered during food processing and later on, during GI transit. A possible approach to this issue could be the application of several pre-adaptation processes to induce stress responses and genetic engineering (Giraffa, 2012). Other challenges do exist regarding the stability of probiotics during their production. These bacteria require specific conditions for growth media high in nutrients and stable environmental conditions. Therefore, it is possible that during probiotic production and storage processes, the viability of probiotics might be compromised as well as their functional properties. It is of utmost importance to choose an optimal culture medium and cell protectants for probiotic preparations to guarantee that the viability will not be affected until industrial use. Furthermore, the selection of probiotics is very important due to post-acidification problems during the shelf-life of the probiotic products (El Hage et al. 2017). In addition, the impact of the

food matrix on probiotic viability must be considered for successful probiotic applications. It is known that different matrices affect differently the added cultures and this is related to the micro-environment conditions present in the food, the nutrients available, the packaging conditions, the shelf-life etc.

In cases of probiotic products where multistrain preparations are utilized, it is crucial that the biochemical and immunological characteristics of the product are maintained during the entire shelf-life, especially when is used for consumers with compromised medical history. This is one of the biggest challenges for the industry to be able to market a probiotic product and have its full control to ensure its uniformity in the market and to guarantee that it continues to provide the benefits claimed until the end of shelf-life. Extra attention, however, must be paid on the matrices used for probiotic delivery due to possible impacts on probiotic viability and efficacy (di Simone, 2019).

Regulatory bodies worldwide classify and evaluate differently the status of probiotic products. In Europe and USA reservations do exist regarding health claims attributed to probiotic foods, whilst in Japan the situation is different. Since the "probiotic concept" has become so popular such discrepancies between regulatory authorities cause confusion among industries and consumers. A more "uniform" approach on probiotic regulations might be useful, and a critical update on this issue is needed now more than ever (El Hage et al. 2017).

1.1.6. Alternative ways for probiotic delivery in foods

(The introduction sections 1.1.6 & 1.1.7 have been included in the review publication: Probiotic Incorporation in Edible Films and Coatings: Bioactive solution for Functional Foods (2018) by

Pavli F., Tassou. C., Nychas G-J.E. and Chorianopoulos, N. International Journal of Molecular Sciences, 19, 150.)

In order to confer a health benefit, a generally recommended dose of probiotic viable cells is that of $10^{8–9}$ per day. In fact, issues are raised regarding the cell viability and probiotic survival in the final products. Several factors, both intrinsic and extrinsic, can affect the behavior of a microorganism within different food environments. Typical examples include the type of the selected culture, the physiological state of the cells, the food matrix, the pH, the temperature, the manufacturing processes and the storage conditions (Soukoulis et al. 2014). Apart from these factors, the viability of probiotics is determined by their passage through the gastrointestinal tract, where the major hurdles are the low pH and bile. To address these limitations and enhance viability, different technological strategies have been suggested and investigated, with the most important being the inclusion of probiotics into edible films and coatings or their microencapsulation into polymeric matrices (De Prisco and Mauriello, 2016; Espitia et al. 2016).

In the particular case of edible coatings containing probiotics, probiotic release is not even required, since the coating itself is supposed to be eaten with the food (Espitia et al. 2016). The first study dealing with the incorporation of probiotics into edible films and coatings was that by Tapia et al. in 2007 (Tapia et al. 2007). Since then, quite a number of studies were available in the literature, investigating the probiotic incorporation in edible films with different approaches. The application of probiotic edible films in food matrices is a tool for effective probiotic delivery, but it is also important in enhancing food stability and food safety by controlling the growth of spoilage microorganisms through competition or through antimicrobial substances produced by probiotics.

Microencapsulation is defined as a technology including ingredients (solid, liquid or gaseous) that are being entrapped or completely surrounded by protective matrices (De Prisco and Mauriello, 2016). Referring to probiotics, the aim of encapsulation techniques is to carry and protect these bacteria from the effects of the low pH and bile that bacteria encounter during their passage through the gastrointestinal tract (Pinto et al. 2015; De Prisco and Mauriello, 2016). Additionally, microencapsulation techniques intend to protect and reassure the viability of the encapsulated probiotics during food processing and storage conditions of the food products until their consumption. Other factors that are involved in the microencapsulation procedure such as low water activity or oxygen exposure can affect the efficacy of this technique. With regard to the materials used for microencapsulation, there are numerous studies available with a major category being biodegradable polymers. These biopolymers, include alginate, chitosan, cellulose, protein, carrageenan, gelatin and pectin which are materials for the manufacturing of edible films and coatings (De Prisco and Mauriello, 2016; Anal and Singh, 2007). Microencapsulation techniques and applications have been reviewed elsewhere (De Prisco and Mauriello, 2016; Martin et al. 2015; Rathore et al. 2013; Burgain et al. 2011; Anal and Singh, 2007; Krasaekoopt et al. 2004).

1.1.7. Edible films and coatings

Edible films can be produced from materials with film-forming ability. The components used for the preparation of edible films and coatings can be classified into three categories of hydrocolloids, lipids and composites (Bourtoom, 2008; Rojas-Grau, 2009). Other compounds such as plasticizers and emulsifiers are added to the film-forming solutions to improve their mechanical properties or to enhance stability when lipids and hydrocolloids are combined (Valencia-Chamorro et al. 2011). Plasticizers are usually required for the manufacture of edible

films and coatings, in particular when polysaccharides or proteins are used as materials. Plasticizers are low molecular weight agents incorporated into film-forming materials that decrease the glass transition temperature. The addition of plasticizers enhances flexibility, toughness and the tear resistance of the film. Amongst the most commonly used plasticizers are glycerol, sorbitol, sucrose and polyethylene glycol (Ramos et al. 2012).

The hydrocolloids group comprises of polysaccharides and proteins. Polysaccharides used for edible films or coatings are cellulose derivatives, dextrans, inulin, alginate, carrageenan, starch derivatives, pectin derivatives, chitosan, seaweed extracts, and galactomannans (Valencia-Chamorro et al. 2011; Cerqueira et al. 2011; Suput et al. 2015; Bourtoom, 2008). Polysaccharide films and coatings serve as good oxygen, odor and oil barriers with good mechanical properties, but their major drawback is the moisture permeability, due to their hydrophilic characteristics (Ramos et al. 2012). Proteins used for edible films and coatings are gelatin, corn zein, wheat gluten, soy protein, collagen and casein. Protein films are generally formed from solutions or dispersions of protein as solvent evaporates. The solvent usually is water, ethanol or their mixture. Proteins must be denatured by heat, acid, base or solvent in order to form a more extended structure that is required for the film formation (Bourtoom et al. 2008). Protein-based films exhibit poor water resistance, but they possess better mechanical and barrier properties than polysaccharides (Suput et al. 2015). From this group of materials, the most important regarding their use are presented below:

i. Cellulose is the major cell-wall component in plants. Cellulose derivatives such as methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) are commonly found in the formulation of edible coatings. Films and coatings based on cellulose and its derivatives are transparent, flexible, odourless and tasteless. MC is more resistant to water, however, the

water vapor permeability is quite high. Both MC and HPMC have the ability to form gelatinous coatings after thermal processes, which gives the opportunity to be used to prevent oil absorption in frying foodstuff (Bourtoom, 2008). Regarding their use as probiotic carriers in edible films and coatings, several studies are available (Tavera-Quiroz et al. 2015; Romano et al. 2014; Sanchez-Gonzalez et al. 2014; Sanchez-Gonzalez et al. 2013).

ii. Chitosan is mainly obtained from the exoskeleton of crustaceans and fungal cell-walls. Chitosan is a deacetylated derivative of chitin (Pillai et al. 2009). Chitosan degree of deacetylation, has been reported as an important parameter that determines many of its physicochemical and biological properties, such as crystallinity, hydrophobicity and degradation. The degree of deacetylation of chitosan is controlled by a relatively aggressive alkaline hydrolysis process applied to chitin, with a combination of exposure time and temperature (Foster et al. 2015; Yuan et al. 2011). The molecular weight of chitosan is depended on the initial source material (shrimp, crab, fungi) and can be decreased with processing to increase the deacetylation. Molecular weight has been proven to be an important factor in chitosan properties such as crystallinity, degradation, tensile strength and moisture content (Yuan et al. 2011). Mechanical and barrier properties of chitosan films can be controlled by selecting a suitable solvent system, the appropriate molecular weight of chitosan as well as the addition of plasticizers (Martinez-Chamacho et al. 2010; Park et al. 2002). Chitosan as a coating material has excellent film-forming abilities, broad antimicrobial activity and compatibility with other substances such as vitamins, minerals and antimicrobial agents. This material receives particular interest for the targeted release of probiotics because of its high compatibility with living cells (De Vos et al. 2010). Furthermore, chitosan has been studied for application as a coating due to its antifungal and

antibacterial abilities (Dos Santos et al. 2012, Raafat and Sahl, 2009; Ribeiro et al. 2007). Chitosan can form semi-permeable coatings, which can modify the internal atmosphere when applied to fruits and vegetables. The main disadvantage of chitosan films is their low moisture barrier, which makes their broad use in food applications difficult (Suput et al. 2015).

iii. One of the most commonly used polysaccharides is alginate. Alginate is a generic term for the salts of alginic acid. Alginates possess good film-forming properties and produce transparent and water-soluble films (Tapia et al. 2008). It has been used mainly for meat products, since it delays dehydration and eliminates lipid oxidation (Suput et al. 2015; Varela and Fiszman, 2011).

iv. Starch and its derivatives have been widely used as food hydrocolloids, because they are inexpensive, abundant, biodegradable and easy to use. Coatings made from starch are usually transparent, odourless, tasteless and colourless with low permeability to oxygen at low-to-intermediate relative humidity (Corrales et al. 2009). Starch films have excellent barrier properties to O_2 and CO_2, but not to water. Starch granules contain the macromolecules amylose and amylopectin, which can form solutions and gels. Amylose is a sparsely branched molecule mainly based on α (1-4) bonds with a molecular weight of 10^5-10^6 anhydroglucose units (Basiak et al. 2017). During gel formation amylose and amylopectin form inter- and intra-molecular crosslinks so that they produce a macromolecular network, and subsequently the film after water evaporation. Physical crosslinks in the macromolecular network of starch are formed mainly by microcrystalline domains of amylose. The more these domains exist in the starch-based film, the higher the tensile strength of the films (Talja et al. 2007). A high amylose content in starch is responsible for the production of strong and

flexible films (Liu et al. 2005). Chemical, physical and functional properties of edible films and coatings depend on the amylose/amylopectin ratio (Basiak et al. 2017). Degree of crystallinity of starch films increases by increasing the amylose content of starch (Talja et al. 2007). Starches with high-amylose content have been used to extend the shelf-life of deep fried foods (Shah et al. 2016). Additionally to the usual starch films, modified starch films have started to be investigated because they possess good solubility and improved mechanical properties. Starches such as cross-linked, substituted, oxidized and acid-hydrolyzed are being produced as a result of chemical modifications. These chemical modifications have been examined regarding their effect on the film characteristics such as their mechanical, barrier and thermal properties. More specifically, starches with increased numbers of cross-linkages exhibit improved water absorption, maintain viscosity and texture. Substitution results in increased water affinity, lower starch gelatinization temperature, better hydration, and less firm gels. Oxidized starches are applied to deep fried food as coatings to improve their eating quality by retaining crispness. These starches usually are corn, potato, cassava and bean starches. Acid-modified starches, are usually applied to jelly candies, processed meats and to extruded cereals and snacks. Acid hydrolysis leads to decreased swelling power, increased solubility and more options regarding the gelatinization temperature compared to native starches (Shah et al. 2016).

v. Pectins are structural components of plant cell walls. These materials are a common type of gelling agents, as they are widely used in jams, jellies and sweets, apart from the production of edible films. Several studies have investigated the potential of pectin as a material in edible films (Soukoulis et al. 2017; Espitia et al. 2014).

vi. Gelatin is obtained by controlled hydrolysis of collagen at high temperatures in the presence of water and it is widely found in nature. Antioxidant and antimicrobial activities are associated with gelatin (Gomez-Guillen et al. 2011). In general, gelatin films and coatings have poor water vapor barrier properties (Suput et al. 2015) and their application includes meats, since they reduce oxygen, oil and moisture transport (Bourtoom et al. 2008). López de Lacey et al. (2012) investigated the incorporation of *L. acidophilus* and *B. bifidum* in gelatin films for fish preservation, while in another study of Soukoulis et al. (2016), binary starch-gelatin edible films were examined regarding their compositional, physicochemical and structural properties on the stability of *L. rhamnosus* GG.

Lipids used for edible films and coatings include natural waxes, vegetable oils, acetoglycerides and fatty acids and resins. These compounds exhibit certain disadvantages regarding their application, showing mechanical and chemical instabilities as well as decreased organoleptic quality (Ramos et al. 2012). Lipids are usually combined with other film-forming materials such as polysaccharides or proteins, to increase the resistance to water penetration (Suput et al. 2015).

Edible films and coatings might be heterogeneous regarding the material used for their manufacture. It is possible that an edible film consists of a blend of polysaccharides, protein and/or lipids. Such a film is defined as a composite (Bourtoom, 2008). The aim of producing composite films is to adjust the characteristics of the film in order to use it for a specific application. Since each individual coating material possesses some unique, but limited functions, a combination of different materials can be more effective (Rojas-Grau, 2009). Studies regarding probiotic edible films and coatings and their materials are presented in Table 2. In the current

thesis, the potential of alginate-based edible films was assessed in delivering probiotic bacteria to sliced ham, whilst the quality characteristics of the products were also evaluated (Chapter 5).

Table 2. Edible films and coatings that have been used for probiotic or LAB incorporation.

Biopolymer Material	Probiotics	Application Matrix	Reference
Alginate/Gellan	*B. lactis* Bb-12	Fresh-cut apples and papayas	Tapia et al. 2007
Sodium caseinate	*L. sakei*	Fresh beef	Gialamas et al. 2010
Alginate/Starch	*C. maltaromaticum*	Smoked salmon	Concha-Meyer et al. 2011
Gelatin	*L. acidophilus, B. bifidum*	Hake fish	Lopez de Lacey et al. 2012
Starch	*L. acidophilus*	Baked bread	Altamirano-Fortoul et al. 2012
Pullulan/Starch	*L. plantarum, L. reuteri, L. acidophilus*	-	Kanmani and Lim, 2013
Isolate Pea Protein/MC/Sodium caseinate/HPMC	*L. plantarum*	-	Sanchez-Gonzalez et al. 2013
Agar	*B. animalis* spp. *lactis, L. paracasei* spp. *paracasei*	Hake fillets	Lopez de Lacey et al. 2014
Methylcellulose (MC)	*L. delbruecki* subsp. *bulgaricus, L. plantarum*	-	Romano et al. 2014
Gelatin	*L. rhamnosus* GG	-	Soukoulis et al. 2014
Alginate, Whey Protein Concentrate	*L. rhamnosus* GG	Bread	Soukoulis et al. 2014
MC, Sodium caseinate	*L. reuteri, L. acidophilus*	-	Sanchez-Gonzalez et al. 2014
Kefiran	*L. plantarum*	-	Piermaria et al. 2015
WPI	*B. animalis* Bb-12, *L. casei*	-	Odila-Pereira et al. 2016
Rice/Corn Starch, Gelatine/Sodium caseinate/ Soy protein concentrate	*L. rhamnosus* GG	-	Soukoulis et al. 2016
Sodium alginate	*L. plantarum, L. pentosus*	Ham slices	Pavli et al. 2017
Sodium alginate/Pectin/κ-Carrageenan-Locust bean gum/Gelatine/Whey protein	*L. rhamnosus* GG	-	Soukoulis et al. 2017

1.1.8. Antimicrobial packaging: The case of Essential Oils

The increasing demand for safe and high quality food products has challenged industries to explore their opportunities to the full. The demand for minimally processed, more "natural" ready-to-eat products has increased dramatically due to modern lifestyle. In order to control the growth of microorganisms in foods, antimicrobial substances can be incorporated in food packaging materials. The major applications in use of antimicrobials involve meat, fish, poultry, fruits and vegetables (Vermeiren et al. 1999). Plenty of antimicrobial agents do exist such as sulfites, nitrites, organic acids, bacteriocins, essential oils and can be applied in food products. Essential oils (EO) are aromatic and volatile extracts obtained from plant tissues with extraction or distillation and they exhibit strong antimicrobial activity. Some of the EO that have been used as antimicrobials in food preparations include EO of oregano, thyme, rosemary, clove, cinnamon, basil and coriander. Due to the fact that EO are GRAS, they are highly acceptable by consumers. It has to be noted that several thermal or non-thermal technologies can be used in combination with EO to prevent or eliminate spoilage and enhance food safety (Jayasena et al. 2013). The antimicrobial activity of EO has been extensively studied and such activity has been linked to certain phenolic compounds such as thymol, carvacrol or eugenol. The mechanisms of action of EO against bacteria, include the degradation of the cell-wall, damage of membrane proteins and coagulation of the cell proteins (Burt, 2004). It has been reported by Skandamis et al. (2002) that EOs from clove, oregano, rosemary, thyme and sage have high inhibitory activity against Gram-positive bacteria. Moreover, it has been proven that EO are effective against foodborne pathogens, including *E.coli* 0157:H7, *Salmonella* Typhimurium, *L. monocytogenes, S. aureus*,

Campylobacter and others (Rivera-Calo et al. 2015). Due to the very strong flavor of the EOs, usually an inhibitory concentration is used, rather than a bactericidal (Chorianopoulos et al. 2006).

Generally, it has to be noted that the interaction of some EOs with food components can reduce the antimicrobial activity of the EOs. Additionally, the composition of a particular EO might vary significantly between different plant varieties or due to the extraction method or due to the part of the plant used for the extraction (Jayasena et al. 2013). Also, the antimicrobial activity of a particular EO might be compromised due to pH, temperature or initial bacterial levels. A drawback on the use of EO, apart from their intense flavor and the impact on the sensory characteristics of the product, is the cost, since the oils are required in high concentrations in order to achieve an efficient antimicrobial activity. To overcome these issues, the incorporation of EO into biopolymers and films/coatings has been investigated for controlled applications. In such cases, lower amounts of EO can be used together with other antimicrobial compounds or other technologies for preservation in order to obtain a synergistic effect. Several studies are available in the literature, where EO were incorporated into biopolymers and edible films/coatings, confirming their unique potential as antimicrobial solutions and highlighting the commercial interest (Acevedo-Fani et al. 2015; Teixeira et al. 2014; Pires et al. 2013; Bonilla et al. 2012; Benavides et al. 2012; Sanchez-Gonzalez et al. 2011; Sanchez-Gonzalez et al. 2010; Atares et al. 2010; Zinoviadou et al. 2009). As a possible drawback in the incorporation of EOs in edible films and coatings can be that the diffusion of the antimicrobial compounds in the product is reduced (Ruiz-Navajas et al. 2013). Furthermore, the release of antimicrobial compounds from the films can be compromised due to electrostatic interactions between the antimicrobial agent and the polymer chains, or environmental conditions etc. On the contrary, smaller amounts of EO

would be required to achieve the expected shelf-life in a particular product compared to direct EO application, since EO in the films would be released gradually on the food surface. Therefore, part of this thesis, was the study of the antimicrobial activity of EO incorporated into alginate edible films against the pathogen *Listeria monocytogenes* in sliced ham (Chapter 6).

1.1.9. Non-thermal Processing Technologies: The example of HPP

Over the last decades, HPP has been employed as a non-thermal, alternative and promising technology, enabling inactivation of pathogenic and spoilage organisms, resulting in a "less-processed" and "more natural" food product. In other words, with HPP, products of high quality and with extended shelf-life can be produced (Panagou et al. 2007). This technology is based on the principle of Le Chatelier and the isostatic rule (Hugas et al. 2002). With HPP, isostatic pressure is transmitted instantly to the product regardless of its size, dimensions and composition (Varela-Santos et al. 2012). The HPP application has a comparable preservation effect on microbial inactivation with the thermal treatment, while keeping flavor, taste and color changes to the minimum, although exceptions have been observed for certain food products in relatively high values of pressure. Pressure levels higher than 400MPa are generally recommended for efficient microbial inactivation (Simonin et al. 2012). HPP enhances shelf-life extension, results in products with high organoleptic quality and better nutrient retention, therefore is considered advantageous for high valued products (Varela-Santos, 2012).

Generally, HPP at low or moderate temperature causes destruction of the microbial vegetative cells and enzyme inactivation, without drastically altering the sensory characteristics of the product, while leaving most of the nutrients intact. However, the resistance of microorganisms on the applied pressure is variable and depends on the strain. Other parameters affecting the result of the treatment are the selected pressure, the temperature of the treatment and

the time spent with the applied pressure (Hugas et al. 2002). HPP has been employed for decontamination purposes against several pathogens including *L. monocytogenes*, *S. aureus*, and *V. parahaemolyticus* (Torres et al. 2005). Spain, is the pioneer in Europe in HP treated meat with the first commercial application in 1998 of processed sliced-cooked ham. After the successful application of HPP in sliced cooked-ham, other meat products followed such as cured ham, pork, chorizo and sausages (Garriga and Aymerich, 2009). This technology offers a unique opportunity for combined applications with new packaging systems and natural antimicrobial substances (Hugas et al. 2002). It has to be highlighted that especially for meat products, HPP might lead to significant changes in the quality attributes of meat or meat products such as inducing protein denaturation and acceleration of lipid oxidation, during storage. These alterations can potentially result in color and texture changes and therefore, decreased consumer acceptability. Consequently, the combined application with other hurdles to control the microbial growth and spoilage could be a solution for applying a milder HPP treatment. Moreover, HPP has been employed for meat tenderization, thus adding an extra application to this technology as a method to develop innovative meat products and not just as an alternative to commercial pasteurization (Simonin et al. 2012). The effect of HPP and the potential benefits of its use were investigated in this study in sliced ham in combination with the application of edible films supplemented with probiotics (Chapter 5) and EO-hurdle concept (Chapter 6).

1.1.10. Meat products as potential probiotic carriers

Fermented meats are linked to tradition and culture and are products with high added value and exceptional sensory characteristics and nutritional value. Their stability and convenience rates them amongst the most popular meat products worldwide. Dry-fermented sausages are prepared by stuffing the meat batter into casings to initiate fermentation, which is

then followed by maturation and ripening. The meat batter is usually composed of minced meat, fat, curing agents, starter cultures, sugar and herbs or spices, while smoking is also an option at a later stage during or after fermentation (Leroy et al. 2006). In order to eliminate the negative image, with which meat products are usually associated (high fat levels, high salt levels ect), and boost their value, functional meat products are gaining industrial interest. Utilisation of functional ingredients such as fibers, antioxidants, prebiotics or probiotics are only some of the options for developing functional meat products. Recently, the possibility of developing probiotic meat products has dominated research on functional meat products. The use of probiotics initiated a revolution in the meat industry, due to the potential health benefits introduced to meat products (Arihara et al. 2006). Dry-fermented sausages constitute an ideal food matrix for probiotic incorporation. The technological properties of fermented sausages such as the fermentation stage and the absence of thermal processing, enable probiotic bacteria to remain alive until the product consumption. It has to be noted that the cell viability in a fermented meat environment is probably strain-dependent (De Vuyst, Falony and Leroy, 2008). Moreover, it is believed that the sausage matrix protects the survival of probiotics during their passage from GIT (Klingberg and Budde, 2006). Sevelar studies are available in the literature investigating the addition of probiotic cultures in dry-fermented sausages (Jofre et al. 2015; Sidira et al. 2014; Rubio et al. 2014; Leroy et al. 2006; Pennacchia et al. 2004; Papamanoli et al. 2003).

The addition of probiotic cultures in dry-fermented sausages would enhance microbial safety, while offering nutritional, organoleptic or health benefits (Leroy et al. 2006). A big challenge in the application of probiotics in fermented sausages is the selection of the suitable culture and the source of isolation. One option is to use bacteria that are usually associated with the meat environment and possess the appropriate physiological requirements and health-

promoting benefits. Such bacteria could be found by screening of the autochthonous sausage isolates (wild-type) or existing commercial meat starter cultures. Alternatively, isolates of human intestinal origin could be evaluated for their viability during the manufacturing processes and storage of the fermented meat. Last but not least, the addition of probiotic cultures in the meat matrix would possibly result in increased pathogen inactivation and therefore, in boosting the product safety. Considering the above, dry-fermented sausages were evaluated in the current study as probiotic vehicles and the effects of the probiotic culture in the technological and sensorial properties of the products were also assessed (Chapter 4).

Another category of meat products are those undergoing thermal processing. Ham is a high quality thermally-processed meat product and one of the most consumed worldwide. Meat industries are trying to produce ham that is of high sensory quality, which is determined by several traits such as color, texture, flavor, juiciness etc., while at the same time extending the shelf-life as much as possible. Important factors that impact the overall quality of ham include the quality of the raw material, the mechanical treatment, the cooking temperature and time and the cooling treatment, the additives, etc (Tomovic et al. 2013). In general, meat is composed of a mixture of different chemical components and each of them affects the quality independently or in combination with other components. Links do exist between chemical constituents such as water, protein, fat, salt and ash and physical attributes, such as tenderness, hardness, cohesion, chewiness and color (Valkova et al. 2013). Aroma, textural firmness and juiciness have been proved to be the most important traits in ham evaluation, and in general it has been reported that the more aromatic is a product, the greater the acceptance is. Usually, products such as ham, are not considered ideal for probiotic incorporation, due to the high spoilage potential of LAB and the specific characteristics of the product. However, alternative methods for probiotic inclusion in

ham could be their application in edible films or microencapsulated, as long as the acidification is controlled and kept to the minimum. The successful probiotic delivery to ham would increase the range of the products available for consumers and would develop a novel product with possibly new sensorial attributes. It has to be highlighted that such information is missing from the literature. Therefore, part of the thesis dealt with the probiotic delivery in sliced ham using sodium-alginate edible films as a carrier. The effect of the probiotic bacteria on several quality characteristics of the ham was examined, as well as the spoilage potential (shelf-life studies) (Chapter 5).

1.1.11. Spoilage of meat products: The case of dry-fermented sausages and sliced ham

Fermented sausage is a meat product prepared by microbial fermentation with a very long shelf-life and exceptional flavor. Usually, in dry-fermented products, the main bacterial group contributing to deterioration is LAB and fungi, while other bacterial groups such as *Brochothrix* spp., *Pseudomonas* spp., and *Enterobacteriaceae* remain below detection limit. Furthermore, due to the stability of these products, spoilage is difficult to be assessed and is mainly determined through chemical reactions occurring in the fermented sausages and are more profound than microbial changes. Protein oxidation can be present during the fermentation and ripening of the sausages can lead to structural and functional modifications of the proteins and increase rancidity or cause meat browning (Bolivar-Monsalve, 2019). These changes might be detrimental on the quality and sensory characteristics of the product and have been correlated to meat tenderness. Protein oxidation can result in a loss of the essential amino acids, reduction of protein digestibility and compromise the nutritional value. Oxidative rancidity is also considered one with the most detrimental effects due to losses in nutritional value, development of off-flavors and formation of potentially harmful chemical compounds. Oxidation can take place where the

meat muscle membranes get disrupted. On the contrary, lipid oxidation can occur due to enzymatic reactions involving enzymes which are naturally present in the meat or are of a microbial origin. Also, autoxidation can occur and is linked to the presence of polyunsaturated fatty acids. Lipases, esterases and phospholipases, are enzymes responsible for phenomena of enzymatic hydrolysis, while non-enzymatic hydrolysis is caused by heme proteins such as myoglobin and hemoglobin. The oxidation of unsaturated fatty acids results in the formation of derivatives of volatile or non-volatile nature, including alcohols, furans, aldehydes, ketones, carbonyls that contribute to the development of rancidity and off-color (Bolivar-Monsalve, 2019).

Completely different is the spoilage activities of thermally-processed meat such as ham. Spoilage bacteria associated with ham include *Lb. sake*, *Lb. curvatus* and *Ln. mesenteroides*. The main undesired effects due to spoilage activity are the pH reduction, the gas and slime production, the formation of off-flavors and the discolorations. Production of cooked ham involves processing at a temperature of around 70°C, which inactivates most of the vegetative cells. On the other hand, massive bacterial proliferation takes place close to the end of the shelf-life, although several preservative methods have been applied (chilling temperature, presence of salt and nitrites) (Raimondi et al. 2019). High bacterial counts can be found in the product after 10-15 days of production, with the composition of the microbiota to depend on several factors such as packaging conditions, hygienic conditions throughout processing, temperature in distribution and domestic refrigeration (Samelis et al. 1998).

1.1.12. FTIR spectroscopy combined with chemometrics as a tool for spoilage evaluation

Nowadays, the demand for food products of high quality requires vast and safe inspection of the entire production. For this reason, the development of fast, accurate and non-destructive

tools for individual sample assessment at a minimum time is of utmost importance (Cortés et al. 2019). The term "vibrational spectroscopy", refers to the techniques of Infrared and Raman spectroscopy. These techniques are deemed as non-destructive, and slightly invasive. The absorbance or intensity values can be representative of the composition, the interactions within the sample and the structure. In both methods, energy levels are measured that are linked to the specific particular bonds present on the sample, and therefore they serve as a fingerprint. In particular, IR spectroscopy enables easy, fast and inexpensive analyses to samples (excluding the initial high cost of the equipment), without prior preparation or pre-treatment to the samples and has a potential to develop instruments for in-line applications. Fourier-Transform Infrared spectroscopy was developed to overcome certain limitations of IR instruments enabling measuring of all the infrared frequencies in a simultaneous way. Both techniques have been used for food analysis with many applications, while most of the studies being focused on investigating the potential of these techniques in evaluating spoilage of food products. Studies dealing with FTIR spectroscopy and the relevant food applications are presented in Table 3.

FTIR spectroscopy, is based on indirect measurements and generates data that are highly complex to handle and visualize. Consequently, the analysis of the FTIR spectra requires an additional support of the so called "chemometrics" to utilize in full the information coded on FTIR data (Cortés et al. 2019). With the term chemometrics, a data-driven extraction of information from chemical systems is implied. Chemometrics in its principle is part of the analytical chemistry, focusing on extracting and visualizing information from chemical analytical data with the application of appropriate mathematical and statistical methods (Wold, 1991). Today, chemometrics are extensively utilized in chemistry, biochemistry, chemical engineering and food science, while some of the methods used include multivariate statistics, applied

mathematics and computer science (Ropodi, Panagou & Nychas, 2016). Generally, for chemometric application three steps are required: i) Pre-treatment of the spectral data, ii) Calibration models development, and iii) Model transfer. The main objective of data pre-treatment or pre-processing is the transformation of data into more concise information enabling a multivariate analysis. Common pre-treatments include smoothening methods; derivation methods; standard normal variate (SNV) transformation; multiplicative scatter correction (MSC); normalization or scaling amongst others. Furthermore, different combinations of these methods can be performed simultaneously for signal processing (Brereton, 2003). On the other hand "machine learning" is a separate area of computer science dealing with machine learning issues in several fields and can be extended beyond programming and can be linked to computational intelligence.

The methods used in chemometrics can be divided in supervised or unsupervised. Unsupervised learning is composed of detecting data-driven structures and groupings without prior knowledge and can be performed with cluster analysis. This technique can give information on how similar is one sample to another. Their aim is to model the underlying structure of the data without prior information about them. They are used to examine the data structure, identify similarities between multiple objects and check for outliers in the dataset (Granato et al. 2018).

In supervised learning, the aim is to model the input variables, based on the output (y-data) (Goodacre, 2003). Normally, supervised methods give good results since the actual output is taken into account. On the contrary, the development and calibration of the model is quite complex and several parameters require adjustments eg. Cross-validation; criteria for Root Mean Square Error (RMSE), etc. Depending on the method, the process of building a model for training or calibration can vary from very simple to very difficult. In the case of supervised techniques,

the models are developed for qualitative and quantitative estimation based on previously collected data. Among qualitative methods, κ-nearest neighbours (κ-NN), Partial Least Squares Discriminant Analysis (PLS-DA), fuzzy rule-building expert system (FuRES), linear or quadratic discriminant analysis (LDA or QDA) are the most common in chemometrics. However, other methods with applications nowadays in the field of food science include classification and influence matrix analysis (CAIMAN), support vector machines (SVM), artificial neural networks (ANN) amongst others. The best modelling method for FTIR spectra is Partial Least Squares (PLS). PLS are the major modelling tools for analytical chemical data, due to their good collinearity in analytical chemical datasets. PLS methods usually enable the identification, quantification and display of the essential relationships between and within chemical datasets (Szymanska, 2018). In the current thesis, the potential of FTIR spectroscopy as a technique for quality evaluation and spoilage assessment was investigated in the meat products, previously produced in Chapter 4, 5 and 6 in tandem with chemometrics (PLS models) (Chapter 7).

Table 3. Studies dealing with FTIR spectroscopy and their relevant food applications.

Food category	Aim	Reference
Chicken breast muscle	Spoilage	Alexandrakis, Downey and Scanell, 2012
Minced beef	Spoilage	Ammor, Argyri and Nychas, 2009
Beef fillets	Spoilage	Argyri et al. 2010
Pork muscle fiber tissue	Processing	Böcker et al. 2007
Muscle foods	Identification	Ellis et al. 2005
Raw and Processed foods	Microbial evaluation	He and Sun, 2015
Minced beef	Adulteration	Meza-Márquez, Gallardo-Velázquez and Osorio-Revilla, 2010
Milk	Spoilage	Nicolaou and Goodacre, 2008
Milk	Enumeration and growth of bacteria	Nicolaou, Xu and Goodacre, 2011
Beef fillets	Spoilage	Panagou et al. 2011
Minced pork	Spoilage	Papadopoulou et al. 2011
Beef meatballs	Adulteration with pork	Rohman, Sismindari and Che Man, 2011
Fresh salmon	Spoilage	Saraiva, Vasconcelos and de Almeida, 2017
Beefburger	Adulteration	Zhao, Downey and O'Donnell, 2014
Meat	Spoilage	Argyri et al. 2013
Minced beef	Frozen-then-thawed	Ropodi, Panagou and Nychas, 2018
Pasteurised vanilla cream	Microbial quality	Lianou et al. 2018
Minced pork	Spoilage	Fengou et al. 2019
Farmed sea bream	Spoilage	Fengou et al. 2019
Raw chicken fillets	Spoilage	Vasconcelos, Saraiva and de Almeida, 2014
Spoilage Fungi	Characterization	Shapaval et al. 2013
-	Volatile detection	Jiao et al. 2019
Sliced ham	Spoilage	Pavli et al. 2018; Pavli et al. 2019
Dry-fermented sausages	Quality characteristics	Pavli et al. 2020

1.2. Objectives of the thesis

The main objective of this study was to investigate the performance of adjunct LAB cultures with probiotic potential on different meat matrices such as dry-fermented sausages and sliced ham, as well as the impact of the adjunct cultures on the spoilage of these products using the FTIR technique. Secondary objectives of the study included the isolation and characterization of potentially probiotic bacteria using *in vitro* tests; studying the GABA-producing ability of the selected LAB as well as studying the contribution of quorum-sensing compounds (Autoinducer-2) under standard growth conditions and also conditions naturally occurring in food matrices (presence of salt, acidic environment, high temperature) and while digestion (extreme acidic conditions and presence of bile). Additional aims of the thesis were the evaluation of HPP treatment in sliced ham as a preservation technology, the efficacy of probiotic-supplemented edible films as probiotic carriers in sliced ham, the effectiveness of antimicrobial edible films (supplemented with essential oil) on pathogen (*Listeria monocytogenes*) inactivation in ham and the assessment of FTIR spectroscopy as a tool for monitoring spoilage and the quality status of the aforementioned meat products.

CHAPTER 2

Molecular characterization of lactic acid bacteria from traditional fermented products and evaluation of their probiotic potential *in vitro*

2.1. Lactic acid bacteria isolated from traditional meat and dairy products and their *in vitro* probiotic potential

In this sub-chapter, the aim was the isolation and characterization of lactic acid bacteria isolated from traditional greek fermented products as well as their probiotic potential evaluation *in vitro*. This work has been included in the following publication:

Pavli FG, *Argyri AA, Papadopoulou OS, Nychas G-JE, Chorianopoulos NG, and Tassou C (2016). Probiotic Potential of Lactic Acid Bacteria from Traditional Fermented Dairy and Meat Products: Assessment by In Vitro Tests and Molecular Characterization. Journal of Probiotics and Health 4: 157. DOI: 10.4172/2329-8901.1000157.*

2.1.1. Abstract

The aim of the present study was to evaluate the probiotic potential of LAB isolated from Greek traditional fermented products. A series of *in vitro* tests that included survival in simulated gastrointestinal conditions (resistance to low pH, bile salts resistance and bile salts hydrolysis) and safety assessment (resistance to antibiotics, haemolytic and antimicrobial activity) were performed to select potential probiotic candidates, while *Lactobacillus rhamnosus* GG and *Lactobacillus casei* Shirota were used as reference strains. Initially, a total of 255 isolates of LAB were recovered from meat and dairy products and screened for their survival in simulated gastrointestinal (GI) tract conditions. 133 isolates that exhibited moderate or good behavior in these tests were subsequently differentiated and characterized at species level with molecular tools. PFGE was applied for strain differentiation, while species differentiation was based on restriction analysis of the amplified 16S rRNA gene. Specific multiplex PCR assay targeting the *rec*A gene was applied to resolve the species level of the isolates of *Lb. plantarum* group. From the 133 isolates, 47 different strains were recovered and were assigned to *Lactobacillus sakei* (14), *Lactobacillus curvatus* (4), *Leuconostoc mesenteroides* (4), *Lactococcus lactis* (4), *Lactobacillus casei* group (1), *Lactobacillus brevis* (1), *Lb. plantarum* (10), *Lb. pentosus* (7) and *Lb. paraplantarum* (2). The identified strains with good behavior to the GI tract tests were selected and further evaluated for their safety aspect. In conclusion, 19 out of the 47 identified strains were assessed as well-behaved, under simulated gastrointestinal conditions and also considered as safe, possessing thus desirable *in vitro* probiotic properties similar or better to that of the reference strains. These strains may be considered as good candidates for further investigation at *in vivo* and *in situ* studies to assess their potential health benefits and their performance as novel probiotic starters or adjunct cultures.

2.1.2. Introduction

The term probiotic is a quite new word meaning “for life” and it is recently used to name bacteria related to positive effects for humans (Fijan, 2014) and animals (Chaucheyras-Durand and Durand, 2010). The first observation of the positive role of some selected bacteria is ascribed to Elie Metchnikoff, the Russian born Nobel Prize holder who was working at the Pasteur Institute at the beginning of the last century. A generally accepted definition of probiotics recognized by the FAO/WHO, proposes that probiotics are "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host” (FAO/WHO, 2001). Members of the genera *Bifidobacterium*, *Lactobacillus*, *Streptococcus* and *Enterococcus* are the most frequently used probiotics, although members of the genera *Streptococcus* (Delorme, 2008) and *Enterococcus* contain some opportunistic pathogens (Ogier and Serror, 2008; Salminen et al. 1998).

Several beneficial functions have been suggested for probiotic bacteria e.g. vitamin production (Le Blanc et al. 2011), cholesterol lowering (Choi and Chang, 2015), alleviation of lactose intolerance (He et al. 2007), cancer prevention (Rafter et al. 2007), stimulation of the immune system (Shraf and Shah, 2014), enhancement of bowel motility (Whelan and Quigley, 2013), relief from constipation (Kim et al. 2015), prevention and reduction of rotavirus and antibiotic associated diarrhea (McFarland, 2007). Some of these benefits have been proven and established, while other have shown a promising potential in animal models, with human clinical studies required to confirm these claims (Vasiljevic and Shah, 2008). It’s of great importance to mention that the biological effects revealed from probiotic bacteria are strain specific and there is

no universal strain that would provide all the suggested benefits, not even strains of the same species (Vasiljevic and Shah, 2008).

Foods containing probiotic bacteria fall within the category of functional foods, which are defined as foods claimed to have a positive effect on health. Such products are gaining more widespread popularity and approval throughout the developed world, while increased commercial interest has contributed significantly to the development and expansion of this market sector (Saad et al. 2013). Despite their increasing economic significance, probiotic functional foods are not specifically regulated by European legislation and currently only Japan, the U.K., the USA and the Scandinavian countries have accomplished substantial evolution (Losio et al. 2015).

Traditional fermented foods represent a rich source of microorganisms. Among fermented foods, dairy products are considered to be the major source of probiotic bacteria isolation with numerous studies confirming this theory (Losio et al. 2015; Zago et al. 2011; Maragkoudakis et al. 2006). Although these products have been exploited in depth as both source and carrier of probiotic lactic acid bacteria, research has been conducted with other fermented products as well, such as fruits and vegetables (Vitali et al. 2012), table olives (Botta et al. 2014; Argyri et al. 2013), fermented cereals (Rivera-Espinoza and Gallardo-Navarro, 2010; Manini et al. 2016) and fermented meat (Pennacchia et al. 2004; Papamanoli et al. 2003).

The aim of the current study was to isolate strains from Greek traditional dairy and meat products and to perform a series of *in vitro* tests to assess their probiotic properties. The isolates that exhibited moderate or good properties at *in vitro* tests, were then differentiated and characterized with molecular tools (PFGE, multiplex PCR), as a part of the selection of new probiotic candidates. The results acquired from this study will be employed in further research

focusing on the assessment of the technological properties of the isolated strains for the selection of potential adjunct cultures with improved characteristics in fermented meat and dairy products and food industry in general.

2.1.3. Materials and Methods

2.1.3.1.Isolation of LAB and pre-selection of most promising probiotic strains

Traditional Greek dairy products such as feta cheese, manouri cheese and xerotyri cheese, and traditional meat products such as sausages, fermented sausages from Lefkada region, cured beefs and soutzouki (a dry spicy product) were obtained from local markets in Greece.

Samples of 25 g were weighted aseptically, added to 225 mL quarter strength Ringer's solution (LABM, Lancashire, UK) and homogenized in a stomacher (Stomacher 400 circulator, SEWARD LIMITED, Norfolk, UK) for 60 s at room temperature. Decimal dilutions were prepared and 1 mL of the sample was mixed on De Man-Rogosa and Sharpe agar (OXOID, Hampshire, UK). MRS Agar was used for selection and quantification of LAB population and was incubated at 30 °C for 48-72 h. 20% of the colonies were randomly selected and purified from each sample from the appropriate dilution of the growth medium. Pure cultures were stored at -80 °C in MRS broth supplemented with 20% (v/v) glycerol (APPLICHEM, Darmstadt, Germany). Before experimental use, each isolate was sub-cultured twice on the appropriate medium and colonies were checked for purity before use. A total of 255 isolates were recovered from feta cheese (9 isolates), manouri cheese (26 isolates) and xerotyri cheese (30 isolates), as well as from sausages (17 isolates), fermented sausages from Lefkada region (89 isolates), cured beefs (67 isolates) and soutzouki (17 isolates). These isolates as well as, 2 reference strains i.e. *Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus casei* Shirota (ACA-DC 6002),

kindly provided by Prof. E. Tsakalidou, Laboratory of Dairy Research, Agricultural University of Athens, were screened for their probiotic potential with a series of *in vitro* tests (screened for their survival in simulated GI tract conditions). 133 out of 255 isolates that exhibited moderate or good behavior in simulated gastrointestinal conditions were subsequently differentiated and characterized at species level with molecular tools.

2.1.3.2. Pulsed Field Gel Electrophoresis

PFGE was performed in order to determine LAB differentiation at strain level. In brief, genomic DNA extraction was performed from all isolates as previously reported (Doulgeraki et al. 2010). The restriction enzyme *Sma*I (10U) (NEW ENGLAND BIOLABS, Ipswich, MA, USA) was used according to manufacturer recommendations for 16 h. Following digestion, restriction fragments were separated in 1% PFGE grade agarose gel in 0.5mM Tris-Borate buffer on a CHEF-DRIII (BIO-RAD, Hercules, CA, USA) equipment with the following running parameters: 6V/cm, 1 s initial switching time, 10 s final switching time and 16 h total run at 14 °C. Gels were then stained with ethidium bromide (0.5mg/L) in water for 1 h and distained for 2 h before being photographed with GelDoc system. Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA clustering with Bionumerics software, version 6.1 (APPLIED MATHS, Sint-Martens-Latem, Belgium).

2.1.3.3.Identification and characterization of strains

Following PFGE differentiation, the different isolates were subjected to sequence analysis of V1-V3 region of 16S rRNA gene (Doulgeraki et al. 2010). DNA was extracted according to Doulgeraki, Paramithiotis and Nychas (2011) and PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to manufacturer

instructions. For the differentiation of *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum*, specific multiplex PCR assay targeting the *rec*A gene was employed, while the sizes of the amplicons were 318 bp for *Lb. plantarum*, 218 bp for *Lb. pentosus*, and 107 bp for *Lb. paraplantarum* (Torriani, Fellis and Dellaglio, 2001). The GenBank closest relative accession numbers for the 16S rRNA gene sequences are given in Table 1 for each strain.

2.1.3.4. Probiotic tests *in vitro*

Survival under simulated human gastrointestinal (GI) tract

The methods that were used to examine resistance of strains to low pH, resistance to bile salts and bile salts hydrolysis are described below and were performed according to Argyri et al. (2013) with slight modifications.

Resistance to low pH

In order to examine resistance of strains to low pH, bacterial cells from overnight cultures (18 h), were harvested by centrifugation (10000 g, 5 min, 4 °C), washed twice with PBS buffer (pH 7.2) before being re-suspended in PBS solution, with a pH adjusted to 2.5. Resistance to low pH was assessed in triplicates in terms of viable colony counts and enumerated on MRS agar (OXOID, Hampshire, UK) after incubation at 37 °C under stirring conditions, for 0, 0.5, 1, 2 and 3 h, reflecting the corresponding time which food spends in the stomach. The isolates that exhibited final counts $\geq 10^3$ CFU/mL or $\geq 10^6$ CFU/mL at low pH for 3 h, were considered to have moderate or good resistance, respectively, to this test and were selected for strain differentiation, characterization and safety assessment tests. For the final selection of the identified strains, the criterion of counts $\geq 10^6$ CFU/mL at low pH for 3 h was set.

Resistance to bile salts

Bacterial cells from overnight cultures (18 h), were harvested by centrifugation (10000 g, 5 min, 4 °C), washed twice with PBS buffer (pH 7.2), before being re-suspended in PBS solution (pH 8.0), containing 0.5% (w/v) bile salts (OXOID, Hampshire, UK). Resistance to bile salts was assessed in triplicates in terms of viable colony counts and enumerated after incubation at 37 °C under stirring conditions, for 0, 1, 2 and 4 h reflecting the corresponding time that food spends in the small intestine. The isolates that exhibited final counts $\geq 10^3$ CFU/mL or $\geq 10^6$ CFU/mL in bile salts for 4 h, were considered to have moderate or good resistance, respectively, to this test and were selected for strain differentiation, characterization and safety assessment tests. For the final selection of the identified strains, the criterion of counts $\geq 10^6$ CFU/mL in bile salts for 4 h was set.

Bile salts hydrolysis

Fresh bacterial cultures were streaked on MRS agar in triplicates containing 0.5% taurodeoxycholic acid-TDCA (SIGMA, Missouri, USA). The hydrolysis effect was evaluated by different colony morphology (partial hydrolysis) in comparison to the control MRS plates, after 48 h of anaerobic incubation at 37 °C.

2.1.3.5.Safety assessment of the selected strains

The strains that had good behavior to the aforementioned GI tract tests were selected and further evaluated for their potential haemolytic activity, antimicrobial activity and resistance to antibiotics according to Argyri et al. (2013).

Antimicrobial activity against pathogens

All strains were tested in triplicates for antimicrobial activity against 3 *Listeria monocytogenes* strains (FMCC-B-129, FMCC-B-131, FMCC-B-133), 1 *Salmonella enterica* subsp. *enterica* serovar Enteritidis strain (FMCC B-56 PT4), 1 *Staphylococcus epidermidis* strain (FMCC B-202 C5M6), kindly provided by the laboratory of Food Microbiology and Biotechnology (Food Microbiology Culture Collection of the Agricultural University of Athens), 1 *Escherichia coli* strain (ATCC 25922) and 1 *Staphylococcus aureus* strain (ATCC-25923). Fresh overnight bacterial MRS culture supernatants of the tested LAB strains were harvested by centrifugation (10000 g, 15 min, 4 °C), adjusted to pH 6.5 and then sterilized by filtration (0.22 μm). The cell free culture supernatants (CFCs) of the tested LAB strains were screened for antimicrobial activity using the well diffusion assay. Initial inoculum of 10^6 CFU/mL of the target strain was incorporated into soft agar (1% w/v) plates of the appropriate for the target strain medium. CFSs (50 μL) were transferred in holes (5 mm diameter) drilled into the agar. The plates were incubated at 37 °C and were examined for growth-free zones (diameter) around the well. The antibiotic kanamycin (30μg/mL) was used as positive control, while MRS broth adjusted to pH 6.5 was the negative control.

Haemolytic activity

Fresh bacterial cultures were streaked on Columbia agar plates (OXOID, Hampshire, UK) in triplicates containing 5% (w/v) of horse blood and incubated for 48 h at 30 °C. Blood agar plates were examined for signs of α-haemolysis (green-hued zones around colonies), β-haemolysis (clear zones around colonies) or γ-haemolysis (no zones around colonies).

Antibiotic Resistance

For testing antibiotic resistance of the strains selected by the previous phenotypic tests, microdilution broth was used. Bacterial strains were inoculated (1% v/v) in MRS broth supplemented with antibiotics (vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, tetracycline, chloramphenicol) at various concentrations (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 μg/mL) and examined in triplicate for growth in a microplate reader (OD 610 nm) following an incubation period of 24 h at 30 °C.

2.1.4. Results and Discussion

2.1.4.1. Isolation of LAB, strain differentiation and characterization

The total of 255 isolates that were recovered, were initially screened for their survival in simulated gastrointestinal conditions and the 133 isolates that exhibited moderate or good behavior, were subsequently differentiated and characterized at species level. The application of PFGE analysis to the 133 isolates resulted in 47 different fingerprints (Figure 1). The cluster analysis of PFGE *Sma*I digestion fragments of the LAB isolates showed two major clusters as seen on Figure 1. From the two clusters, the upper cluster was found to contain 4 strains belonging to *Ln. mesenteroides,* which were recovered from both dairy and meat samples. On the other hand, no specific information could be provided from the clustering in the second branch, which included isolates of different genus and species recovered from different sources of dairy and meat products.

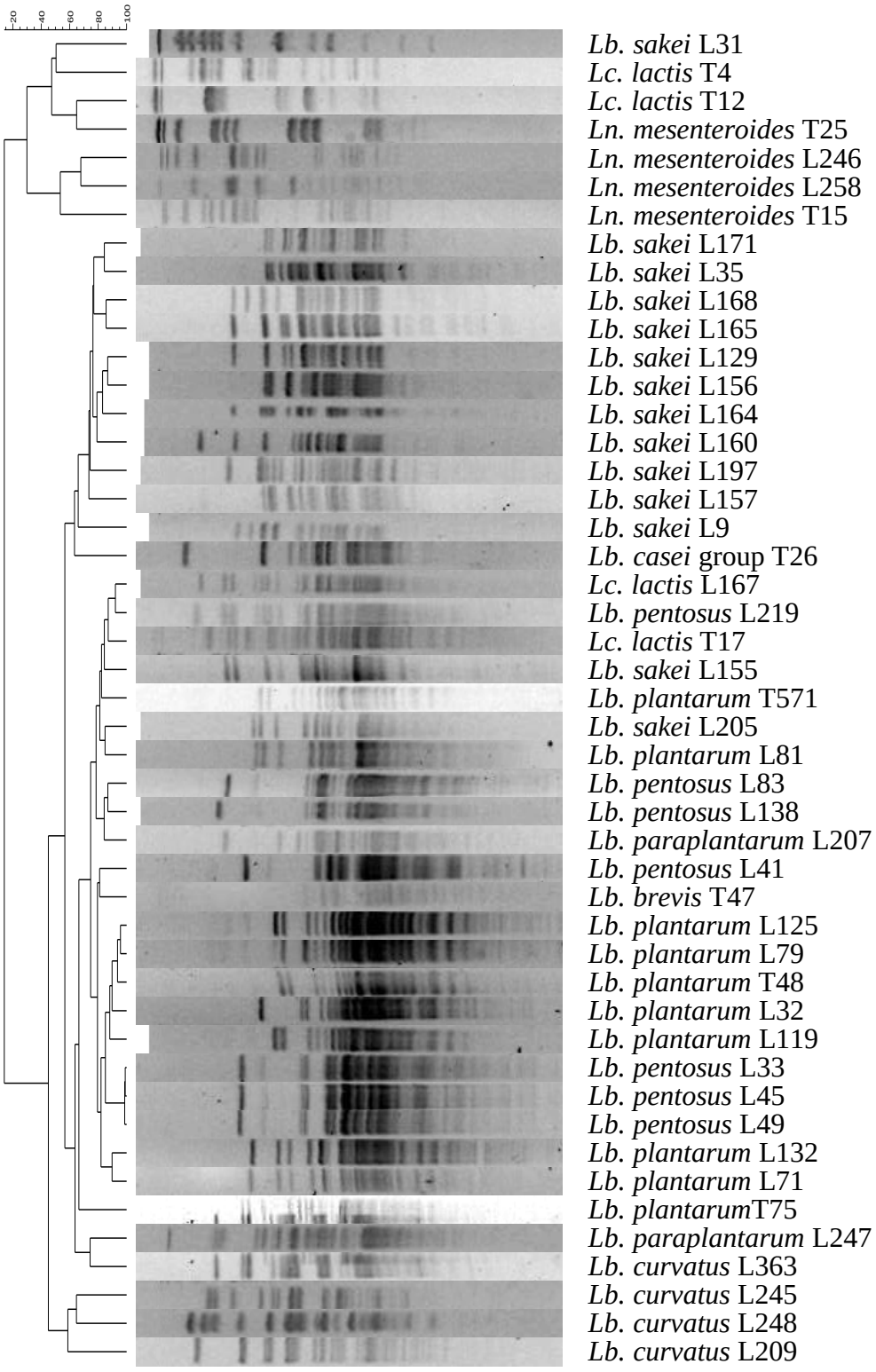

Figure 1. Cluster analysis of PFGE results. *Sma*I digestion fragments of the lactic acid bacteria recovered from different dairy and meat samples calculated by the unweighted

average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Table 1. Species identification. Results obtained after sequencing of the variable V1-V3 region of the 16S rRNA gene as well as the closest relative and its accession number from the GenBank.

Strain	**Closest Relative**	**GenBank Accession Number of the closest relative**	**Identity**
Lc. lactis T4	*Lc. lactis* subsp. *lactis*	LC153549	100%
Lc. lactis T12	*Lc. lactis*	KU248781	100%
Lc. lactis T17	*Lc. lactis* subsp. *lactis*	LC153549	100%
Lc. lactis L167	*Lc. lactis* subsp. *cremoris*	LC129537	100%
Ln. mesenteroides T15	*Lc. mesenteroides*	KT722833	99%
Ln. mesenteroides T25	*Lc. mesenteroides*	KT722833	99%
Ln. mesenteroides L246	*Lc. mesenteroides*	KT722833	100%
Ln. mesenteroides L258	*Lc. mesenteroides*	KT722833	100%
Lb. plantarum L32	*Lb. plantarum*	KX082943	100%
Lb. plantarum T48	*Lb. plantarum*	KX074205	99%
Lb. plantarum T71	*Lb. paraplantarum*	LC090476	100%
Lb. plantarum T75	*Lb. plantarum*	KR078354	100%
Lb. plantarum L79	*Lb. paraplantarum*	LC090476	100%
Lb. plantarum L81	*Lb. plantarum*	KR025393	100%
Lb. plantarum L119	*Lb. plantarum*	KX074205	100%
Lb. plantarum L125	*Lb. plantarum* subsp. *plantarum*	KP763941	100%
Lb. plantarum L132	*Lb. plantarum* subsp. *plantarum*	KP763941	100%
Lb. plantarum T571	*Lb. paraplantarum*	LC090476	100%
Lb. pentosus L33	*Lb. plantarum*	KP763939	100%
Lb. pentosus L41	*Lb. plantarum*	KR025402	100%
Lb. pentosus L45	*Lb. plantarum*	KX082943	99%
Lb. pentosus L49	*Lb. plantarum*	KP764187	100%
Lb. pentosus L83	*Lb. plantarum*	KX074205	99%
Lb. pentosus L138	*Lb. plantarum* subsp. *plantarum*	KP763946	100%
Lb. pentosus L219	*Lb. plantarum*	KP887104	100%
Lb. paraplantarum L207	*Lb. plantarum*	KX082940	99%
Lb. paraplantarum L247	*Lb. plantarum*	KT722828	99%
Lb. sakei L9	*Lb. sakei*	EU755262	99%
Lb. sakei L31	*Lb. sakei*	KT351714	99%
Lb. sakei L35	*Lb. sakei*	LC129551	100%
Lb. sakei L129	*Lb. sakei*	EU755262	99%
Lb. sakei L155	*Lb. sakei*	LC129551	99%
Lb. sakei L156	*Lb. sakei*	KT351714	99%
Lb. sakei L157	*Lb. sakei*	LC129551	100%

Lb. sakei L160	*Lb. sakei*	LC129551	99%
Lb. sakei L164	*Lb. sakei*	LC129551	99%
Lb. sakei L165	*Lb. sakei*	LC129551	100%
Lb. sakei L168	*Lb. sakei*	LC129551	99%
Lb. sakei L171	*Lb. sakei*	LC129551	99%
Lb. sakei L197	*Lb. sakei*	LC129551	100%
Lb. sakei L205	*Lb. sakei*	KT351714	99%
Lb. curvatus L209	*Lb. curvatus*	LC129556	100%
Lb. curvatus L245	*Lb. curvatus*	LC129556	100%
Lb. curvatus L248	*Lb. curvatus*	LC129556	100%
Lb. curvatus L363	*Lb. curvatus*	LC129556	100%
Lb. brevis T47	*Lb. brevis*	KT285603	100%
Lb. casei group T26	*Lb. casei*	KU315405	99%

The sequence analysis of the different 47 strains, revealed the presence of *Lactobacillus sakei* (14), *Lactobacillus curvatus* (4), *Leuconostoc mesenteroides* (4), *Lactococcus lactis* (4), *Lactobacillus casei* group (1), *Lactobacillus brevis* (1) and *Lactobacillus plantarum* group (19). For the differentiation of isolates assigned to *Lb. plantarum* group, multiplex PCR assay targeting the *rec*A gene was employed and resulted in 10 *Lb. plantarum*, 7 *Lb. pentosus* and 2 *Lb. paraplantarum* strains. The prevalence of different identified species detected in the different samples is summarized in Table 2.

Table 2. Source of the selected strains. LAB strains isolated from different dairy and meat products and selected according to their probiotic potential

Species	**Source**		
	Dairy samples	**Meat samples**	**Total**
Lactobacillus sakei	-	14	14
Lactococcus lactis	3	1	4
Lactobacillus curvatus	-	4	4
Leuconostoc mesenteroides	2	2	4
Lactobacillus casei group	1	-	1
Lactobacillus brevis	1	-	1
Lactobacillus plantarum	4	6	10
Lactobacillus pentosus	-	7	7
Lactobacillus paraplantarum	-	2	2
Total	**11**	**36**	**47**

The aforementioned species are related with the microbiota of spontaneous fermentation of dairy and meat products in previous studies. More specifically, *Lc. lactis*, *Ln. mesenteroides* and *Lb. plantarum* are identified as the most frequently isolated species in fermented dairy products (Yu et al. 2011; Bizzarro et al. 2000). Furthermore, *Lb. casei* group strains as well as *Lb. brevis* are recovered from dairy samples in previous studies (Yu et al. 2011; Bizzarro et al. 2000). It has to be noted that *Leuconostoc* strains naturally play an important role in the development of flavor in fermented products, although they display a weak competitive ability during milk fermentation, because of their complex nutritional requirements (Yu et al. 2011). Several researchers have investigated the biodiversity of fermented meat products and most of the studies reveal that *Lb. sakei* and *Lb. curvatus* are the predominant microflora of such products (Greppi et al. 2015; Pisacane et al. 2015; Cocolin, Dolci and Rantsiou, 2011; Villani et al. 2007). Additionally, *Ln. mesenteroides* (Greppi et al. 2015; Urso, Comi and Cocolin, 2006; Drosinos et

al. 2005) and *Lc. lactis* (Comi et al. 2005) are detected in fermented meat samples. Other species isolated include *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* (Cocolin, Dolci and Rantsiou, 2011; Villani et al. 2007; Urso, Comi and Cocolin, 2006; Drosinos et al. 2005; Comi et al. 2005; Leroy, Verluyten and De Vuyst, 2006; Rantsiou et al. 2006).

2.1.4.2. *In vitro* tests related to probiotic potential

Probiotics must remain viable during their passage in the gastrointestinal tract in population levels of 10^6-10^7 CFU/g in order to deliver the health benefits (Argyri et al. 2013). The acid environment of the stomach and the inhibitory effects of bile salts secreted in the duodenum are the major obstacles against probiotic survival. The *in vitro* evaluation of the survival of the potential probiotic strains in simulated GI tract conditions may only be necessary in predicting the actual *in vivo* survival of a strain when consumed in a non-protected way (Maragkoudakis et al. 2006).

Survival under simulated human GI tract conditions

The isolates that exhibited final counts $\geq 10^3$ CFU/mL at low pH for 3 h and $\geq 10^3$ CFU/mL in bile salts for 4 h were considered to have moderate or good resistance to these tests and were selected for strain differentiation, characterization and safety assessment tests. Since bile salts resistance test resulted in <3 log reduction for the total of isolates, the main criterion for the selection of the isolates was the resistance to low pH. As a result, 133 isolates out of 255 met both criteria and were further characterized with molecular tools, resulting to 47 identified strains that were selected and further studied.

Resistance to low pH

133 isolates out of 255, exhibited final counts ≥ 10^3 CFU/mL at low pH for 3 h. Regarding the 47 identified strains, the viable counts of most *Lb. plantarum* and *Lb. pentosus* strains showed higher resistance to low pH than *Ln. mesenteroides* and most of *Lc. lactis* strains which their final counts indicated the lowest resistance (10^3 CFU/mL). Furthermore, variability in the final viable counts of *Lb. sakei* strains after exposure to low pH for 3 hours was observed. Totally, 19 strains showed good resistance (>6 log CFU/ml) to low pH (*Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83) (Figure 2). These results are in agreement with other studies, where *Lactobacillus* strains are able to maintain their viability when exposed to low pH values (2.5-4.0) (Maragkoudakis et al. 2006; Argyri et al. 2013), while other researchers have reported strains of *Lb. plantarum* with lower ability to survive at low pH (Manini et al. 2016). *In vitro* assays propose to select acid resistant strains including exposure to pH-adjusted PBS (Maragkoudakis et al. 2006; Pennacchia et al. 2004), incubation in gastric juice (Charteris et al. 1998; Fernández, Boris and Barbés, 2003), or the use of GIT simulator (Minekus et al. 1999). The survival of potential probiotic strains to stomach juice is determined by their intrinsic resistance to the hostile environment, but also on the ingestion vector and its contents. As a result, foods with a high level of fat and the presence of certain proteins in the food may provide additional protection to the bacteria from gastric acid and therefore increase survival to gastric transit (Monteagudo-Mera et al. 2012). In the current study, pH value of 2.5 was used, in order to select potential probiotic strains. Such low pH value is very selective and although it is not the most common pH value encountered in the stomach, it guaranties the isolation of the very acid-tolerant strains (Pennacchia et al. 2004).

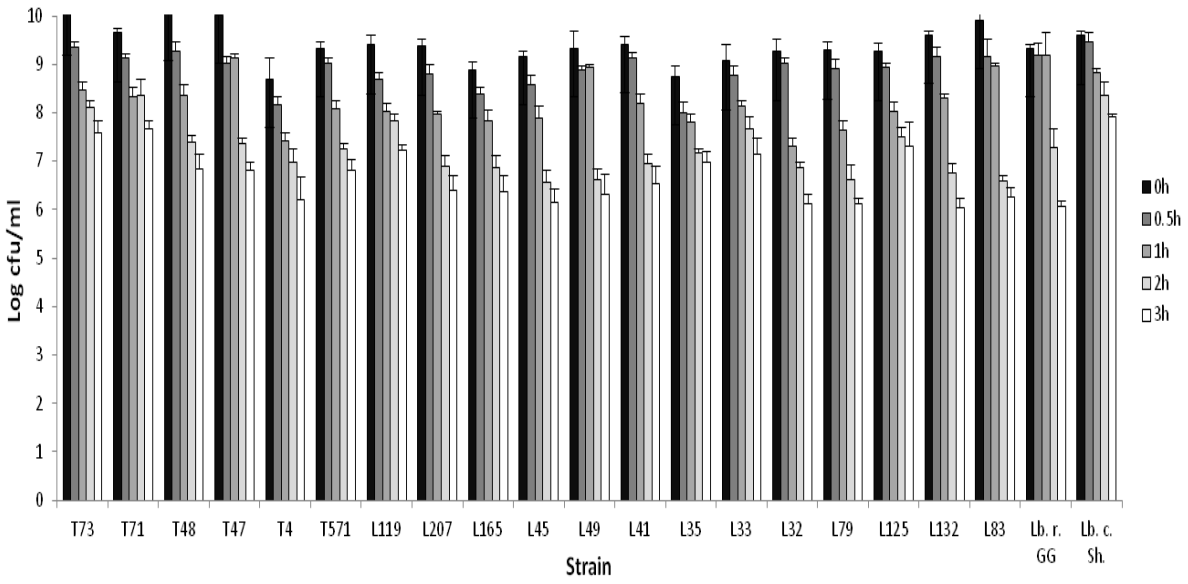

Figure 2. Results for low pH resistance for the selected strains. Resistance to low pH after 0, 0.5, 1, 2 and 3h of the selected strains *Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83 and the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG (Error bars indicate standard deviation from three replications).

Resistance to bile salts

The majority of the isolates were found to be highly resistant to bile salts even after 4 hours of exposure. Amongst the 47 identified strains, the viability of 40 strains was retained with minor reduction in viable counts (<1 log cycle), while 7 strains (*Lb. sakei* L168, L165, L155, *Lb. curvatus* L363, *Lb. casei* group T26, *Lb. plantarum* L132 and *Lb. paraplantarum* L207) showed approximately a reduction of <2.5 logs after 4 h of exposure to bile salts.

Tolerance to bile is one of the most essential attributes for probiotic bacteria, as it ascertains their ability to survive in the small intestine, and accordingly their ability to play a

functional role as probiotics (Ruiz, Margolles and Sánchez, 2013). Bile response is a complex phenomenon, involved in a variety of processes. Active efflux of bile salts/acids, bile salt hydrolysis and changes in the design/composition of cell membrane and cell wall, seem to be the most basic bile-specific mechanisms for resistance in *Lactobacillus* species (Ruiz, Margolles and Sánchez, 2013).

Suggested concentration of bile salts for probiotics is between 0.15 - 0.5%, as it is the range of the physiological concentrations that are met in the GIT (Papadimitriou et al. 2015). It has to be noted that, the majority of the strains survive well in such bile conditions, suggesting a potential recovery of the initial levels during the passage of the small intestine (Maragkoudakis et al. 2006). Furthermore, studies point out the huge variability in bile resistance that can be encountered within a species or genus (Jacobsen et al. 1999), revealing that bile tolerance is a strain-dependent feature and tolerances of species cannot be universal (Begley, Gahan and Hill, 2005).

Bile salts hydrolysis

Concerning bile salt hydrolysis (BSH), 11 strains demonstrated partial bile salt hydrolase activity, recorded as differentiated colony morphology on TDCA-MRS agar when compared to the control MRS agar plates. These strains were *Lb. plantarum* L132, L125, L81, L32, T48, T71, T73, *Lb. pentosus* L83, *Lb. sakei* L35 and L168 and *Lc. lactis* T12. The rest of the tested strains did not exhibit bile salt hydrolase activity, while the growth of 2 strains (*Lb. curvatus* L363, *Ln. mesenteroides* T25) was completely inhibited in the presence of 0.5% (w/v) taurodeoxycholic acid.

There are many studies confirming that BSH activity of probiotics is associated with hypocholesterolemic effect (Ooi and Liong, 2010; Pavlovic, Stankov and Mikov, 2012). BSH-active probiotic strains exert the aforementioned effect through deconjugation that leads to decreased solubility and lower reabsorption of bile salts and in the excretion of larger quantities of free bile acids in feces. Complementary, deconjugation of bile salts could result in a decrease in serum cholesterol to substitute that misplaced in feces or by decreasing the cholesterol solubility following absorption of cholesterol through the intestinal lumen (Pavlovic, Stankov and Mikov, 2012). Furthermore, microbial BSH function in the detoxification of bile salts, increase the intestinal survival and persistence of producing strains and possibly the profitable effects related to the strain (Begley, Hill and Gahan, 2006). On the other hand, there is still essential work to be carried out on BSH activity, concerning its mechanism of action in order to prevent other risks that may be caused by the excessive use of probiotics, including sepsis or colon cancer due to the secondary bile salts that are produced (Ishimwe et al. 2015).

2.1.4.3.Safety Assessment

Antimicrobial activity against pathogens

None of the supernatants of the selected LAB strains and the 2 reference probiotic strains obtained at adjusted pH of 6.5, inhibited the growth of the pathogenic strains tested (3 *Listeria monocytogenes*, 1 *Salmonella enterica* subsp. *enterica* serovar Enteritidis, 1 *Staphylococcus epidermidis*, 1 *Escherichia coli* and 1 *Staphylococcus aureus*) by the use of well-diffusion assay, leading to the assumption that no bacteriocin-like action exists. These results are in accordance to previous studies (Maragkoudakis et al. 2006; Argyri et al. 2013; Rubio et al. 2014). One of the functional properties involved in the characterization of probiotic bacteria is the capability of

producing antimicrobial compounds such as organic acid, short chain fatty acids and bacteriocins (Argyri et al. 2013). Antimicrobial ability of probiotics is also associated with the enhancement of the intestinal barrier function (Papadimitriou et al. 2015). Nonetheless, the *in vitro* production of antimicrobial substances alone, cannot provide us with reliable outcomes concerning the probiotic behavior *in vivo* (Papadimitriou et al. 2015).

Haemolytic activity

Absence of haemolytic activity is considered as safety criterion for the selection of a probiotic strain. In our study, none of the selected examined strains exhibited α- or β-haemolytic activity, when grown in Columbia blood agar, whereas all strains were γ-haemolytic (no haemolysis). These results are similar with previous observations where all of the tested strains (Pisano et al. 2014; Zoumpopoulou et al. 2008) or most of them are γ-haemolytic (Maragkoudakis et al. 2006; Argyri et al. 2013).

Antibiotic Resistance

The Minimum Inhibitory Concentrations (MICs) detected for the selected strains and the 2 reference probiotic strains, are presented in Tables 3 and 4. Strains are considered resistant when they exhibit MIC values higher than those established by the European Food Safety Authority (EFSA, 2012). Variable susceptibility to antibiotics was observed, according to the breakpoints set by EFSA (2012), even for strains of the same species. All LAB strains showed resistance to vancomycin, similarly to the findings of previous reports (Maragkoudakis et al. 2006; Argyri et al. 2013; Ruiz-Moyano et al. 2009), although a specified breakpoint is absent for these genus strains. 5 strains were found to be resistant to gentamycin and 4 to tetracycline, including the reference strains. Lower resistance to erythromycin and chloramphenicol was

observed for the majority of the tested strains with the reference strain *Lb. casei* Shirota to be the only resistant for both antibiotics. For kanamycin and streptomycin moderate susceptibility was exhibited with 3 strains to be resistant to kanamycin and 1 to streptomycin, despite the fact that MIC's were not low enough.

The antibiotic resistance of potentially probiotic bacteria is controversial and various opinions have been stated so far. For instance, the natural resistance to specific antibiotics might be desirable for some probiotic strains in order to develop antibiotic/probiotic combination therapies for certain conditions such as diarrhea (Charteris et al. 1998; Temmerman et al. 2003). On the other hand, LAB as probiotics enter human intestines in large numbers and are able to interact with the intestinal microbiota and therefore, they have the potential to transfer genes to other bacteria, even to pathogenic ones (Mathur and Singh, 2005). For safety reasons, the resistance observed to specific antibiotics has to be chromosomally encoded and not inducible or transferable. As accepted by EFSA (2008), intrinsic resistance and resistance due to mutation of chromosomal genes exerts low risk of horizontal dissemination and such probiotic strains should be acceptable for food consumption, whereas acquired resistance mediated by added genes may confer a risk for public health (Bernardeau et al. 2008). In lactobacilli, intrinsic resistance to vancomycin and kanamycin has been reported, whilst resistance to tetracyclines, chloramphenicol and erythromycin requires attention regarding safety (Temmerman et al. 2003).

Table 3. Antibiotic resistance of the 19 selected strains. MIC values for the selected strains according to the breakpoints set by EFSA (2012).

Strain	**MICs[a] (μg/mL)**						
	V	**G**	**K**	**S**	**E**	**T**	**C**
Lc. lactis T4	32[R]	32	32	32	<1	2	1
Lb. plantarum L32	512	32[R]	128[R]	256	<1	16	1
Lb. plantarum T48	512	8	32	16	<1	16	1
Lb. plantarum T71	≥ 1024	32[R]	64	256	<1	128[R]	1
Lb. plantarum T73	≥ 1024	32[R]	32	256	<1	16	1
Lb. plantarum L79	512	4	32	32	<1	8	1
Lb. plantarum L119	≥ 1024	4	16	256	<1	128[R]	1
Lb. plantarum L125	≥ 1024	8	32	64	<1	32	2
Lb. plantarum L132	512	32[R]	128[R]	256	<1	32	1
Lb. plantarum T571	≥ 1024	2	32	64	<1	32	1
Lb. pentosus L33	≥ 1024	16	32	64	<1	8	1
Lb. pentosus L41	≥ 1024	32[R]	64	256	<1	64	1
Lb. pentosus L45	512	4	32	64	<1	8	1
Lb. pentosus L49	≥ 1024	4	32	64	<1	8	1
Lb. pentosus L83	≥ 1024	4	32	32	<1	8	1
Lb. paraplantarum L207	512	8	64	64	<1	8	0.5
Lb. sakei L35	512	8	32	64	<1	4	0.5
Lb. sakei L165	256	16	32	64	<1	2	1
Lb. brevis T47	64	8	32	16	<1	16[R]	1
Lb. casei Shirota	≥ 1024	16	4	128[R]	2[R]	16[R]	8[R]
Lb. rhamnosus GG	≥ 1024	16	256[R]	32	<1	2	4

[R] Resistant according to the EFSA's breakpoints (EFSA, 2012).

V: vancomycin, G: gentamycin, K: kanamycin, S: streptomycin, E: erythromycin, T: tetracycline, C: chloramphenicol.

[a] MIC: minimum inhibitory concentration.

Table 4. Results from all tests *in vitro* for the 19 selected strains. Detailed results from the strains with probiotic potential according to *in vitro* tests in comparison with the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG.

Strains	**Test**				
	Low pH (SR%)	**Bile Salts (SR%)**	**Bile Salts Hydrolase**	**Haemolytic activity**	**Antibiotic Resistance**
Lc. lactis T4	71.46	90.02	0	γ	V
Lb. plantarum L32	66.20	94.20	1	γ	G,K
Lb. plantarum T48	67.89	98.81	1	γ	-
Lb. plantarum T71	79.48	97.36	1	γ	G,T
Lb. plantarum T73	74.51	96.17	1	γ	G
Lb. plantarum L79	65.88	93.90	1	γ	-
Lb. plantarum L119	76.91	95.95	0	γ	T
Lb. plantarum L125	78.94	96.49	1	γ	-
Lb. plantarum L132	62.81	78.65	1	γ	G
Lb. plantarum T571	73.18	94.87	0	γ	-
Lb. pentosus L33	78.83	93.96	0	γ	-
Lb. pentosus L41	69.43	93.35	0	γ	G
Lb. pentosus L45	67.18	91.40	0	γ	-
Lb. pentosus L49	67.74	94.22	0	γ	-
Lb. pentosus L83	63.10	91.46	1	γ	-
Lb. paraplantarum L207	68.20	87.11	0	γ	-
Lb. sakei L35	79.86	94.14	1	γ	-
Lb. sakei L165	71.65	87.27	0	γ	-
Lb. brevis T47	69.93	98.63	0	γ	T
Lb. casei Shirota	82.54	98.18	0	γ	S,E,T,C
Lb. rhamnosus GG	65.11	99.26	0	γ	K

V: vancomycin, G: gentamycin, K: kanamycin, S: streptomycin, E: erythromycin, T: tetracycline, C: chloramphenicol.

[a] Survival rate after 3 h in low pH

[b] Survival rate after 4 h in bile salts

2.1.5. Conclusions

In onclusion, certain strains were found to possess desirable probiotic properties *in vitro*. In more detail, 19 strains (*Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83) were found to have desirable probiotic properties alike or superior of the 2 reference probiotic strains examined, too. The selected strains are good candidates for further investigation with *in vivo* and *in situ* studies, to elucidate their potential health benefits and their performance as novel probiotic starters and adjunct starters in food fermentation processing.

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2.2. *In vitro* screening of γ-aminobutyric acid and autoinducer-2 signalling in lactic acid bacteria exhibiting probiotic potential from natural black Conservolea olives

In this sub-chapter, the aim was to investigate the probiotic potential of LAB, previously isolated from table olives var. Conservolea. Furthermore, their ability to produce AI-2 signal molecules and extracellular γ-aminobutyric acid ($GABA_e$) under standard growth conditions was also examined. This work has been included in the following publication:

Pavli F., *Gkana E., Adebambo O., Karatzas K-A, Panagou E. and Nychas G-J. (2019). In vitro screening of γ-aminobutyric acid and autoinducer-2 signalling in lactic acid bacteria exhibiting probiotic potential from natural black Conservolea olives. Foods, 8 (12), 640.*

2.2.1. Abstract

In the present study, 33 strains of lactic acid bacteria (LAB) previously isolated from natural black Conservolea olives were assessed for their probiotic potential *in vitro*, as well as for their autoinducer-2 (AI-2) activity under standard growth conditions and the production of γ-aminobutyric acid (GABA). The probiotic tests included the *in vitro* resistance to low pH and resistance to bile salts, the evaluation of bile salt hydrolase activity, as well as safety tests regarding their possible haemolytic activity and their antimicrobial activity against pathogens. The results indicated that 17 strains were able to survive in low pH and in the presence of bile, with 15 of them also exhibiting partial bile salt hydrolase activity. None of the strains exhibited haemolytic activity or inhibited the growth of any of the examined pathogens. Moreover, the strains displayed generally low AI-2 activity under the growth conditions tested, regardless of the species. Interestingly, in contrast to what has been found in most foods, none of the isolates were found to produce GABA after 48 h of growth. The results from the AI-2 activity and extracellular GABA detection were considered as unexpected for LAB with probiotic attributes.

2.2.2. Introduction

Lactic acid bacteria (LAB) are the most important group of probiotic bacteria, followed by *Bifidobacteria*, some species of *Enterococci* and *Bacillus*, although the safety of the latter ones remains in controversy. Many beneficial effects of probiotics have been associated with lowering of cholesterol, controling symptoms of lactose intolerance, stimulating the immune system, relieving constipation, preventing antibiotic-associated and traveller's diarrhoea and prevention of *Clostridium difficile* infections (Choi and Chang, 2015; He et al. 2007; Kim et al.

2015; McFarland, 2015; McFarland, 2007). Such bacteria have been isolated from various sources, including fermented dairy products (Maragkoudakis et al. 2006; Zago et al. 2011), non-dairy products (Pavli et al. 2016; Botta et al. 2014; Argyri et al. 2013; Vitali et al. 2012; Pennacchia et al. 2004) and from the intestinal tract of healthy individuals or infants (Kirtzalidou et al. 2011; Vizoso-Pinto et al. 2006; Martin et al. 2006). Fermented products of plant origin, such as table olives, are considered as an excellent source of probiotic bacteria and also great vehicles for probiotic delivery, with many studies confirming their potential (Peres et al. 2012; Hurtado et al. 2012; Argyri et al. 2014; Blana et al. 2014). A series of *in vitro* tests could be performed as a first step in order to assess the probiotic potential of these strains. Such tests could include acid and bile resistance, the production of antimicrobial compounds, and bile salt hydrolase activity, although further tests and clinical studies are also required at a later stage.

Cell-to-cell communication, also called quorum sensing, is the process of signalling that enables bacteria to control their gene expression and regulate their activities. A "universal" signal-molecule, called autoinducer-2 (AI-2), could be present in Gram-positive and Gram-negative bacteria, supporting inter-species communication. The expression of various phenotypes including virulence factors, biofilm formation, light production and stress resistance are only some of the functions that are linked to AI-2 in many bacterial species (Xavier and Bassler, 2003; Lebeer et al. 2007). This is of a particular interest in the case of probiotic bacteria, due to their exposure to a variety of environmental challenges, including low pH, the presence of bile salts and enzymes. Lebeer (2007) reported that AI-2 production had an impact on several physiological functions of the probiotic *Lactobacillus rhamnosus* GG. In another study, *Lb. paraplantarum* L-ZS9 was found to overexpress *luxS* gene and produce AI-2 that enhances heat, bile, and salt resistance, while also having an effect on its biofilm formation (Liu et al. 2018).

Furthermore, other studies have related AI-2 activity with acid tolerance in probiotic bacteria (Moslehi-Jenabian, Gori and Jespersen, 2009).

γ-Aminobutyric acid (GABA) is an important metabolite produced by the glutamate decarboxylase system (GAD) in various bacteria through the decarboxylation of glutamate under acidic conditions. Thus, quantification of GABA could be an important tool to assess acid resistance of some probiotic bacteria, which is important because survival in the acidic pH of the stomach is required for gut colonization in the host (O'Byrne et al. 2011). Antianxiety, antidepressant, antihypertensive and tranquilizing roles have been attributed to GABA, thus far (Foster and Kemp, 2006; Möhler 2012). Due to the aforementioned beneficial effects, much attention has been paid to the development of foods enriched with GABA. Several microorganisms have been examined for their potential to produce GABA through the GAD system, such as *Lb. brevis* and *Lactococcus lactis*. Furthermore, it has been reported that strains of *Lb. buchneri*, *Lb. paracasei* and *Lb. plantarum* isolated from traditional cheeses have the ability to produce GABA (Franciosi et al. 2015; Siragusa et al. 2007). It has to be noted that the ability of LAB to produce GABA varies among species and strains (Park and Oh, 2006). Therefore, screening of LAB for their ability to synthesize GABA, is of a special interest for the development of novel GABA-enriched functional foods, and this ability of LAB might be considered as an important probiotic trait in the near future (Ohmori, Tahara and Ohshima, 2018).

In the present study, 33 strains of LAB, were examined for their probiotic potential using a series of *in vitro* tests. The strains that exhibited good performance in the probiotic tests were then screened for AI-2 activity under standard growth conditions, followed by another screening with regard to their ability to produce GABA.

2.2.3. Materials and Methods

A total of 33 strains of LAB, previously isolated from natural black Conservolea olives during storage in different packaging conditions, as well as two reference strains, namely *Lb. casei* Shirota (ACA-DC 6002) and *Lb. rhamnosus* GG (ATCC 53103), kindly provided by Prof. E. Tsakalidou (Laboratory of Dairy Research, Agricultural University of Athens) were screened for their probiotic potential following a series of *in vitro* tests. The studied strains were 8 *Lb. plantarum*, 20 *Lb. pentosus*, 1 *Lb. paraplantarum*, 2 *Lb. corinyformis* and 2 *Pediococcus ethanolidurans* (Table 1), that were previously isolated, identified and characterized (Doulgeraki et al. 2012). Strains were stored in 20% glycerol at -80 °C for long term storage and were routinely cultured at 30 °C in de Man, Rogosa and Sharpe (MRS) broth (Biolife, Milan, Italy) or agar (Biolife, Milan, Italy) for 24 or 18 h and 72h, respectively.

2.2.3.1.Low pH Assay, Bile Salts Assay, and Bile Salt Hydrolase (BSH) Activity

The tests to assess the resistance of the strains to low pH and bile salts, as well as the bile salt hydrolase (BSH) activity, were performed according to Argyri et al. (2013). Briefly, for the resistance to low pH, bacterial cells from overnight cultures (18 h) were harvested by centrifugation (5000 x g, 15 min, 4 °C), washed twice with phosphate-buffered saline (PBS) (pH 7.2), and finally re-suspended in PBS solution with a pH of 2.5. After incubation for 0, 1, 2 and 3 h at 37 °C under stirring conditions, resistance to low pH was assessed in terms of viable colony counts enumerated on MRS agar. The strains that exhibited final counts ≥ 6 log CFU/mL at a pH of 2.5 for 3 h were selected to be screened for bile salt resistance. For the bile salt resistance test, the same procedure was applied, with the final resuspension being in PBS solution with a pH 8, containing 0.5% bile salts (Oxoid, Hampshire, UK). After incubation for 0, 1, 2, 3 and 4 h at 37 °C under stirring conditions, resistance was assessed in terms of viable colony counts enumerated

on MRS agar. The strains that exhibited final counts ≥ 6 log CFU/mL under these conditions, were selected to be screened for BSH activity. For the BSH activity, the bacterial cultures were streaked on MRS agar containing 0.5% taurodeoxycholic acid (TDCA; Sigma, St. Louis, MO, USA). The hydrolysis effect was assessed by different colony morphology in comparison to the control MRS plates (without TDCA), after anaerobic incubation at 37 °C for 48 h. The results were expressed as no hydrolysis or partial hydrolysis. The assays of low pH and bile were performed in triplicate, whereas the test for BSH activity was performed in duplicate.

2.2.3.2.Safety assessment of the selected strains

The selected strains from the previous tests were further evaluated regarding their potential haemolytic activity and antimicrobial activity according to Argyri et al. (2013). Fresh bacterial cultures were streaked on Columbia agar plates (LabM Limited, Lancashire, UK) containing 5% w/v horse blood and incubated for 48 h at 30 °C. After incubation the plates were examined for signs of α-haemolysis (green-hued zones around colonies), β-haemolysis (clear zones around colonies) or γ-haemolysis (no zones around colonies). With regard to the antimicrobial activity, all strains were tested against the following pathogens: *Listeria monocytogenes* ATCC 13932; *Listeria monocytogenes* FMCC B-129; *Listeria monocytogenes* 23UD, kindly provided by Prof. L. Cocolin (Cocolin et al. 2005); *Listeria monocytogenes* H7550, kindly provided by Prof. S. Kathariou; *Salmonella enterica* subsp. *enterica* serovar Enteritidis P167807; *Salmonella enterica* subsp. *enterica* serovar Typhimurium 4/74 (Hoiseth and Stocker, 1981); *Salmonella enterica* FMCC B-64; *Salmonella enteritidis* ATCC 13076; *Escherichia coli* FMCC B-13; *Escherichia coli* NCTC 13127; *Escherichia coli* ATCC 35150 and *Escherichia coli* ATCC 25922.The tests for haemolytic and antimicrobial activity were performed in duplicate.

2.2.3.3.Screening for AI-2 activity

For this assay, two strains of *Vibrio harveyi* were used: *V. harveyi* BAA-1117 (ATCC BB-170) as biosensor and *V. harveyi* BAA-1119 (ATCC BB-152) as positive control. *Vibrio* strains were cultured in Autoinducer Bioassay (AB) broth (Lu, Hume and Pillai, 2004), and incubated with agitation (160 rpm) at 30 °C for 24 h. The cultures used directly in the bioassay, were prepared by transferring a single colony from AB agar in 10 mL of AB broth, and incubating with agitation (160 rpm) at 30 °C for 16 h. For the growth of the LAB strains, quarter-strength Brain Heart Infusion (BHI) broth (LabM Limited, Lancashire, UK) was used as previously reported (Blana, Doulgeraki and Nychas, 2011). The AI-2 activity bioassay was performed as described previously (Surette and Bassler, 1998). In this study, 10 μL of sterile growth medium was used as a negative control, whereas 10 μL of the cell-free supernatant (CFS) from *V. harveyi* BA-1119 strain, was used as a positive control to verify the bioassay. The microplates were incubated at 30 °C and luminescence was measured every 15 min using a Synergy HT multi-mode microplate reader (Biotek, Winooski, VT, USA). Measurements were collected until the negative control exhibited an increase in luminescence. AI-2 activity was expressed as relative AI-2 activity, which was calculated as the ratio of luminescence of the test sample (CFS_{LAB}) to that of the control (negative) sample. The bioassay was performed in triplicate with four technical replicates each.

2.2.3.4.Detection of extracellular GABA

Single colonies from each strain, previously grown on MRS agar, were obtained and inoculated in MRS broth, followed by incubation anaerobically at 37 °C overnight. These cultures were used as the inoculum 1% (v/v) to prepare the cultures used for the GABA determination in MRS broth supplemented with 10mM L-glutamic acid (Sigma-Aldrich, Poole,

UK) and were incubated under the same conditions. The population of each strain together with the pH values with the presence of L-glutamic acid after 48 h incubation were recorded, whereas the supernatant was collected from each culture with centrifugation (13000 rpm for 10 min). Then, 10 μL of the supernatant were incubated with 90 μL of the assay mixture in each well of a 96-well microtiter plate, as described previously (Tsukatani, Higuchi and Matsumoto, 2005; Karatzas et al. 2010). Additionally, standard solutions containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM GABA were added to 90 μL of the assay mixture on the same microtiter plate, in order to obtain a standard curve for GABA determination. The microtiter plate was incubated at 37 °C for 180 min using a Sunrise Spectrophotometer (Tecan, Männedorf, Switzerland), and the optical density (OD) at 340 nm was measured every 120 s. The concentration of GABA in the supernatant was calculated using the calibration curves generated by the standard solutions. The test for GABA detection was performed in triplicate.

2.2.3.5.Statistical analysis

The statistical analysis was performed using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA). Regarding the resistance to low pH and resistance to bile salts, analysis of variance (ANOVA) for final bacterial counts of each strain (per hour and in total) was performed and means were separated with Tukey's HSD test. The Tukey post hoc test was also used to compare the means of GABA concentration and AI-2 activity, expressed as the ratio of luminescence of the test sample to the control sample. All differences were reported at a significance level of 0.05.

2.2.4. Results and Discussion

2.2.4.1.Low pH Assay, Bile Salts Assay, and BSH Activity

Out of the 33 LAB strains, 18 exhibited high population counts after exposure to pH of 2.5 for a total of 3 h. Amongst these, eight strains (5 *Lb. pentosus* and 3 *Lb. plantarum*) showed the highest population ≥7 log CFU/mL under the acidic conditions tested (Figure 1). Statistically significant differences in the bacterial counts were detected after 1 h in low pH ($p<0.05$). Results from low pH resistance were in agreement with other studies, where *Lb. pentosus* and *Lb. plantarum* strains were able to maintain their viability after exposure to low pH; however a variation in the acid resistance among different strains was also observed (Maragkoudakis et al. 2006; Pavli et al. 2016; Argyri et al. 2013). Regarding the resistance to bile salts, out of the 18 strains, only one (*Pediococcus ethanolidurans* B389) showed final population <6 log CFU/mL after exposure to bile salts for 4 h. A total of 16 out of 17 strains exhibited a very low log reduction <1, whereas one strain, *Lb. pentosus* B362, showed a slightly higher log reduction (1.14 log CFU/mL; Figure 2). The resistance to bile salts is a prerequisite for probiotic characterization, and guarantees that the cells could reach the intestinal tract alive (Taranto, Perez-Martinez and Font de Valdez, 2006). Although, generally *lactobacillus* species are able to tolerate bile concentrations normally encountered in the host (0.1-0.5%), great variability in bile resistance has been reported at genus and species level (Jacobsen et al. 1999; Papadimitriou et al. 2015). These observations confirm the hypothesis that bile resistance is a strain-dependent characteristic (Begley, Gahan and Hill, 2005). It has to be noted that bacterial resistance to low pH and bile salts *in vitro* is not necessarily similar to the *in vivo* behaviour (Morelli, 2007).

Table 1. List of lactic acid bacteria (LAB) strains used in the study and their final viable counts after exposure to pH 2.5 for 3 h.

Species	Strain	Final Counts (log CFU/ml)
Lactobacillus plantarum	B355	<1
Lactobacillus plantarum	B359	2.57±1.14
Lactobacillus plantarum	B372	6.05±0.41
Lactobacillus plantarum	B373	7.31±0.63
Lactobacillus plantarum	B374	4.15±0.15
Lactobacillus plantarum	B375	7.00±0.83
Lactobacillus plantarum	B380	7.68±0.16
Lactobacillus plantarum	B384	6.62±0.43
Lactobacillus pentosus	B356	6.69±1.08
Lactobacillus pentosus	B357	<1
Lactobacillus pentosus	B360	1.79±1.18
Lactobacillus pentosus	B361	3.96±0.73
Lactobacillus pentosus	B362	7.08±1.29
Lactobacillus pentosus	B363	6.81±1.07
Lactobacillus pentosus	B364	6.34±1.32
Lactobacillus pentosus	B366	6.78±0.22
Lactobacillus pentosus	B368	7.01±1.01
Lactobacillus pentosus	B369	5.48±0.07
Lactobacillus pentosus	B370	7.61±0.14
Lactobacillus pentosus	B371	6.26±0.52
Lactobacillus pentosus	B377	7.85±0.11
Lactobacillus pentosus	B378	6.92±0.83
Lactobacillus pentosus	B383	4.61±0.31
Lactobacillus pentosus	B385	7.24±0.73
Lactobacillus pentosus	B399	6.48±0.01
Lactobacillus pentosus	B400	2.07±0.32
Lactobacillus pentosus	B401	<1
Lactobacillus pentosus	B402	<1
Lactobacillus paraplantarum	B365	3.22±1.56
Pediococcus ethanolidurans	B389	6.46±0.25
Pediococcus ethanolidurans	B397	<1
Lactobacillus coryniformis	B395	2.57±0.11
Lactobacillus coryniformis	B403	<1

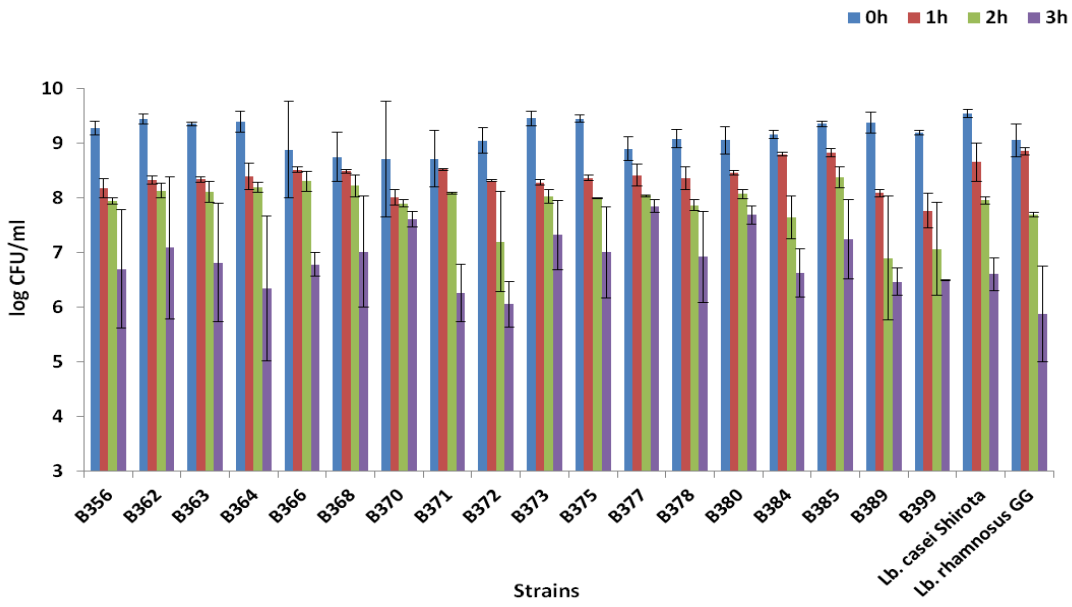

Figure 1. Resistance to low pH after 0, 1, 2 and 3 h of the selected strains *Lb. pentosus* B356, B362, B363, B364, B366, B368, B370, B371, B377, B378, B385, B399, *Lb. plantarum* B372, B373, B375, B380, B384, *Pediococcus ethanolidurans* B389 and the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG.

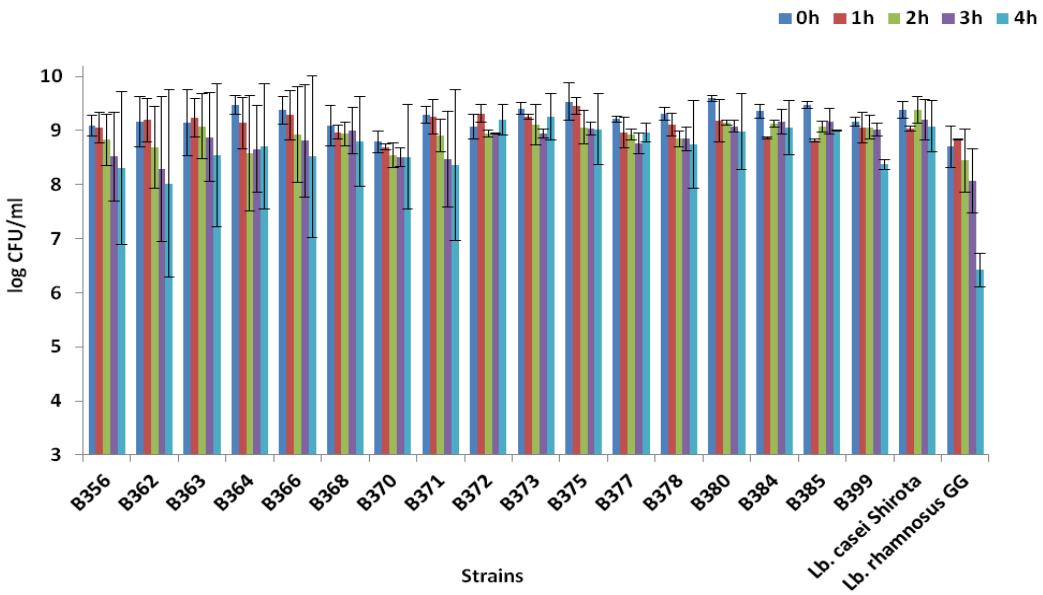

Figure 2. Resistance to bile salts after 0, 1, 2, 3, and 4 h of the selected strains *Lb. pentosus* B356, B362, B363, B364, B366, B368, B370, B371, B377, B378, B385, B399; *Lb. plantarum* B372, B373, B375, B380, B384 and the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG.

BSH was examined on those strains that exhibited satisfactory performance in the low pH and bile resistance tests. A total of 15 out of 17 strains, exhibited partial BSH activity, expressed as differentiated colony morphology recorded on TDCA-MRS agar plates compared to the control MRS agar plates. BSH activity might be a beneficial attribute for a potentially probiotic strain and is often associated with the resistance to bile salts due to the reduction in serum cholesterol or cholesterol solubility and absorption (Begley, Hill and Gahan, 2006). However, further studies are needed to confirm that other risks for the host are kept to a minimum, when excessive amount of probiotic bacteria is consumed. Furthermore, apart from live cells, dead or

non-growing lactobacilli cells have been reported to have the ability to reduce cholesterol from media (Liong and Shah, 2005).

2.2.4.2.Safety Assessment of the selected strains

Haemolytic activity is one of the screening tests performed for probiotic characterization. Absence of haemolytic activity is considered as a safety requirement for the selection of probiotic strains. In the present study, none of the 17 strains, previously selected for their good performance in low pH and bile assays, exhibited α- or β-haemolysis, whereas all of them exhibited γ-haemolysis (no haemolysis) (Table 2). The findings are similar to those of previous studies regarding the safety of the LAB (Maragkoudakis et al. 2006; Argyri et al. 2013; Zoumpopoulou et al. 2008; Ruiz-Moyano et al. 2009; Tejero-Sariňena et al. 2012), although some exceptions do exist.

The antimicrobial activity against pathogens is deemed as a desirable trait, although not obligatory for probiotic characterization. From the 17 selected LAB strains, none of them were found to inhibit the growth of the 12 pathogens tested, according to the well-diffusion method. These results are in agreement with previous studies (Maragkoudakis et al. 2006; Argyri et al. 2013; Rubio et al. 2014), where no antimicrobial activity was reported. On the contrary, significant antimicrobial activity of the LAB against two strains of *Listeria monocytogenes* and low or moderate antimicrobial activity against *Bacillus cereus* was reported in another study (Ruiz-Moyano et al. 2009).

Table 2. Selected strains with probiotic potential according to *in vitro* tests.

Strains	Test				
	Low pH (SR%)[A]	Bile Salts (SR%)[B]	Bile Salts Hydrolase[C]	Haemolytic Activity	Antimicrobial Resistance
Lb. pentosus B356	72.17	91.38	1	γ	-
Lb. pentosus B362	75.05	87.47	1	γ	-
Lb. pentosus B363	72.84	93.42	1	γ	-
Lb. pentosus B364	67.55	91.88	1	γ	-
Lb. pentosus B366	76.38	90.83	1	γ	-
Lb. pentosus B368	80.16	96.78	1	γ	-
Lb. pentosus B370	87.38	96.83	1	γ	-
Lb. pentosus B371	71.86	90.04	1	γ	-
Lb. plantarum B372	66.93	101.29	1	γ	-
Lb. plantarum B373	77.40	98.46	1	γ	-
Lb. plantarum B375	74.12	94.60	1	γ	-
Lb. pentosus B377	88.19	97.26	1	γ	-
Lb. pentosus B378	76.20	93.88	0	γ	-
Lb. plantarum B380	84.88	93.53	1	γ	-
Lb. plantarum B384	72.35	96.76	1	γ	-
Lb. pentosus B385	77.41	95.03	1	γ	-
Lb. pentosus B399	70.56	91.32	0	γ	-

[A] Survival rate after 3 h in low pH.

[B] Survival rate after 4 h in bile salts.

[C] 0: no hydrolysis; 1: partial hydrolysis.

2.2.4.3.AI-2 activity

The AI-2 activity for the tested strains exhibited values ranging from 0.32- to 1.57-fold compared to the negative control, whereas the bacterial counts were estimated from 6.42 to 8.30

log CFU/mL after 20 h of incubation time in ¼ strength BHI (Table 3). According to the results obtained, the selected strains exhibiting probiotic potential did not show detectable AI-2 activity under these growth conditions. Generally, diverse results have been reported with regard to the ability of LAB to produce AI-2 molecules (Moslehi-Jenabian, Gori and Jespersen, 2009; Blana, Doulgeraki and Nychas, 2011; Park et al. 2016). The detection of AI-2 molecules has been proven to be growth-medium dependent (Surette and Bassler, 1999; De Keersmaecker and Vanderleyden, 2003). The *lux*S gene, responsible for AI-2 production, is subject to catabolic repression by glucose, thus AI-2 molecules are difficult to be detected in a growth medium containing glucose (Ammor, Michaelidis and Nychas, 2008). De Keersmaecher & Vanderleyden (2003) suggested that a final concentration of 2 mM of glucose present in the CFS, could cause an inhibition to the light production in the bioassay, whereas other sugars such as galactose did not. Furthermore, the acidic conditions present in CFS could also have an impact on the bioassay, as reported in the previous study (De Keersmaecher & Vanderleyden, 2003). Another critical factor in the detection of AI-2 molecules is the growth stage. The best point in growth to detect AI-2 molecules in LAB is during the late exponential phase and/or the stationary phase, as the molecules are still considered to be present. This information was taken into consideration in the current study, and the supernatant was collected after 20 h of incubation at 30 °C, whereas the bacterial population was confirmed with plate counting.

Many potentially probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* having a *lux*S homologue can produce AI-2 molecules. *Lb. rhamnosus* GG has been thoroughly studied regarding its ability to produce AI-2 and regulate its physiology (Lebeer et al. 2007), suggesting that the *lux*S gene has a central metabolic role in this strain. In a previous study (Moslehi-Jenabian, Gori and Jespersen, 2009), *Lb. rhamnosus* GG and *Lb. salivarius* UCC118 were found

to produce AI-2 signal molecules under standard growth conditions, reaching the maximum concentration at the late exponential and stationary phase, respectively. In the same study, the AI-2 activity after an acidic shock with pH of 3 and 4, showed an increase in *Lb. acidophilus* NCFM and *Lb. rhamnosus* GG. Such an observation supported the hypothesis that the LuxS- mediated quorum sensing via AI-2 activity possibly plays an important role in the stress tolerance response of *Lactobacillus* species. Park et al. (2016), reported various intensities of AI-2 activity in fermented kimchi products and also from the LAB obtained from these products. Strains of *Lb. plantarum*, *Lb. brevis*, *Lb. fermentum* and *Lb. garlicum* exhibited significant AI-2 activity, which was considered by the authors as an interesting characteristic for the future of fermented foods (Park et al. 2016). Although QS is generally reported in LAB participating in food fermentations (Johansen and Jespersen, 2017), it is possible that the most dominant QS system is that of autoinducing peptides (AIP), especially for the case of fermented vegetables such as table olives (Ruiz-Barba et al. 2010; Caballero-Guerrero et al. 2013; Rizzello et al. 2014; Doulgeraki et al. 2013).

2.2.4.4.Detection of extracellular GABA

Screening of LAB for their ability to produce GABA is important for the food industry, as GABA-producing strains could be utilised as starters or adjunct cultures in fermented foods, developing GABA-enriched functional products. GABA is synthesized and exported by the glutamic acid decarboxylase (GAD) system, which is a very potent acid resistance mechanism. The GAD enzyme catalyzes the proton-consuming decarboxylation of L-glutamate to GABA, which subsequently is exported by a glutamate/GABA antiporter that also imports another glutamate molecule to initiate another cycle of glutamate decarboxylation (Karatzas et al. 2010). Furthermore, the GAD system can decarboxylate intracellular L-glutamate pools to produce

intracellular GABA which can be metabolised to succinate through the GABA shunt (Feehily, O'Byrne and Karatzas, 2013). Several studies have indicated the presence of GAD system in lactic acid bacteria (Siragusa et al. 2007). Generally, GABA synthesis in bacteria is related to enhanced resistance under acidic conditions. In the present study, 17 strains of LAB, with good probiotic attributes, were screened for their ability to produce GABA *in vitro*. Extracellular GABA was not detected in any of the strains tested (concentrations between 0.16-0.66 mM/mL), under the growth conditions tested, as presented in Table 3.

Table 3. Relative autoinducer-2 (AI-2) activity and GABA values of the selected LAB exhibiting probiotic potential.

Strains	**AI-2 activity**		**GABA determination**		
	Bacterial Counts[A]	**Relative AI-2 activity[B]**	**Bacterial Counts[C]**	**pH[D]**	**GABA[E]**
Lb. pentosus B356	7.57±0.15	0.68±0.22[a]	9.52±0.17	3.92±0.00	0.30±0.05[a]
Lb. pentosus B362	7.55±0.06	0.57±0.15[a]	9.40±0.17	3.91±0.00	0.16±0.03[a]
Lb. pentosus B363	7.13±0.22	0.70±0.27[a]	9.52±0.17	4.09±0.01	0.52±0.49[a]
Lb. pentosus B364	7.26±0.08	0.58±0.28[a]	9.44±0.17	4.00±0.04	0.40±0.11[a]
Lb. pentosus B366	7.47±0.09	0.48±0.11[a]	9.66±0.42	3.92±0.00	0.30±0.07[a]
Lb. pentosus B368	7.51±0.37	0.50±0.16[a]	9.32±0.06	4.02±0.03	0.27±0.06[a]
Lb. pentosus B370	8.30±0.24	0.56±0.17[a]	9.49±0.15	4.00±0.04	0.39±0.06[a]
Lb. pentosus B371	7.73±0.15	0.58±0.24[a]	9.48±0.19	3.93±0.00	0.46±0.08[a]
Lb. plantarum B372	7.28±0.14	1.39±0.45[b]	9.51±0.07	4.05±0.03	0.66±0.05[a]
Lb. plantarum B373	8.11±0.52	1.28±0.33[b]	9.31±0.01	3.95±0.03	0.56±0.51[a]
Lb. plantarum B375	8.15±0.22	1.34±0.68[b]	9.60±0.43	3.93±0.00	0.53±0.13[a]
Lb. pentosus B377	6.98±0.13	0.53±0.27[a]	9.28±0.24	3.99±0.06	0.30±0.07[a]
Lb. pentosus B378	7.18±0.50	0.32±0.12[a]	9.41±0.42	3.92±0.00	0.14±0.10[a]
Lb. plantarum B380	7.34±0.01	1.57±1.16[b]	9.29±0.16	3.92±0.00	0.40±0.05[a]
Lb. plantarum B384	6.42±0.16	0.38±0.14[a]	9.27±0.27	4.06±0.01	0.42±0.17[a]
Lb. pentosus B385	7.15±0.22	0.54±0.15[a]	9.22±0.32	3.95±0.04	0.34±0.21[a]
Lb. pentosus B399	6.94±0.15	0.48±0.19[a]	9.49±0.02	3.95±0.03	0.23±0.02[a]

[A] Bacterial counts (log CFU/mL) after 20 h in ¼ strength brain heart infusion (BHI) at 30 °C are presented as mean ± standard deviation.

[B] Relative AI-2 activity was calculated as the ratio of the luminescence of the test sample (CFS_{LAB}) to that of the control (negative) and is presented as mean ± standard deviation. Values with different letters are significantly different ($p<0.05$).

[C]Bacterial counts (log CFU/mL) after 48 h in MRS broth supplemented with L-glutamic acid at 37 °C are presented as mean ± standard deviation.

[D] pH after 48 h in MRS broth supplemented with L-glutamic acid at 37 °C is presented as mean ± standard deviation.

[E]γ-Aminobutyric acid (GABA) values (mM) are presented as mean ± standard deviation. Values with different letters are significantly different ($p<0.05$).

Measurements of extracellular GABA ($GABA_e$) as means of quantification of the GAD system activity could potentially indicate the acid resistance of a specific microorganism. However, the quantification of the intracellular GABA ($GABA_i$) is also important for the investigation of the GAD system (O'Byrne et al. 2011). It has to be noted that although the GAD system is widely distributed in LAB, the ability of LAB to produce GABA varies significantly (Komatsuzaki et al. 2005). Similar conclusions were made previously, for GABA production in *Listeria monocytogenes*, where different strains export GABA in different media and environmental conditions, suggesting that diverse activation signals present in different niches might activate the GAD system in different strains (Karatzas, Suur and O'Byrne, 2012).

Several factors are considered to have a detrimental effect on GABA synthesis *in vitro*, such as the incubation temperature, the incubation time, and the glutamate concentrations (Komatsuzaki et al. 2005; Villegas et al. 2016). The optimum temperature range for GABA synthesis is 30-37 °C, whereas at 45 °C or more, GABA is not detected, possibly due to

difficulties in bacterial growth. For the detection of the highest GABA concentrations, the optimum incubation time is 48 h, whereas extra incubation time does not result in an increase in GABA values (Villegas et al. 2016). In our study, the bacterial counts of the examined strains, clearly showed that the presence of L-glutamic acid did not affect their growth (9.22-9.66 log CFU/mL) (Table 3).

In a study of Yunes et al. (Yunes et al. 2016), 135 human-derived strains of lactobacilli and bifidobacteria were isolated and screened for their potential to synthesize GABA. A total of 43% of the isolates were determined as GABA-producers, with the strains assigned to *Lb. plantarum*, *Lb. brevis*, *B. adolescentis*, *B. angulatum*, and *B. dentium.* On the contrary, Barrett et al. (2012), reported that from the 91 human-derived lactobacilli and bifidobacteria, only 4% were GABA-producers, including *Lb. brevis*, *B. dentium*, *B. infantis*, *B. adolescentis*, with *Lb. brevis* being the one with the highest conversion ability of monosodium glutamate to GABA. Furthermore, isolates from various foods such as Italian cheeses (Siragusa et al. 2007), selected dairy products (Valenzuela et al. 2019), artisanal Zlatar Cheese (Sokovic Bajic et al. 2019) or Nostrano cheeses made from raw alpine milk (Franciosi et al. 2015) were positive in GABA production by 13.86%, 50%, 28% or 70% respectively. The work on the Italian cheeses has shown that gorgonzola and pecorino harboured a high number of GABA-producing LAB. In addition, the type of milk used for cheese manufacturing, together with the ripening period, had an impact on the GABA concentrations.

Interestingly, in our work we found no isolate producing any GABA. This is in contrast with the situation in isolates from human colon and dairy products. This might be related to the fact that LAB strains isolated from dairy products might have a higher potential in GABA production compared to strains isolated from non-dairy products (Shan et al. 2015). Cheese

possesses specific characteristics that favor the natural presence of GABA-producing LAB. The high content of L-glutamate (17.5% of the total amino acid content) in milk caseins is metabolized from LAB during the ripening process (Renes et al. 2017). In addition to strain variability, GABA production is affected by many other factors such as the temperature, the pH, the medium composition, and other environmental factors (Siragusa et al. 2007; Zhuang et al. 2018; Mazzolli and Pessione, 2016). Work by Villegas et al. (2016), has shown that 3 out of 19 strains (15%) originated from amaranth and quinoa showed GABA producing ability that was low but still significantly higher than the absence of GABA-producers found in this study. It is well-known that the amount of GABA available in fruits and vegetables is relatively low compared to other sources such as dairy and meat products (Villegas et al. 2016; Kim et al. 2009). In a study of Karatzas et al. (2010), it was reported that *L. monocytogenes*, although producing GABA in rich media, is unable to export GABA in a defined medium supplemented with L-glutamate. This suggests that additional factors present in a nutrient-rich environment play an important role in the function of the GAD system.

2.2.5. Conclusions

A total of 17 strains were chosen as good candidates for potentially probiotic applications, as adjunct or co-starter cultures, although more tests are required to further assess their potential as probiotics. AI-2 signal molecules and extracellular GABA were not detected under the *in vitro* growth conditions tested; however, further research is needed to better understand the systems involved and the mechanisms triggering their production in LAB. The lack of GABA-producing strains isolated from natural black olives seems unique among various fermented foods, although the functionality of the GAD system or the genes involved have not yet been studied.

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CHAPTER 3

Effect of different stress factors on the expression of the *lux*S gene and the production of Autoinducer-2 signal molecules of potentially probiotic *Lactobacillus* species

3.1. Abstract

Thirty-four *Lb. plantarum* and *Lb. pentosus* strains, all isolated from fermented food products, were initially studied for the presence of *lux*S gene. From the results obtained, 4 strains -2 *Lb. plantarum* and 2 *Lb. pentosus*- were selected for further analysis on the production of Autoinducer-2 (AI-2) signal-molecules, using the AI-2 activity bioassay and *lux*S gene expression by Real-Time PCR under selected stress factors. The stress factors applied to the strains included different pH, with values 2, 3, 4 and 6.5 (control), temperature with values 10, 50 and 37 °C (control), NaCl with concentrations of 0.2, 0.4M and 0M (control) and bile salt concentrations of 2 % w/v and 0 % (control). With regard to the AI-2 activity, higher values were estimated for the pH 2, followed by pH 4 and pH 3, for all the strains, whereas in the rest of the treatments the estimated AI-2 activity was low. The housekeeping gene *fus*A_2 was used as internal control for the Real Time-PCR and from the results obtained no significant influence of the stress factors on the *lux*S gene expression was revealed, however slight differences were observed amongst the different LAB strains under certain stresses.

3.2. Introduction

The probiotic potential of LAB has been thoroughly investigated during the last years. It has been documented that the probiotic properties of different bacterial isolates vary between the same species and different strains of the same species might exhibit different behavior amongst stresses. A requirement for an efficient probiotic delivery is the tolerance to the conditions typically encountered in the human GIT. The probiotic survival under stress depends on the strains' ability to sense and respond to the challenging conditions by regulating the expression of certain stress-response genes.

Bacteria frequently communicate via quorum-sensing mechanisms, based on the recognition of a signal-molecule called autoinducer (AI), resulting in changes in the gene expression. One of the quorum-sensing signals, which is responsible for the communication among and between species, is called AI-2, with the LuxS enzyme catalyzing AI-2 synthesis to be present in many Gram-positive and Gram-negative species, including species of the genus *Lactobacillus*. Plenty of data are available in the literature regarding the role of AI-2 in bacterial virulence, motility, light production, adhesion to CaCO-2, biofilm formation and increased resistance to stresses (Xavier and Bassler, 2003; Lebeer et al. 2007). Potentially probiotic bacteria such as *Lactobacillus*, possess a *lux*S homologue and have the ability to produce AI-2 (Sun et al. 2014, De Keersmaecker, 2003).

Moslehi-Jenabian et al. (2009), reported increased AI-2 activity in *Lactobacillus* strains when exposed to acidic shock, while in another study of Lebeer et al. (2008), a relation was observed between AI-2 signals and gastric stress tolerance and colonization in *Lb. rhamnosus* GG. Furthermore, the *lux*S gene in *Lb. rhamnosus* is considered to play an important role on bacterial growth, biofilm formation and in the central metabolism, although other factors such as the growth medium might have an effect (Lebeer, 2007, 2008). Although *lux*S in some *Lactobacillus* species is related to acid resistance, there is lack of information regarding the relation of *lux*S to other stresses encountered in the GIT or other niches.

The aim of the present study was to investigate the transcription of the *lux*S gene of 4 potentially probiotic strains after their exposure to certain stress conditions using quantitative RT-PCR. Additionally, the AI-2 activity was also examined after exposure to the same stress conditions.

3.3. Materials and Methods

3.3.1. Bacterial strains and growth conditions

Thirty-four *Lb. plantarum* and *Lb. pentosus* strains, previously isolated and characterized by Doulgeraki et al. 2012 and Pavli et al. 2016, were used for the detection of the *lux*S gene. Pure cultures were stored in MRS broth (Biolife, Italy) supplemented with 20% (v/v) glycerol. Before use, each isolate was subcultured twice in MRS broth at 30 °C for 24 and 18 h respectively. For the rest of the experiments, strains were grown in modified MRS medium supplemented with MES buffer, according to Moslehi-Jenabian (2009), in which glucose was replaced by galactose (De Keersmaecker et al. 2003).

The *Vibrio harveyi* BAA-117 biosensor strain and the AI-2 producing *V. harveyi* BAA-1119 strain were used for the AI-2 activity bioassay. The strains were revived from the frozen stock in 10 mL autoinducer (AB) broth (Lu et al. 2004) and incubated with agitation (160 rpm) at 30 °C for 24 h, while working cultures were stored refrigerated into AB agar for a maximum of 2 weeks. Cells from a single colony, were then transferred into 10 mL AB broth and incubated for 16 h under the same conditions.

3.3.2. Detection of *lux*S gene

DNA was extracted according to Doulgeraki et al. (2011) and was then subjected to PCR analysis with DNA primers as listed by Lin et al. (2015). Reactions were carried out in a final volume of 25 μL containing: 10 mM Buffer (5x), 25 mM $MgCl_2$, 10 mM dNTP's, 10 μM primer LuxS F and 10 μM of LuxS R and 5U μL^{-1} Taq polymerase. PCR reactions consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 30 s and primer extension at 72 °C for 1 min and concluded by a

final extension step at 72 °C for 5 min. Amplicons were then separated in a 2% (w/v) agarose gel in TAE 1x at 80 V for 2 h. After the run, gels were stained with ethidium bromide 0.5 μL mL^{-1} for 30 min, before being photographed with GelDoc system.

3.3.3. Transcription of the *lux*S gene after exposure to stresses

Samples of cells exposed for 1 h to each stress factor as well as control cells were collected with centrifugation. RNA Stabilization Reagent (Qiagen) was added to eliminate RNA degradation and the samples were kept at -20°C until use. RNA extraction was performed with PureLink RNA Kit (Ambion) according to the manufacturer's instructions and the samples were kept at -80 °C until further use.

Primescript Reverse Transcriptase (Takara) was used for the cDNA synthesis, according to the protocol described by the manufacturer in a final volume of 20 μL. The cDNA was stored at -20 °C until further use. cDNA synthesis was performed with the following conditions: Reaction mixture at 65 °C for 5 min, 30 °C for 10 min (incubation), 42 °C for 60 min (reverse transcription) and 95 °C for 5 min (enzyme inactivation). Real-Time PCR assays were performed in 96-well plates in a StepOnePlus detection system (Applied Biosystems). The SYBR Green chemistry was used to monitor cDNA amplification. The housekeeping gene *fus*A_2 was used as a reference gene (internal control). Each well contained SYBR green Master Mix (KapaBiosystems), 200 nM of each primer and 1μL template. PCR amplification was initiated at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Template-free negative controls were included to detect background contamination. Data analysis was undertaken by StepOnePlus RT-PCR System Software v2.1.

3.3.4. AI-2 activity under standard growth conditions

The four selected strains were propagated twice in modified MRS broth supplemented with 0.1MES buffer (pH 6.5) at 37 °C for 24 and 18 h, respectively. Supernatants from the late exponential and stationary phase (4, 8, 12, 16, 18, 20, 22, 24) were examined for AI-2 activity. Bacterial cells were removed from the growth medium by centrifugation at 5000 x g for 15 min at 4 °C, pH was adjusted to 6.5 with NaOH followed by filtration through 0.2μm-pore-size filters (Whatman, USA). The supernatant was then kept refrigerated for a maximum of 48 h until AI-2 bioassay was conducted.

3.3.5. Effect of stress factors on bacterial population and AI-2 activity

The effect of several stresses on the bacterial population and AI-2 activity was examined. For that reason, the bacterial cultures were inoculated into pre-warmed (37 °C) modified MRS broth (pH 6.5) supplemented with 0.1 MES buffer and incubated at 37 °C for 18 h. Cells were then harvested by centrifugation (5000 x g for 5 min at 4 °C), washed twice with PBS (pH 7.2) and finally re-suspended and incubated with each stress condition for 1 h. Each stress condition was as follows: pH stress (pH 2.0, pH 3.0, pH 4.0 and pH 6.5 as control); osmotic stress (0.2 M, 0.4 M and without NaCl as control), bile salt stress (2 % w/v and 0% as control) and temperature stress (10 °C, 50 °C and 37 °C as control). After 1 h incubation, the cells were harvested by centrifugation, the supernatants were collected, adjusted to a pH of 6.5 with NaOH and filter sterilized as previously described. The supernatants were kept refrigerated for a maximum of 48 h until the AI-2 bioassay was performed. For each of the treatments the bacterial population was determined both before and after the exposure stress, whilst the pH of the medium was also recorded.

3.3.6. AI-2 Bioassay

The AI-2 activity bioassay was performed as described by Surette and Bassler (1998). An overnight culture of *V. harveyi* BAA-1117 was diluted 1:5000 with fresh AB medium. 90 μL of this cell suspension were mixed with 10 μL of the tested supernatant (samples from each strain and time point) in a 96-well polystyrene microplate (μ-Clear, Greiner Bio-One, Germany). 10 μL of sterile growth medium (modified MRS) were used as negative control. Supernatant of *V. harveyi* BAA-1119 was used as a positive control for the bioassays.

The microplates were incubated at 30 °C and luminescence was measured every 15 min using Synergy HT multi-mode microplate reader (Biotek, Winooski, USA) and AI-2 activity was expressed as "relative AI-2 activity", which was calculated as the ratio of luminescence of the tested sample to that of the control (negative) sample.

3.4. Results and Discussion

3.4.1. Presence of *lux*S

As a first step, the presence or absence of *lux*S gene was investigated in a total of 34 strains (13 *Lb. plantarum* and 21 *Lb. pentosus*), all isolated from fermented products. The primers used in this study, are given in Table 1. From the PCR results it was revealed that the *lux*S gene was present in 33 out of 34 strains. The *lux*S gene was absent from only one *Lb. pentosus* strain (Table 2), however, it was found that other LAB strains also do not possess this gene (strains of *Lb. sakei*, *Lb. paraplantarum* and *Lb. brevis*). Generally, the *lux*S gene has been detected in several Gram-positive and Gram-negative bacteria, although only a few have been reported to use an AI-2/LuxS QS system (Lin et al. 2015).

Table 1. DNA primers used in the study.

Primers for PCR and gene cloning	*lux*S Forward[a]	ATGGCTAAAGTAGAAAGTTTTAC
	*lux*S Reverse[a]	CTATTCAACGACTTTGCGTACAA
qPCR primers	*lux*S Forward[a]	CATTTGGTTGTCGGACTGGTT
	*lux*S Reverse[a]	TCCCTTGTACGTCTTCCCACTT
	fusA_2 Forward[b]	CCCATGATGGTGCTTCACAA
	fusA_2 Reverse[b]	TCGTGGCAGCAGAGGTAATG

[a] Lin et al. 2015

[b] Doulgeraki et al. 2013

Table 2. LAB strains, source of isolation and presence of *lux*S gene

Stain	Source of isolation	*lux*S gene
L. pentosus L33[a]	Fermented sausage from Lefkada region	+
L. pentosus L41[a]	Fermented sausage from Lefkada region	+
L. pentosus L45[a]	Fermented sausage from Lefkada region	-
L. pentosus L49[a]	Fermented sausage from Lefkada region	+
L. pentosus L83[a]	Fermented sausage from Lefkada region	+
L. pentosus B356[b]	Conservolea table olives	+
L. pentosus B362[b]	Conservolea table olives	+
L. pentosus B363[b]	Conservolea table olives	+
L. pentosus B364[b]	Conservolea table olives	+
L. pentosus B366[b]	Conservolea table olives	+
L. pentosus B368[b]	Conservolea table olives	+
L. pentosus B370[b]	Conservolea table olives	+
L. pentosus B371[b]	Conservolea table olives	+
L. pentosus B376[b]	Conservolea table olives	+
L. pentosus B377[b]	Conservolea table olives	+
L. pentosus B378[b]	Conservolea table olives	+
L. pentosus B381[b]	Conservolea table olives	+
L. pentosus B382[b]	Conservolea table olives	+
L. pentosus B385[b]	Conservolea table olives	+
L. pentosus B387[b]	Conservolea table olives	+
L. pentosus B399[b]	Conservolea table olives	+
L. plantarum B372[b]	Conservolea table olives	+
L. plantarum B373[b]	Conservolea table olives	+
L. plantarum B375[b]	Conservolea table olives	+
L. plantarum B380[b]	Conservolea table olives	+
L. plantarum B384[b]	Conservolea table olives	+
L. plantarum L32[a]	Fermented sausage from Lefkada region	+
L. plantarum T71[a]	Xerotyri cheese	+
L. plantarum T75[a]	Xerotyri cheese	+
L. plantarum L79[a]	Fermented sausage from Lefkada region	+
L. plantarum L119[a]	Fermented sausage from Lefkada region	+
L. plantarum L125[a]	Fermented sausage from Lefkada region	+
L. plantarum L132[a]	Fermented sausage from Lefkada region	+
L. plantarum T571[a]	Feta cheese	+

[a] Culture Collection of Institute of Technology of Agricultural Products, Hellenic Agricultural Organization-DEMETER

[b] Food Microbiology Culture Collection-FMCC, Agricultural University of Athens

3.4.2. AI-2 activity under standard growth conditions

Out of all the tested strains, 4 (2 *Lb. pentosus* and 2 *Lb. plantarum* strains) were selected to be further studied. AI-2 activity was monitored under standard growth conditions between 4 and 24 h (modified MRS broth-MES at 37 °C). As shown in Figure 1, all 4 strains exhibited similar relative AI-2 activities with the highest to be observed at 4 h (early exponential phase). It has to be noted that although the highest AI-2 activity was observed at the 4th hour of growth, still the results indicate that the activity was very low when compared to other studies (Moslehi-Jenabian, Gori and Jespersen, 2009; Yeo et al. 2015). These 4 LAB strains, due to their low AI-2 levels, were further selected for monitoring any AI-2 activity under stress conditions.

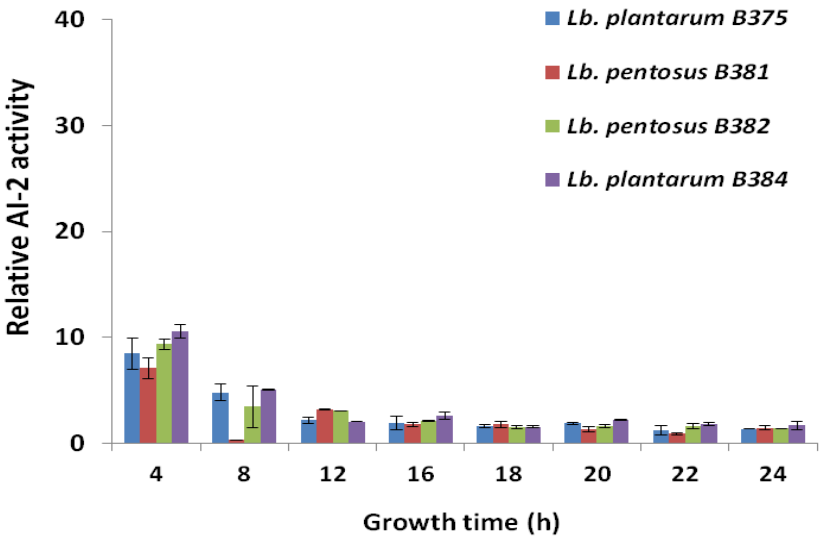


Figure 1. Relative AI-2 activity of LAB strains under standard growth conditions (modified MRS broth-MES, 37 °C) in 4, 8, 12, 16, 18, 20, 22 and 24h.

3.4.3. Effects of stress factors on bacterial population and AI-2 activity

The effect of each stress factor on the survival of each LAB strain is given in Table 3. All the strains were able to proliferate in the control (pH 6.5, 37 °C) and treatment with NaCl. Variable survival rates were estimated for 10 and 50 °C, while lower survival rates were observed in the treatments with low pH values. Regarding the relative AI-2 activity, all the 4 strains exhibited similar values for each treatment. The acidic shock affected the AI-2 activity, and higher values were estimated for the pH 2, while for pH 3 and 4 similar values were observed. The low temperature (10 °C) affected slightly the AI-2 activity in a similar way to 0.4M NaCl.

Table 3. Survival rates (%) of the strains due to the stress factor applied for 1h.

Strain	**Survival Rates %**							
	pH 6.5	pH 4	pH 3	pH 2	10 °C	50 °C	0.2M NaCl	0.4M NaCl
Lb. plantarum B375	101.40	95.30	79.56	51.11	98.31	101.64	95.33	104.12
Lb. pentosus B381	105.37	92.79	88.88	49.74	94.05	79.13	94.94	108.60
Lb. pentosus B382	104.01	93.38	85.81	46.76	98.53	90.78	103.23	104.85
Lb. plantarum B384	105.68	94.30	90.44	51.55	104.41	99.83	102.04	103.09

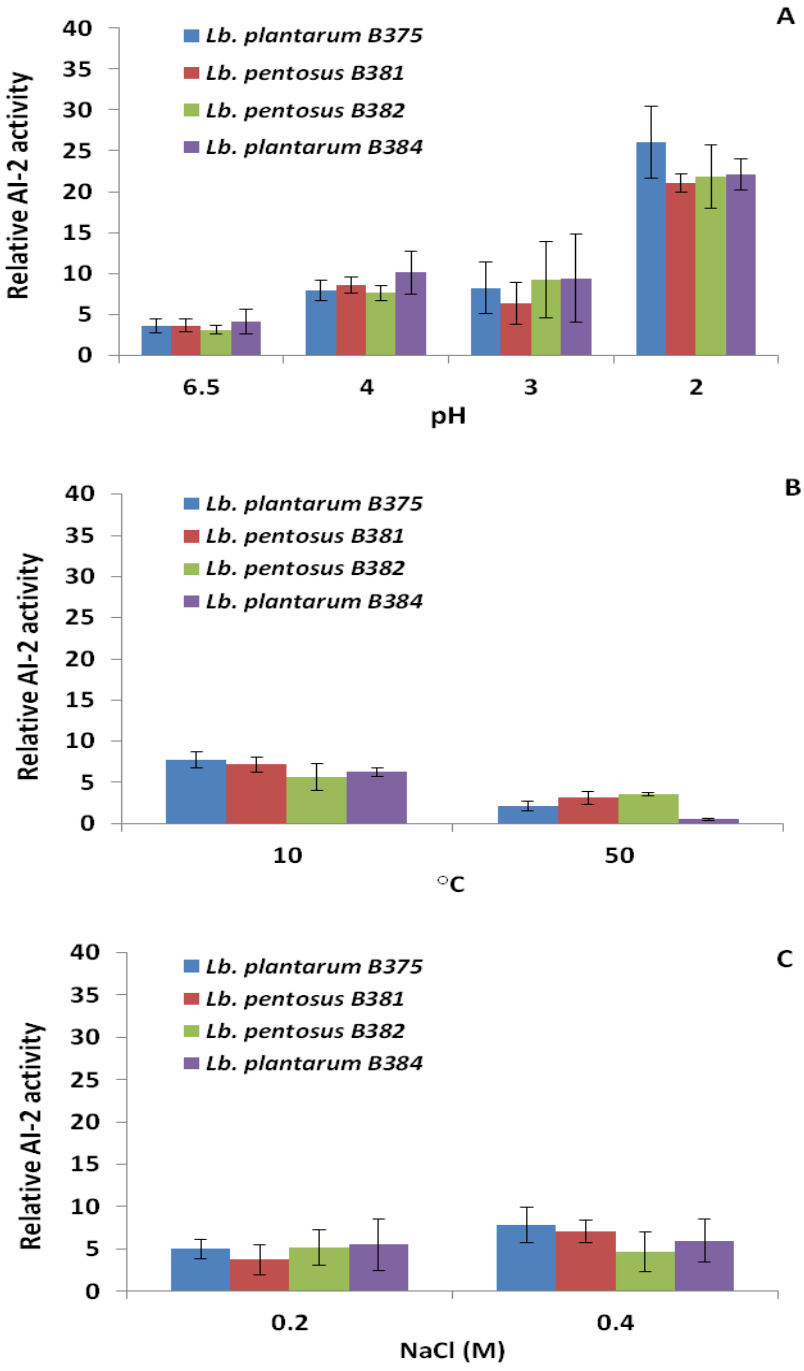


Figure 2. Relative AI-2 activity of LAB strains after treatment application for 1 h. (A) Relative AI-2 activity in pH of 2, 3, 4, 6.5 (control), (B) Relative AI-2 activity in 10 and 50 °C and (C) Relative AI-2 activity for 0.2 and 0.4 M NaCl.

3.4.4. Transcription of *lux*S after stress exposure

The relative expression of the *lux*S gene is given in Figure 3. In general, it was observed that the stress factors applied did not have a significant effect on the *lux*S expression. However, differences were noted among the strains and treatments, which support the hypothesis that the contribution of *lux*S in stress resistance is highly strain-dependent. Another important observation is that the gene transcription did not follow the same pattern as the relative AI-2 activity, indicating firstly, that the bioassay is not always suitable to extract solid conclusions, since it is affected by many parameters and secondly that the *lux*S transcription is more complicated and other genes might be playing an important role.

In a study of Lin et al. (2015), it was found that *lux*S gene was up-regulated in two strains of *Lb. plantarum* and a strain of *Lb. sakei*, after exposure to high nitrate concentrations. In another study of Moslehi-Jenabian, Gori and Jespersen (2009), *L. rhamnosus* GG and *L. acidophilus* NCFM were monitored for *lux*S transcription after acidic shock in pH of 3, 4, 5 and 6.5 (used as a control pH). It was found that acidic shock induced *lux*S expression in both bacteria. Furthermore, the findings of the study indicated that the time of the exposure is a critical factor when monitoring the gene expression. It was reported that the highest transcription levels were observed after 60 min of exposure, followed by medium levels after 90 and 30 min, while the minimum expression levels were observed after 15 min of exposure. Another critical point highlighted in the aforementioned study was the importance of the physiological state of the bacterial cells the time where the shock is applied. It was reported that acid-adapted cells responded differently to the stress compared to the non-adapted cells. These observations were taken into consideration in the present study.

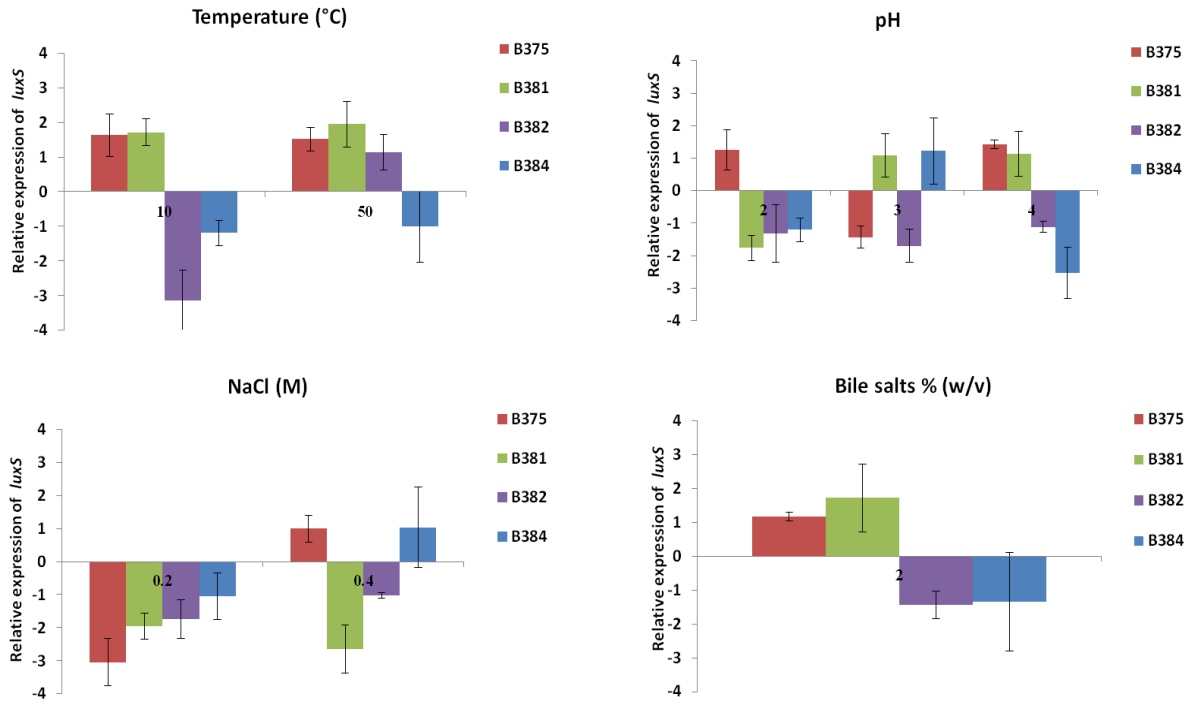


Figure 3. Relative expression of *lux*S gene for the different stress factors for the 4 LAB strains.

3.5. Conclusions

The results of the study, indicate that LuxS/AI-2 system might not be involved in the growth of the LAB strains under standard conditions. Furthermore, the transcription of the *lux*S gene under the stress conditions applied, showed differences among the strains and the stress factors, however, it was estimated at low levels. This observation possibly indicates that LuxS/AI-2 system, might not be taking any part in the resistance mechanisms of certain LAB, although further studies are required to better explain the role of this QS system.

Acknowledgements

I would like to cordially thank the Assistant Researcher Dr. Doulgeraki for the design and supervision in the molecular part of the study and Ms. Eleni Komita for the technical assistance.

CHAPTER 4

Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages

In this chapter, the aim was the manufacturing and production of dry-fermented sausages supplemented with a LAB strain that exhibited probiotic potential and was selected from the previous tests. This work has been included in the following publication:

***Pavli FG**, Argyri, A.A, Chorianopoulos N, Nychas G-J.E. and Tassou C. (2020). Effect of Lactobacillus plantarum L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. LWT-Food Science and Technology, 118, 108810.*

The supplementary material for this study is provided in the Appendix I.

4.1. Abstract

The performance of the *Lactobacillus plantarum* L125 strain with probiotic potential, previously isolated from a traditional Greek fermented sausage, was assessed as an adjunct culture for the production of dry-fermented pork sausages. The probiotic was added in the meat batter with the commercial starter cultures and the microbial populations were estimated during fermentation, ripening and storage at 4 and 12 °C, respectively. Physicochemical analyses (pH and a_w), and sensory evaluation were also performed, while the presence of L125 strain was monitored by PFGE. Lactic acid bacteria (LAB) counts at the inoculation time were 8.21 and 6.44 log CFU/g for the probiotic and control samples, respectively, while the L125 strain maintained high population levels (> 6 log CFU/g) during storage. At the end of the ripening, the products exhibited similar pH values of 4.46 and 4.42 and a_w values of 0.87 and 0.85 for the control and probiotic case, respectively. Overall, the *Lb. plantarum* L125 was found to possess desirable technological characteristics, indicating its effectiveness to be used in fermented sausage manufacturing.

4.2. Introduction

The production of dry-fermented sausages is characterized by three well-defined stages: mixing of ingredients, fermentation and ripening (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008). The most commonly used ingredients for the production of dry-fermented sausages include minced meat, fat, salts, curing agents and spices. When mixed these ingredients are stuffed into casings and the product is then fermented and ripened under controlled conditions (Ammor & Mayo, 2007; Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007). The characteristics of the final product depend on the ripening process occurring during drying and this in turn will define the slicing ability, color and flavor of the sausage. Lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) are the most popular starter cultures for the production of dry-fermented sausages. LAB are very important in the fermentation process due to the production of lactic and acetic acids through the fermentation of carbohydrates that enhances the microbial stability with the pH reduction (Drosinos et al., 2007; Urso, Comi, & Cocolin, 2006). CNC, are the main contributors to color stabilization, decomposition of peroxides, proteolysis and lipolysis, while certain species of staphylococci, such as *S. xylosus* and *S. carnosus*, give the desired aroma to the product through the conversion of amino acids and free-fatty acids. Moreover, CNC are weak competitors compared to LAB, and only a slight increase in their population occurs, during the ripening stage of the sausages (Leroy, Verluyten, & De Vuyst, 2006).

Fermented meats are important constituents of human diet due to their stability, convenience, special sensorial attributes and nutritional value (Leroy et al., 2018). The increased consumers' interest in functional foods, presents an excellent opportunity to the meat industry to ameliorate the quality and improve the image of meat products. Probiotic foods are a group of

health promoting functional products with their health benefits being based on the presence of selected strains of LAB, that confer a health benefit, when consumed in adequate amounts (FAO/WHO, 2002).

Due to the fact that fermented meat products are not heat-treated, they could serve as suitable matrix for assessing probiotic LAB as starter cultures (Rubio et al., 2013). Moreover, it is believed that the sausage matrix promotes the survival of probiotic bacteria during the passage through the gastrointestinal tract (Klingberg & Budde, 2006). Despite these, the intrinsic factors of the meat environment, such as low pH, water activity, curing salts and the competition of the starter cultures, have a potential negative impact on the cell viability of the probiotic strains, although this is deemed to be strain-specific (De Vuyst, Falony, & Leroy, 2008). It also generally accepted that different starter cultures affect differently the sensory characteristics of dry-fermented sausages (Erkkilä et al., 2001; Cavalheiro et al., 2015).

The aim of the present study was to assess the performance of a potentially probiotic strain, *Lb. plantarum* L125, that was previously isolated from a traditional Greek dry-fermented sausage (Pavli et al., 2016), as an adjunct culture for sausages fermentation. This was monitored by evaluating the strain's: (i) competitiveness during manufacturing and storage, (ii) effects on physicochemical and sensory quality characteristics of the final products, (iii) viability during storage conditions to adequate levels.

4.3. Materials and Methods

4.3.1. Bacterial cultures

The *Lb. plantarum* L125 strain was previously isolated from traditional Greek fermented sausages from Lefkada region (Pavli et al., 2016). The *in vitro* probiotic potential of this strain was assessed in the latter study regarding several probiotic traits such as resistance in simulated gastrointestinal conditions, hydrolytic and haemolytic activity, resistance to antibiotics and antimicrobial activity against pathogens. The pure culture was stored at −80 °C in MRS broth supplemented with 20% (v/v) glycerol. The bacterial strain was cultured twice in de Man, Rogosa and Sharpe (MRS) broth at 30 °C for 24 h and 18 h, respectively, before use. After incubation the cells were harvested by centrifugation at 10000 g for 10 min at 4 °C, washed twice with ¼ strength Ringer solution (composition: Sodium Chloride 2.25 g/L, Potassium Chloride 0.105 g/L, Calcium Chloride 0.12 g/L, Sodium Bicarbonate 0.05 g/L, LAB100Z, LabM, UK) and used directly for inoculation of the meat batter.

4.3.2. Preparation of sausages

Two types of sausages were manufactured according to traditional processing, one with the addition of the commercial starter cultures i.e. *Pediococcus pentosaceus* and *Staphylococcus carnosus* (BACTOFERM® BFL-F02, CHR. HANSEN, Nienburg, Germany) -control case- and another with the addition of commercial cultures plus the potentially probiotic strain *Lb. plantarum* L125 -probiotic case-. For each manufacture batch lean pork meat (42%), pork shoulder (38%) and pork fat (lard) (20%) of Greek origin were grounded in a meat mixer. The meat batter was then mixed with the following ingredients: garlic 5 g/Kg, collagen emulsion 2.5 g/Kg, spices 2 g/Kg, salt 20 g/Kg, dextrose 5 g/Kg and sodium nitrite 0.15 g/Kg, all provided

by Provil SA, Greece. The initial inoculum of the probiotic strain in the meat batter was approximately 8 log CFU/g. In brief, after the mixing of all the ingredients (according to the industrial recipe) including starter cultures, stuffing in synthetic cellulose casings was carried out, followed by fermentation (8 days) and ripening (14 days) according to the industrial procedures. After the ripening process the sausages had a diameter of approximately 4 cm and the weight varied from 160 to 180 g. The nutrition facts per 100 g of final product were: energy 416 Kcal, fat 36 g (of which saturated 15 g), carbohydrates 2 g (of which sugars 1 g), fibre 0 g, proteins 21 g, salt 4 g. The sausage manufacturing was performed twice in different periods of time (different batches) with three replicates each. The samples were subsequently stored for 160 days (i.e. 182 days after production) at 4 and 12 °C, while the suggested shelf life indicated by the industry was 3 months at 4 °C. The temperature of 4 °C was investigated since it is the expected temperature of refrigeration, while the temperature of 12 °C was selected as a temperature abuse condition. The aim of the study was also to identify possible differences between these two storage temperatures on the microbiological, physicochemical and organoleptic characteristics of the products, and to compare the probiotic survival through PFGE.

4.3.3. Microbiological analysis

Microbiological analysis was performed to monitor the population changes at the beginning and end of fermentation (days 0 and 8), end of ripening (day 22) and storage period (days 48, 74, 100, 126, 152 and 182) at 4 °C and 12 °C. At every sampling point, the casings were aseptically removed and 25 g of sausage was diluted 1/10 in ¼ strength Ringer's solution (LAB100Z, LabM, UK) and homogenized in a stomacher (Stomacher 400 Circulator, Seward, UK) for 60 s. *Lactobacillus* counts were determined by plate counting on MRS agar (MRS ISO, Biolife, Italy) after 72 h of incubation at 30 °C, micrococci and staphylococci were determined on

Mannitol Salt agar (MSA, Biolife, Italy) after 48 h at 30 °C and Total Viable Counts were determined on Plate Count Agar (PCA, Biolife, Italy) after 72 h at 30 °C. For the enumeration of mesophilic lactococci and streptococci, M17 agar (M17, Biokar Diagnostics, France) was used after incubation at 30 °C for 72 h, for Enterobacteriaceae Violet Red Bile Glucose agar (VRBG, Biolife, Italy) was used after 24 h of incubation at 37 °C, while for yeasts and molds Rose Bengal Chloramphenicol agar (RBC, LabM, UK) was used after incubation at 25 °C for 5 days. To evaluate the safety of the sausages, the presence of *Listeria monocytogenes* was determined by plate counting on Palcam Agar Base (LabM, UK) incubated at 30 °C for 48 h (detection limit of the method: 2 log CFU/g).

4.3.4. Physicochemical analysis

pH and water activity (a_w) were measured in every sampling point at both test temperatures. The pH value was recorded with a digital pH meter (HI 2211 pH-ORP Meter, HANNA Instruments, USA) by immersing the electrode in the stomacher homogenate (first decimal dilution), while a_w was recorded on sausage slices, using an AquaLab LITE device (Decagon Devices, Inc., USA).

4.3.5. Monitoring probiotic strain survival

To monitor the presence of the inoculated *Lb. plantarum* L125 strain, colonies were isolated from the highest countable (30-300 colonies) dilution of MRS agar from different time points (beginning, middle and end of storage time) at both temperatures. From each sampling point, 20% of the colonies were randomly selected and purified. Pure cultures were stored at −80 °C in MRS supplemented with 20% (v/v) glycerol. Before use, each isolate was subcultured

twice, while the purity of the culture was always checked. A total of 200 isolates were picked at 3 different sampling times (beginning, middle and end of storage) at both temperatures and batches.

Pulsed Field Gel Electrophoresis was applied to screen the succession of the inoculated strain according to Doulgeraki, Paramithiotis, Kagkli, and Nychas (2010). In brief, the restriction enzyme *Sma*I (10U) (New England Biolabs, Ipswich, MA, USA) was used according to the recommendations of the manufacturer. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris-Borate buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA) on CHEF-DRIII equipment (Bio-Rad, Hercules, CA, USA) with the following running parameters: 6 Vcm^{-1}, 1 s initial switching time, 10 s final switching time and 16 h of total run at 14 °C. The obtained profiles of the isolates were compared to the PFGE fingerprints of the inoculated *Lb. plantarum* L125 strain.

4.3.6. Sensory evaluation

At the end of the ripening stage and during storage at 4 and 12 °C, fermented sausages were organoleptically assessed by a 10-member trained laboratory panel in a sensory room allocated in the Institute of Technology of Agricultural Products. In brief, the sausage casings were removed and the samples were cut into slices of approximately 5 mm thickness. Sausage samples were served individually to each panelist randomly, while water and unsalted crackers were provided between samples for palate cleansing. The same individuals were used in all evaluations and all were unaware of the tested sample. Eighteen attributes related to odor, taste, appearance and texture were scored on a non-structured evaluation sheet in 10 cm hedonic scale with intensity descriptors. The direction was from left to right with increasing intensities (1: low, 5: high) (Fernández-López et al., 2008). For the sensory assessment, the overall conception of odor, taste, appearance and texture of the sample was scored, as well as certain individual

characteristics namely redness, paleness, oily appearance; acidic, smoking and raw meat odor; acidic, salty, sweet, spicy, aftertaste and juicy taste; and with regard to texture cohesion and hardness, while any defects such as easy peel of casing, liquid fat or presence of mold were also noted. An additional attribute regarding the overall sensory preference of each sample was given on the same scale, defining an overall assessment.

4.3.7. Statistical analysis

All experiments were carried out with two independent batches of sausages and with three samples being analysed for each batch. Microbiological, physicochemical (pH and a_w) and sensory results were analyzed for statistical significance by performing analysis of variance (ANOVA). Significance was established at $p < 0.05$. The statistical analysis was conducted using SPSS Statistics for Windows software, Version 16.0 (SPSS for Windows, Version 16.0. Chicago, SPSS Inc.).

4.4. Results

4.4.1. Microbiological results

Lb. plantarum L125 was employed as adjunct culture in the meat batter and microbiological analysis was performed during the stages of fermentation, ripening and storage for both selected storage temperatures, 4 and 12 °C. All the microbial populations of fermented sausages at the storage temperature of 4 °C are presented in Figure 1, for control and probiotic case, respectively, while the corresponding results for the storage temperature of 12 °C are presented in Appendix I- Figure 1. As a general result, all bacterial populations were affected by the treatment (control or probiotic) and the time ($p < 0.05$), while the temperature did not affect the populations significantly ($p > 0.05$). LAB were characterized as the dominant population

group both immediately after the fermentation-ripening and during the storage period and the TVC population followed the dynamics of LAB during the aforementioned stages at both the probiotic and control cases (Figure 1). Due to the probiotic inoculation, LAB counts were higher ($p < 0.05$) in the probiotic case than those of the control, starting with a population of 8.21 log CFU/g, while for the control case the population was 6.44 log CFU/g. Counts of LAB for probiotic case at 4 °C remained almost constant (*ca* 7-8 log CFU/g) during storage of the products, while for the control case, LAB increased during the fermentation stage, remained stable during ripening, and then significantly decreased at the end of storage period (days 152 and 182) ($p < 0.05$) (Figure 1). The final LAB counts for the control case were 5.01 and 5.49 log CFU/g at 4 and 12 °C, respectively. Regarding the probiotic case, LAB showed the higher population levels at 4 °C (Figure 1), when compared to 12 °C (Appendix I - Figure 1), especially near the end of the storage period. More specifically, at the end of the storage time (day 182), the LAB had a population of 7.46 log CFU/g in the probiotic case at 4 °C, while at 12 °C the population decreased at 6.60 log CFU/g. Staphylococci, decreased at the end of the ripening reaching average levels of 5.57 and 4.44 log CFU/g, for control and probiotic case, respectively. The population of staphylococci in probiotic cases decreased more than in control cases ($p < 0.05$) in the initial stage of fermentation, probably due to the strong competition between LAB cultures (*P. pentosaceus* and *Lb. plantarum* L125). Despite this fact, staphylococci levels were higher in probiotic samples at the end of the storage period (day 182) for both temperatures compared to the control case. Specifically, the counts of staphylococci in the probiotic case were 4.47 and 4.25 log CFU/g for 4 and 12 °C, while in the control case were 2.89 and 2.30 log CFU/g, respectively. In general, LAB were the predominant bacterial group, which is evident from the microbiological results. It has to be noted that LAB had the ability to grow well in M17

medium too, explaining the similar counts observed in MRS and M17 media, in the probiotic case. In this context, similar observations were made in the control case, where the starter culture *Pediococcus pentosaceus* had the ability to grow in both media, without significant difference. Spoilage bacteria such as *Pseudomonas* spp., *Brochothrix* spp., Enterobacteriaceae and yeasts/molds, as well as *Listeria monocytogenes* were below the detection limit of the enumeration method (<2 log CFU/g) throughout the fermentation, ripening and storage period at both temperatures (4 and 12 °C).

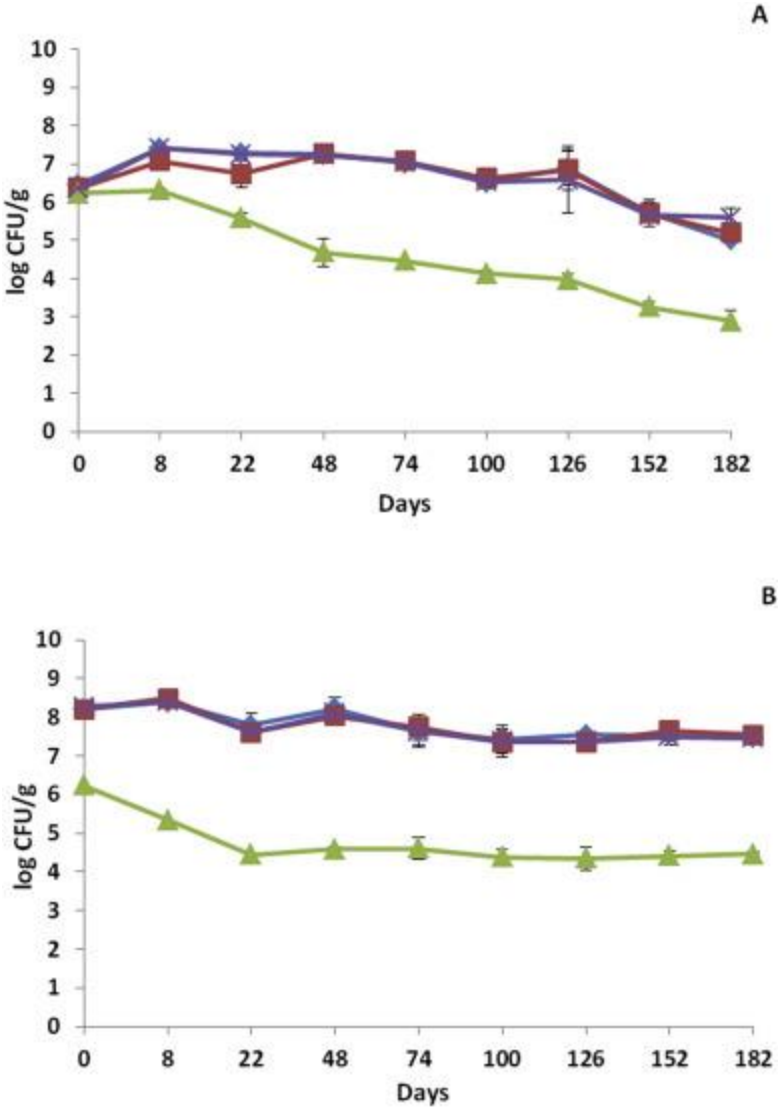


Figure 1. Growth curves of the examined bacterial populations during fermentation, ripening and storage period of dry-fermented sausages for control (A) and probiotic (B) samples stored at 4 °C. (♦) LAB, (■) TVC, (▲) Staphylococci, (✕) Mesophilic cocci/streptococci. The bars represent the mean values ± standard deviation.

4.4.2. pH and a_w measurements

The evolution of pH and water activity is presented in Figure 2 and Figure 2 in Appendix I, respectively, for 4 and 12 °C. The addition of *Lb. plantarum* L125 had no effect ($p > 0.05$) on the pH of dry-fermented sausages. Post-fermentation of the probiotic inoculated sausages was similar to the control one ($p > 0.05$). pH values ranged in usual levels, without significant differences between the tested temperatures. Although, the time in general significantly affected the pH, due to the fermentation process, no significant differences were observed throughout the storage period ($p > 0.05$). Water activity values were similar in control and probiotic case ($p > 0.05$), falling from an initial value of 0.98 to 0.87 and 0.85 for control and probiotic case, respectively. Also, no differences were noted on the water activity between 4 and 12 °C ($p > 0.05$). Water activity was significantly reduced during ripening ($p < 0.05$), as it is expected for these products. It has to be mentioned that, both pH and a_w values did not change significantly ($p > 0.05$), after the ripening process (day 22) and remained almost stable during storage. All the samples met the requirements for pH and water activity that confirm completion of sausage fermentation and ripening.

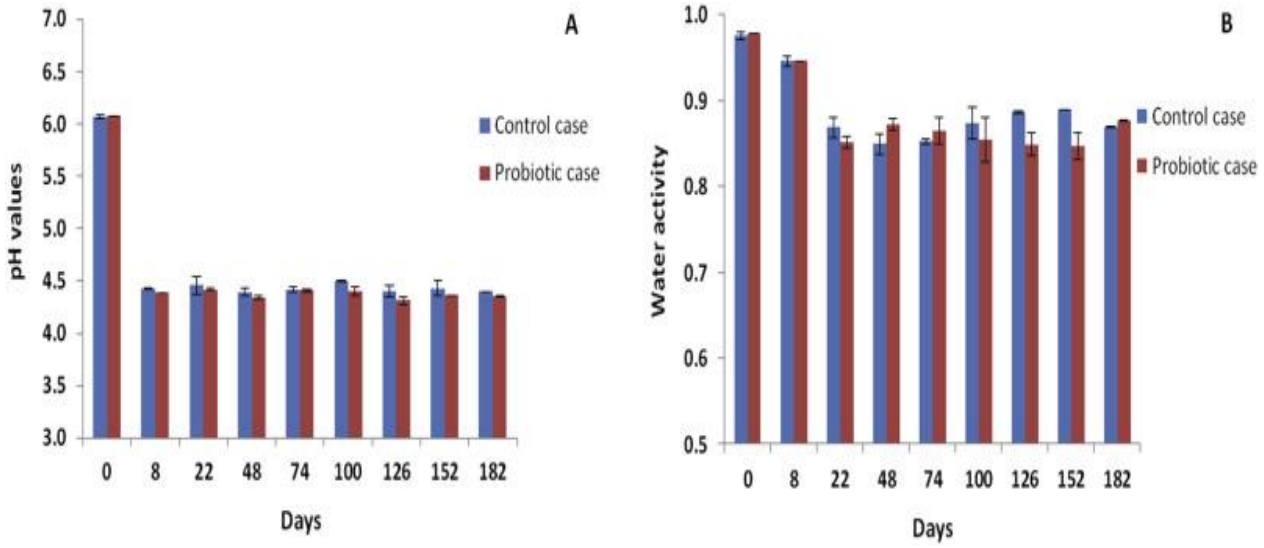
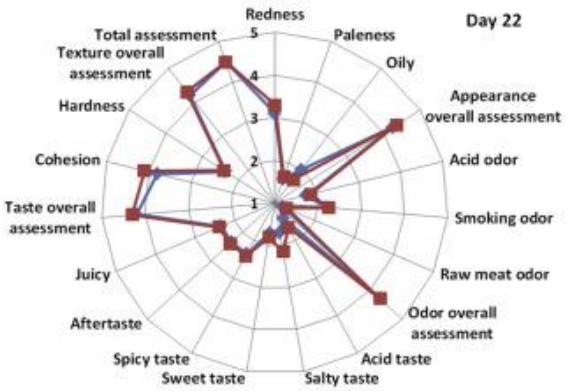


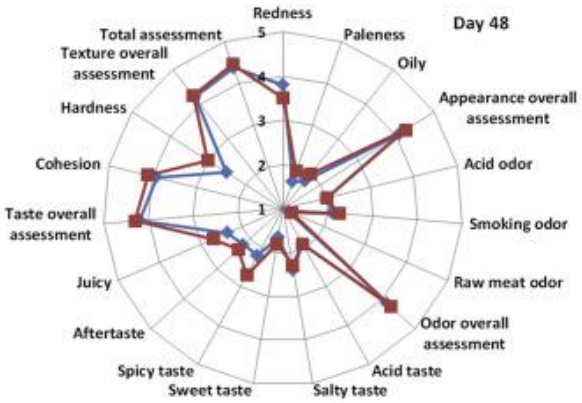
Figure 2. pH (A) and water activity (B) values during fermentation, ripening and storage of dry-fermented sausages at 4 °C. The bars represent the mean values ± standard deviation.

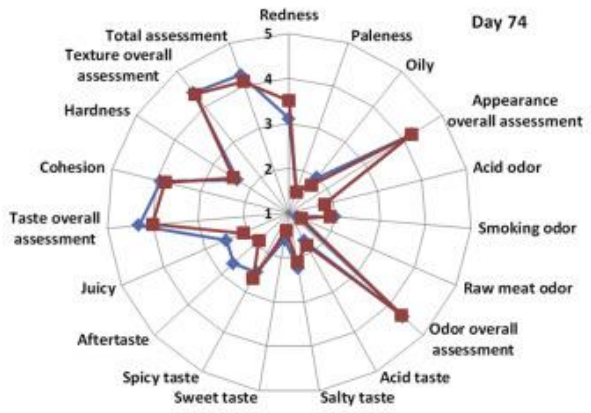
4.4.3. Sensory assessment

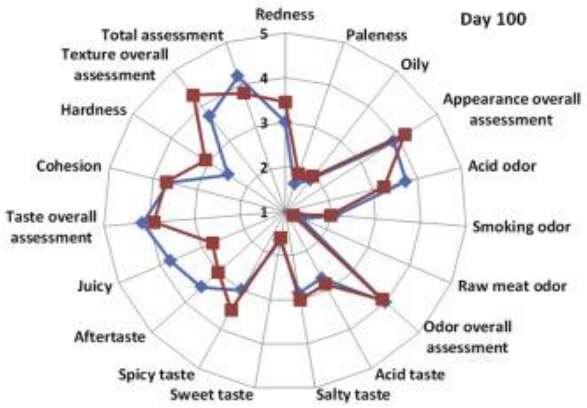
The median scores of the sensorial characteristics of dry-fermented sausages at 4 and 12 °C, are presented in Figure 3 and Figure 3 in the Appendix I, respectively. Results represent sampling points at 22 (end of ripening/beginning of storage), 48, 74, 100, 128, 152 and 182 days of storage. In general, small differences were observed between the control and probiotic cases, mainly in the attributes of redness, raw odor and acidic taste ($p < 0.05$). The acidic taste was slightly more intense in the probiotic samples, however it was not considered as unpleasant. Characteristics that were found to be significantly affected by the temperature ($p < 0.05$) include paleness, odor overall assessment, taste overall assessment, appearance overall assessment and total assessment. The storage period significantly affects all the sensorial characteristics ($p < 0.05$), except from the smoking odor. The oily appearance was noted to be more intense on day 152 and 182 for both cases and temperatures, while the most acidic taste was reported on the day 182 for the probiotic case at 12 °C. With regard to the aftertaste, the probiotic sample at 4 °C

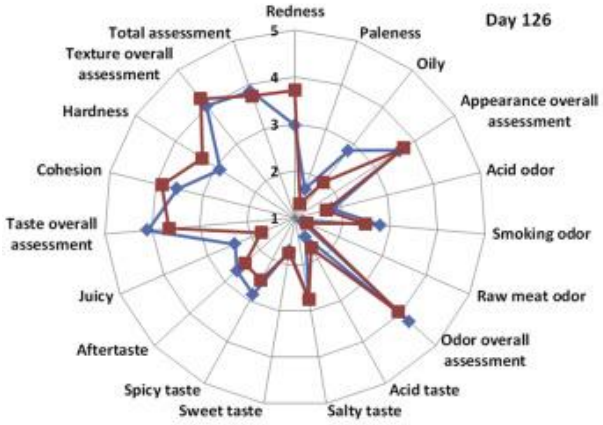
was evaluated with similar scores with the control, while higher scores were given to the probiotic sample stored at 12 °C in comparison with the control of the same temperature. The total assessment scores between the control and probiotic cases were similar, however, higher score was given to the probiotic samples at 4 and 12 °C compared to the controls for the day 182, which was the end of the storage period.

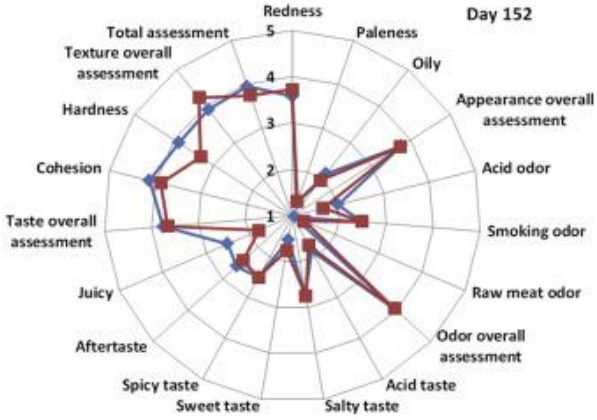

Day 22
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5


Day 48
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5


Day 74
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5


Day 100
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5


Day 126
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5


Day 152
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5

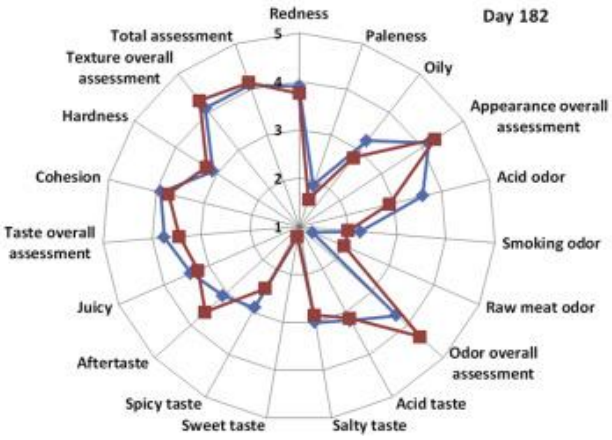

Day 182
Redness
Paleness
Oily
Appearance overall assessment
Acid odor
Smoking odor
Raw meat odor
Odor overall assessment
Acid taste
Salty taste
Sweet taste
Spicy taste
Aftertaste
Juicy
Taste overall assessment
Cohesion
Hardness
Texture overall assessment
Total assessment
1
2
3
4
5

Figure 3. Sensory evaluation of dry-fermented sausages during storage at 4 °C (22nd, 48th, 74th, 100th,128th, 152nd and 182nd day) —•—: Control sample, —■—: Probiotic sample.

4.4.4. Pulsed Field Gel Electrophoresis

After cell enumeration, the presence of *Lb. plantarum* L125 in petri dishes was confirmed by PFGE. *Lb. plantarum* L125 was identified at levels > 6 log CFU/g in all samples in the beginning (22nd day), middle (100th day) and end (182nd) of storage period. The results demonstrated that 100% of the LAB recovered from the samples were *Lb. plantarum* L125 isolates, at both storage temperatures (4 and 12 °C). The levels of the potential probiotic strain were found to be similar with those required for conferring a probiotic effect (> 6 log CFU/g) throughout storage (Table 1).

Table 1. Percentage of isolates of *Lb. plantarum* L125 recovered during storage of fermented sausage based on to the PFGE profiles at 4 and 12 °C.

Temperature	Inoculated Strain	Beginning of storage (22nd day)	Middle of storage (100th day)	End of storage (182nd day)
4 °C	*Lb. plantarum* L125	100%	100%	100%
	Other LAB	Not detected	Not detected	Not detected
12 °C	*Lb. plantarum* L125	100%	100%	100%
	Other LAB	Not detected	Not detected	Not detected

4.5. Discussion

The overall performance of a newly isolated *Lb. plantarum* L125 strain with probiotic potential, was examined in the present study, as an adjunct culture in the production of dry-fermented sausages. LAB counts of the probiotic case were found to remain almost constant for the storage temperature of 4 °C, while for the temperature of 12 °C, a slight decrease was observed throughout the storage period ($p < 0.05$). It is known that fermentations carried out in Greece and Italy are characterized by a rapid increase of the LAB population reaching values of 7-8 log CFU/g, after which they remain stable (Rantsiou et al., 2005). Also in our study, it was noted that the levels of staphylococci, although decreased at the end of the ripening process in both cases, in the control samples were significantly higher ($p < 0.05$) compared to that of the probiotic ones. Despite this fact, the staphylococci levels in the probiotic case maintained their levels throughout storage, whereas in the control case the initial higher levels of staphylococci, were decreased in earlier time points ($p < 0.05$). In general, the microbiological results were in accordance with those observed in other studies of sausages of other Mediterranean countries (Fernández-López et al., 2008; Ruiz-Moyano et al., 2011; Sayas-Barberá, Viuda-Martos, Fernández-López, Pérez-Alvarez, & Sendra, 2012). Population levels of *Pseudomonas* spp., *Brochothrix* spp., Enterobacteriaceae and *Listeria monocytogenes*, were found to be constantly below the detection limit of the enumeration method, which is in agreement with previous studies (Baka, Papavergou, Pragalaki, Bloukas, & Kotzekidou, 2011; Drosinos et al., 2005).

Regarding the pH and a_w values, the results of this study can be considered satisfactory, since the requirements for this type of product were met, while no statistically significant differences were observed between the treatments ($p > 0.05$). Additionally, *Lb. plantarum* L125 exhibited satisfactory scores regarding the sensory characteristics, with differences to be

observed between the probiotic and the control samples at certain time points. The probiotic samples stored both at 4 and 12 °C, received higher scores in the total assessment in the end of the storage period (day 182), which was a positive result. Other studies, also reported high similarity regarding the flavor and taste profiles of probiotic and traditional fermented sausages (Erkkilä et al., 2001; Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005; Trząskowska, Kolozyn-Krajewska, Wójciak, & Dolatowski, 2014).

At the end of the storage period (day 182), LAB levels in the probiotic samples were 7.46 and 6.60 log CFU/g, at 4 and 12 °C, respectively. Although the information about the minimum recommended dose is still not clear, it is generally agreed that probiotic products should have a minimum concentration of 10^6-10^7 CFU/mL or g and that a total of 10^8-10^9 probiotic bacteria should be administered daily to confer a probiotic effect to the consumer (Burgain, Gaiani, Linder, & Scher, 2011; De Prisco & Mauriello, 2016; Kechagia et al., 2013). Hence, the presence of the inoculated probiotic strain was confirmed in adequate levels throughout the storage period at both temperatures (4 and 12 °C). The consumption of 10 g per day of this sausage, which is generally realistic and compatible with a healthy diet, would be adequate to achieve the minimum recommended dosage for probiotic use (~10^8 CFU).

4.6. Conclusions

The results of the current study demonstrated that the potential probiotic strain *Lb. plantarum* L125 can be used in the dry-fermented sausages manufacture, since it leads to equal or better technological characteristics of the produced sausages. *Lb. plantarum* L125 survived and competed well with the starter cultures used (*P. pentosaceus* and *S. carnosus*) and it was detected in adequate amounts during the storage period at both temperatures. Furthermore, the sausages

inoculated with this strain received similar or higher sensory scores compared to the control (commercial) ones. Although further research is required in terms of clinical studies, *Lb. plantarum* L125 can be considered as a promising probiotic adjunct culture to develop functional dry-fermented sausages.

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CHAPTER 5

Alginate-Based Edible Films Delivering Probiotic Bacteria to Sliced Ham Pretreated with High Pressure Processing

In this chapter, the aim was the incorporation of potentially probiotic bacteria into edible films in order for them to be applied to thermally-processed meat products. Specifically, the trials were performed with RTE sliced ham. This work has been included in the following publication:

Pavli FG, *Kovaiou I, Apostolakopoulou G, Kapetanakou A, Skandamis P, Nychas G-J.E, Tassou C, Chorianopoulos N (2017). Alginate-Based Edible Films Delivering Probiotic Bacteria to Sliced Ham Pretreated with High Pressure Processing. International Journal of Molecular Sciences, 18, 1867, doi:10.3390/ijms18091867.*

5.1. Abstract

The aim of the present work was to evaluate the efficacy of Na-alginate edible films as vehicles for delivering probiotic bacteria to sliced ham with or without pretreatment using High Pressure Processing (HPP). Three strains of probiotic bacteria were incorporated in Na-alginate forming solution. Ham slices (with or without pretreatment using HPP at 500MPa for 2 min) were packed under vacuum in contact with the films and then stored at 4, 8 and 12 °C for 66, 47 and 40 days, respectively. Microbiological analysis was performed in parallel with pH and color measurements. Sensory characteristics were assessed, while the presence and the relative abundance of each probiotic strain during storage was evaluated using PFGE. In ham slices without HPP treatment, probiotic bacteria were enumerated above 10^6 CFU/g during storage at all temperatures. Same results were obtained in cases of HPP treated samples, but pH measurements showed differences with the latter ones exhibiting higher values. Sensory evaluation revealed that probiotic samples had a more acidic taste and odor than the control ones, however these characteristics were markedly compromised in samples treated with HPP. Overall, the results of the study are promising since probiotic bacteria were successfully delivered in the products by edible films regardless of the HPP treatment.

5.2. Introduction

During the last years, new trends are being observed in consumer demands regarding food products and diet habits. Interest is more focused on the active role of foods in well-being and life prolongation, as well as in their impact in the prevention of chronic diseases. As a result, a relatively new term “functional foods” is of great interest for both the industry and the consumers. Within the category of functional foods, probiotic supplemented foods obtain a remarkable position, with their market increasing annually (Granato et al. 2010). Probiotic foods have been marketed mainly in the dairy and infant-food market, but the development of new non-dairy probiotic foods is considered essential. This is because lactose intolerance, cholesterol content, and allergenic milk proteins are the major drawbacks related to the intake of dairy products.

Additionally, consumers preferences in minimally processed foods have prompted researchers to focus on the application of innovative and alternative technologies for developing better quality products without compromising food safety (Kapetanakou, Karyotis and Skandamis, 2016). High pressure processing (HPP) has a great potential in producing foods with an extended shelf-life by rendering food products microbiologically safer and hence ameliorating their quality. This emerging technology enables the reduction of spoilage microorganisms, while the population level of the surviving microbiota is kept in low levels, during storage (Fuentes et al. 2010). Recently, different HPP treatments have been applied in the food industry on various food products such as meat, fruits, fruit juices and vegetables. Advancements in the efficiency of HPP equipment have allowed this technology to be used in a wide variety of industrial applications with pressures ranging from 100 to 800 MPa depending on the objective (Argyri et al. 2014). With regard to the application of this technology on meat, HPP has been proven to

promote lipid oxidation, volatile formation and induce color changes in sliced dry-cured ham (Rivas-Cañedo, Fernández-García and Nuñez, 2009; Andres et al. 2006). Furthermore, HPP has an impact on the sensory characteristics of meat products (Simonin, Duranton and de Lamballerie, 2012), however, limited research has been conducted to investigate the influence of HPP on the sensory properties of dry-cured ham (Fuentes et al. 2010).

Active packaging has been defined as a method of packaging in which the packaging material, the product and the environment interact during storage. Accordingly, the shelf-life is increased and the quality and safety of the products are improved (Kapetanakou, Karyotis and Skandamis, 2016). Improvements in active packaging technologies, have led to the development of bioactive food packaging systems that have the ability of presenting health benefits to the consumers. Bioactive agents can be incorporated into packaging materials, which can result in their gradual (and sometimes controlled) release to the food product. In the particular case of bioactive edible films and coatings, this release is not even required since the film or coating itself is supposed to be eaten with the food (Lopez-Rubio, Gavara and Lagaron, 2006).

Due to the sensitivity of probiotics to common processing conditions such as heat treatment, acidic environment, high osmotic pressure and high redox potential, the design of an effective physicochemical barrier to stabilize the organisms is essential (Soukoulis et al. 2017). Such a possible solution was the incorporation of probiotic cultures into edible coatings, which was first proposed in 2007 by Tapia et al. (2007) for application on frsh fruits. This concept may be expandable also to other surface contaminated foods, e.g., fresh meat and cooked meat products including frankfurters and ham slices. Since there are limited studies on this area, the development of films and coatings supplemented with probiotics still needs a lot of research.

In a previous study, we demonstrated that out of 47 strains that were screened for probiotic potential, 19 showed good behavior under simulated gastrointestinal conditions and were considered safe, thus possessing desirable in vitro probiotic properties. Based on the above, a subsequent challenge is the application of these strains in various foods, both for exploiting their probiotic properties and/or their antimicrobial effect for food preservation and food safety. The objectives of the present study were: (i) to develop a Na-alginate edible film based on the incorporation of probiotic cultures in the matrix; (ii) to examine the effectiveness of such films in probiotic delivery; (iii) to investigate the effect of HPP treatment; and (iv) to evaluate the effect of the probiotic cultures on the physicochemical and sensory characteristics of the ham slices.

5.3. Materials and Methods

5.3.1. Probiotic Strains and Ham Slices

Three strains of potentially probiotic bacteria were used in the present study: *Lactobacillus plantarum* B282, *Lactobacillus plantarum* L125 and *Lactobacillus pentosus* L33 which were previously isolated from table olives and meat products (Pavli et al. 2016; Doulgeraki et al. 2013). The pure cultures were stored at -80 ^{o}C in De Man-Rogosa and Sharpe broth (LabM, Lancashire, UK) supplemented with 20% (v/v) glycerol and the strains were subcultured twice before use. Commercial packages of ham slices were purchased from a local supermarket (Athens, Greece) (10 x 10 cm; 20 g). Three storage temperatures were used in the study (4, 8 and 12 °C), while two independent experiments were performed (ham slices produced by different manufacturer were used) and duplicate samples were studied in each experiment.

5.3.2. High Pressure Processing (HPP) Treatment

HPP treatment (when applied) was conducted at the pressure of 500 MPa for 2 min at room temperature (20 °C). Pressure and temperature were constantly monitored and recorded (in 1 s intervals) during the process. Pressurization time reported does not include the pressure come-up and release times. Further details of the high pressure system and operating conditions can be found in previous papers (Tassou et al. 2008; Panagou et al. 2007).

5.3.3. Preparation of Na-Alginate Edible Films

The preparation of Na-alginate edible films was conducted as reported previously by Kapetanakou et al. (Kapetanakou, Karyotis and Skandamis, 2016). Briefly, quantity of 2 g of Na-alginate (Applichem GmbH, Darmstadt, Germany) was added gradually in 100 mL of pre-warmed (65 °C) distilled sterile water and under stable agitation for complete dissolution. One mL of glycerol (plasticizer) was added in order to improve film flexibility and the forming solution was kept at 4 °C for 30 min to lower the temperature, until the addition of the probiotic cultures. A mix of the three probiotics was added with agitation, in a final population of 10^9 CFU/ml in the forming solution (probiotic- supplemented edible films-PS). Na-alginate solution without the addition of probiotic cultures was also prepared (probiotic-free edible films-PF). Films were produced in different Petri-dishes using 20 g of Na-alginate solution and then were placed in a laminar flow cabinet to dry at ambient temperature for 12 h. Following drying process, aliquots of 20 mL of 2% w/v $CaCl_2$ were added for 1 min, in order to detach the square films (*ca.* 0.5 g) from the Petri-dishes.

5.3.4. Microbiological Analysis

Samples (10 g) of ham slices were weighed aseptically, added to sterile quarter strength Ringer's solution (LabM, Lancashire, UK) (90 ml), and homogenized in a stomacher (Stomacher 400, Circulator, Seward) for 60 s at room temperature. The resulting suspensions were serially diluted in the same diluent and 1 or 0.1 mL samples of the appropriate dilutions were poured or spread, respectively, on the following agar media: de Man–Rogosa–Sharpe Agar (MRS, Oxoid, Hampshire, UK) for LAB, incubated at 30 °C for 72 h; Plate Count Agar (LabM, Lancashire, UK) for TVC, incubated at 30 °C for 48 h; STAA Agar Base (Oxoid, Hampshire, UK) for *Brochothrix thermosphacta*, incubated at 25 °C for 48 h; Rose Bengal Chloramphenicol Agar (LabM, Lancashire, UK) for yeasts/molds incubated at 25 °C for 5 days; Violet Red Bile Glucose Agar (Oxoid, Hampshire, UK) for Enterobacteriaceae, incubated at 37 °C for 24 h; Pseudomonas agar base (LabM, Lancashire, UK), for *Pseudomonas* spp. incubated at 25 °C for 48 h, as well as Palcam Agar Base (LabM, Lancashire, UK), for *Listeria* spp. incubated at 30 °C for 48 h.

5.3.5. Viability of the Probiotic Strains Incorporated within the Film

The viability of the incorporated strains was tested in films that were in contact with the ham samples at the same time intervals with theham samples. The films were removed aseptically from the slices and placed in a sterile stomacher bag and homogenised for 120 s. Decimal dilutions were prepared in the same medium and 1 mL of the appropriate dilutions were poured on MRS agar and incubated at 30 °C for 72 h.

5.3.6. pH Values

The pH value of the samples was measured with a digital pH meter (HI 2211 pH-ORP Meter, HANNA Instruments, Woonsocket, RI, USA). The pH of the ham slices was measured in the ham homogenate (stomacher homogenate) after the end of the microbiological analysis.

5.3.7. Color Measurements

The ham color was assessed by taking at least 5 random measurements from the surface of the different ham samples using a Minolta Chroma Meter fitted with a CR-300 measuring head (Minolta, Osaka, Japan). The CIE (Commission Internationale de l' Eclairage) *L**, *a**, *b**, colorimetry system was used for color determination with *L** indicating lightness, *a** indicating redness and *b** indicating yellowness. Measurements of the instrument were standardized with respect to a white calibration plate. Color measurements avoiding the area with excess fat were taken and the values were recorded in order to determine C* (chroma).

5.3.8. Sensory Evaluation

Sensory evaluation of ham slices was performed during storage in all temperatures according to Gill and Jeremiah (1991) by a sensory panel composed of five members (staff from the laboratory) at the same time intervals with microbiological sampling points. The same trained personnel were used in each evaluation and were all blinded to the sample tested. The evaluation was carried out under artificial light at ambient room temperature. The descriptors selected were based on the perception of aroma, taste and appearance. Each attribute was scored on a three-point hedonic scale ranging from 1 (fresh) to 3 (unacceptable). We used this scale because our aim was to evaluate the changes in aroma, taste and appearance regarding the spoilage, and not the preference. The same hedonic scale and with a panel of 5 members was used in many other

studies aiming to evaluate spoilage status or shelf-life of meat products such as minced beef and pork (Papadopoulou et al. 2011; Argyri et al. 2015). Intermediate sensory qualities were attributed to scores of 1.5, 2 and 2.5. Specifically, a score of 1.5 was characterized deteriorated and was the first indication of change from that of typical fresh ham (i.e., less vivid color, aroma and taste slightly changed, but still acceptable by the consumer). Scores > 2 characterized the product spoiled and indicated the end of shelf-life.

5.3.9. PFGE for Monitoring Probiotic Survival and Strain Differentiation

In total, 476 isolates (237 from ham samples and 239 from films) were recovered from the highest dilution in MRS agar and were then screened with PFGE to determine the survival of the inoculated probiotic strains in levels ≥ 10^6 CFU/g and the differentiation during the storage period at the three temperatures tested without or after the HPP treatment. In brief, genomic DNA extraction was performed from all isolates, as previously reported (Doulgeraki et al. 2010). The restriction enzyme *Sma*I (10U) (New England Biolabs, Ipswich, MA, USA) was used according to manufacturer recommendations for 16 h. Following digestion, restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris-Borate buffer on a CHEF-DRIII (BIO-RAD, Hercules, CA, USA) equipment with the following running parameters: 6V/cm, 1 s initial switching time, 10 s final switching time and 16 h total run at 14 °C. Gels were then stained with ethidium bromide (Sigma-Aldrich, Schnelldorf, Germany) (0.5mg/L) in water for 1 h and distained for 2 h before being photographed with 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).

5.3.10. Statistical Analysis

All experiments were carried out in duplicate with two independent batches of ham slices each. Analysis of variance was performed and means were separated with Duncan's multiple range test. Significance was established at $p < 0.05$. The differences of the dependent variables regarding the factor "Pressure" were evaluated using the Student T-test in order to compare the means of the two different groups (with and without HPP treatment) to verify statistically significant differences between them. The statistical analysis was conducted using IBM® SPSS® Statistics for Windows software, Version 24.0 (IBM Corp., New York, NY, USA).

5.4. Results

5.4.1. Microbiological Analysis

The initial population of sliced ham was approximately 10^3 CFU/g for both studied batches, which was relatively low and enabled the probiotic strains to be the dominant population in the ham. Lactic acid bacteria (LAB) and total viable counts (TVC) population levels for control samples, for samples in contact with probiotic-free edible films (PF) and for samples in contact with probiotic-supplemented edible films (PS) without prior HPP treatment for all storage temperatures, are presented in Figure 1. Regarding the samples with the probiotic-supplemented edible films, the LAB and TVC counts were maintained at levels of $>10^6$ CFU/g in all temperatures during the storage period of the products. In Figure 2, changes in the population levels of LAB and TVC are presented for the samples treated with HPP during storage at 4, 8 and 12 °C. The storage temperature affected the LAB and TVC counts for all cases tested, and the differences were statistically significant ($p<0.05$).

HPP treatment caused a reduction in the initial microbial population of the ham, therefore LAB and TVC were below detection levels in the beginning of the storage. After the first sampling for each storage temperature, LAB population in samples of probiotic-supplemented edible films was $>10^6$ CFU/g and the levels remained constant during the shelf-life at 4, 8 and 12 °C. In the cases without HPP treatment, higher microbial counts were detected closer to the end of shelf-life ($>10^8$ CFU/g) in control samples and samples with the probiotic-free edible films, whereas, for the HPP treated samples, the counts were always lower at the same time points. The pressure affected both the microbial populations, in all of cases apart for the case of 12 °C, and the differences were significant ($p<0.05$). In every sampling point, other microbial populations were tested such as *Brochothrix thermosphacta*, *Pseudomonas* spp., Enterobacteriaceae, yeasts/moulds and *Listeria monocytogenes*, but they were always below the detection limit.

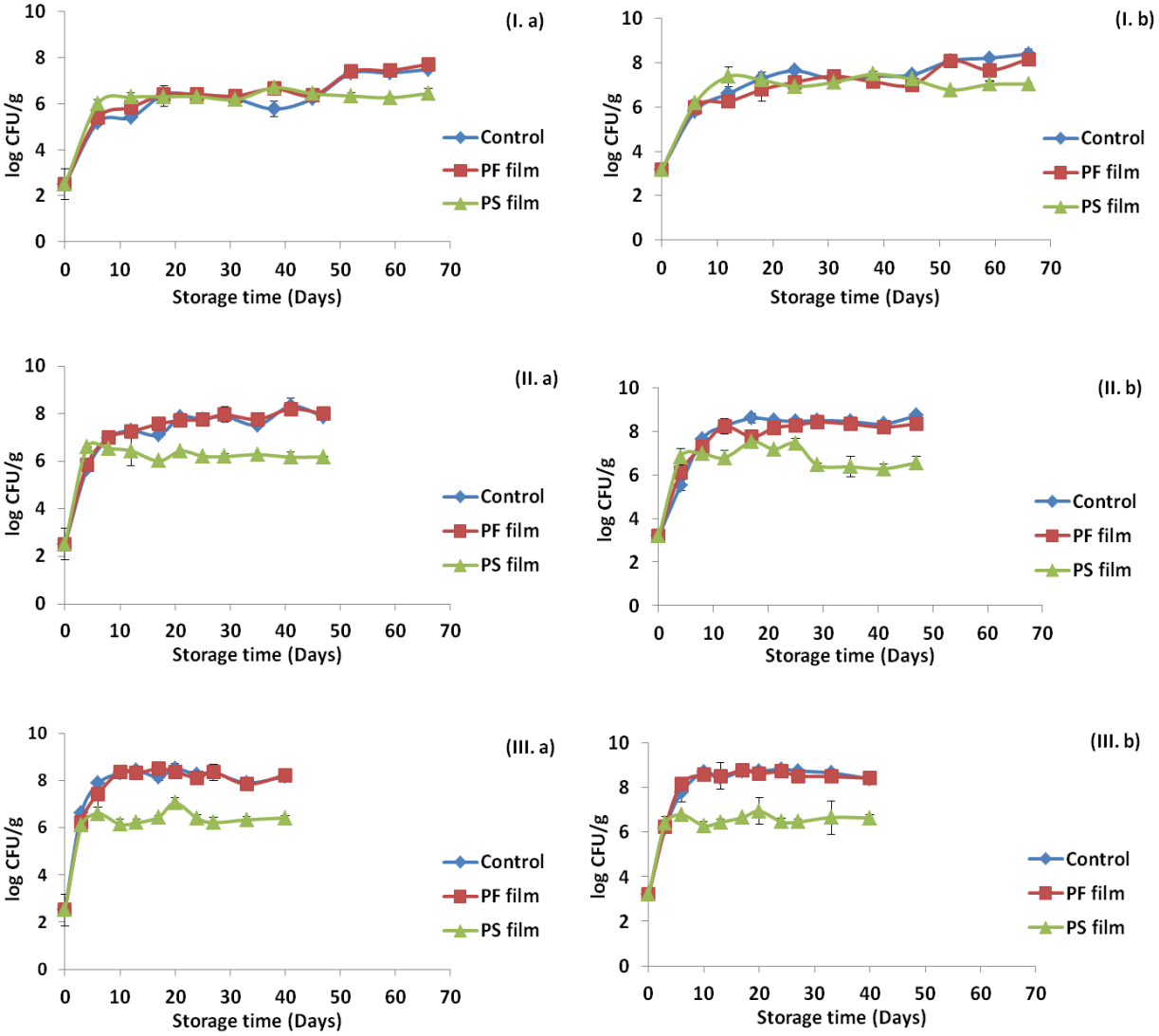

Figure 1. Changes in population levels of: (**a**) Lactic acid bacteria; (**b**) Total Viable Counts in ham slices with probiotic-supplemented films (PS) without high pressure processing (HPP) treatment during storage at: 4 °C (I); 8 °C (II); and 12 ° C (III). Samples without edible films (controls) or with probiotic-free edible films (PF) were also studied.

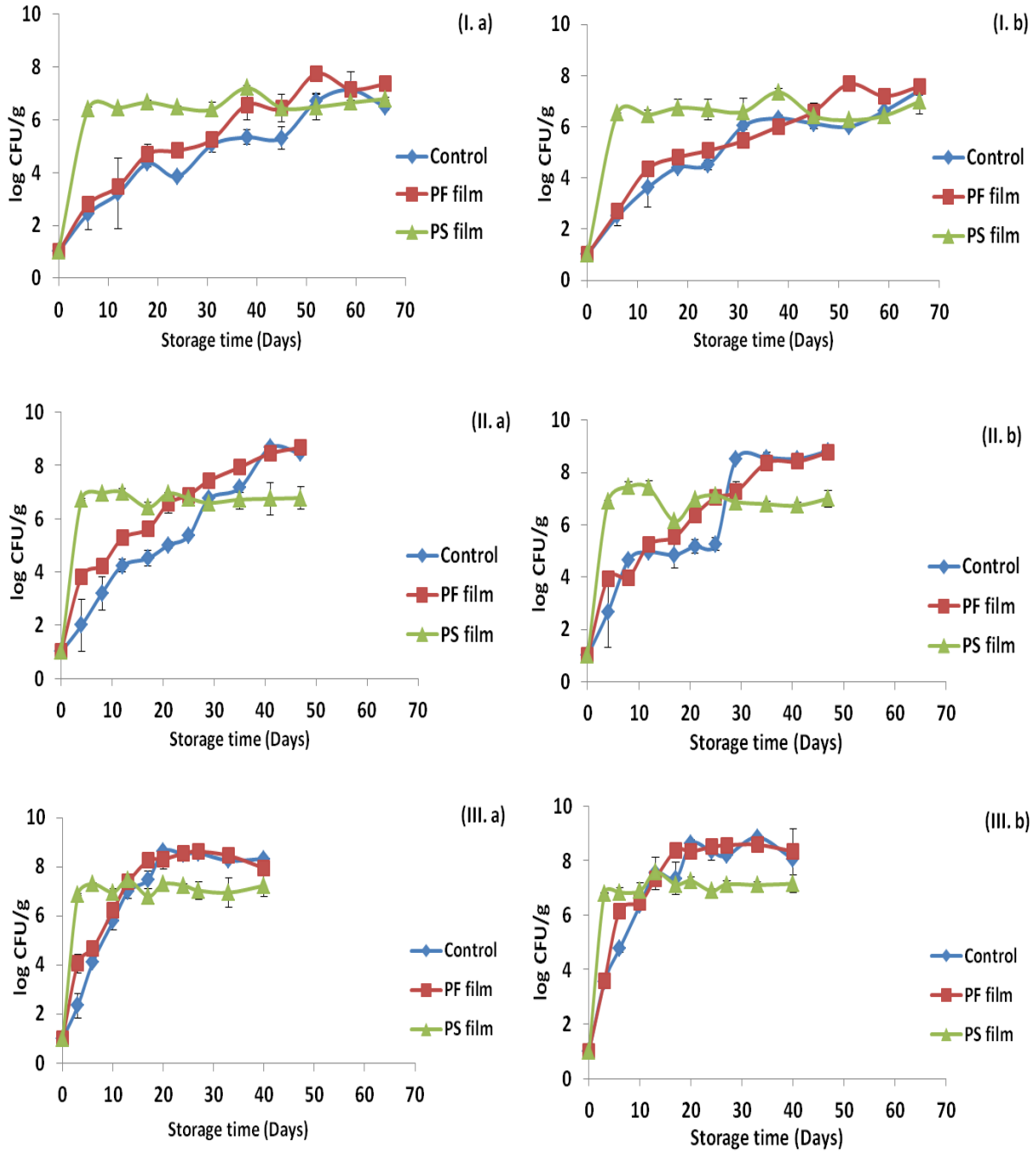

Figure 2. Changes in population levels of: (**a**) Lactic acid bacteria; and (**b**) Total Viable Counts on ham slices with PS after HPP treatment during storage at: 4 °C (I), 8 °C (II) and 12 °C (III). Samples without edible films (controls) or with PF were also studied.

5.4.2. Viability of the Incorporated Strains in the Na-Alginate Films

The viability of the incorporated strains was examined throughout the storage period in contact with the ham slices (66 days at 4 °C, 47 days at 8 °C and 40 days at 12 °C). Populations of LAB and TVC were determined for edible films stored at the three tested temperatures and the results are presented in Figure 3. As it can be seen, the storage temperature and the HPP treatment of the ham slices had no effect on the viability of the inoculated strains. In general, a reduction was detected in the films in the sampling point after their application in ham for all temperatures, however, it can be assumed that the drying process and subsequent stress had limited effect on the probiotic survival in adequate levels ($>10^6$ CFU/g).

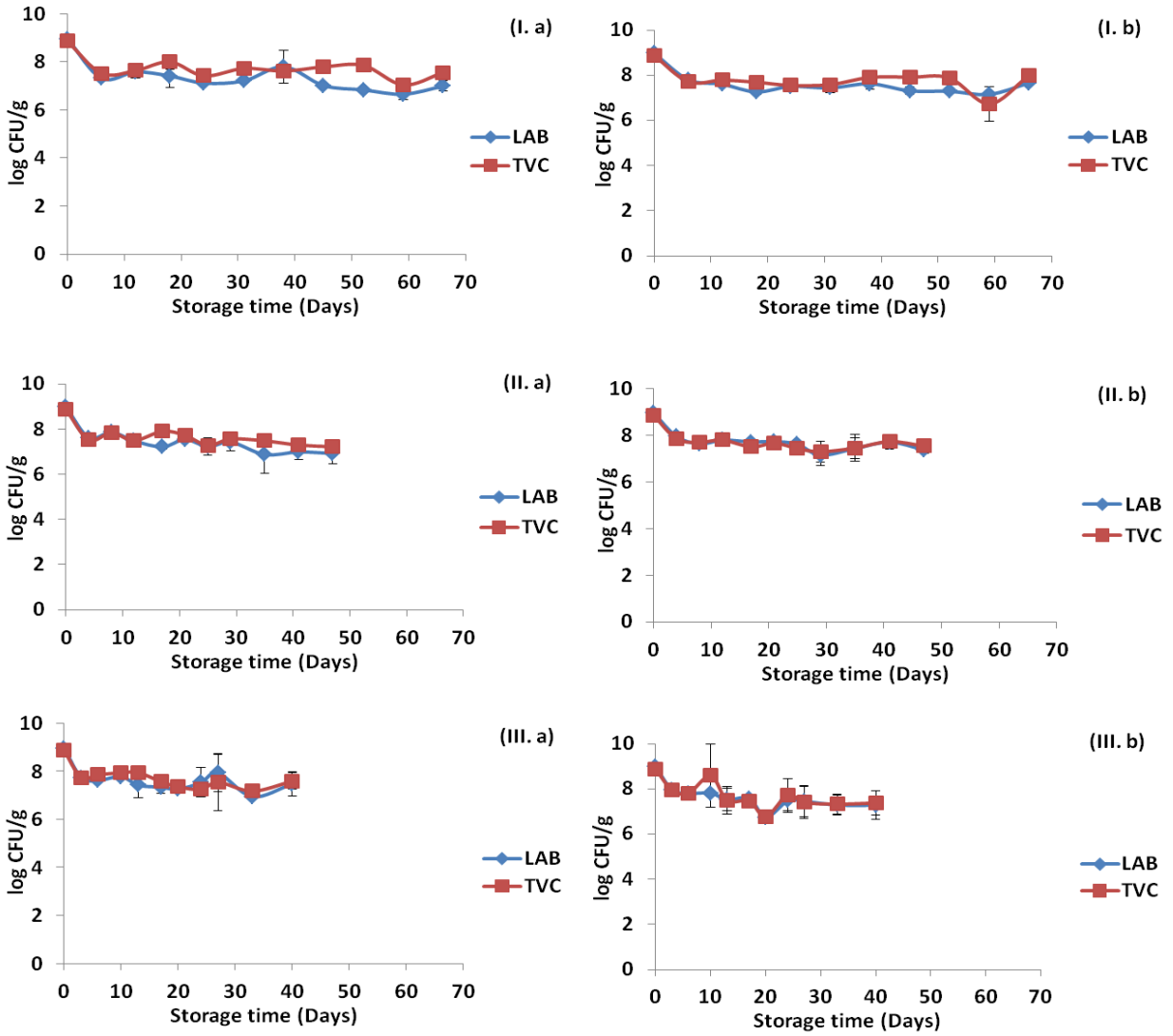

Figure 3. Changes in population levels of lactic acid bacteria (LAB) and total viable counts (TVC) in PS edible films in contact with ham slices: (**a**) Without HPP treatment; and (**b**) After HPP treatment and during storage at: 4 °C (I), 8 °C (II) and 12 °C (III).

5.4.3. pH Determination

The pH results are presented in Tables 1-3. In the cases of the application of PS edible films, the pH values were affected and a rapid decrease was observed in all temperatures, regardless of the HPP treatment (p<0.05). Overall, the application of HPP resulted in samples with higher pH values, while pH values were significantly (p< 0.05) affected by all factors (edible films, HPP, storage time). The pH values recorded for samples with PS films are quite low for this type of ready-to-eat (RTE) ham and this was due to the high population levels of the probiotic bacteria.

Table 1. Changes in pH values for ham slices treated or not with HPP, during storage at 4 °C.

pH values at 4 °C						
	Without HPP treatment			With HPP treatment		
Days	**Control**	**PF film**	**PS film**	**Control**	**PF film**	**PS film**
0	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$
6	$6.53^{A,a} \pm 0.06$	$6.24^{B,a} \pm 0.01$	$5.22^{B,b} \pm 0.23$	$6.52^{A,a} \pm 0.00$	$6.30^{B,b} \pm 0.01$	$5.43^{B,c} \pm 0.07$
12	$6.35^{B,a} \pm 0.03$	$6.11^{C,b} \pm 0.01$	$4.77^{C,c} \pm 0.04$	$6.45^{AB,a} \pm 0.01$	$6.30^{B,b} \pm 0.01$	$5.23^{C,c} \pm 0.01$
18	$5.76^{C,b} \pm 0.01$	$6.04^{C,a} \pm 0.02$	$4.44^{D,c} \pm 0.01$	$6.42^{AB,a} \pm 0.03$	$6.31^{B,b} \pm 0.01$	$5.05^{D,c} \pm 0.01$
24	$5.54^{D,b} \pm 0.03$	$5.66^{D,a} \pm 0.01$	$4.32^{DE,c} \pm 0.02$	$6.40^{AB,a} \pm 0.00$	$6.14^{C,b} \pm 0.01$	$4.91^{EF,c} \pm 0.01$
31	$5.55^{D,a} \pm 0.05$	$5.44^{E,a} \pm 0.04$	$4.36^{DE,b} \pm 0.01$	$6.48^{AB,a} \pm 0.01$	$5.72^{D,b} \pm 0.02$	$4.77^{G,c} \pm 0.03$
38	$5.52^{D,a} \pm 0.04$	$5.44^{E,b} \pm 0.00$	$4.40^{DE,c} \pm 0.01$	$5.76^{D,a} \pm 0.07$	$5.75^{D,a} \pm 0.01$	$4.93^{E,b} \pm 0.00$
45	$5.52^{D,a} \pm 0.01$	$5.44^{E,a} \pm 0.07$	$4.36^{DE,b} \pm 0.01$	$6.01^{C,a} \pm 0.01$	$5.43^{E,b} \pm 0.07$	$4.84^{FG,c} \pm 0.03$
52	$5.39^{E,a} \pm 0.01$	$5.25^{F,a} \pm 0.16$	$4.22^{E,b} \pm 0.01$	$5.83^{D,a} \pm 0.04$	$5.26^{G,b} \pm 0.02$	$4.78^{G,c} \pm 0.01$
59	$5.27^{F,a} \pm 0.03$	$5.17^{F,b} \pm 0.01$	$4.26^{E,c} \pm 0.01$	$5.73^{D,a} \pm 0.11$	$5.33^{F,b} \pm 0.07$	$4.68^{H,c} \pm 0.04$
66	$5.36^{E,a} \pm 0.01$	$5.25^{F,b} \pm 0.01$	$4.25^{E,c} \pm 0.03$	$5.62^{E,a} \pm 0.03$	$5.40^{EF,b} \pm 0.07$	$4.66^{H,c} \pm 0.04$

^{A,B,C,D,E,F,G,H} Means with different uppercase letters within the same treatment are significantly different (p< 0.05).

^{a,b,c} Means with different lowercase letters within the same storage day are significantly different (p< 0.05).

Table 2. Changes in pH values for ham slices treated or not with HPP, during storage at 8 °C.

	pH values at 8 °C					
	Without HPP treatment			With HPP treatment		
Days	**Control**	**PF film**	**PS film**	**Control**	**PF film**	**PS film**
0	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$
4	$6.35^{B,a} \pm 0.06$	$6.36^{B,a} \pm 0.00$	$4.87^{B,b} \pm 0.02$	$6.52^{A,a} \pm 0.01$	$6.26^{B,b} \pm 0.01$	$5.40^{B,c} \pm 0.03$
8	$5.49^{CD,a} \pm 0.01$	$5.46^{C,a} \pm 0.05$	$4.52^{C,b} \pm 0.00$	$6.51^{A,a} \pm 0.02$	$6.32^{B,b} \pm 0.01$	$4.98^{C,c} \pm 0.00$
12	$5.56^{C,a} \pm 0.02$	$5.37^{D,b} \pm 0.04$	$4.33^{D,c} \pm 0.04$	$6.52^{A,a} \pm 0.01$	$6.04^{C,b} \pm 0.04$	$4.72^{D,c} \pm 0.03$
17	$5.47^{CD,a} \pm 0.04$	$5.25^{E,b} \pm 0.01$	$4.37^{D,c} \pm 0.01$	$6.50^{A,a} \pm 0.07$	$5.96^{D,b} \pm 0.04$	$4.66^{E,c} \pm 0.01$
21	$5.45^{DE,a} \pm 0.06$	$5.19^{EF,b} \pm 0.01$	$4.27^{E,c} \pm 0.03$	$6.26^{B.a} \pm 0.06$	$5.92^{DE,b} \pm 0.01$	$4.63^{EF,c} \pm 0.04$
25	$5.43^{DEF,a} \pm 0.04$	$5.23^{EF,b} \pm 0.01$	$4.14^{H,c} \pm 0.03$	$6.18^{B,a} \pm 0.11$	$5.88^{E,b} \pm 0.02$	$4.63^{EF,c} \pm 0.02$
29	$5.35^{EF,a} \pm 0.07$	$5.21^{EF,b} \pm 0.01$	$4.19^{GH,c} \pm 0.02$	$5.69^{C,a} \pm 0.01$	$5.61^{F,b} \pm 0.01$	$4.61^{EF,c} \pm 0.00$
35	$5.33^{F,a} \pm 0.06$	$5.17^{F,b} \pm 0.01$	$4.19^{FGH,c} \pm 0.03$	$5.55^{D,a} \pm 0.04$	$5.40^{G,a} \pm 0.06$	$4.57^{FG,b} \pm 0.04$
41	$5.34^{EF,a} \pm 0.01$	$5.04^{G,b} \pm 0.04$	$4.24^{EF,c} \pm 0.00$	$5.31^{E,a} \pm 0.01$	$5.29^{H,a} \pm 0.01$	$4.58^{FG,b} \pm 0.02$
47	$5.22^{FG,a} \pm 0.01$	$5.02^{G,b} \pm 0.01$	$4.24^{EFG,c} \pm 0.01$	$5.24^{E,a} \pm 0.04$	$5.12^{I,b} \pm 0.01$	$4.54^{G,c} \pm 0.01$

^{A,B,C,D,E,F,G,H,I} Means with different uppercase letters within the same treatment are significantly different ($p < 0.05$).

^{a,b,c} Means with different lowercase letters within the same storage day are significantly different ($p < 0.05$).

Table 3. Changes in pH values for ham slices treated or not with HPP, during storage at 12 °C.

pH values at 12 °C						
	Without HPP treatment			With HPP treatment		
Days	**Control**	**PF film**	**PS film**	**Control**	**PF film**	**PS film**
0	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.49^{A,a} \pm 0.07$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$	$6.54^{A,a} \pm 0.11$
3	$6.01^{B,a} \pm 0.01$	$5.93^{B,b} \pm 0.03$	$4.75^{B,c} \pm 0.03$	$6.43^{A,a} \pm 0.06$	$6.19^{B,b} \pm 0.02$	$5.28^{B,c} \pm 0.04$
6	$5.62^{C,a} \pm 0.01$	$5.30^{C,b} \pm 0.08$	$4.19^{CD,c} \pm 0.02$	$6.42^{A,a} \pm 0.01$	$6.10^{C,b} \pm 0.03$	$4.50^{CD,c} \pm 0.00$
10	$5.46^{D,a} \pm 0.01$	$4.92^{E,b} \pm 0.07$	$4.06^{E,c} \pm 0.01$	$6.15^{B,a} \pm 0.02$	$6.03^{D,b} \pm 0.01$	$4.42^{E,c} \pm 0.04$
13	$5.42^{D,a} \pm 0.01$	$5.17^{CD,b} \pm 0.00$	$4.08^{E,c} \pm 0.04$	$5.76^{C,a} \pm 0.05$	$5.60^{E,b} \pm 0.02$	$4.50^{CD,c} \pm 0.02$
17	$5.35^{D,a} \pm 0.01$	$4.96^{E,b} \pm 0.11$	$4.12^{DE,c} \pm 0.01$	$5.54^{D,a} \pm 0.02$	$5.49^{F,a} \pm 0.01$	$4.44^{DE,b} \pm 0.02$
20	$5.45^{D,a} \pm 0.04$	$5.02^{DE,b} \pm 0.13$	$4.13^{DE,c} \pm 0.04$	$5.25^{E,a} \pm 0.06$	$5.09^{G,b} \pm 0.01$	$4.53^{C,c} \pm 0.04$
24	$5.24^{E,a} \pm 0.02$	$5.07^{DE,a} \pm 0.04$	$4.26^{C,b} \pm 0.08$	$5.16^{F,a} \pm 0.01$	$5.03^{G,b} \pm 0.04$	$4.53^{C,c} \pm 0.04$
27	$5.23^{E,a} \pm 0.12$	$5.02^{DE,a} \pm 0.03$	$4.09^{E,b} \pm 0.01$	$5.12^{FG,a} \pm 0.05$	$4.87^{H,b} \pm 0.07$	$4.40^{E,c} \pm 0.03$
33	$5.06^{F,a} \pm 0.05$	$5.00^{E,a} \pm 0.01$	$4.08^{E,b} \pm 0.03$	$5.06^{GH,a} \pm 0.04$	$4.91^{H,b} \pm 0.05$	$4.46^{DE,c} \pm 0.03$
40	$5.04^{F,a} \pm 0.04$	$4.95^{E,a} \pm 0.06$	$4.13^{DE,b} \pm 0.03$	$4.98^{H,a} \pm 0.01$	$4.87^{H,b} \pm 0.02$	$4.51^{C,c} \pm 0.01$

^{A,B,C,D,E,F,G,H} Means with different uppercase letters within the same treatment are significantly different ($p < 0.05$).

^{a,b,c} Means with different lowercase letters within the same storage day are significantly different ($p < 0.05$).

5.4.4. Color Measurements

In Figure 4, the C* values are presented for each case and for the three temperatures. The application of edible films affected the mean values of C* and such differences were observed in all storage temperatures, however, only slightly. On the other hand, pressure did not affect the C* values in any case ($p>0.05$). Previous studies suggest that HPP processing, affects the C* value by causing an increase in L^* value, a decrease in a^* and no changes to b^* values (Souza et al. 2012), although in this study, similar results were not observed.

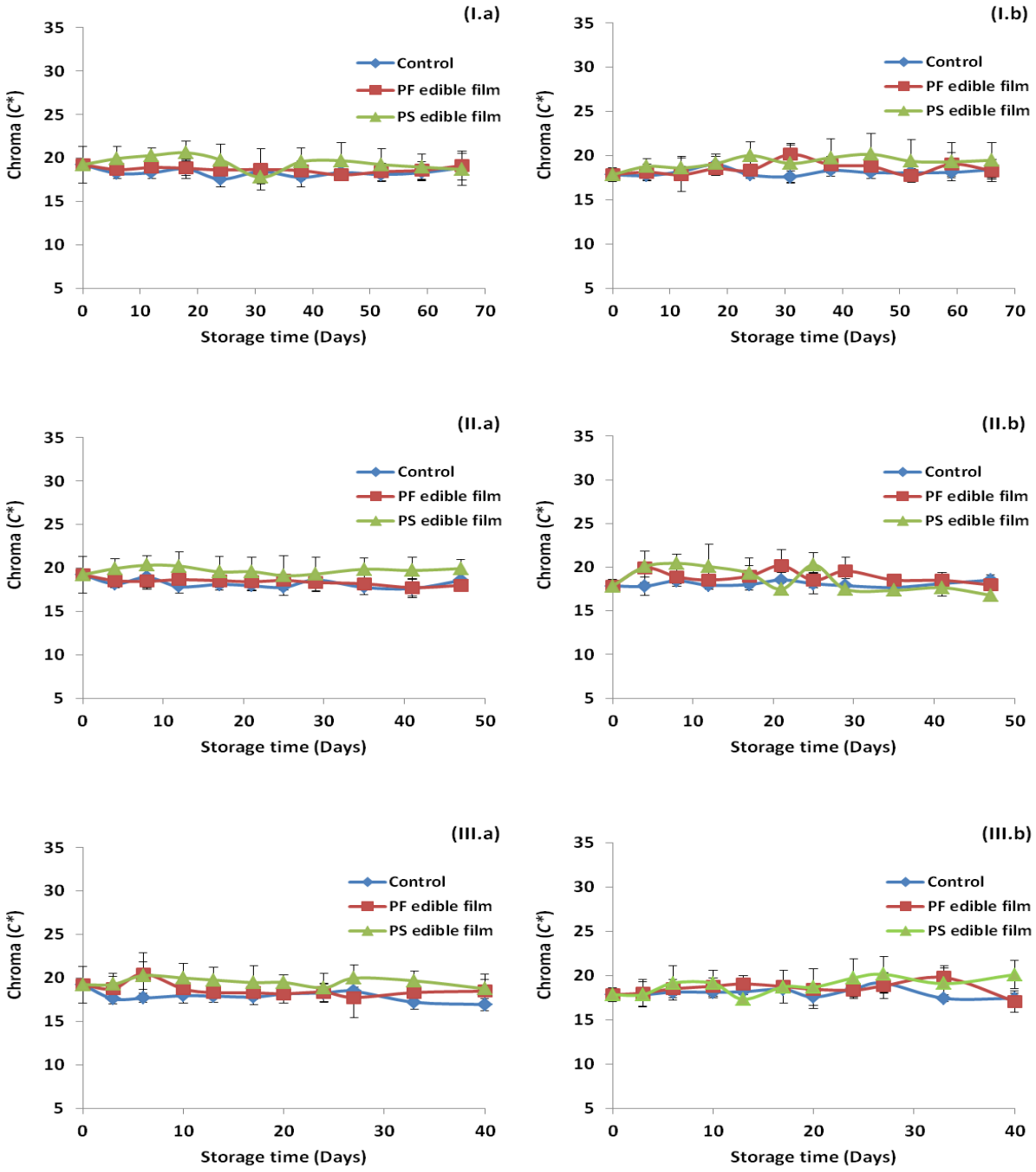

Figure 4. Changes in C* values for ham slices without (a) and after (b) high pressure processing treatment, during storage at: 4 °C (I), 8 °C (II) and 12 °C (III).

5.4.5. Sensory Evaluation

The results of the sensory assessment are presented in Figure 5. The presence of the PS film affected significantly the aroma and taste parameters and, as a result, the total scores in all the temperatures tested. The HPP treatment, however, resulted in the production of less acidic samples with PS films and in better total score values from the panelists. Regarding the appearance scores, similar values were observed between pressurised and non-pressurised ham slices, while, at some time points the appearance of the treated ones was evaluated as better. The period with ham showing acceptable sensory characteristics and overall appearance was consideredtypically as “shelf-life”. Total scores >2 indicated unacceptable quality. Since no major microbiological changes had occurred by that time, shelf-life was determined by only sensory evaluation. HPP application enhanced the sensory characteristics of the ham slices and lower scores (more fresh/less spoiled) were given from the panelists at the same time points compared to the untreated samples.

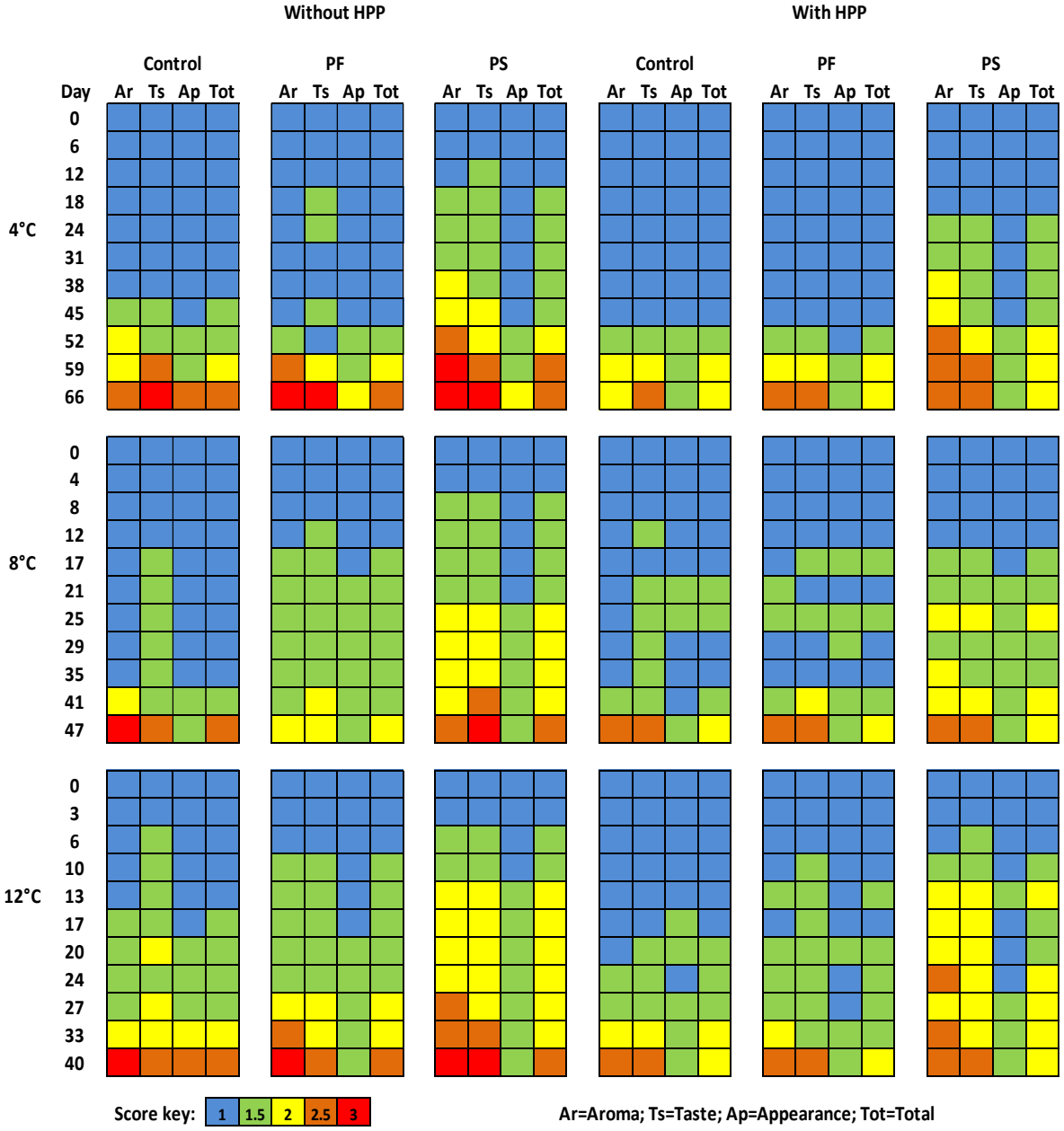

Figure 5. Sensory scores for ham slices treated or not with HPP, during storage at 4, 8 and 12 °C. The "Total" value represents the mean value of the aroma, taste and appearance of each sample rounded to the closest value.

5.4.6. Probiotic Survival and Strain Differentiation in the Ham Slices and Edible Films

As shown previously, Figures 1-3 represent the LAB and TVC counts for the ham slices and the probiotic-supplemented edible films, respectively, during the storage at the three temperatures. Counts of LAB remained high (>10^6 CFU/g) in both ham slices and films, therefore it was crucial to verify the presence of the film-incorporated probiotic strains. A total of 476 isolates (237 from ham samples and 239 from films), were recovered from petri dishes corresponding to the 6^{th} or 7^{th} dilution, and the presence of the incorporated strains was confirmed by PFGE. The results demonstrated that up to 100% of the microorganisms recovered, all belonged to the three probiotic strains that had been incorporated in the films with the distribution of each strain in each case to be presented in Figures 6 and 7 for the ham slices and the edible films, respectively. The design of the experiment was such to have the same initial levels of the three strains in the ready-to-use edible films; however this was not achieved, as it is presented in Figure 7. The initial distribution of the incorporated strains in the edible films before their direct application in ham slices were 26.1% for *Lb. plantarum* B282, 52.2% for *Lb. plantarum* L125 and 21.7% for *Lb. pentosus* L33. Results from the edible films revealed that *Lb. pentosus* L33 was present in the initial films prepared before their application, while it was not detected throughout the storage in cases of 4 °C without HPP treatment and 12 °C for both HPP treated or not. Regarding the other cases, the aforementioned strain was detected in low percentages in the beginning and middle of storage time, whereas it was detected at the end of storage time, only in the case of HPP treatment. Higher percentages of presence in the films, observed for the strain *Lb. plantarum* L125 in all cases, a fact that was confirmed from the high percentage of this strain in the initial film concentration. With regard to the distribution of the

strains in the ham slices, similar results were found with those obtained from the films. The strain *Lb. pentosus* L33 was detected in the beginning of storage in the cases of 8 °C (HPP or not) and in the case of the samples with or without pretreatment using HPP and stored at 12 °C, but with relatively low percentages, 15.4 %, 8.3% and 7.7%, respectively. The strain *Lb. plantarum* L125, was the one with the higher percentage, similar to the results from the edible films and generally it is proven that the strain distribution for each case of the ham samples is similar to that of the edible films.

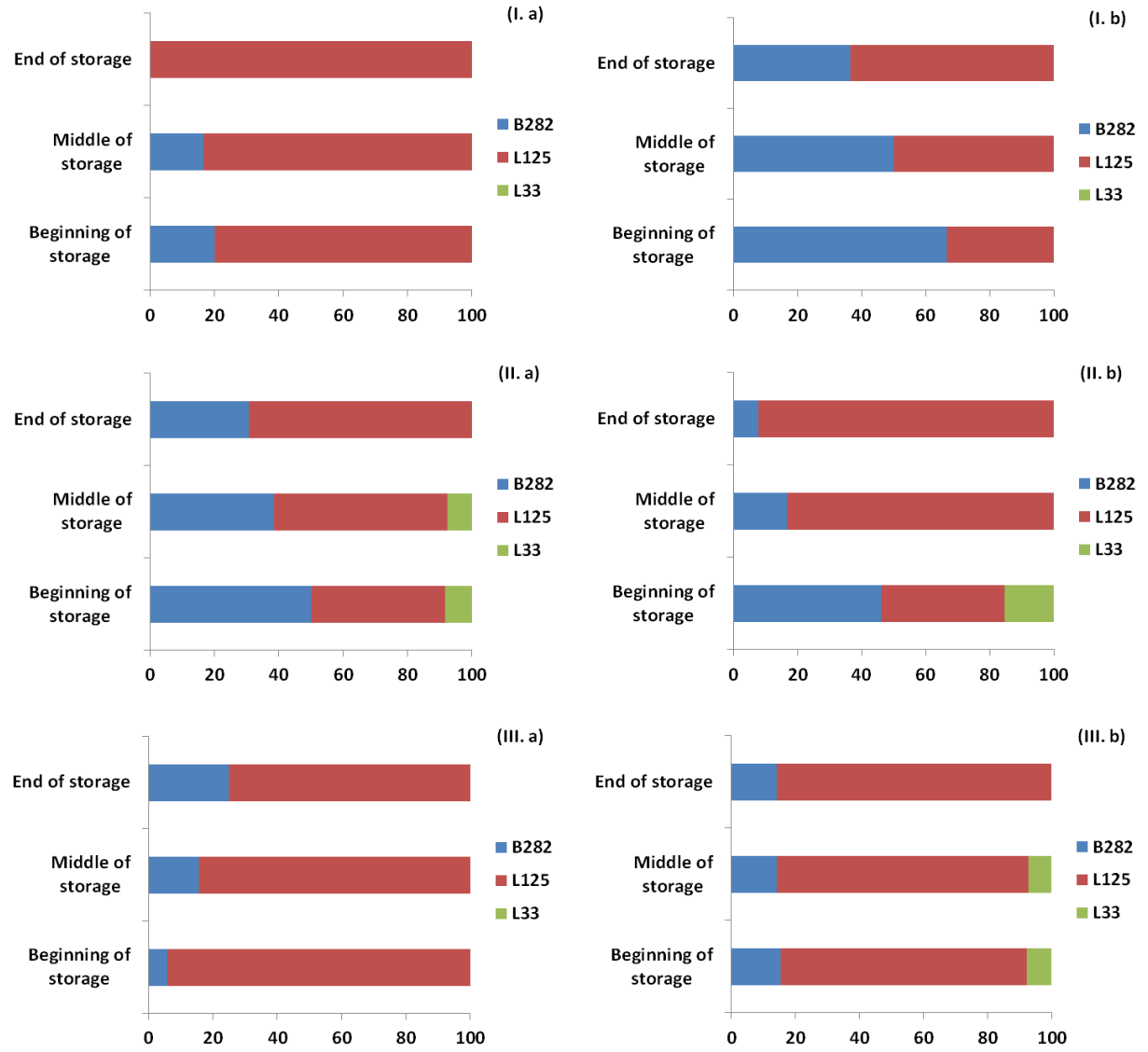

Figure 6. Distribution of isolates (%) of probiotic strains recovered from ham slices in three time points (beginning, middle and end) during storage at: 4 °C (I); 8 °C (II); and 12 °C (III); and (a) without HPP; or (b) with HPP treatment based on the PFGE profiles.

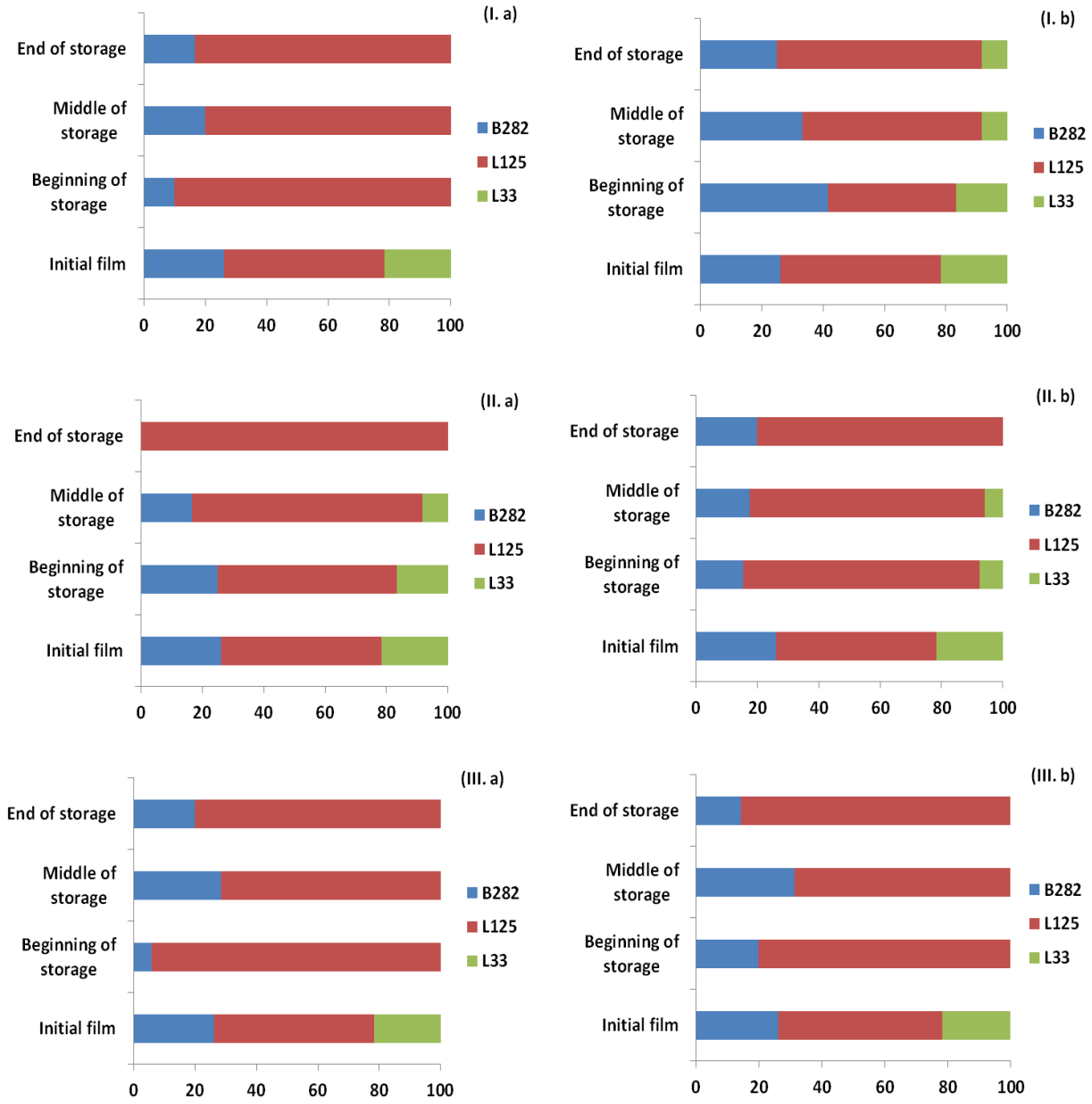

Figure 7: Distribution of isolates (%) of probiotic strains recovered from films in contact with ham slices in three time points (beginning, middle and end) during storage at: 4 °C (I); 8 °C (II); and 12 °C (III); and (a) without HPP; or (b) with HPP treatment based on the PFGE profiles.

5.5. Discussion

Ham is among to the most popular RTE meat products and is mainly processed thermally during its production. HPP is an attractive preservation technology, and is relatively mild for meat products such as sliced ham, when low or moderate temperature and pressure combinations are applied. The efficacy of this technology has been reported previously for many different products including ham (San Martin et al., 2002; Trujillo et al. 2002; Garriga et al. 2004; Aymerich, Picouet and Monfort, 2008; Marcos et al., 2008; Ferrari, Maresca and Ciccarone, 2010; Vercammen et al. 2011; Han et al. 2011; Chawla, Patil and Singh, 2011; Hereu et al. 2012; Liu et al. 2012; Varela-Santos et al. 2012) . Findings of our study confirmed the hypothesis that HPP can be efficient in reducing the microbial populations in the pressure values tested (500 MPa for 2 min) in cooked ham slices.

Much research, on the other hand, has been conducted regarding novel packaging materials, especially biopolymer edible films, that can be applied in food products to increase the shelf-life or enhance food safety, by possessing antimicrobial substances (Juck, Neetoo and Chen, 2010; Lim, Hong and Song, 2010; Ravishankar et al. 2012; Pattanayaiying, Kittikun and Cutter, 2015; López de Lacey et al. 2012). Recent advances in this field include the incorporation of heat-sensitive bioactive materials, one such example being that of probiotic bacteria, although to the best of our knowledge there is scarcity of studies (Soukoulis et al. 2017; Soukoulis et al. 2014; Kanmani and Lim, 2013). None of the latter studies examined the efficiency of the

probiotic supplemented edible films on meat products, and thus the data obtained from this work are of major importance for the practical potential of applying probiotic cultures on cooked meat products.

The probiotic-supplemented films, were found to be efficient for probiotic delivery on the sliced ham, regardless its previous HPP treatment, in the desirable levels (>10^6 CFU/g). This is crucial, since probiotic beneficial effects are dose dependent and the suggested daily intake ranges from 10^6 to 10^9 viable cells. Based on the capacity of producing biomass of probiotic cultures and the required amount of these cultures per product/unit/package, it is anticipated that the augmentation of the price of package is negligible. As such, we believe that the proposed technology is sustainable and cost effective for the food industry. From the microbiological results, it was observed that the major spoilage organisms of ham products were LAB, since no other microbial populations were detected during the experiment. Such results are justified due to the packaging under vacuum that was used. LAB in samples with PS films after the first sampling point exceeded the level 10^6 CFU/g in all temperatures and their levels remained high until the end of shelf-life. It is notable that in contrary to non-treated samples (Figure 1), in HPP-treated samples, LAB population in PS films is lower than in PF, suggesting possible competitive effect of probiotic on natural LAB. Together with the microbiological analyses of the ham samples, analysis was also performed to the PS films that were in contact with the ham to monitor the possible reduction in the viability of the incorporated probiotic bacteria. The obtained results were promising, since only 1-1.5 log reduction was observed, regardless the storage temperature. Such results are in agreement with those of a previous study (Gialamas et al. 2010), where the viability of *Lactobacillus sakei* remained almost unaffected when it was incorporated into sodium-caseinate edible films and stored at 4 and 25 °C for 30 days. Another study with

similar results regarding the viability of the incorporated probiotic bacteria was that of Lopez de Lacey (2012), in which the gelatin edible films were stored at 2 ±1 °C and the viability of the tested *Bifidobacterium bifidum* and *Lactobacillus acidophilus* was fairly constant throughout storage time. However, to confirm the probiotic strains presence in both ham samples and edible films, PFGE was performed and strain distribution was assessed in specific time points for the three temperatures examined. All the isolates belonged to the incorporated probiotic strains, although analysis revealed that the performance of the inoculated probiotic strains in ham slices and edible films was strain specific. These results highlight that the selection of a probiotic strain to be incorporated, should be thoroughly tested in order to achieve its successful delivery to the food products.

Results from pH indicated that ham samples with PS films had significantly (p<0.05) lower values compared to the control ones and this can be associated with the high LAB population of these samples. When HPP was applied, the pH values of the aforementioned samples were low, but higher than before and this can be explained due to the changes that occur after the HPP process in meat samples. In a previous study of Souza et al. (2012), higher values in the pH of pork meat were observed after HPP with a difference of 0.46 compared to the control samples. It needs to be noted, however, that by the 2nd sampling day at all temperatures, PS samples had an unexpected low pH (<5.5) for cooked meat products, contrary to PF films with the natural LAB present, without though having any sensory impact (Figure 5). Color values were determined in our study, since it is an important parameter that affects the evaluation of the ham quality by consumers. Color index was affected by the application of edible films (p<0.05) and was slightly increased in all storage temperatures, whereas HPP treatment did not affect the color significantly (p>0.05). Many parameters contribute to the final color of the HPP treated

ham such as the fat and water content, the salt level, the applied values of pressure as well as the duration of the treatment. In other studies, HPP application, affected the color of the treated meat samples (Bak et al. 2012), while in another study no differences were mentioned (Souza et al. 2011).

Regarding their sensorial attributes, ham slices packed with PS edible films were evaluated as more acidic in aroma and taste than the control ones, as it can be assumed also from the pH values. Similar sensorial results were observed in the samples treated with HPP, but, in this case, they were characterized as less acidic. The intense acidification that occurred in these samples due to the inoculated probiotic strains is the major drawback of such film applications. The appearance, on the other hand, was always evaluated with similar values to the control samples and regardless the HPP treatment. In general, HPP resulted in producing samples with better sensorial characteristics during the storage time, even for the temperatures of 8 and 12 °C in comparison with the untreated ones. Sensory acceptance of HPP treated meat products in general, depends on color, texture, aroma and taste modifications induced by the process. Problems of sensory acceptance occur with raw products, mainly because of visible color changes. Thermal processed or cured products such as ham are less modified by pressure (Simonin, Duranton and de Lamballerie, 2012). Results obtained from the sensory assessment contribute to the better understanding of the effects of the different technologies studied in this work and the consumers should always consider such results since they are crucial for the future acceptability of the products.

5.6. Conclusions

The application of sodium-alginate edible films supplemented with probiotic bacteria as a vehicle for delivery in RTE ham slices was found to be successful. The new products of ham had

different organoleptic characteristics from the control samples, with the major difference being their acidic aroma and taste, while the appearance remained the same. HPP application was efficient in reducing the microbial population levels prior to edible film application and it did not affect negatively the sensory attributes of the products. Furthermore, HPP increased the shelf-life of the ham slices in all cases tested, exhibiting at the same time points better organoleptic attributes.

Acknowledgements

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CHAPTER 6

Antimicrobial activity of oregano essential oil incorporated in sodium alginate edible films: Control of *Listeria monocytogenes* in ham slices treated with High Pressure Processing

In this chapter, the effect of oregano essential oil incorporated into edible films was examined in sliced ham contaminated with *Listeria monocytogenes*. This work has been included in the following publication:

Pavli FG, *Argyri A.A., Skandamis P., Nychas G-J.E, Tassou C, Chorianopoulos N. (2019). Antimicrobial activity of oregano essential oil incorporated in sodium alginate edible films: Control of Listeria monocytogenes in ham slices treated with High Pressure Processing. Materials, 12 (22), 3726.*

The supplementary material for this study is provided in the Appendix I.

6.1. Abstract

The aim of the study was to evaluate the efficacy of oregano essential oil (OEO) incorporated in Na-alginate edible films when applied to sliced ham inoculated with a cocktail of *Listeria monocytogenes* strains, with or without pretreatment by high pressure processing (HPP). Microbiological, physicochemical and sensory analyses (in *Listeria*-free slices) were performed, while, the presence/absence and the relative abundance of each *Listeria* strain, was monitored by pulsed field gel electrophoresis (PFGE). The OEO incorporation in the films, caused approximately 1.5 log reduction in *Listeria* population at 8 and 12 °C at the end of the storage period, and almost 2.5 log reduction at 4 °C. The HPP treatment caused 1 log reduction to the initial *Listeria* population, while levels kept on decreasing throughout the storage for all the tested temperatures. The pH of the samples was higher in the cases where HPP was involved, and the samples were evaluated as less spoiled. Furthermore, the presence of OEO in the films resulted in color differences compared to the control samples, whilst the aroma of these samples was improved. In conclusion, the combined application of HPP and OEO edible films on the slices, led to a significant reduction or absence of the pathogen.

6.2. Introduction

Listeria monocytogenes is a widespread food contaminant of major safety concern especially in ready-to-eat (RTE) food products. In 2017, 2480 cases of listeriosis were reported in Europe, with 1.8% overall occurrence of *Listeria monocytogenes* in RTE meat products (EFSA Report, 2018), although other pathogenic bacteria such as *Salmonella*, Shiga-toxin producing *E. coli* and *Staphylococcus aureus*, could also be found in such products. *Listeria monocytogenes* is a psychrotrophic bacterium and has the ability to proliferate at various temperatures and environmental conditions (Dussault, Dang and Lacroix, 2016). This pathogen can spread within food processing plants via raw materials, equipment and human activities, and is able to adhere to many food contact surfaces. Furthermore, the prevalence of *Listeria monocytogenes* in RTE products which are preserved under refrigeration such as deli products, is of major concern due to the fact that are generally consumed without further processing (Zilelidou, Manthou and Skandamis, 2016).

RTE meat products that have undergone thermal treatment during processing are usually deemed safe and free of pathogens (Liu et al. 2012). Among RTE meat products, sliced ham remains on demand, due to consumers' modern lifestyle. Although ham is a thermally-processed product, other activities such as slicing and handling considerably increase the likelihood of post-processing contamination. Consequently, ensuring safety during the entire shelf-life, without adding or increasing food additives or preservatives is a challenge for food manufacturers worldwide (Ramaroson et al. 2018). On the other hand, particularly for sliced ham, the quality can be easily compromised, due to the development of off-aromas, rancidity, and issues related to the appearance of the product (Amaro-Blanco et al. 2018). Such issues have encouraged research

in new technologies for RTE products in order to inhibit microbial growth, while ensuring quality, freshness and nutritional value (Odila Pereira et al. 2018).

Several methods have been investigated to extend shelf-life or decontaminate food products. Non-thermal alternative processing technologies have been widely accepted throughout the food industry (Varela-Santos et al. 2012). Especially for sliced cooked meat products a promising technology is high pressure processing (HPP). HPP is a processing technique, in which microorganism inactivation occurs with the use of high pressure; usually above 300 MPa, while the processing temperature does not increase beyond 40 °C (Varela-Santos et al. 2012; Bak et al. 2012). HPP causes destruction of microbial vegetative cells and enzyme inactivation, without adverse effects on the sensory characteristics of the product (Hugas, Garriga and Monfort, 2002). However, the efficacy of the treatment depends on the applied pressure, the time of exposure and the treatment's temperature (Bak et al. 2012; Hugas, Garriga and Monfort, 2002). Details regarding the technology of HPP for meat products have been reported previously (Hugas, Garriga and Monfort, 2002; Cheftel and Culioli, 1997; Torres and Velazquez, 2005; Simonin, Duranton and de Lamballerie, 2012). HPP treatment can be satisfactorily combined with new packaging systems or natural antimicrobial compounds in the sense of a "hurdle concept", where all the factors can act synergistically, to reach the objective of minimal processing, without compromising the products' safety (Liu et al. 2012).

Active packaging is a system where the product, the package and the package environment interact with each other (McMillin et al. 2017). Active packaging technologies aim to extend the shelf-life and enhance safety by retarding or even inhibiting the spoilage or the growth of pathogenic microorganisms, and also maintain or improve the properties of the packaged food (EC 450/2009; Kapetanakou and Skandamis, 2016). The mechanism of this type

of packaging is based on the incorporation of active compounds into the packaging material to absorb substances from the food or its environment, or to release agents from the packaging to the food (McMillin et al. 2017; Ribeiro-Santos et al. 2017). Antimicrobial active packaging is a type of active packaging, which has received a lot of attention by both researchers and industries, with numerous commercial applications so far. Antimicrobial substances may be released through evaporation or migrated into food through diffusion and partitioning, directly with application on the food surface or indirectly with their incorporation into carrier materials such as coatings or edible films (Kapetanakou, Karyotis and Skandamis, 2016). Other antimicrobial effects can also be provided with this technology through oxygen scavenging systems, moisture absorbing systems, carbon dioxide generation, ethanol generation, and humidity buffering (McMillin et al. 2017). Active packaging and antimicrobial packaging technologies have been reviewed elsewhere in detail (McMillin, 2017; Kapetanakou, Karyotis and Skandamis, 2016; Vermeiren et al. 1999; Coma, 2008; De Azeredo, 2013; Ahmed et al. 2017; Wyrwa and Barska, 2017; Zanetti et al. 2018; Yildirim et al. 2018).

Natural or chemical antimicrobial compounds may be used in antimicrobial active packaging. Due to the fact that the use of chemical substances raises safety concerns to the consumers and efforts have been made to eliminate their use, the application of natural antimicrobial compounds is of great interest (Atares and Chiralt, 2016). Antimicrobial compounds such as bacteriocins, enzymes, ethanol or essential oils, have been studied thoroughly to determine their effectiveness against spoilage and pathogenic microorganisms in food matrices (Skandamis, Tsigarida and Nychas, 2002; Deegan et al. 2006; Kykkidou et al. 2009; Gutierrez, Barry-Ryan and Bourke, 2009; Jayasena and Jo, 2013; Muriel-Galet et al. 2013; Calo et al. 2015). Essential oils (EO) are aromatic natural substances extracted by steam distillation from aromatic

plants, herbs and spices and most of them are classified as generally recognised as safe (GRAS). Many studies are available regarding the *in vitro* antimicrobial properties of certain EOs, with quite low values of minimal inhibitory concentrations (MIC) against pathogens, including *Listeria monocytogenes*. Reports have shown that higher concentrations of EOs are required for food applications compared to laboratory media, due to their interactions with food components (Pesavento et al. 2015). Numerous studies have confirmed that the EO of oregano (*Origanum vulgare*) is very effective against food spoilage and pathogenic microorganisms (Dimitrijevic et al. 2007; Menezes et al. 2018) and it has been used as antimicrobial agent in meat and meat products to control *Listeria monocytogenes* (Pesavento et al. 2015; Tsigarida, Skandamis and Nychas, 2000; Dussault, Vu and Lacroix, 2014; Paparella et al. 2016). The chemical composition of oregano EO includes a mixture of p-cymene, α-terpinene, thymol and carvacrol, with the latter two components being the most important due to their effects on bacterial membranes (Muriel-Galet et al. 2015). Additionally, it has been reported that these compounds have the ability to protect against oxidation processes (Yanishlieva et al. 1999).

It has to be noted that the use of EOs as food preservatives is still limited because of their intense flavour (Kapetanakou and Skandamis, 2016; Atares and Chiralt, 2016; Acosta et al. 2016). An alternative methodology to minimize the sensory effects can be the incorporation of EOs into polymer matrices such as edible films/coatings, leading to the reduction of the organoleptic impact of the substances, while their diffusion to the product can be controlled (Acosta et al. 2016; Ruiz-Navajas et al. 2013). As an edible film can be defined a layer of material that is applied to the surface of a food product and can normally be consumed with it. Edible films can be particularly useful as carriers of essential oils, due to their ability to maintain high concentrations of these substances on the food surface (Oussalah et al. 2007). The

incorporation of essential oils in edible films has a great impact on the control of bacterial growth during the storage of meat and meat products, since contamination usually occurs on the surface of such products (Pattanayaiying, Kittikun and Cutter, 2015). Sodium alginate is a polysaccharide that has received a lot of attention as a film matrix due to its biocompatibility and low toxicity. This material is usually obtained from seaweeds and has the ability to form a strong film that presents better performances when compared to other materials regarding water permeability and mechanical properties (Costa et al. 2018). It is important to be mentioned that alginate has been applied to meat and meat products, since it delays dehydration and eliminates the effects of lipid oxidation (Varela and Fiszman, 2011).

Since sublethally injured pathogens could be more susceptible to antimicrobial compounds such as EOs, the combined application of HPP and other preservation technologies could theoretically increase pathogen inactivation, while at the same time the use of relatively low pressure levels enhances the preservation of the quality characteristics of the product. The objective of the present study was to evaluate the effectiveness of different treatments and their combination to control *Listeria monocytogenes* during storage of vacuum-packed sliced ham at different temperatures (4, 8 and 12 °C). The treatments used were i) application of HPP, ii) antimicrobial packaging using Na-alginate edible films with oregano essential oil, and iii) a combination of these two technologies. The effect of the different treatments on the shelf-life of the product was also assessed.

6.3. Materials and Methods

6.3.1. Bacterial strains and cocktail preparation

Three strains of *Listeria monocytogenes*, kindly provided by the laboratory of Microbiology and Biotechnology of Foods of Agricultural University of Athens (Food Microbiology Culture Collection-FMCC), were used in the present study. The strains, namely FMCC-B-129, FMCC-B-131and FMCC-B-133, were originated from Greek industries. More specifically, the three strains were isolated from RTE frozen minced meat meal, conveyor belt of RTE frozen food and from soft cheese, respectively. The pure cultures were stored in -80 °C in brain heart infusion broth (BHI, LabM, Lancashire, UK) supplemented with 20% (v/v) glycerol. The strains were subcultured twice in BHI broth for 24 h and 18 h at 37 °C, before use. Bacterial cells were harvested separately by centrifugation at 10.000 g for 10 min, washed in 1/4 strength Ringer's solution (this step was performed twice), and finally resuspended in 10 mL of the aforementioned solution. The three-strain cocktail was prepared by mixing the three strains in equal volumes. The final mixture was used to inoculate ham slices at an approximate level of 4 log CFU/g. Inoculum size was confirmed by serial dilutions and plating on Palcam Agar base (LabM, Lancashire UK), for *Listeria* spp., incubated at 30 °C for 48 h.

6.3.2. Ham Slices

Sliced ham in commercial packages was purchased from a local supermarket (Athens, Greece) with dimensions 10 cm×10 cm, 2 mm thick and 20 g approximate weight per slice. Due to a possible contamination, the first 5 slices coming out from the cutting machine were removed completely, as well as all the slices that were in direct contact with the packaging material provided by the supermarket. Ham slices were inoculated with the *Listeria* cocktail at levels of

ca. 4 log CFU/g, and were then processed according to each case scenario. Ham slices were also prepared, without the addition of *Listeria monocytogenes* and were used for sensory evaluation. Three storage temperatures were examined in the present study (4, 8 and 12 °C), while two independent experiments were performed with duplicate samples in each experiment. On each independent experiment, ham slices by different manufacturer were used.

6.3.3. High Pressure Processing (HPP) Treatment

HPP treatment was conducted at the pressure of 500 MPa for 2 min at room temperature (20 °C), when applied according to the case. Both pressure and temperature were monitored and recorded during the process with 1 s intervals, while for the pressurization time reported (2 min) the pressure come-up and release times were excluded. Details regarding the high pressure equipment and operating conditions are available elsewhere (Panagou et al. 2007; Tassou et al. 2008).

6.3.4. Preparation and Application of Na-Alginate Edible Films

The preparation of Na-alginate edible films was performed as previously described by Kapetanakou, Karyotis and Skandamis (2016). Briefly, 2 g of Na-alginate (Applichem GmbH, Darmstadt, Germany) was added gradually in 100 mL of pre-warmed (65 °C) distilled sterile water and under constant agitation for complete dissolution. A total of 1 mL of glycerol was used as a plasticizer in order to improve the film's flexibility. After the addition of the glycerol, the forming solution was kept at 4 °C for approximately 30 min to lower the temperature, until the addition of the essential oil. Oregano essential oil (Ecopharm, Hellas) was added under stable agitation at a final volume of 1% (v/v) in the forming solution, for the preparation of the oregano essential oil-supplemented edible films (OEOS). Na-alginate solution without the addition of

oregano essential oil was also prepared for the oregano essential oil-free edible films (OEOF). Films were produced in square Petri-dishes using 20 g of Na-alginate forming solution and were immediately placed in a laminar flow cabinet at room temperature to dry for 12 h. Once the films were dry, aliquots of 20 mL of 2% (w/v) $CaCl_2$ were added in the Petri-dishes for 1 min, so that the films get detached (*ca*. 0.5 g). After the preparation, the films were stored under refrigeration until their final application (less than 24 h), when needed.

6.3.5. Microbiological Analysis

Microbiological analyses were carried out throughout the storage period in all temperatures tested. Samples of ham slices (10 g) were weighed aseptically, added to sterile 1/4 strength Ringer's solution (90 mL) (LabM, Lancashire, UK), and finally homogenized in a stomacher (Stomacher 400 Circulator, Seward Limited, Norfolk, UK) for 1 min at room temperature. Serial decimal dilutions were prepared in the same diluent and the appropriate dilutions were poured or spread using 1 or 0.1mL, respectively. The agar media used were the following: de Man-Rogosa-Sharpe (MRS) medium (CM1153, Oxoid, Hampshire, UK) for lactic acid bacteria (LAB), overlaid with the same medium and incubated at 30 °C for 72 h; M17 Agar (4017192, Biolife, Milano, Italy) for lactococci/streptococci, incubated at 30 °C for 72 h; Plate Count Agar (LAB149, LabM, Lancashire, UK) for total viable counts (TVC), incubated at 30 °C for 48 h; Streptomycin Thallous Acetate-Actidione Agar (STAA, CM0881, supplemented with selective supplement SR0151, Biolife, Milano, Italy) for *Brochothrix thermosphacta*, incubated at 25 °C for 48 h; Rose Bengal Chloramphenicol Agar (LAB036, supplemented with selective supplement X009, LabM, Lancashire, UK), for yeasts/molds, incubated at 25 °C for 5 days; Violet Red Bile Glucose Agar (CM0485, Oxoid, Hampshire, UK) for *Enterobacteriaceae*, incubated at 37 °C for 24 h; Pseudomonas Agar base (LAB108 supplemented with selective

supplement X108, LabM, Lancashire, UK), for *Pseudomonas* spp., incubated at 25 °C for 48 h; as well as Palcam Agar base (LAB148 supplemented with selective supplement X144, LabM, Lancashire, UK), for *Listeria* spp., incubated at 30 °C for 48 h. In cases where the levels of *Listeria* were below the detection limit of the enumeration method, enrichment was followed according to ISO 11290-1:1996/Amd 1:2004, using the following media: Half Fraser broth (Biolife, Milano, Italy) incubated at 30 °C for 24 h, Fraser broth (Biolife, Milano, Italy), incubated at 37 ° C for 48 h and Agar Listeria Ottaviani and Agosti (ALOA, Biolife, Milano, Italy), incubated at 37 °C for 24 or 48 h.

6.3.6. Isolation of *Listeria* Cells and Strain Differentiation

Ham slices were analyzed at specific time intervals during their storage at the different temperatures and from all the cases tested (with or without HPP treatment, with or without edible films-either OEOF or OEOS). From specific time points, approximately 20% of the colonies were randomly collected from the appropriate dilution on Palcam agar base or ALOA, after the enrichment step. The isolates, after checked for their purity, were stored in -80 °C in BHI broth, supplemented with 20% (v/v) glycerol. Before further analysis, each isolate was grown twice in BHI broth at 37 °C for 24 h. For *Listeria monocytogenes* typing, pulsed field gel electrophoresis (PFGE) was used according to Kagkli et al. (Kagkli et al. 2009). The restriction enzyme *Apa*I (10U) (New England Biolabs, Ipswich, MA, USA) was used in line with the recommendations from the manufacturer for 18 h. After the digestion step, restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris-Borate buffer on a CHEF-DRIII (BIO-RAD, Hercules, CA, USA) equipment with the following running parameters: 6 V cm^{-1}, 1 s initial switching time, 40 s final switching time and 18 h total run at 14 °C. The obtained restriction profiles were then compared to the PFGE fingerprints of the inoculated *Listeria* strains.

6.3.7. pH and Color Determination

The pH value of the samples was recorded with a digital pH meter (HI 2211 pH-ORP Meter, HANNA Instruments, Woonsocket, RI, USA), during storage at different temperatures at different time intervals. After finishing the microbiological analysis, the ham homogenate (stomacher homogenate) was used to measure the pH of the samples. The color of the ham samples was evaluated by taking at least five random readings from the surface of the different samples using a Minolta Chroma Meter fitted with CR-300 measuring head (Minolta, Osaka, Japan). Measurements of the instrument were standardized with respect to a white calibration plate, every time before use. The CIE (Commission Internationale de l'Eclairage), L^*, a^*, b^*, colorimetry system was used for color determination with L^* representing lightness, a^* representing redness and b^* representing yellowness. All of the measurements were collected from areas on the ham surface without visual excess fat, and the values were recorded for C* (chroma) calculation using the following equation: $C^* = (a^{*2} + b^{*2})^{1/2}$.

6.3.8. Sensory Evaluation

Sensory evaluation of ham slices (non-pathogen inoculated samples) was performed during storage at all temperatures, according to Gill and Jeremiah (Gill and Jeremiah, 1991), while further details regarding the design of the sensory evaluation were previously reported (Pavli et al. 2017). Briefly, a trained sensory panel of 5 members was used and sensory analysis was performed at the same time intervals with the microbiological sample points. The same 5 trained members were used throughout the sensory evaluation and were always blinded to the tested sample. Sensory evaluation was conducted under artificial light in individual booths in a sensory analysis room allocated in the Institute of Technology of Agricultural Products of Hellenic Agricultural Organization-DEMETER. The selected descriptors were based on the

perception of aroma, taste and appearance. Each attribute was scored on a three-point hedonic scale ranging from 1 (fresh) to 3 (unacceptable), since the aim of the sensory evaluation was the detection of changes with regards to aroma, taste and appearance that are related to spoilage. Intermediate sensory qualities were attributed to scores of 1.5, 2 and 2.5, while scores > 2 indicated the end of shelf-life and classified the product as spoiled. A total value was calculated as a mean value of the aroma, taste and appearance, rounded to the closest value.

6.3.9. Statistical Analysis

All experiments were carried out in duplicate with two independent batches of ham slices each. The results from different treatments were analyzed using multifactor analysis of variance in order to test the effect of independent main factors (treatment, bacterial populations, pH, color, sensory scores) on the dependent variable (*Listeria monocytogenes* counts). Means were compared with Duncan post-hoc tests and differences were considered as significant at a 5% level. The differences regarding the factor "pressure" were evaluated using the student T-test to compare the means of the two different groups (with and without HPP treatment). The statistical analysis was performed with IBM®SPSS®Statistics for Windows software, Version 24.0 (IBM Corp., New York, NY, USA).

6.4. Results

6.4.1. Microbiological Results

Microbiological analysis was performed in all samples for each treatment and the results are presented in Figures 1-3, for the storage temperature of 4, 8 and 12 °C, respectively. The initial population of ham was 2.51 ± 0.67 for LAB, 3.15 ± 0.11 for lactococci/streptococci and 3.19 ± 0.09 log CFU/g for TVC. When pressure was applied the initial population of ham

decreased to 1 log CFU/g for LAB, lactococci/streptococci and TVC. As shown in Figures 1-3, all bacterial populations increased in counts during the storage in all temperatures. However, in the cases of HPP treatment, all microbial groups reached their maximum population in later time points depending on the storage temperature. In general, differences were observed between the counts of the bacterial populations in some of the cases, with the lowest counts exhibited by the LAB, while counts of lactococci/streptococci and TVC were very similar throughout the storage period. The application of HPP treatment resulted to intense variations in the standard deviations at each time point. Furthermore, slightly lower bacterial counts were observed in the case of OEOS edible films application. LAB, lactococci/streptococci and TVC were affected significantly ($p < 0.05$) by "pressure" and "time" at 4, 8 and 12 °C. The factor "treatment" affected significantly ($p < 0.05$) the LAB and lactococci/streptococci at 4 °C, but not the TVC counts ($p > 0.05$). Differently, all bacterial counts were affected ($p < 0.05$) by "treatment" at storage temperatures of 8 and 12 °C. When OEOS edible film was used, the bacterial counts of all populations were significantly lower ($p < 0.05$), regardless the storage temperature. It has to be noted that in every sampling point, other microbial populations such as *Brochothrix thermosphacta*, *Pseudomonas* spp., *Enterobacteriaceae* and yeasts/molds were always below the detection limit (<1 log CFU/g).

Growth curves of *Listeria* are presented in Figure 4, for every treatment and storage temperature. The initial inoculum of *Listeria* was 3.91 ± 0.11 log CFU/g in the ham slices, with the application of HPP treatment reducing it to 2.74 ± 0.65 log CFU/g. For the cases without HPP treatment, *Listeria* mean counts were higher ($p < 0.05$) compared to the samples with OEOS edible films for the three temperatures. *Listeria* growth was not observed in any case throughout the storage time. Additionally, the application of HPP treatment, led to a decrease in *Listeria*

counts in undetectable levels, before or at the end of the shelf-life, while the combination of HPP and OEOS edible films led to such decrease far before the end of the shelf-life at all storage temperatures. All the factors; “pressure”, “treatment” and “time” were found to affect significantly ($p < 0.05$) the counts of *Listeria monocytogenes* at all storage temperatures. Regarding the factor “treatment” lower mean values were observed for the case of OEOS edible films application. However, slightly higher mean counts of *Listeria* were observed in the control case, compared to the case of OEOF edible films application.

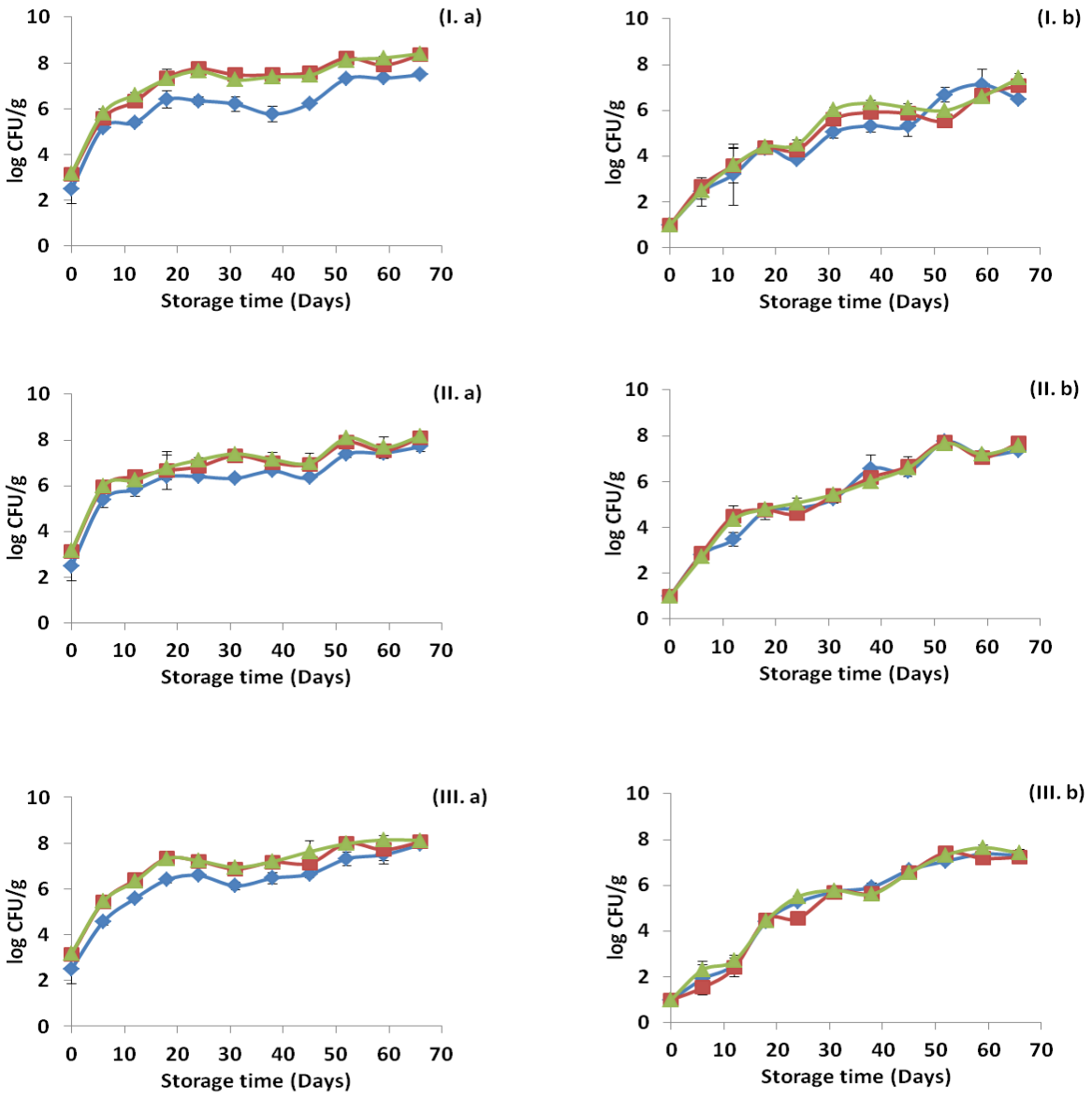

Figure 1. Growth curves of the different bacterial populations in ham stored at 4 °C for the control samples (**I**), samples with edible film free from oregano essential oil- (OEOF) (**II**) and samples with edible film supplemented with oregano essential oil- OEOS (**III**), without (**a**) and after (**b**) the high pressure processing treatment. (♦) Lactic acid bacteria, (■) lactococci/streptococci and (▲) total viable counts. The bars represent the mean values ± standard deviations.

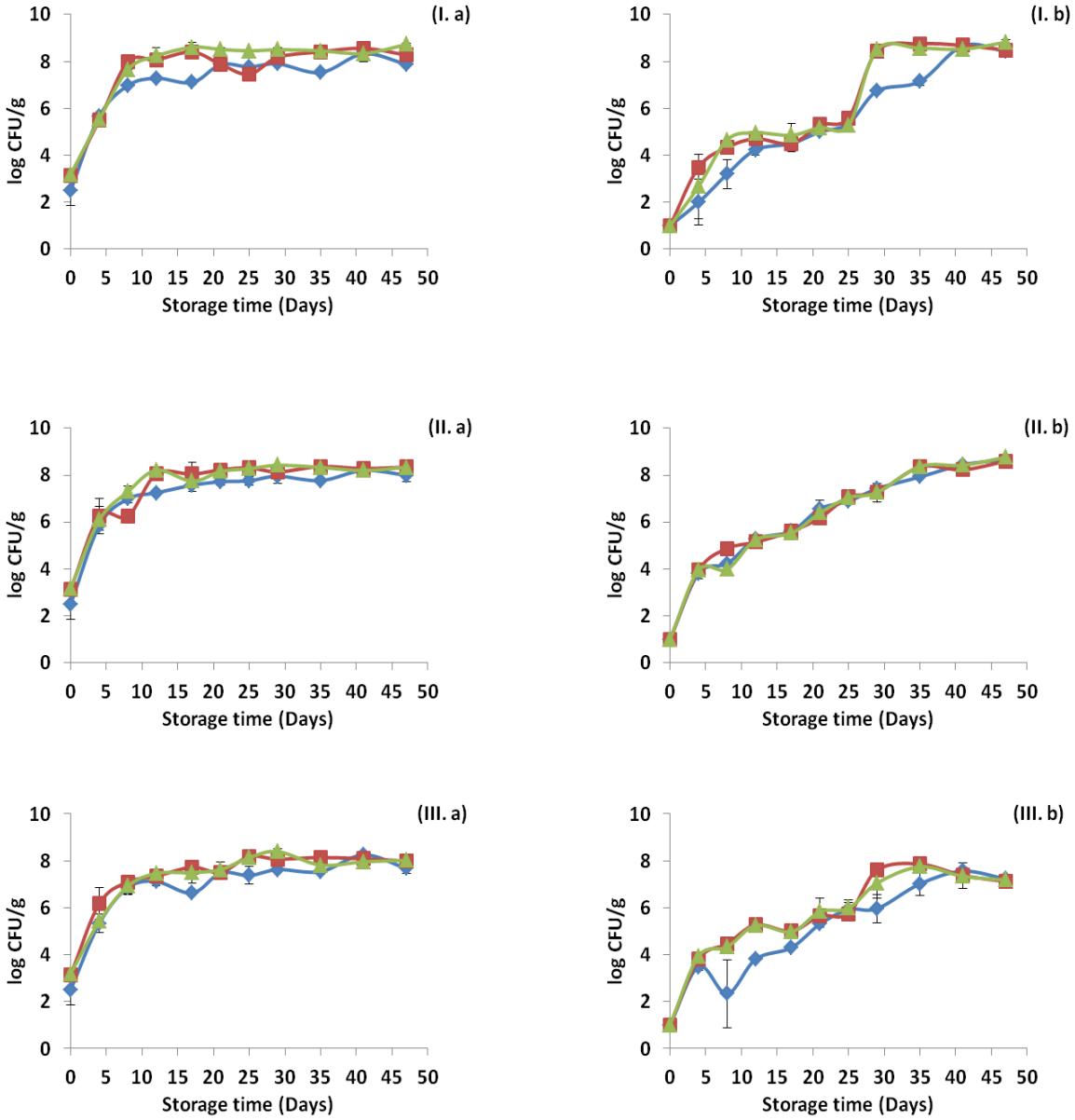

Figure 2. Growth curves of the different bacterial populations in ham stored at 8 °C for the control samples (**I**), samples with edible film free from oregano essential oil-OEOF (**II**) and samples with edible film supplemented with oregano essential oil-OEOS (**III**), without (**a**) and after (**b**) the high pressure processing treatment. (♦)

Lactic acid bacteria, (■) lactococci/streptococci and (▲) total viable counts. The bars represent the mean values ± standard deviations.

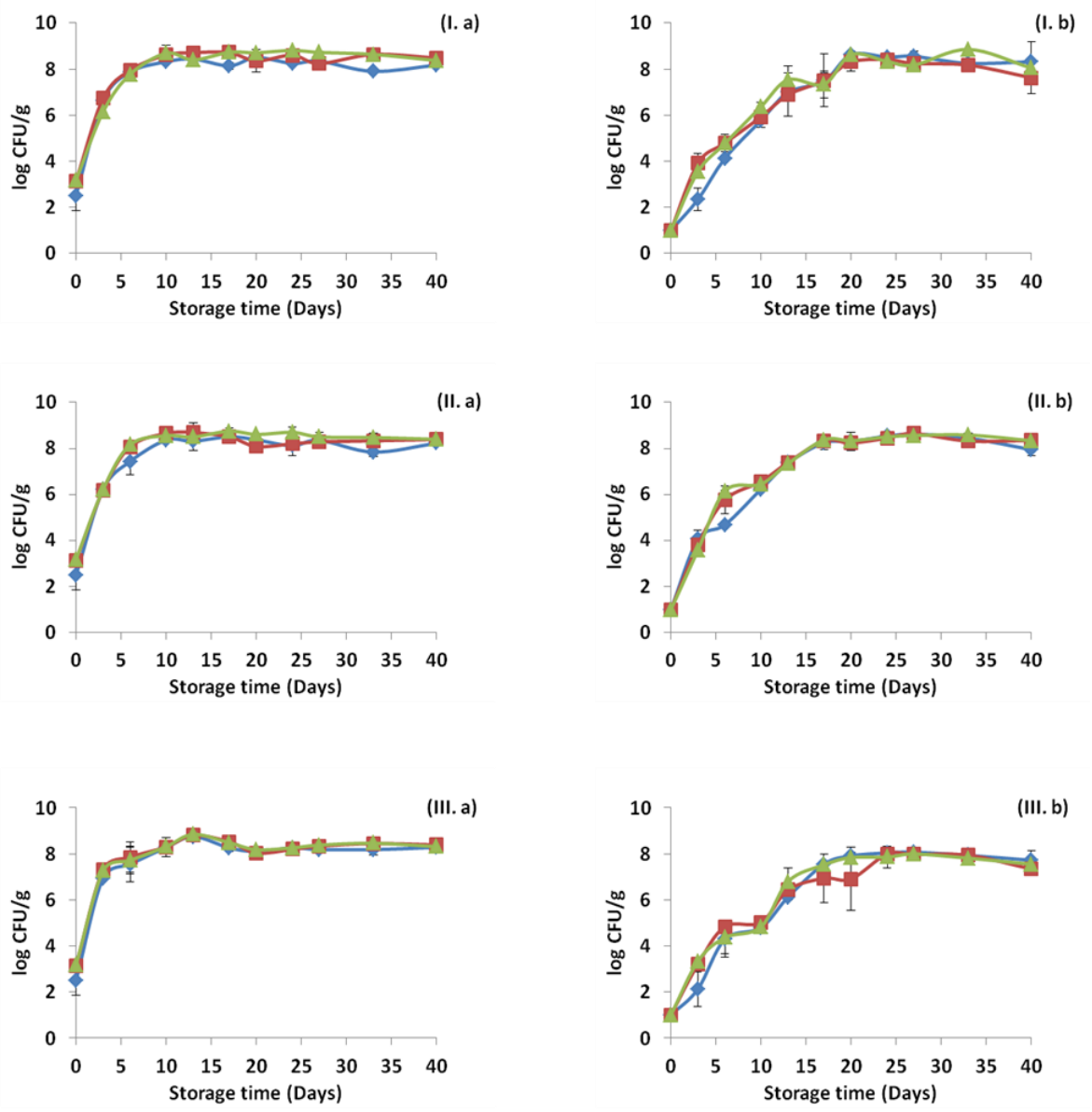

Figure 3. Growth curves of the different bacterial populations in ham stored at 12 °C for the control samples (**I**), samples with edible film free from oregano essential oil-OEOF (**II**) and samples with edible film supplemented with oregano essential oil-

OEOS (**III**), without (**a**) and after (**b**) the high pressure processing treatment. (♦) Lactic acid bacteria, (■) lactococci/streptococci and (▲) total viable counts. The bars represent the mean values ± standard deviations.

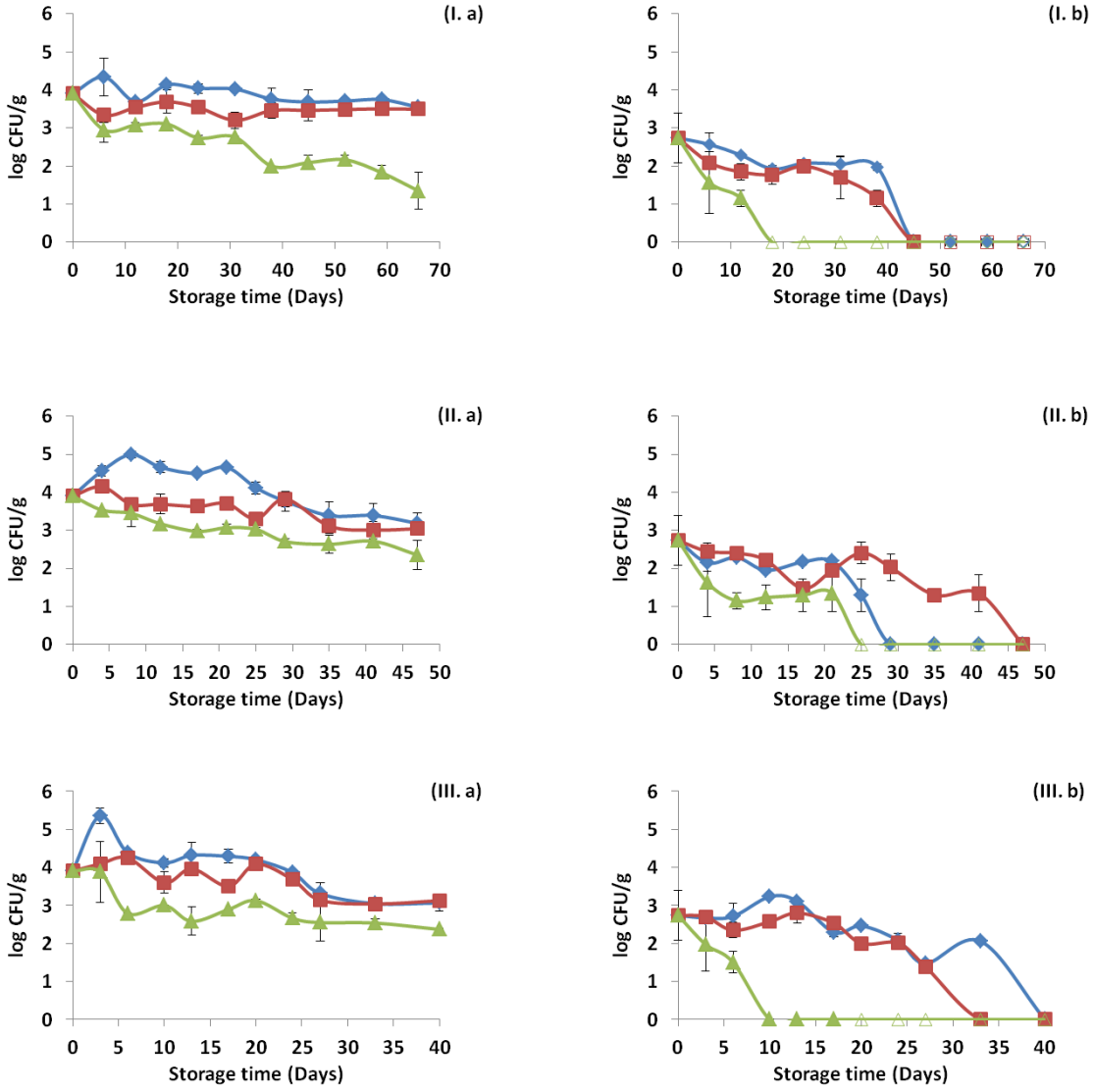

Figure 4. Survival curves of *Listeria monocytogenes* cocktail strains in ham stored at 4 °C (**I**), 8 °C (**II**) and 12 °C (**III**), without (**a**) and after (**b**) high pressure processing treatment. (♦) Control samples, (■) samples with edible film free from

oregano essential oil-OEOF and (▲) samples with edible film supplemented with oregano essential oil-OEOS. Open symbols (◊, □, Δ), indicate absence of *Listeria monocytogenes* after application of the enrichment method. The bars represent the mean values ± standard deviations.

6.4.2. pH and Color Measurements

Figure 5 demonstrates the changes in the pH during storage at the three storage temperatures and for all the cases tested. The pH values for all the cases were decreased by time, and the decrease was more intense in the cases where HPP treatment was not applied. In addition, storage temperature affected the final values of the pH, with the lowest ones to be observed at the storage temperature of 12 °C (abuse temperature). The pH was affected by the factors "pressure", "treatment" and "time" ($p < 0.05$) in all temperatures tested (4, 8 and 12 °C). In Figure 6, the C* values are presented for each case and for the three storage temperatures. The application of OEOS edible films affected the mean values of C* (higher values) and such differences were observed in all temperatures. Differently, the factors "pressure" and "time" did not affect significantly ($p > 0.05$) the C* values at any temperature.

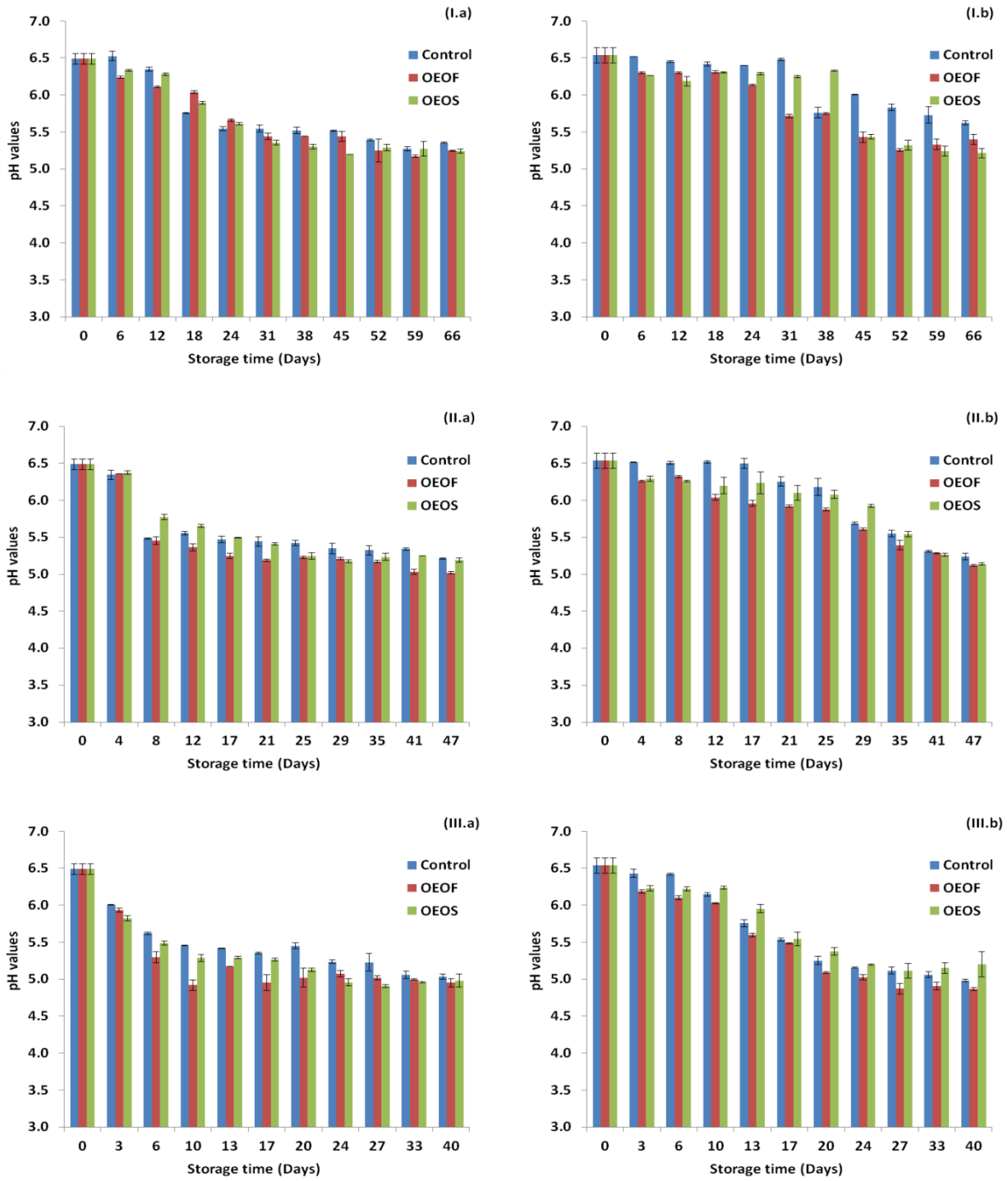

Figure 5. Changes in pH values for ham slices without (**a**) and after (**b**) high pressure processing treatment, during storage at 4 °C (**I**), 8 °C (**II**) and 12 °C (**III**).

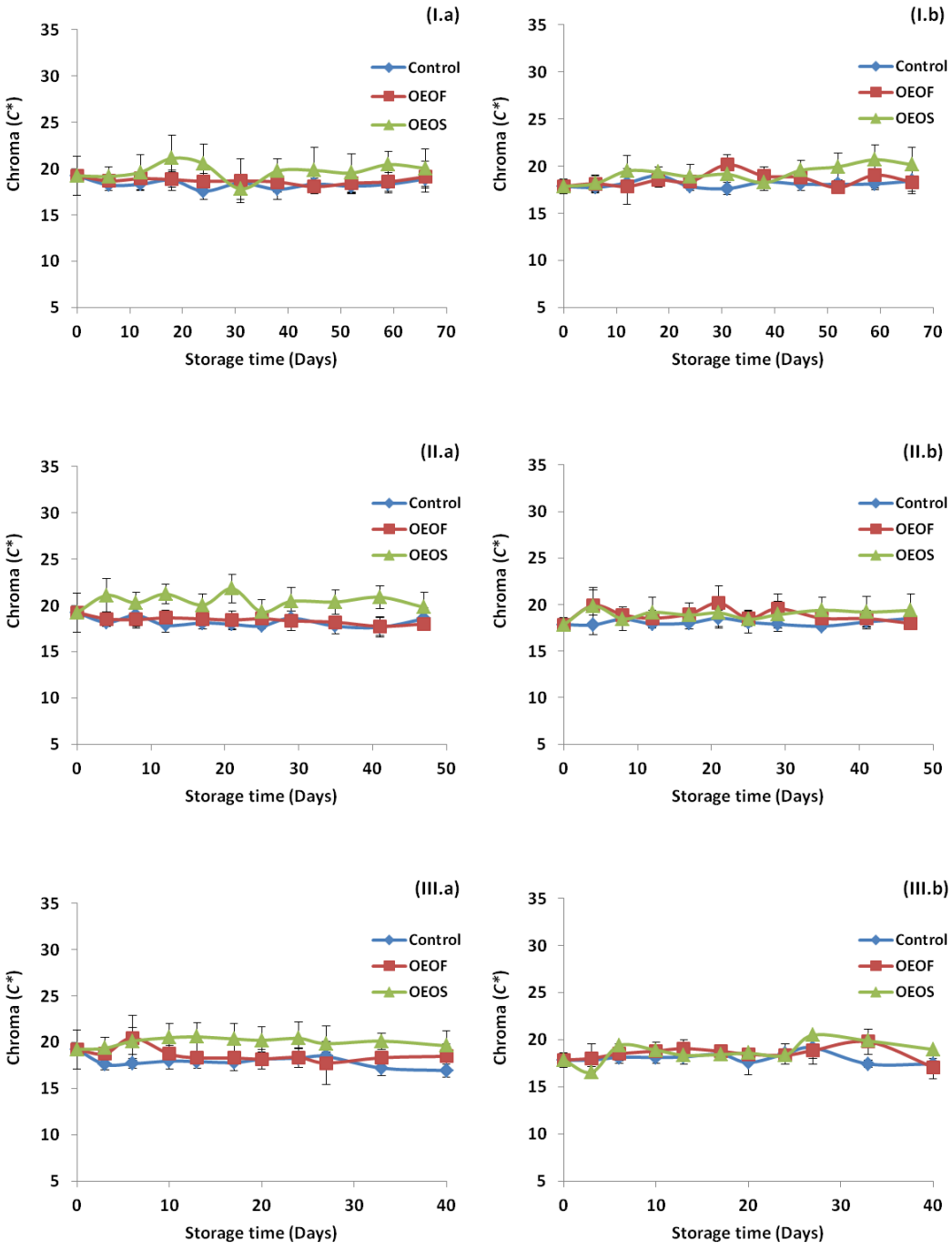

Figure 6. Changes in C* values for ham slices without (**a**) and after (**b**) high pressure processing treatment, during storage at 4 °C (**I**), 8 °C (**II**) and 12 °C (**III**).

6.4.3. Sensory Assessment

The results of the sensory evaluation of the different treatments and cases are presented in Figure 7. The application of HPP affected significantly the sensory characteristics of the ham slices in all storage temperatures and lower scores (more fresh/less spoiled) were given by the panelists compared to the untreated samples for the same time points. The presence of edible films supplemented with oregano essential oil (OEOS), improved the aroma scores given by the panelists in most of the cases. In addition, the appearance was evaluated better (lower values) in the HPP treated samples compared to the non-treated. As "shelf-life" was established the period of time when the ham slices showed acceptable sensory characteristics with regard to aroma, taste and appearance, whereas total scores > 2 specified the end of the shelf-life (Figure 7).

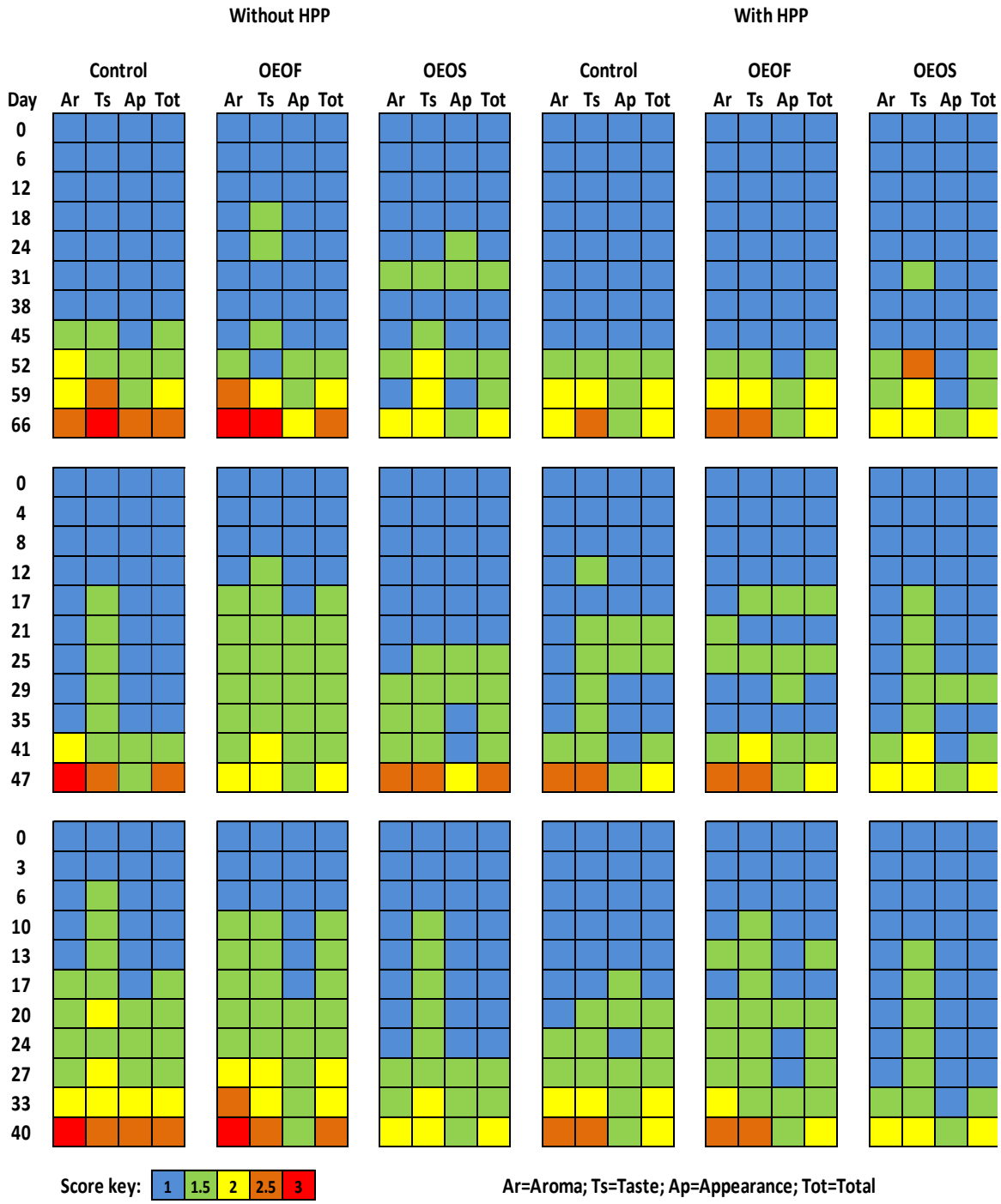

Figure 7. Sensory scores for ham slices without and after the high pressure processing treatment, during storage at 4 (upper line), 8 (middle line) and 12 °C (bottom line). The

"total" value represents the mean value of aroma, taste and appearance of each sample rounded to the closest value.

6.4.4. Monitoring Survival and Strain Differentiation of *Listeria monocytogenes*

As showed previously, Figure 4 represents the *Listeria* counts for the ham slices for each treatment and storage temperature. Isolates of *Listeria* were recovered from petri dishes of Palcam agar base from the highest dilution or after the enrichment step for each time point and treatment and the presence or absence, as well as the percentage of each of the inoculated strains was confirmed with PFGE. The results are presented in Figures 8-10 for the storage temperature of 4, 8 and 12 °C, respectively.

The initial distribution of the inoculated strains was 41.9% for B129, 22.6% for B131 and 35.5% for B133. The application of OEOS films on the ham slices affected the percentages of recovery of the strains, with the strain B129 being the only one surviving at 4 and 8 °C, while at 12 °C for the same treatment all the strains were detected. In general, it has to be noted that the results obtained from the control and OEOF samples were very similar for the samples without HPP treatment.

In the case of the HPP application, the initial distribution of the strains was totally different. Specifically, exactly after the HPP treatment the percentages of recovery in the ham slices were 5.9% for B129, 94.1% for B131, while B133 was not detected at all in the beginning of storage period in any case. The only case when the strain B133 was detected after HPP treatment, was in control samples and in the middle of the storage period at 8 and 12 °C. From the results, it is proven that the strain that managed to be recovered widely after the HPP application was B131. *Listeria monocytogenes* strains were not detected in the cases of the combined application of HPP and OEOS edible films, at 4 °C in the middle and end of storage

period, with the only exception being the case of 8 and 12 °C, when the strain B131 was recovered after enrichment in the middle of storage, but not in the end.

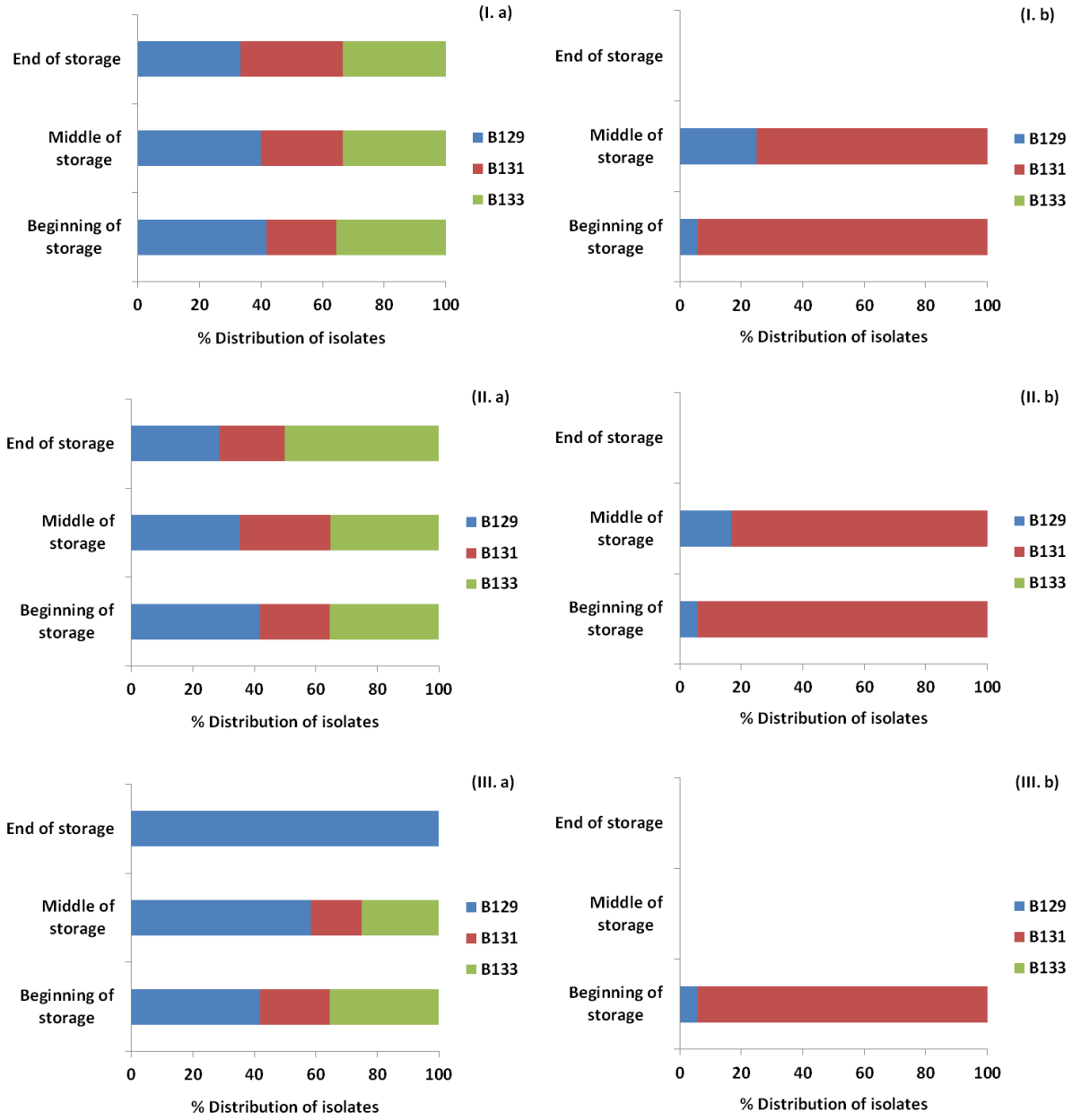

Figure 8. Distribution (%) of *Listeria monocytogenes* strains recovered from ham slices at three time points (beginning, middle, end) during storage at 4 °C based on the PFGE profiles, for control samples (**I**), samples with edible film free from oregano

essential oil-OEOF (**II**), samples with edible film supplemented with oregano essential oil-OEOS (**III**), without (**a**) or after (**b**) high pressure processing treatment.

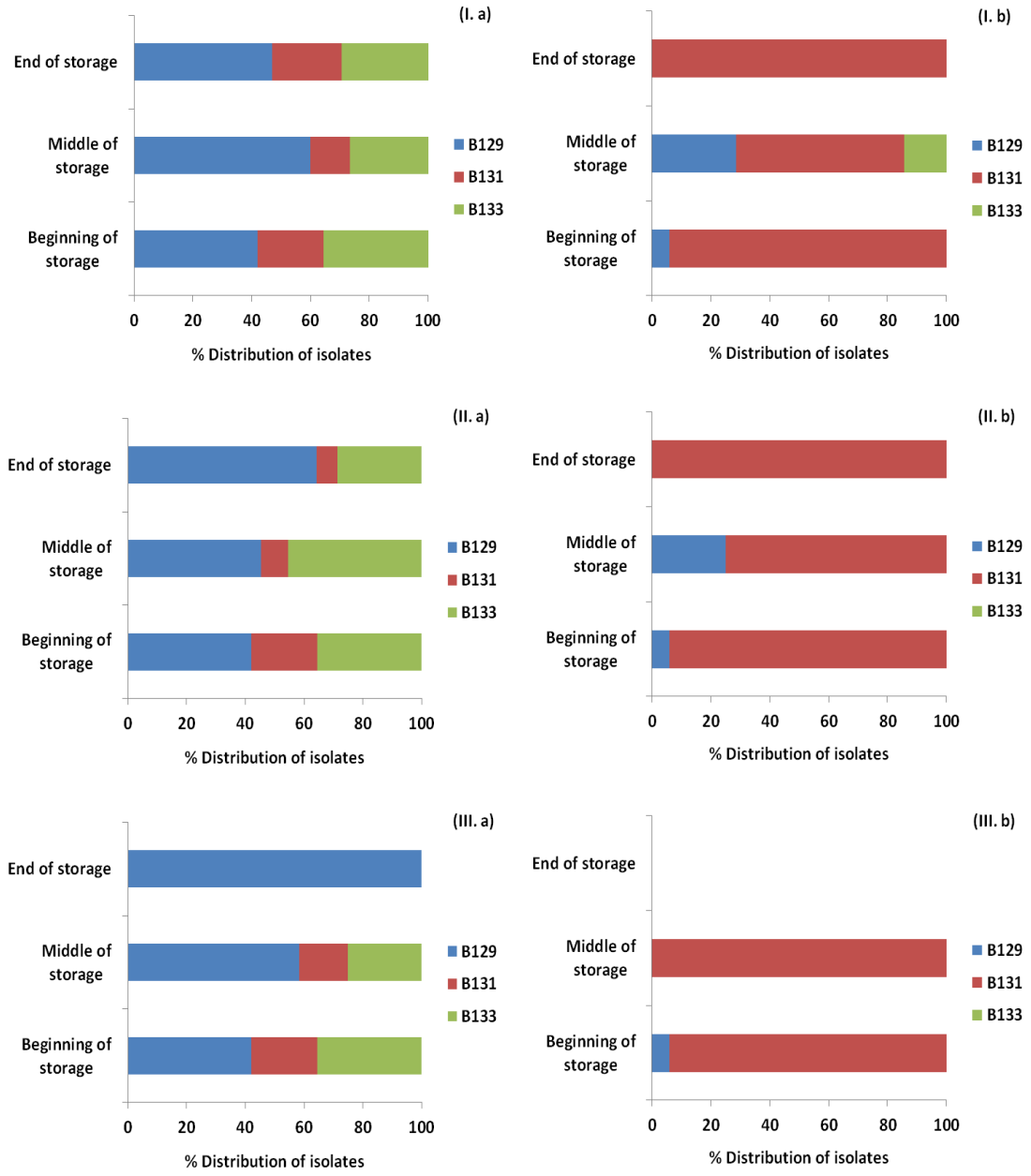

Figure 9. Distribution (%) of *Listeria monocytogenes* strains recovered from ham slices at three time points (beginning, middle, end) during storage at 8 °C based on the PFGE

profiles, for control samples (**I**), samples with edible film free from oregano essential oil-OEOF (**II**), samples with edible film supplemented with oregano essential oil-OEOS (**III**), without (**a**) or after (**b**) high pressure processing treatment.

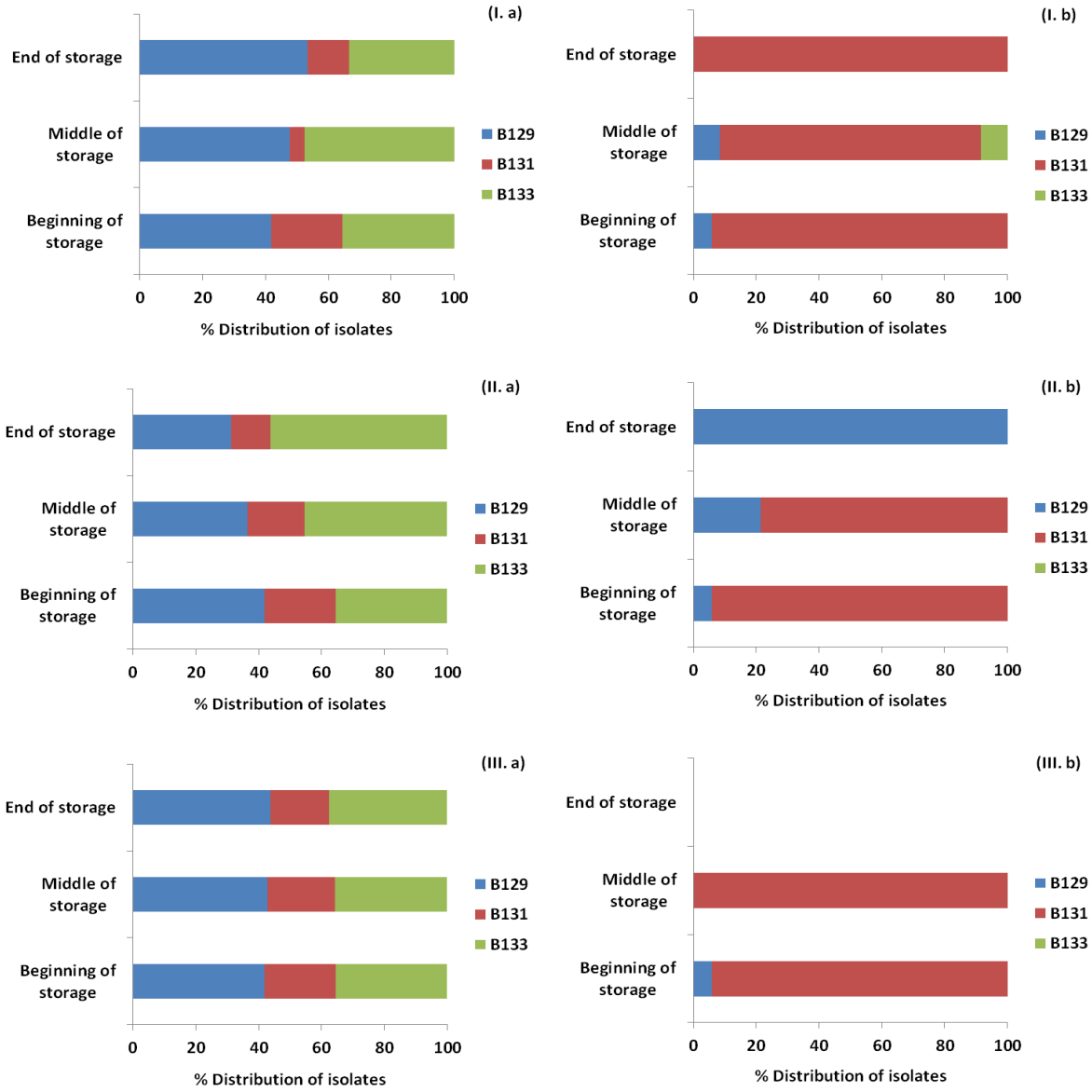

Figure 10. Distribution (%) of *Listeria monocytogenes* strains recovered from ham slices at three time points (beginning, middle, end) during storage at 12 °C based on the PFGE

profiles, for control samples (**I**), samples with edible film free from oregano essential oil-OEOF (**II**), samples with edible film supplemented with oregano essential oil-OEOS (**III**), without (**a**) or after (**b**) high pressure processing treatment.

6.5. Discussion

Sliced ham is one of the most popular and widely consumed RTE meat products in the market. The production of ham normally entails a thermal or curing processing, before it is suitable for consumption. The application of HPP as an alternative method instead of the thermal pasteurization gained a lot of attention the past decade, especially to be used for meat products and ham. A variety of HPP products can be found in USA and Japan, as well as in Spain, which is a pioneer in high pressure processed meat (Simonin, Duranton and de Lamballerie, 2012). There are many studies available in the literature with regard to the efficacy of the HPP application in meat products and the limitations observed for each product category, including ham (Liu et al. 2012; Jofré et al. 2009; Han et al. 2011; Vercammen et al. 2011; Myers et al. 2013; Belletti et al. 2013; Hereu et al. 2014; Pietrasik, Gaudette and Johnston, 2016; Pingen et al. 2016; Rubio et al. 2018).

HPP treatment is deemed to be efficient for pathogen inactivation, especially for *Listeria monocytogenes* in meat and meat products and the findings of this study support this claim. *Listeria* counts were reduced from 3.91 to 2.74 log CFU/g after HPP at 500MPa for 2 min at an ambient temperature. Similar results were obtained by Stollewerk (Stollewerk et al. 2012), investigating the effect of HPP in sliced cured ham inoculated with *Listeria monocytogenes* at a level of 20 CFU/g. In this study, the application of 600 MPa for 5 min at 13 °C caused a 1.1 log

reduction to the initial pathogen level, which remained below detection limit during storage period at 4 °C for 112 days. Interestingly, in the control samples of the same study (samples without HPP treatment) *Listeria* did not exhibit any growth despite the optimum pH, which is in accordance with the findings of the present study. In another study of Jofre et al. (Jofré et al. 2009), HPP at 600 MPa for 6 min at 31 °C, caused a reduction from 3.5 log CFU/g to < 10 CFU/g in cooked ham and dry cured ham, while during refrigeration *Listeria* although was present, remained below the detection limit. In the control samples of the same study, *Listeria* in cooked ham exhibited a mild growth; 1-2 log increase, while in dry cured ham remained in the same levels throughout the refrigerated storage. On the contrary, different observations were made by Koseki, Misuko and Yamamoto (2007) when HPP treatment did not eliminate the presence of *Listeria monocytogenes* in dry cured ham. Specifically, the use of HPP caused an initial reduction to *Listeria* levels from 5 log CFU/g to <10 CFU/g (detection limit), however, the pathogen showed a gradual growth and reached the levels of 7-8 log CFU/g after 70 days of storage at 10 °C. It has to be noted that HPP treatment below 450 MPa did not seem to affect *Listeria* counts and reduction of less than 1 log was observed, regardless of the treatment time and temperature (Bover-Cid et al. 2011).

To enhance the efficiency of HPP treatment on *Listeria monocytogenes* inactivation, other hurdles can also be applied, in combination with HPP, such as the presence of antimicrobials or active antimicrobial packaging, to enhance a pathogen-free product. Several studies are available dealing with the combined application of both technologies with generally promising results for pathogen inactivation in meat products (Marcos et al. 2008a; Marcos et al. 2008b; Jofre, Garriga and Aymerich, 2008; Stratakos et al. 2014; Teixeira et al. 2018). In the present study, oregano essential oil was incorporated into Na-alginate edible films and its efficiency against *Listeria*

monocytogenes and spoilage microbiota was examined with or without the application of HPP. When only HPP was used, an initial decrease of 1.2 log CFU/g in *Listeria* counts was observed, while the use of OEOS edible films led to a reduction of 1.5 logs in the end of storage period at 8 and 12 °C with the highest reduction observed at 4 °C (2.5 logs). The combination of HPP and OEOS films led to the reduction of *Listeria* counts below the detection limit and also in absence of the pathogen from almost the middle of the storage period. These results are similar with those previously reported by Jofré, Garriga and Aymerich (2008), where HPP treatment of 600 MPa for 5 min was combined with the use of the antimicrobials nisin and potassium lactate to inactivate *Listeria monocytogenes* in sliced cooked ham. Consequently, HPP caused a 3 log reduction to *Listeria* counts, while with the use of the antimicrobials no growth was observed throughout the storage time at 1 and 6 °C (<10 CFU/g). In another study, the efficiency of enterocins as natural antimicrobials together with HPP against *Listeria monocytogenes* in sliced cooked ham was investigated. The authors used a pressure of 400 MPa for 10 min and the antimicrobials with a *Listeria* inoculum of 4.5-5.0 log CFU/g and storage temperatures of 1 and 6 °C. The combination of HPP, enterocins and storage at 1 °C led to a reduction of *Listeria* levels, while the use of enterocins was the most effective when combined with HPP compared to the use of lactate-diacetate and HPP (Marcos et al. 2008).

In the present study, *Listeria* growth in ham samples was not observed in any case, including the control samples. A possible explanation can be the levels of the nitrates/nitrites present in the samples. It has to be noted that another critical factor for the growth of *Listeria* is the pH. The pH of the control samples without HPP, was found to be reduced faster compared to the control samples with HPP. The relatively fast drop of the pH, as a result of the increase of the population of the spoilage microbiota (LAB, lactococci/streptococci), could have also affected

the fate of *Listeria*. Generally, the pH values for all the cases were decreased by time, while the decrease was less intense in the cases of HPP application. The storage temperature affected the final pH values, with the lowest ones to be observed at the storage temperature of 12 °C (abuse temperature).

Supplementary in this study, PFGE was used in order to monitor the distribution or presence/absence of the different inoculated strains of *Listeria monocytogenes* in each tested case. Based on the results, it was evident that *Listeria* survival was a strain-dependent attribute. Differences were observed in the survival of each strain due to HPP and OEOS edible films application. Strain B133, which was isolated from soft cheese, was barely detected after the HPP application. Due to the fact that the other two strains (B129 and B131) were detected after HPP and where isolated from RTE food, similarly to the matrix used in the current study, it can be assumed that the source of isolation of each strain is important for the fate of *Listeria* when present in other ecological niches. However, it has to be noted that during the selective enrichment step, the strain competition of *Listeria* could have led to false-negative detection results (Zilelidou, Manthou and Skandamis, 2016).

The color values of the samples were determined in the study, due to their importance on quality assessment of the ham by the consumers. The color values were affected by the application of OEOS edible films ($p < 0.05$) and exhibited higher scores in all temperatures, while HPP treatment had no effect ($p > 0.05$) on the color. In a previous study (Cao, Yang and Song, 2018), it was reported that the incorporation of oregano and thyme essential oils in inulin/chitosan blend films resulted in decreased L^* (lightness) value and increased both a^* (redness) and b^* (yellowness) values of the films. In addition, in another study (Petrou et al.

2012), the addition of carvacrol and cinnamaldeyde into carrot, apple and hibiscus-based edible films against *Listeria monocytogenes* in contaminated ham and bologna, showed differences in color compared to the control films. Different results, however, were observed in a study of Muriel-Galet et al. (2015), when ethylene vinyl alcohol polymer (EVOH) supplemented with oregano essential oil at a 5% w/w dry polymer weight was used and no differences were noted in the color value of the EVOH film, after the addition of the essential oil. Regarding the effect of the HPP on the color of the sliced ham, many reports are available in the literature supporting that HPP can alter the color of the treated meat products, something that was not observed in our study (Hugas, Garriga and Monfort, 2002; Cheftel and Culioli, 1997; Simonin, Duranton and de Lamballerie, 2012). The final color of the treated ham can be also affected by the fat, salt and water content of the product, as well as from the parameters involved in the HPP treatment (pressure applied and duration).

The HPP treated samples were evaluated with overall better scores (lower) with regard to the sensorial characteristics compared to the samples without HPP treatment. It has to be highlighted that the HPP had no negative effect on the appearance of the ham slices. One of the disadvantages in the use of HPP is the potential effect of the pressure in the color and texture of the fresh products. Usually, HPP has low impact on thermal processed or cured products including ham, although the pressure values are considered critical for the extent of the effects (Simonin, Duranton and de Lamballerie, 2012). For the case of the OEOS edible films, the aroma of the ham slices was assessed positively (lower scores/more fresh) and are considered as very promising for future applications. This observation was unexpected, due to the intense flavor of the OEO which could potentially have led to a rejection of these samples from the tasting panel. Pesavento et al. (2015), reported that essential oil concentrations more than 0.5% (v/w) in beef

meatballs, resulted in very intense odor of the essential oil, while concentrations more that 2% (v/w) were deemed as unacceptable by the panelists. In the present study, the intensity of the essential oil flavor was masked by the presence of edible film, which mitigated the adverse effect of the oregano essential oil addition to the ham. In another study (Petrou et al. 2012), the addition of OEO to poultry meat resulted in more acceptable aroma and flavor in comparison with samples without OEO addition. In addition, Qin (Qin et al. 2013), reported that chitosan with tea polyphenols enhanced the aroma and the acceptability of pork patties.

6.6. Conclusions

The application of HPP and OEOS edible films, when used separately on ham slices affected the growth and survival of *Listeria monocytogenes* at 4, 8 and 12 °C. The combined application of the aforementioned treatments was found to be the most successful and resulted in a reduction of the pathogen's counts in a shorter time with the lowest final levels. The HPP did not affect negatively the sensory characteristics of the ham slices, while the addition of OEOS edible films enhanced the aroma of the new products. The results of the study support the initial hypothesis that HPP and the addition of OEOS edible films in RTE meat products can eliminate the growth and even the presence of *Listeria monocytogenes*, and thus constitute an intriguing outcome for the food industry.

The HPP is a technology that could be utilized in the meat industry, as a second step, i.e., after the slicing stage of the ham, in order to eliminate spoilage and prevent the product from post-thermal cross-contamination. The selection of the ideal time-pressure combination is a critical parameter in a successful HPP application, and requires further customized investigation. Furthermore, the application of Na-alginate films supplemented with antimicrobials in sliced ham

could be a good choice providing special organoleptic attributes to the product, whilst prolonging the shelf-life, due to the antimicrobial potential of these films. However, a possible difficulty in the use of edible films is the time required to prepare such films and the addition of the essential oils, even if added in small volumes. Considering these, such a technology would lead to an increase in the cost of the final product, which probably would affect the consumers' options.

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CHAPTER 7

Fourier Transform Infrared Spectroscopy as a prediction tool for the quality of functional foods

In this chapter, the potential of FTIR spectroscopy as a tool to determine the quality of meat products supplemented with probiotics was assessed. The products examined were the ones produced in the previous chapters of this thesis, such as dry-fermented sausages, ham slices with probiotic edible films and ham slices with edible films supplemented with oregano essential oil. Parts of this work has been included in the following publications:

Pavli FG, *Argyri, A.A, Chorianopoulos N, Nychas G-J.E. and Tassou C. (2020). Effect of Lactobacillus plantarum L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. LWT-Food Science and Technology, 118, 108810.*

Pavli FG, *Argyri A, Skandamis P, Nychas G-J.E, Tassou C, Chorianopoulos N (2019). Antimicrobial activity of oregano essential oil incorporated in sodium alginate edible films: Control of Listeria monocytogenes in ham slices treated with High Pressure Processing. Materials, 12, 3726.*

Pavli FG, *Argyri A, Nychas G-J.E, Tassou C and Chorianopoulos N. (2018). Use of Fourier Transform Infrared Spectroscopy for monitoring the shelf-life of ham slices packed with probiotic supplemented edible films after treatment with high pressure processing. Food Research International, 106, 1061-1068.*

The supplementary material for this chapter is provided in the Appendix I.

7.1. Abstract

The aim of the present study was to investigate the potential use of Fourier transform infrared (FTIR) spectroscopy to quantify biochemical changes occurring in: i) dry-fermented sausages, ii) ham slices packed with probiotic supplemented edible films and treated with High Pressure Processing (HPP) and iii) ham slices packed with edible films supplemented with oregano essential oil. Details regarding the data collection and experimental design were presented in the previous chapters. A series of Partial Least Squares (PLS) models were developed to correlate spectral data from FTIR analysis with spoilage during storage at different storage temperatures (4, 8 and 12 °C), according to the product studied. FTIR spectra were collected from the surface of the samples in parallel with microbiological analysis. Qualitative interpretation of spectral data was based on a sensory evaluation, using a hedonic scale. The scope of the modeling approach was to discriminate the products in their respective quality class and additionally to predict the microbial population directly from spectral data. The results obtained demonstrated that the processing of the samples affected the performance of classification in the sensory classes. The performance of PLS regression models on providing quantitative estimations of microbial counts were based on graphical plots and statistical indices (B_f, A_f, RMSE, % PE). The results of this study demonstrated for the first time that although FTIR can be used reliably for the rapid assessment of probiotic foods, additional processes such as fermentation, additives or HPP can affect its performance.

7.2. Introduction

Nowadays, the meat industry is facing new challenges related to processing technologies of meat products together with the constant consumer demands for safer and healthier products. The increased consumers' interest in high quality and fresh meat products with natural flavor and taste has urged industries to focus on the application of alternative or innovative technologies for developing products with higher quality, without compromising product safety (Kapetanakou et al., 2016). Dry-fermented sausages and sliced thermally-processed meat products, such as ham, show the greatest rate of increase in sales within the meat market segment. These products are particularly susceptible to cross-contamination during the pre-packaging stage. Therefore, the need for applying new preservation technologies in such products is of paramount importance (Hugas, Garriga, & Monfort, 2002; Simonin, Duranton, & de Lamballerie, 2012).

Indeed, spoilage status i.e. indication of shelf-life of meat products, is crucial for both retailers and consumers (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Thus, it is essential for the meat industry to use rapid analytical methods or tools for quantification of spoilage detection, regardless of the packaging system the storage condition(s) and even the applied processes used. Assessment of food spoilage is of major importance for both food industries and consumers worldwide. More specifically, for certain food commodities such as meat and meat products characterized with limited shelf-life, there is a need to develop accurate and reliable systems to determine their respective quality and safety. Currently, food safety and quality relies heavily on regulatory inspection and sampling regimes (Nychas, Panagou & Mohareb, 2016).

Nowadays a wide range of audits and inspections in which chemical and microbiological analyses have been proposed to evaluate the quality or safety of raw or processed materials and

food products. New, fast and non-invasive analytical instrumental techniques, such as FTIR, Raman, multispectral imaging sensors etc., that entail the least possible sample pre-treatment, have been applied to evaluate food products (including meat) in terms of quality and/or safety, with the interpretation of the output (huge amount of data generated in each sampling point) being an extremely complex work (Ellis, Broadhurst, Clarke, & Goodacre, 2005; Nicolaou & Goodacre, 2008; Ammor, Argyri & Nychas, 2009; Argyri Panagou, Tarantilis, Polysiou, & Nychas, 2010; Meza-Márquez, Gallardo-Velázquez, & Osorio-Revilla, 2010; Panagou, Mohareb, Argyri, Bessant, & Nychas, 2011; Rohman, Sismindari, Erwanto, & Che Man, 2011; Alexandrakis, Downey, & Scanell, 2012; Zhao, Downey, & O'Donnell, 2014; He, & Sun, 2015; Saraiva, Vasconcelos, & de Almeida, 2017). Indeed, the vast range of information provided by spectral data requires an advanced data analysis approach. This methodology involves the integration of modern analytical platforms with computational and data analytical techniques. Multivariate statistical analyses, such as partial least square (PLS) regression, discriminant function analysis (DFA) and cluster analysis, have led to the development of decision support systems for prompt determination of safety and quality of meat products (Ropodi, Panagou & Nychas, 2016).

Thus, the general purpose of this study was to investigate the potential of FTIR spectroscopy used in conjunction with chemometric analysis as a rapid and accurate method for monitoring the shelf-life of probiotic meat products such as dry-fermented sausages and ham slices regardless the process, type of packaging and storage conditions. To our knowledge this information is lacking in the literature.

7.3. Materials and Methods

7.3.1. Dry-fermented sausages

Details regarding the experimental design of this study are given in Chapter 4.

7.3.1.1. FTIR spectroscopy analysis

Samples were analyzed in parallel with the microbiological and sensory analysis. FTIR spectra were collected using an AMTIR 45° ATR (Attenuated Total Reflectance) crystal, with a HATR sampling accessory, on a PerkinElmer Frontier FTIR Spectrometer equipped with a DLaTGS detector with KBr beamspliter and controlled by PerkinElmer Spectrum v10.4.2 software. FTIR-ATR measurements were collected directly from the samples' surface (25 °C) in the spectral range of 4000-870 cm^{-1}, while the scans per measurement were 10 with a resolution of 4 cm^{-1}. The samples were placed on the AMTIR 45° ATR crystal, so that the surface of the sample was held in intimate contact with the crystal, and pressed with a gripper to have the best possible contact with the crystal surface. Reference spectra were obtained by collecting an air background (spectrum from the cleaned blank crystal) prior to the presentation of each sample replicate. At the end of each sampling, the crystal surface was first cleaned with detergent, washed with distilled water, cleaned with ethanol and finally dried with lint-free tissue at the end of each sampling interval. Two (2) replicate FTIR spectra were collected from each of the 2 different batches. The range of the FTIR spectra used for further analysis was between 4000 and 870 cm^{-1}.

7.3.1.2. Data analysis and Partial Least Squares (PLS) modelling

Data analysis was performed using Unscrambler software (version 9.7, CAMO, Norway). Specifically, FTIR spectral data collected between 4000 and 870 cm^{-1} were initially standardized

using standard normal variate (SNV) transformation. The database was partitioned into a training and a validation dataset. The measurements of the first batch samples for each examined bacterial group, sampling day and storage temperature were gathered in the training dataset and the remaining measurements of the second batch were gathered in the validation set. Subsequently, standardized data were subjected to Partial Least Squares (PLS) analysis for the counts prediction of the different microbial groups and the sensory scores of the different sensory parameters. PLS regression model was evaluated by the number of latent components that were extracted from the data and this was determined by using a leave-one-out cross validation procedure on the training set only. The number of latent components needed to yield the lowest root mean square error (RMSE) of the cross-validation was evaluated for the modelling, examining in parallel the plot of cross-validation residual variance against the number of latent components, with up to 20 components included. In the case that the residual variance no longer decreased with additional components, the number of latent components of the first minimum value of residual variance was selected to avoid overfitting.

7.3.1.3.Evaluation of model performance

Regarding the prediction of the microbial counts in each sample, four performance indices were calculated namely accuracy (A_f) and bias (B_f) factors (Ross, 1996) together with root mean squared error (RMSE), and the acceptable prediction error APE (%APE) (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006; Oscar, 2009). Prediction errors (PE, in log 10) or residuals for individual prediction classes were calculated as PE= O–P, where O is the observed value (log), P is the predicted value (log), $PE < 0$ are fail-safe predictions, whereas $PE > O$ are fail-dangerous predictions. To determine whether individual prediction errors are acceptable, a prediction zone (APZ) (-1.0 log < acceptable $PE < 0.5$ log) was used (Oscar, 2009). The APZ

was twice as wide in the fail-safe direction, since a greater error can be tolerated in the fail-safe direction when a model is used to predict food safety (Oscar, 2009). The percentage of acceptable prediction errors (%APE) in the APZ served as an overall measure of the model's performance. It is worth mentioning that a %PE of >70%, indicated a simulation model that provided acceptable predictions for the test data set (Oscar, 2005). For the prediction of the sensory attributes PLS regression was applied as a first step to predict the scores in a continuous scale of 1-5, as assessed from the panelists. In a second step (classification), each sensory score (actual or predicted) was rounded to the closest class membership in a 0.5 step (i.e. 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0), in order to aid interpretability of the results. The overall correct classification (%) of the models was determined as the number of correct classifications in all classes divided by the total number of samples analyzed (Panigrahi, Balasubramanian, Gu, Logue, & Marchello, 2006).

7.3.2. Ham slices with probiotic edible films

Details regarding the experimental design of this study are given in Chapter 5.

FTIR analysis was performed using a ZnSe 45° ATR (Attenuated Total Reflectance) flat plate crystal on a Perkin Elmer Frontier FTIR spectrometer equipped with a DLaTGS detector with KBr window. The measurements were collected directly from the sample surface at 25 °C. The samples used for FTIR analysis were the same samples used for microbiological analysis. The samples were placed on the ZnSe 45° ATR crystal so that the surface of the ham was held in intimate contact with the crystal and pressed with a gripper to have the best possible contact with the crystal surface. The spectrometer was programmed with PerkinElmer Spectrum v10.4.2 software to collect spectra over the wavenumber range 4000-650cm^{-1}, while the scans per measurement were 10 with a resolution of 4cm^{-1}. Reference spectra were acquired by collecting an air background spectrum from the cleaned blank crystal prior to the presentation of each

sample replicate. At the end of each sampling, the crystal surface was first cleaned with detergent, washed with distilled water, cleaned with ethanol and finally dried with lint-free tissue at the end of each sampling interval. For each case and time interval, two (2) replicate FT-IR spectra were collected from each of the 2 biological replicates of each different batch. The number of FTIR spectra collected were 1416.

7.3.2.1.Data analysis and Partial Least Squares (PLS) modeling

Data analysis was performed using Unscrambler software (version 9.7, CAMO, Norway). FTIR spectral data collected between 4000 and 650 cm^{-1}, were initially standardized using standard normal variate (SNV) transformation. SNV was applied to minimize the effect of baseline shift and other interferences that may impair multivariate calibrations. The database was partitioned into a training and a test dataset. The measurements of the first batch samples for each examined group, sampling day and storage temperature were gathered in the training dataset and the remaining measurements of the second batch were gathered in the test set. Subsequently, the normalized data were mean centered and subjected to partial least squares (PLS) analysis to investigate the relationships between FTIR spectral data and quality class as well as microbiological data. The complexity of the PLS regression model was determined by the number of latent components that were extracted from the data and this was determined by using a leave-one-out cross validation procedure on the training set only. The number of latent components needed to yield the best root mean square error (RMSE) of the cross-validation was chosen for the modeling. PLS regression (PLS-R) models were built for the quantitative analysis of the LAB population and TVC, using FTIR measurements of the samples as input variables and the counts of each microbial group as output variables.

7.3.2.2.Evaluation of model performance

In this study, a 2-step approach was followed to predict the sensory scores. PLS regression was applied as a first step to predict the sensory scores at all stages of spoilage (1, 1.5, 2, 2.5, 3) as assessed from sensory analysis, thereby using a linear continuous scale. In a 2nd step (classification), each sensory score (actual or predicted) was rounded to the closest class membership in a 0.5 step, in order to aid interpretability of the results. The overall correct classification (accuracy %) of the models was determined as the number of correct classifications in all classes divided by the total number of samples analyzed (Panigrahi, Balasubramanian, Gu, Logue, & Marchello, 2006). Regarding the prediction of LAB and TVC in each ham sample, 3 performance indices were calculated namely accuracy (A_f) and bias (B_f) factors (Ross, 1996) together with root mean squared error (RMSE). Moreover, to overcome the limitations attributed to the aforementioned indices that may result in inaccurate assessment of model performance, other expressions for performance were used such as the percentage of prediction error (%PE).

Prediction errors (PE, in $\log_{10}$) or residuals for individual prediction classes were calculated as PE= O-P, where O is the observed value (log), P is the predicted value (log), PE<0 are fail-safe predictions, whereas PE>O are fail-dangerous predictions. To determine whether individual prediction errors are acceptable, a prediction zone (APZ) was used (Oscar, 2009).

-1.0 log <acceptable PE < 0.5 log, where the APZ was twice as wide in the fail-safe direction, since a greater error can be tolerated in the fail-safe direction when a model is used to predict food safety (Oscar, 2009). The percentage of PE (%PE) in the APZ served as an overall measure of the model's performance. It has to be noted that, a %PE of >70% indicated a simulation model that provided acceptable predictions for the test data set (Oscar, 2005).

7.3.3. Ham slices with edible films supplemented with OEO

7.3.3.1.FTIR Analysis

The FTIR analysis was performed as previously described in 7.3.2.

7.3.3.2.Data Analysis and Partial Least Squares (PLS) Modeling

The data analysis and modeling was performed as previously described in 7.3.2.1.

7.3.3.3.Evaluation of Model Performance

The evaluation of the model performance was performed as previously described in 7.3.2.2.

7.4. Results and Discussion

7.4.1. Dry-fermented sausages

In the current study, typical FTIR spectra from 4000 to 870 cm^{-1} collected from both treatments, in the beginning (day 22) and end (day 182) of storage period at 4 °C are presented in Figure 1. Spectral data served as metabolic fingerprints of dry-fermented sausages providing useful information regarding biochemical changes occurring during the storage of the products. As it is shown in Figure 1, a major peak at 3460-3280 cm^{-1} was apparent in the sausage samples due to the presence of O–H stretching and N–H stretching vibrations (Nicolaou, Xu, & Goodacre, 2011; Pedersen, Morel, Andersen, & Engelsen, 2003). A second small peak was observed at 2920 cm^{-1} due to saturated aliphatic C–H stretching (Pedersen et al., 2003), followed by the peak at 2854 cm^{-1} due to CH_2 symmetric stretch (Böcker et al., 2007). Other peaks were observed at 1740 cm^{-1} (C–O peak due to carbonyl group in the ester linkages of fat molecules), 1653 cm^{-1} (amide I group), 1563 cm^{-1} (amide II group), 1470 cm^{-1} (C–H deformation of CH_2, CH_3 asymmetric deformation or bending, CH_2 scissoring or bending), 1440 cm^{-1} (CH_2 bending),

1382 cm^{-1} (amide III bands), 1248 cm^{-1} (amide III group), 1166 cm^{-1} (amines, free amino acids, C–N stretch), 1136–1053 cm^{-1} (complex C–O–C ether stretchings) and 973 cm^{-1} (P–O bond from phosphorus) (Böcker et al., 2007; Ellis, Broadhust & Goodacre, 2004; Pedersen et al., 2003).

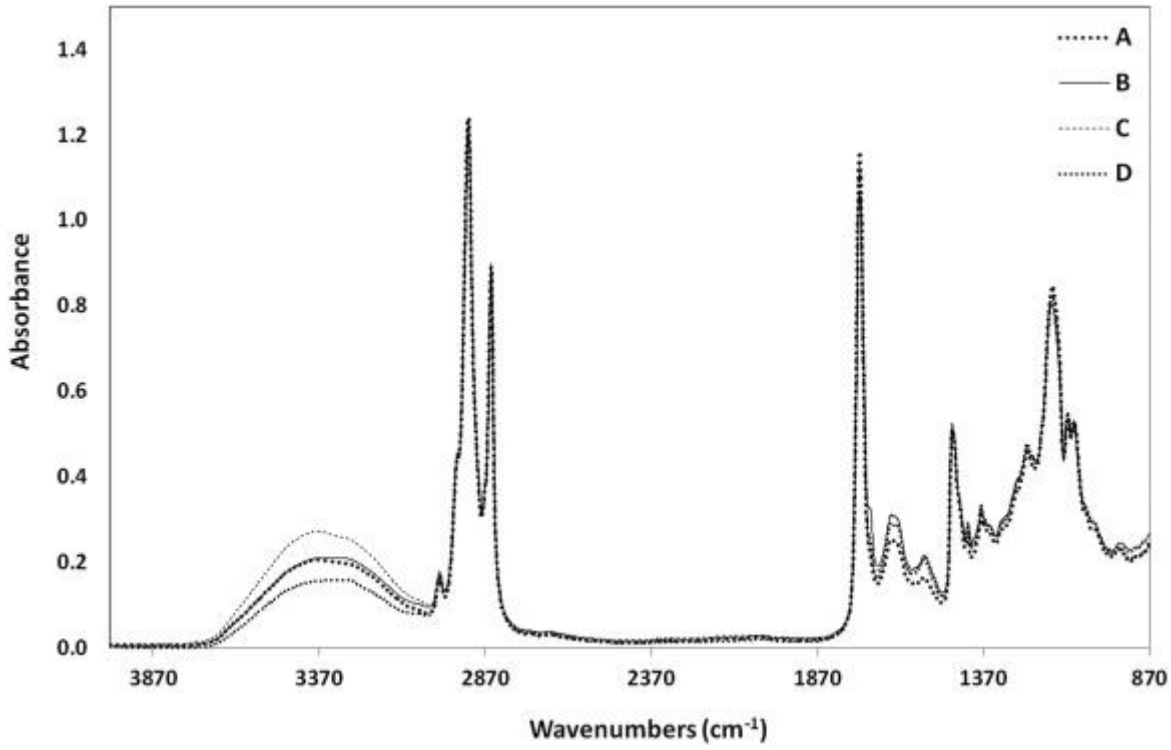


Figure 1. Typical FTIR-ATR spectra of dry-fermented sausages in the range of 4000–870 cm^{-1}, in the beginning and end of storage at 4 °C. A: Control sample in the beginning, B: Control sample in the end, C: Probiotic sample in the beginning, D: Probiotic sample in the end of storage period.

PLS regression models were developed for LAB, mesophilic cocci/streptococci, TVC and staphylococci, using FTIR responses as input variables and the microbial counts as the output ones. These models were built so that the first batch of the experiment was used for the training, while the second was used for the testing process. In general, a relatively good relationship was

observed between counts estimated from FTIR and those reported by the microbiological analysis. The performance indices of the PLS-R models for model testing are presented in Table 1. Overall, the bias factor (B_f) in all microbial groups was close to 1, indicating agreement between observations and predictions, with almost no underprediction ($B_f < 1$) or overprediction ($B_f > 1$). The accuracy factor (A_f) was found to be close to 1, with the average deviation between prediction and observations being 7.8, 7.9, 7.9 and 11.5% for LAB, mesophilic cocci/streptococci, TVC and staphylococci, respectively. Acceptable prediction error (%) values are presented in Table 1, while in Figure 4 (Appendix I), the residuals for the prediction of each of the microbial groups are presented. The acceptable prediction error (APE%), was evaluated as unsatisfactory for PLS-R models to predict the counts of the different microbial groups (66–68% depending on the case), except of the case of LAB (78%) where an %APE>70% was observed. With regard to the qualitative prediction of the sensory scores, the PLS-R models displayed low overall classification rates of 36, 50, 46, 48 and 48% for Overall Appearance, Odor, Taste, Texture and Total Assessment attributes, respectively (Table 2). According to Table 2, the misclassifications of sensory scores were included in the closest sensory class (lower or higher) in most of the cases. Moreover, a trend of overestimation was observed for low classes (3.0 and 3.5), by misclassifying the sensory scores to the next higher class, while for higher classes (4.0 and 4.5) an underestimation was apparent, by misclassifying the sensory scores to the next lower class.

Table 1. Performance indices for the prediction of the microbial counts of the samples using the predicted estimates (external validation) from the PLS-R.

Microbial group	LC[a]	A_f[b]	B_f[c]	RMSE[d]	APE (%)[e]
LAB	2	1.078	1.031	0.633	78
Mesophilic cocci/streptococci	2	1.079	1.028	0.629	68
TVC	2	1.079	1.028	0.641	66
Staphylococci	3	1.115	0.993	0.391	68

[a] Number of latent components used for the PLS model.
[b] Accuracy factor.
[c] Bias factor.
[d] Root Mean Square Error.
[e] Acceptable Prediction Error.

Table 2. Confusion matrix of the PLS model for the sensory attributes of the samples using the predicted estimates (external validation) from the PLS-R.

	True Class	Predicted Class						Correct Classification (Sensitivity %)
		2.5	3.0	3.5	4.0	4.5	5.0	
Overall Appearance (LC[a]:4)	2.5 ($n=0$)	0	0	0	0	0	0	–
	3.0 ($n=2$)	0	0	2	0	0	0	0.00
	3.5 ($n=12$)	0	0	3	9	0	0	25.00
	4.0 ($n=26$)	0	0	11	15	0	0	57.69
	4.5 ($n=10$)	0	0	2	8	0	0	0.00
	5.0 ($n=0$)	0	0	0	0	0	0	–
	Total ($n=50$)	0	0	18	32	0	0	**36.00**[b]
	Specificity %	–	0.00	16.67	46.88	0.00	–	
Overall Odor (LC:2)	2.5 ($n=0$)	0	0	0	0	0		–
	3.0 ($n=0$)	0	0	0	0	0	0	–
	3.5 ($n=12$)	0	0	2	10	0	0	16.67
	4.0 ($n=26$)	0	0	3	23	0	0	88.46
	4.5 ($n=12$)	0	0	0	12	0	0	0.00
	5.0 ($n=0$)	0	0	0	0	0	0	–
	Total ($n=50$)	0	0	5	45	0	0	**50.00**
	Specificity %	–	–	40.00	51.11	0.00	–	

	True Class	Predicted Class						Correct Classification (Sensitivity %)
		2.5	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	
Overall Taste (LC:5)	2.5 ($n = 0$)	0	0	0	0	0	0	0.00
	3.0 ($n = 4$)	0	3	1	0	0	0	75.00
	3.5 ($n = 8$)	0	1	4	3	0	0	50.00
	4.0 ($n = 32$)	0	1	13	14	4	0	43.75
	4.5 ($n = 6$)	0	0	0	4	2	0	33.33
	5.0 ($n = 0$)	0	0	0	0	0	0	–
	Total ($n = 50$)	0	5	18	21	6	0	**46.00**
	Specificity %	–	60.00	22.22	66.67	33.33	–	
Overall Texture (LC:6)	2.5 ($n = 0$)	0	0	0	0	0	0	–
	3.0 ($n = 2$)	0	0	2	0	0	0	0.00
	3.5 ($n = 4$)	0	0	0	4	0	0	0.00
	4.0 ($n = 22$)	0	0	2	15	5	0	68.18
	4.5 ($n = 22$)	0	0	0	12	9	1	40.91
	5.0 ($n = 0$)	0	0	0	0	0	0	–
	Total ($n = 50$)	0	0	4	31	14	1	**48.00**
	Specificity %	–	0.00	0.00	48.39	64.29	0.00	
Total Assessment (LC:2)	2.5 ($n = 0$)	0	0	0	0	0	0	–
	3.0 ($n = 2$)	0	0	1	1	0	0	0.00
	3.5 ($n = 10$)	0	0	4	6	0	0	40.00
	4.0 ($n = 26$)	0	0	9	17	0	0	65.38
	4.5 ($n = 12$)	0	0	0	9	3	0	25.00
	5.0 ($n = 0$)	0	0	0	0	0	0	–
	Total ($n = 50$)	0	0	14	33	3	0	**48.00**
	Specificity %	–	0	28.57	51.52	100.00	–	

[a] Number of latent components used for the PLS model.

[b] Overall correct classification.

The use of FTIR spectroscopy to detect changes during the storage period of dry-fermented sausages gave a substantial amount of data and some observations were made for the first time. So far, several studies have assessed the performance of FTIR methodology to monitor the spoilage in fresh beef (Ammor, Argyri, & Nychas, 2009; Argyri, Panagou, Tarantilis, Polysiou, & Nychas, 2010; Panagou, Mohareb, Argyri, Bessant, & Nychas, 2011), fresh pork (Papadopoulou, Panagou, Tassou, & Nychas, 2011), milk (Nicolaou & Goodacre, 2008), salmon

(Saraiva, Vasconcelos, & de Almeida, 2017) and ham slices (Pavli, Argyri, Nychas, Tassou, & Chorianopoulos, 2018). Regarding fermented food products such studies are limited and mostly refer to cheeses (Lanciotti, Vannini, Lopez, Gobbetti, & Guerzoni, 2005; Papadopoulou, Argyri, Varzakis, Tassou, & Chorianopoulos, 2018; Subramanian, Alvarez, Harper, & Rodriguez-Saona, 2011).

The basic concept underlying the FTIR methodology is based on the principle that the different microbial groups metabolize nutrients from the substrate (food) and this metabolic activity results in a specific biochemical fingerprint. The metabolic fingerprint of a food sample reflects several changes that occur in the food matrix (due to the microbial activity and other physicochemical changes) that may indirectly be correlated with the microbial counts and sensory attributes through the use of data analytics (Argyri et al., 2010; Ellis, Broadhurst, & Goodacre, 2004; Ellis & Goodacre, 2001; Papadopoulou et al., 2018).

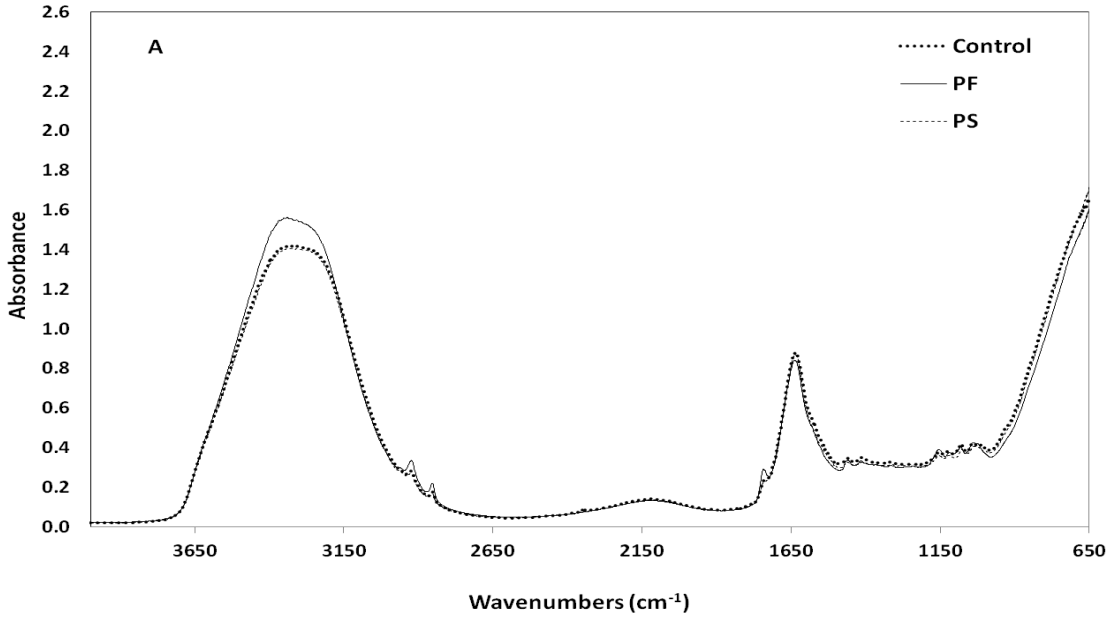
Given the aforementioned robust data available in the literature regarding the potential of FTIR on detecting microbial population changes, an attempt to investigate such a potential on probiotic fermented sausages was made in this study. From the results obtained, the performance indices used, A_f, B_f and RMSE were acceptable for all the microbial groups tested, however, APE % was found to be slightly lower than 70% in the majority of the cases, apart from the case of LAB (where acceptable APE% > 70% was observed). Regarding the potential of FTIR spectroscopy to be applied for the detection of the organoleptic changes occurring in the dry-fermented sausages, higher overall classification rates were shown for the 'Overall Odor' attribute, but in general the dynamics of the PLS-R models to predict the sensory scores were poor. Various hypotheses can be stated to explain the performance of the models, which are mostly related to that particular type of product. In the case of fermented foods and especially in

the case of potentially probiotic foods, the starter cultures are added in a high population in the beginning of fermentation and their numbers may not change dramatically through fermentation and storage. Thus, it is difficult to mathematically correlate the FTIR profile which may be fluctuating considerably in comparison with the microbial counts that are minimally changing. In the recent work of Papadopoulou et al. (2018) the FTIR analysis was found to be very promising regarding a fermented food product that was Feta cheese, so it was of interest to investigate the potential of this analytical method in a different fermented food. In the current case, the addition of a potential probiotic culture might have complicated even more the food matrix and process. Fermented sausage is a complex food matrix that has undergone fermentation and ripening which are processes that modify its physicochemical characteristics. It has to be noted that in the meat batter used to manufacture these sausages, apart from pork meat, other ingredients are added such as lard, sugar, spices, nitrates, so that the final substrate has been altered thoroughly and the impact of each ingredient in the final fermented product is difficult to be evaluated. Moreover, after fermentation and ripening, the product is considered as shelf-stable and this statement can be supported by the levels of the microbial groups during storage. In total, the predictions of the microbial groups and sensory scores were not satisfactory and trustworthy and thus further studies or alternative approaches on data analyses are needed to improve the performance of this technique.

7.4.2. Ham slices with probiotic edible films

In the current study, typical FTIR spectra from 4000 to 650 cm^{-1} collected from each group of ham slices stored at 4 °C and in the middle of shelf-life are presented in Figure 2 for both HPP treated and untreated samples. Spectral data served as metabolic fingerprints of ham

providing information on biochemical changes occurring during storage in an attempt to monitor spoilage.



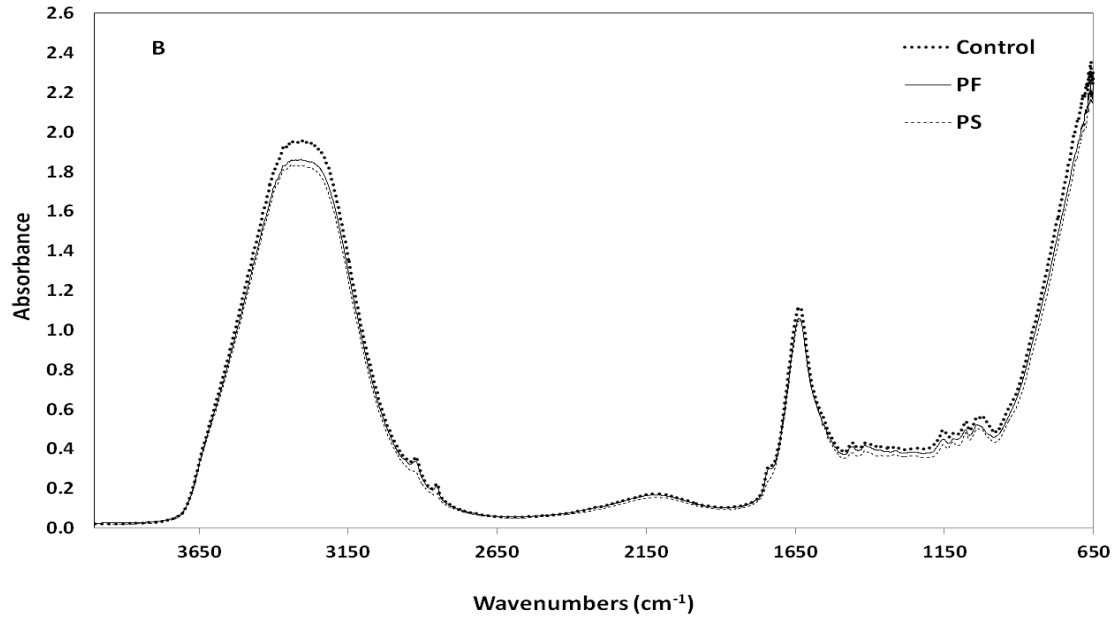


Figure 2. Typical FTIR-ATR spectra of different ham samples in the range of 4000-650cm^{-1}, in the middle of shelf life at 4 °C, without HP treatment (A) or after HP treatment (B).

As it is shown in Figure 2, a major peak at 3350-3326 cm-1 was apparent in the ham samples due to the presence of -OH stretching and –NH stretching vibrations (Nicolaou, Xu & Goodacre, 2011). A second peak was observed at 1640 cm-1 due to moisture (O-H) and contribution of amide I bands of the proteins. Other minor intensity peaks were observed at 2925 cm-1 (-CH2 assymetric stretch), 2853 cm-1 (aliphatic –CH2 groups of fatty acids), 1458 cm-1 [explained by the aliphatic groups (-CH2- and -CH3- scissoring), fat], 1154 cm-1 (CO-O-C assymetric stretch, glycogen and nucleic acids), 1125 cm-1 (C-O stretch of riboses), 1080 cm-1 (PO2 symmetric stretch (nucleic acids and phospholipids/ C-O stretch) and 1040-1042 cm-1 (primary amines, C-N stretch, C-O stretch, polysaccharides) (Bocker et al., 2007; Ammor et al., 2009; Osorio et al., 2009).

7.4.2.1.Correlation of microbiological data with FTIR spectra

PLS regression (PLS-R) models were built for TVC and LAB counts using FTIR responses as input variables and the microbial counts as the output ones. The models were developed so that the first batch of the experiment was used for the training, while the second was used for the testing process. In general, a relatively good relationship was observed between counts estimated from FTIR and observed by microbiological analysis. The performance indices of the PLS regression models for model testing are presented in Tables 3 and 4, depending on each group of samples for TVC and LAB.

Table 3. Performance indices of the PLS regression (PLS-R) model correlating the Total Viable Counts (TVC) in each group of ham samples on the basis of FTIR spectral data.

	Non Pressurized			Pressurized		
	Control	PF	PS	Control	PF	PS
B_f	1.015	1.016	0.979	1.096	0.963	1.009
A_f	1.078	1.064	1.051	1.309	1.124	1.050
RMSE	0.706	0.613	0.450	1.758	0.860	0.433
PE (%)	70.58	81.73	80.00	37.60	55.76	89.09

Table 4. Performance indices of the PLS regression (PLS-R) model correlating the lactic acid bacteria counts (LAB) in each group of ham samples on the basis of FTIR spectral data.

	Non Pressurized			Pressurized		
	Control	PF	PS	Control	PF	PS
B_f	0.966	0.995	0.979	1.036	0.979	1.018
A_f	1.095	1.083	1.051	1.332	1.134	1.048
RMSE	0.859	0.734	0.450	1.523	0.901	0.467
PE (%)	59.50	64.10	79.13	35.59	56.14	93.75

With regard to TVC predictions from the test dataset (estimates presented in Table 3), the bias factor (B_f) in all sample groups, was close to 1, indicating optimum correlation between observations and predictions. Concerning the accuracy factor (A_f), this was found to be close to 1 in non HPP treated samples, indicating that predictions were close to observations, while this was not the case with samples treated with HPP. In the latter case, the A_f value was relatively higher and more specifically, the average deviation between predictions and observations for TVC was 30.9% for the control and 12.4% for PF ham samples. However, this result was not observed for the case of PS samples, where the average deviation was only 5%. Additionally, RMSE value, which is an indication of the goodness of fit of the modeling approach, was relatively low in cases of ham without HPP, with the lowest value being observed in PS case (0.450), whereas the

higher value was observed for the control case in samples treated with HPP (1.758). Analogous results were observed for LAB counts as presented in Table 4. The B_f was close to 1 for either HPP treated or not cases, while the A_f in cases of HPP was 1.332 and 1.134 for control and PF cases, respectively. RMSE values for LAB were similar and slightly higher than the values obtained for TVC, with the lowest values obtained for PS ham samples. The B_f for both TVC and LAB, regardless the case, was close to 1, indicating no underprediction (B_f <1) or overprediction (B_f >1) (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006; Oscar, 2009). On the contrary, although the A_f values were for all untreated with HPP group samples close to 1, an increase was observed for the cases involving HPP, with the exception of PS samples. This can be also shown from the estimated values for prediction error.

Prediction error (%) values are also presented in Tables 3 and 4 for TVC and LAB counts. Indicatively, Figures 3 and 4 show the residuals for the prediction cases of the microbial counts for untreated or treated with HPP samples, respectively. The line y=0.5 determines the fail-dangerous zone direction, whilst the line y= -1.0 determines the fail-safe zone (Oscar, 2009). PLS regression models for the TVC case presented acceptable predictions (%PE>70%) for all the 3 HPP untreated groups. Despite that, in the case of HPP, the percentages for control and PF samples were lower than the PS ones. In the case of LAB predictions the PE % values were a slightly lower than 70% for cases of control and PF samples without HPP, while the values for the HPP treated were much lower than the aforementioned percentage. Similar results were not observed for the case of PS ham samples.

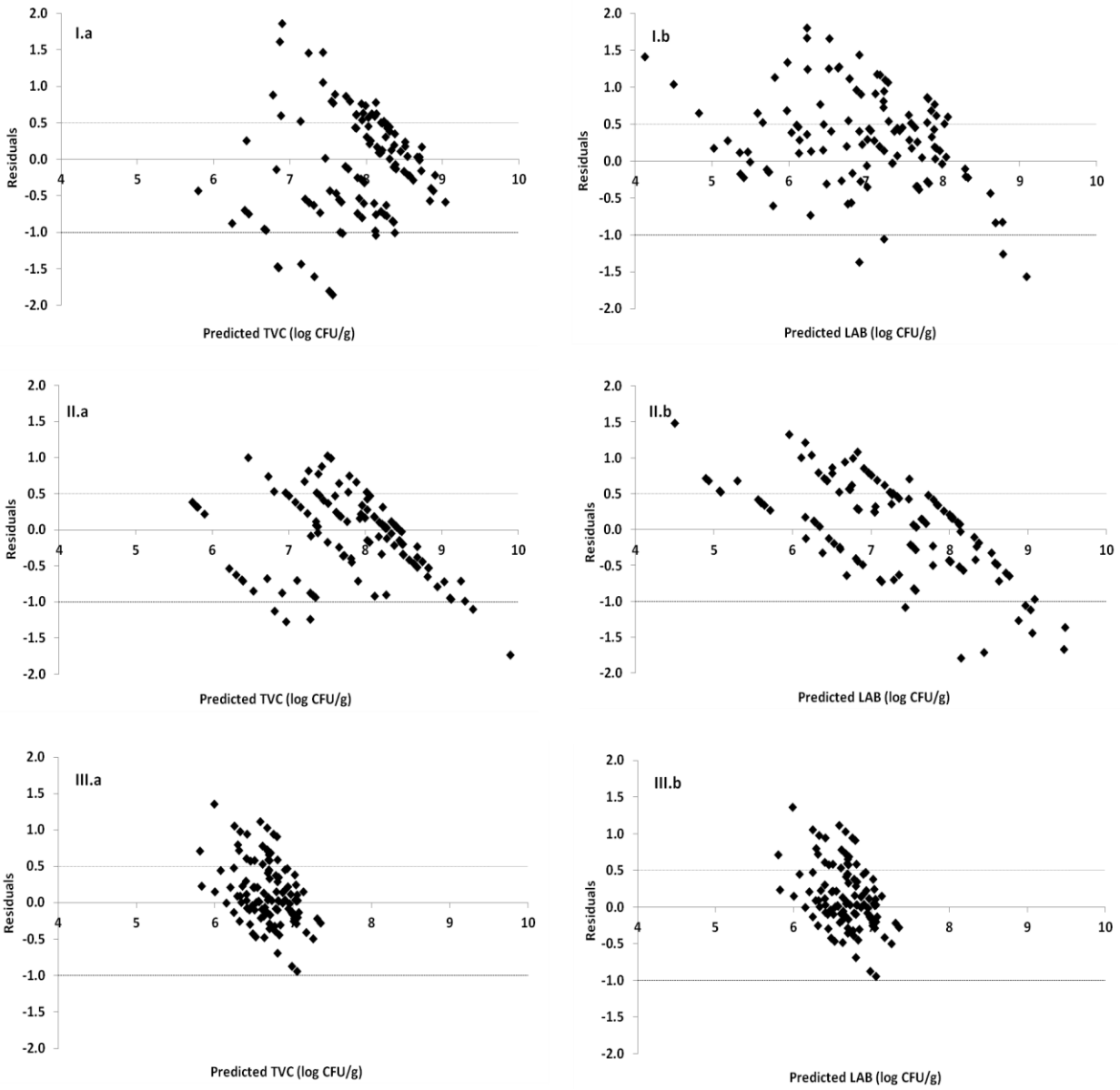


Figure 3. Residuals vs. predicted values of TVC (a) and LAB (b) of the test data set, as estimated by the PLS regression model based on FTIR spectral data, for the cases of control (I), PF (II) and PS (III) for non-pressurized samples. The horizontal lines represent the acceptable prediction zone (APZ).

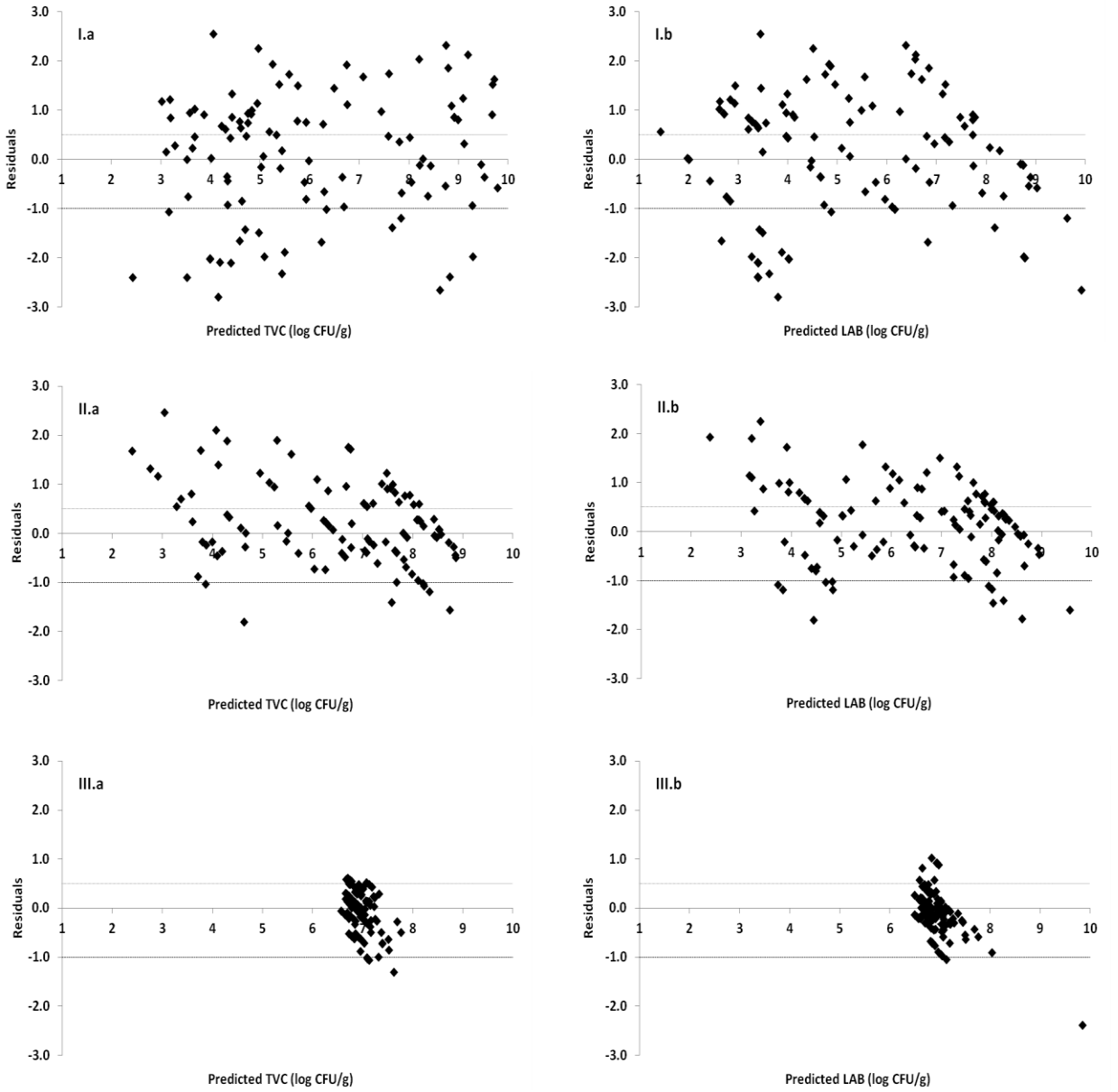


Figure 4. Residuals vs. predicted values of TVC (a) and LAB (b) of the test data set, as estimated by the PLS regression model based on FTIR spectral data, for the cases of control (I), PF (II) and PS (III) for pressurized samples. The horizontal lines represent the acceptable prediction zone (APZ).

HPP seemed to affect the performance of the models, apart from the case of PS ham slices. This result can be explained, since HPP may alter certain characteristics of the texture or aroma of the products, thus contributing to different spectral characteristics of these samples compared to the control ones (without HPP). It is worth to be mentioned that ham is a complex food matrix that has already been processed thermally during its production, so in this case the initial 'raw' product has been altered thoroughly.

7.4.2.2.Sensory class evaluation

The PLS models evaluation was performed using leave-one-out cross validation for the prediction of sensory the sensory scores for each different treatment of ham slices. In a 2nd step (classification), each predicted sensory score was rounded to the closest class membership, in order to aid interpretability of the results. The accuracy of the models for the test subset are presented in Tables 5 and 6, for samples without HPP and for those treated with HPP, respectively. With regard to the control samples without HPP, the highest percentage of correct classification was observed in fresh (78.08%) and spoiled (77.27%) samples, when the overall correct classification was estimated as 72.73%. The lowest performance was obtained in semi-fresh samples (53.85%). In the case of PF ham samples, the highest correct classification was observed in spoiled samples (89.29%), with only 3 misclassifications out of 28 spoiled samples, but with lower percentages being estimated for fresh and semi-fresh classes. Results obtained for PS ham samples, showed relatively high model performances for fresh samples (93.33%), with lower classification percentages being obtained for semi-fresh and spoiled samples (Table 5).

Classification percentages for the ham samples treated with HPP were different than those without this treatment. The correct classification for fresh control samples was 77.97%, which was similar to the value obtained without HPP. However, the correct classification percentage for

the estimation of spoiled controls was low, with a value of 35%. This indicates that HPP on its own affects the model's performance and reduces its sensitivity on predicting the spoiled samples. It was noted, that from the 20 spoiled samples, 7 were categorized correctly as spoiled, 14 were misclassified as semi-fresh, and none of them was misclassified as fresh, which is quite important for the model's evaluation. Despite the fact that in control samples with HPP a lack of sensitivity was observed in predicting spoiled samples, in the case of PS ham samples the observed sensitivity was higher. More specifically, from the 40 spoiled samples, only 5 were misclassified as semi-fresh, increasing the sensitivity percentage to a value of 87.50%.

Acceptable classification accuracies were obtained for fresh and spoiled ham samples for almost all groups, demonstrating the effectiveness of the method to discriminate samples between these two classes. The lower percentages of sensitivity obtained from all the cases tested for semi-fresh class was not unexpected and it has also been observed in other studies (Argyri et al., 2010; Panagou et al., 2011). Additionally, it must be stressed out that the number of examined samples within each class was not equal due to the different spoilage level of the different groups of ham samples and the different storage temperatures, therefore this might have affected the training process. Moreover, the lack of complete discrimination into different sensory groups could be due to the uncertainty of sensory evaluation and the variability of the samples ("raw ham"), which in our case was high (batches of ham slices from different suppliers for training and validating the models).

Table 5. Confusion matrix of the PLS model for the sensory estimates (test data set) of each group of ham slices without HPP treatment.

	True class	Predicted class			Correct Classification (Sensitivity %)
		Fresh	Semi-fresh	Spoiled	
Control	Fresh (*n*=73)	57	11	5	78.08
	Semi-fresh (*n*=26)	6	14	6	53.85
	Spoiled (*n*=22)	1	4	17	77.27
	Total (*n*=121)	64	29	28	72.73*
	Specificity (%)	89.06	48.27	60.71	
PF	Fresh (*n*=46)	30	14	2	65.22
	Semi-fresh (*n*=43)	4	22	17	51.16
	Spoiled (*n*=28)	0	3	25	89.29
	Total (*n*=117)	34	39	44	65.81*
	Specificity (%)	88.23	56.41	56.81	
PS	Fresh (*n*=15)	14	1	0	93.33
	Semi-fresh (*n*=43)	4	28	11	65.12
	Spoiled (*n*=57)	1	18	38	66.67
	Total (*n*=115)	19	47	49	69.57*
	Specificity (%)	73.68	59.57	77.55	

*Overall correct classification (accuracy) for PLS test dataset

Table 6. Confusion matrix of the PLS model for the sensory estimates (test data set) of each group of ham slices HPP treatment.

	True class	Predicted class			Correct Classification (Sensitivity %)
		Fresh	Semi-fresh	Spoiled	
Control	Fresh (*n*=59)	46	13	0	77.97
	Semi-fresh (*n*=40)	14	22	4	55.00
	Spoiled (*n*=20)	0	13	7	35.00
	Total (*n*=119)	60	48	11	63.03*
	Specificity (%)	76.66	45.83	63.63	
PF	Fresh (*n*=54)	32	20	2	59.26
	Semi-fresh (*n*=44)	8	28	8	63.64
	Spoiled (*n*=16)	0	5	11	68.75
	Total (*n*=114)	40	53	21	62.28*
	Specificity (%)	80.00	52.83	52.38	
PS	Fresh (*n*=29)	24	5	0	82.76
	Semi-fresh (*n*=43)	10	22	11	51.16
	Spoiled (*n*=40)	0	5	35	87.50
	Total (*n*=112)	34	32	46	72.32*
	Specificity (%)	70.58	68.75	76.08	

*Overall correct classification (accuracy) for PLS test dataset

7.4.3. Ham slices with edible films with OEO

Typical FTIR spectra from 4000 to 650 cm^{-1} collected from each group of ham slices at 12 °C and at the end of the shelf-life are presented in Figure 5, for samples with or without HPP treatment. As shown in Figure 5, major peaks are observed at 3650-3100 cm^{-1}, due to the presence of water in the samples, at 1748 cm^{-1} due to esters from lipids and at 1650 cm^{-1} due to moisture and amide I bands of the proteins. The spectra collected were similar to those reported previously for ham in the spectral region from 1730 to 850 cm^{-1} (Moreirinha, 2015). Other minor intensity peaks are observed at 2925 (–CH_2 asymmetric stretch), 2853 (aliphatic –CH_2 groups of fatty acids), 1154 (CO–O–C asymmetric stretch, glycogen and nucleic acids), and 1040–1042

cm^{-1} (primary amines, C–N stretch, C–O stretch, polysaccharides) (Böcker et al. 2007; Ammor, Argyri and Nychas, 2009; Osorio et al. 2009). The spectral changes were more intense during the storage temperature at 12 °C and less intense, in the cases were HPP treatment was applied.

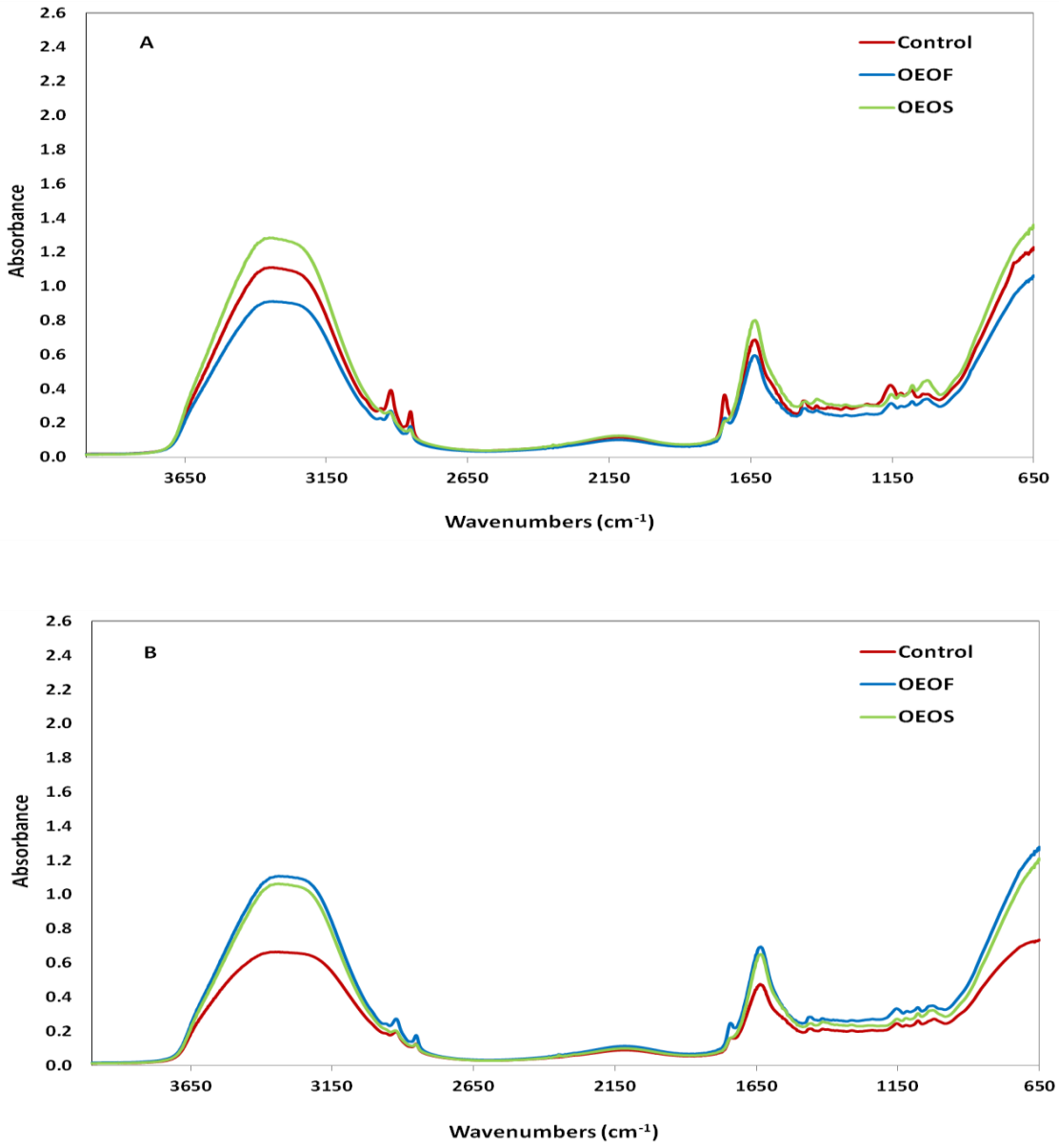


Figure 5. Typical raw FTIR spectra in the range of 4000-650 cm^{-1}, at the end of the shelf-life at 12 °C, without (A) or after (B) HPP treatment for control samples (−), samples with

oregano essential oil-free edible films - OEOF (−), and samples with edible films supplemented with oregano essential oil- OEOS (−).

The performance indices of the validation PLS regression models are presented in Table 7, for each group of samples (without or with HPP treatment) and for each bacterial group (LAB, lactococci/streptococci and TVC). The bias factor (B_f) was estimated close to one for all the cases, showing good correlation between observations and predictions. On the other hand, the accuracy factor (A_f) was estimated to be different between the cases with regard to HPP. Values close to one were observed for the samples without the HPP treatment, which shows that predictions were generally close to observations, while in the cases with HPP treatment, the values were quite higher. Specifically, the average deviation between predictions and observations were 8.2% for LAB, 6.6% for lactococci/streptococci and 5.9% for TVC for the non HPP treated samples, while the corresponding numbers for HPP samples were 30.5% for LAB, 31.1% for lactococci/streptococci and 23.5% for TVC. Analogous results were observed for the RMSE index, with low estimated values for the cases without the HPP treatment (lowest value observed for TVC: 0.56), but quite higher for the cases when HPP treatment was applied, with the highest value estimated for lactococci/streptococci (1.735). For the cases without HPP treatment, the percentage of prediction error was higher than the acceptable (≥70%) for lactococci/streptococci and TVC, while for LAB, was estimated as 66.38%. For the cases with HPP treatment the PE% were quite low; 30.72 for LAB, 37.57 for lactococci/streptococci and 47.54% for TVC.

Table 7. Performance indices of the PLS regression (PLS-R) model, correlating the Lactic Acid Bacteria (LAB), Lactococci/Streptococci and Total Viable Counts (TVC) in each group of ham samples with regard to the HPP application, on the basis of FTIR spectral data.

Performance Index	Without HPP Treatment			With HPP Treatment		
	LAB	**Cocci**	**TVC**	**LAB**	**Cocci**	**TVC**
Prediction error (PE, %)	66.38	74.29	77.40	30.72	37.57	47.54
Root mean square error (RMSE)	0.730	0.624	0.560	1.548	1.735	1.452
B_f	0.973	0.987	0.988	0.942	0.961	1.001
A_f	1.082	1.066	1.059	1.305	1.311	1.235

FTIR spectroscopy revealed differences in samples treated and not treated with HPP and the estimated performance indices of the PLS-R models were found to be slightly different between the cases. A_f values for the non-treated samples were close to one, indicating low average deviations between predictions and observations of the examined microbial groups, however the values of the same index were higher in the case of HPP. On the other hand, the estimated values for B_f were in both cases very close to one, especially for the prediction of TVC, revealing good correlation between observations and predictions. Prediction error (%) values were found to be better for the cases without HPP treatment, with acceptable levels (>70%) for the prediction of lactococci/streptococci and TVC, while for the LAB the value was slightly lower than the acceptable level. Differently, in the samples treated with HPP, the PE values were quite low for all microbial groups, and thus not acceptable. Based on the results, it is evident that HPP treatment affected significantly the performance of the FTIR models to be used as a tool for the prediction of the actual microbial counts. Similar results were also observed in the previous study of our group, where probiotic-supplemented edible films were used instead of OEOS ones

(Pavli et al. 2018). These results can be explained since HPP as a treatment could lead to changes in the matrix of the sliced ham, while ham during its production has received other additions or processes. Generally, it is worth to be mentioned that FTIR spectroscopy has been extensively used to monitor spoilage in raw meat (Ammor, Argyri and Nychas, 2009; Papadopoulou et al. 2014; Panagou et al. 2011; Argyri et al. 2013; Estellez-Lopez et al. 2017), or to detect frozen-then-thawed meat (Ropodi, Panagou and Nychas, 2018), but applications and reports in food products such as ham are limited.

7.5. Conclusions

The FTIR analysis in conjunction with chemometrics was applied for the first time to assess the microbiological and sensory status of the probiotic dry-fermented sausages, however further studies or alternative approaches on data analyses are needed to improve the performance of this technique.

Furthermore, FTIR spectral data exhibit a possible potential in monitoring spoilage as can be measured with microbial counts and/or organoleptic characteristics, of ham slices treated or not with HPP and packed with probiotic edible films, however, revealed difficulties when HPP treatment was applied. In the case of the sensory evaluation models the performance varied between the different cases and classes. Further research is required for this method to be applicable in cases of novel packaging and processing methods that are rising nowadays and consecutively change the food matrix and the character of the spoilage.

CHAPTER 8

Conclusions and Future Plans

8.1. Conclusions

Extended discussion and conclusions are given for the individual experiments in Chapters 2-7. Overall, with this thesis, several LAB strains obtained from fermented food products were assessed as potential probiotics, increasing the number of cultures that can be possibly used in the future for probiotic applications. The addition of these cultures in dry-fermented sausages, resulted in products of high quality, that can support the survival of the added culture until the end of their shelf-life. The probiotic incorporation in edible films for their application in sliced ham, was considered as a good alternative for probiotic delivery, whilst, edible films supplemented with oregano essential-oil exhibited significant antimicrobial activity against the pathogen *Listeria monocytogenes*, when applied to sliced ham. It is evident from the results that the technology of edible films could be very promising for future applications, although it might be costly and time-consuming. Furthermore, the use of FTIR spectroscopy as a tool for monitoring spoilage, revealed difficulties for the case of dry-fermented sausages, especially when predicting the organoleptic attributes of the products. Similarly, FTIR analysis for the sliced ham, was not satisfactory in cases where High Pressure Processing or edible film application was involved, however better performance was observed in the control sliced ham (no further processing). Last but not least, significant information was revealed regarding the possible mechanism of cell-to-cell communication of the potentially probiotic LAB strains. The gene expression of *lux*S revealed that quorum-sensing based on AI-2 signal-molecules is not a critical factor in the resistance of LAB in stress conditions, although differences among LAB strains and stresses were observed. Also, some of the LAB strains were found to be GABA-negative, which was an unexpected result according to the existing literature.

8.2. Future plans

The future plans after the completion of the current thesis could be categorised according to the work included in each Chapter of reference, and are presented hereunder:

Chapter 2: Further *in vitro* tests regarding the adherence of the LAB strains to cell lines (CaCO-2 or HT-29) would be important to better evaluate the probiotic properties *in vitro*. Also, at a later stage, clinical trials on animals are deemed necessary to investigate the effects of the individual LAB strains.

Chapter 3: The role of AI-2 signal molecules in the physiology of the potentially probiotic LAB could be further investigated, under different stress conditions. Furthermore, it would be interesting to investigate the expression of *lux*S gene and AI-2 production in cases when certain pathogens are present, such as *Listeria monocytogenes*. In addition, the detection of AIPs could provide a better understanding on how communication takes place in such bacteria.

Chapter 4: The use of probiotic cultures as starter cultures in dry-fermented sausages would have a special interest for the industry. In the current thesis, the commercial starter cultures were present while adding the probiotic ones, however, the performance of the probiotic cultures as solely starter cultures was not investigated.

Chapter 5: The incorporation of probiotic in edible films has a great potential and can be utilised in many other food products. Furthermore, the probiotic edible films could be used as bioprotective materials against contamination from pathogens-biopreservation.

Chapter 6: Edible films can be supplemented with many antimicrobial substances, apart from essential oils with many different applications in food products.

Chapter 7: Since the analysis of the FTIR data, although quite promising in some cases, failed to correlate accurately all the FTIR spectra with the bacterial and sensorial data, different alternative approaches on data analyses are needed to improve the performance of the FTIR technique.

CHAPTER 9

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Appendix I

Chapter 4:

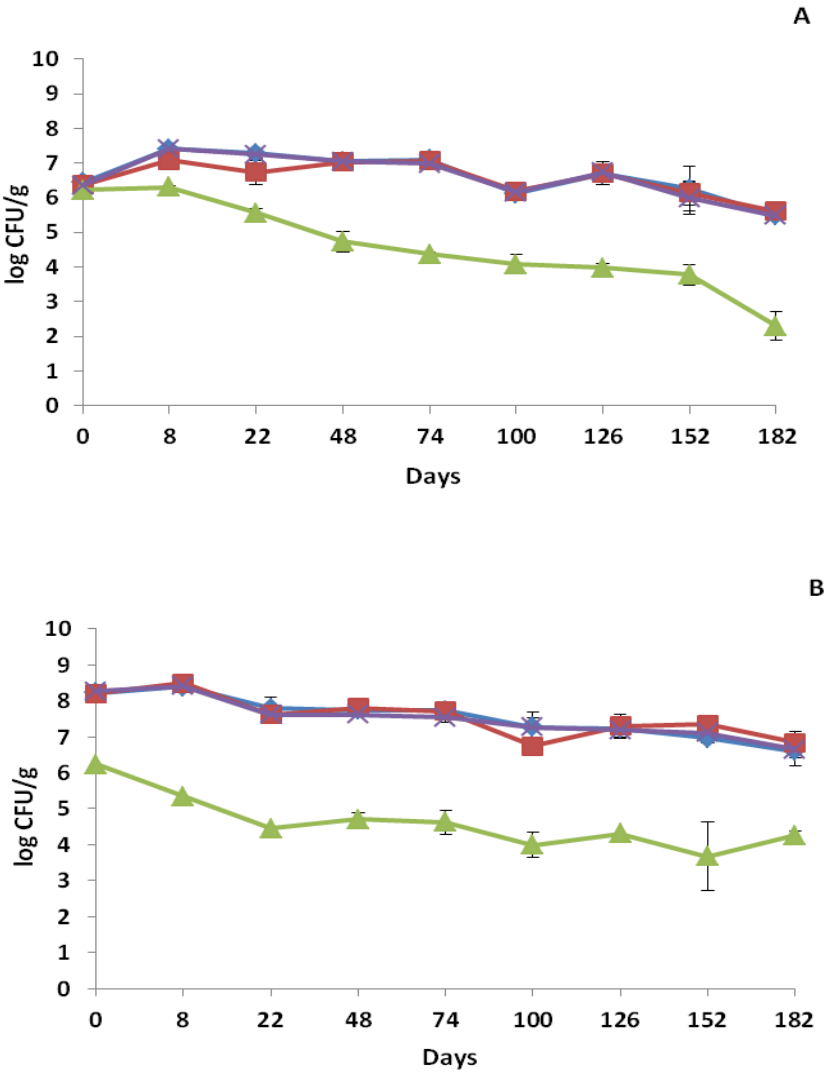

Figure 1. Growth curves of the examined bacterial populations during fermentation, ripening and storage period of dry-fermented sausages for control (A) and probiotic (B) samples stored at 12 °C. (♦) LAB, (■) TVC, (▲) Staphylococci, (✕) Mesophilic cocci/streptococci. The bars represent the mean values ± standard deviation.

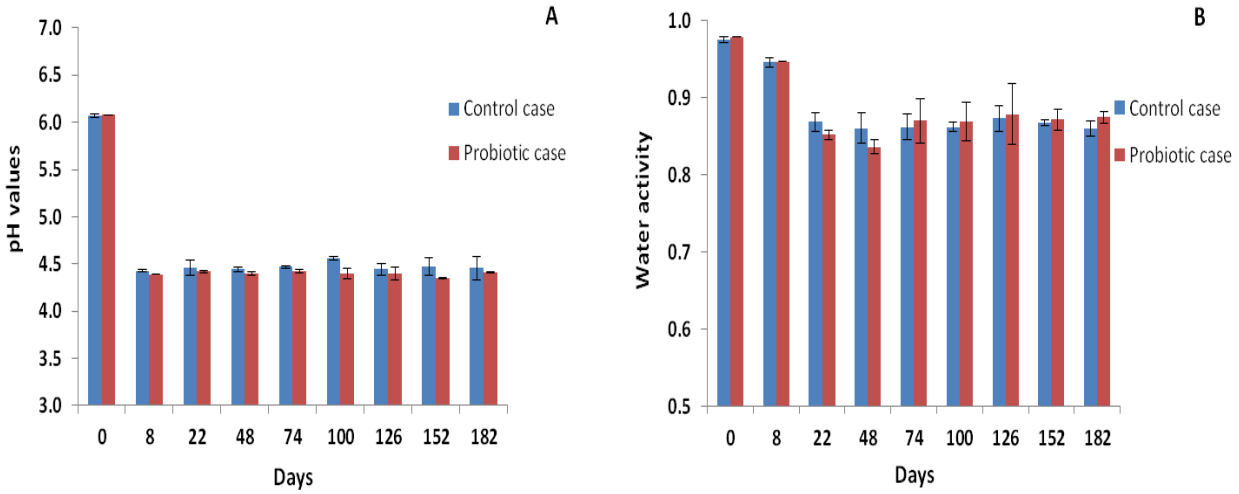

Figure 2: pH (A) and water activity (B) values during fermentation, ripening and storage of dry-fermented sausages at 12 °C. The bars represent the mean values ± standard deviation.

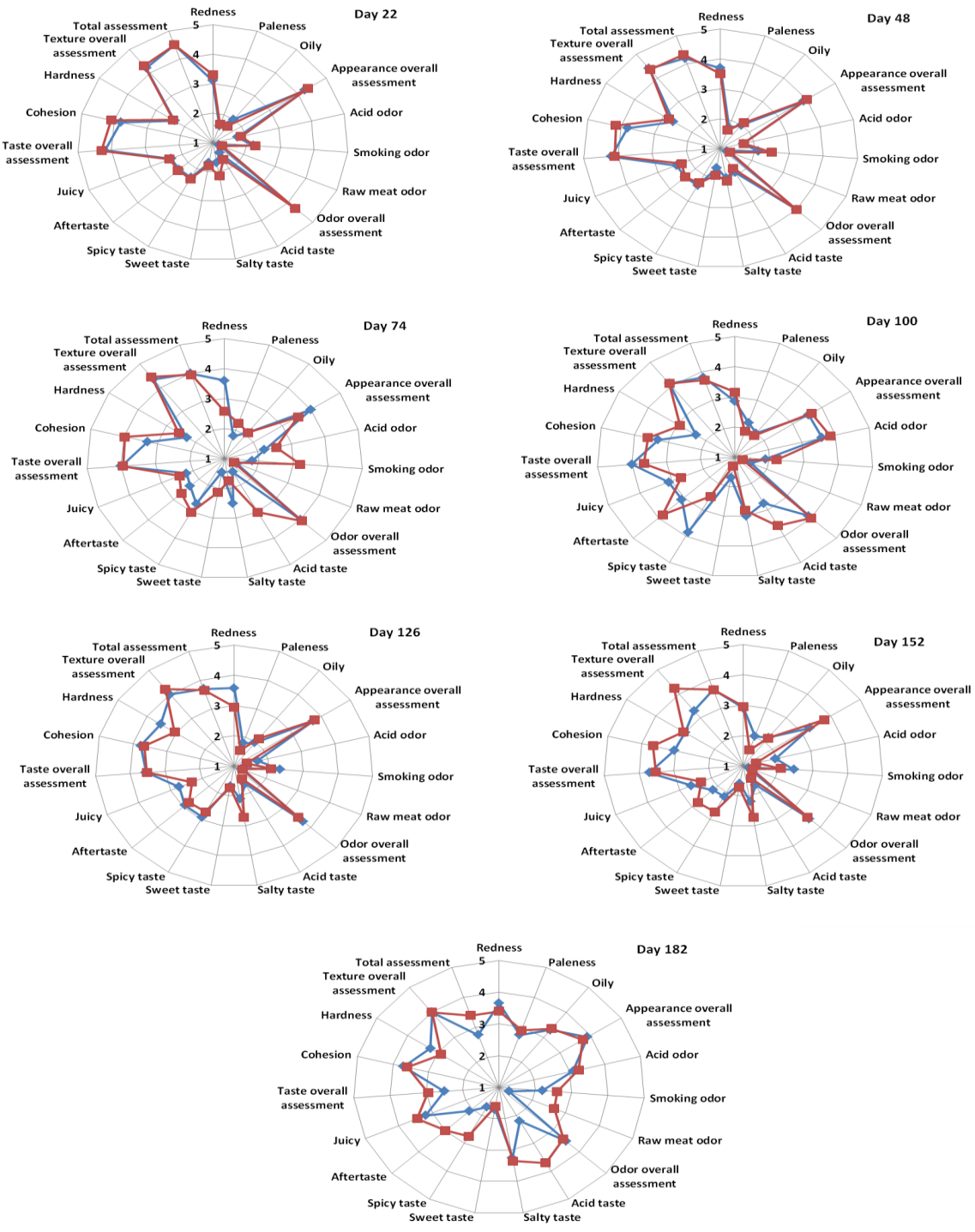

Figure 3: Sensory evaluation of dry-fermented sausages during storage at 12 °C (22nd, 48th, 74th, 100th, 128th, 152nd and 182nd day).

Chapter 7:

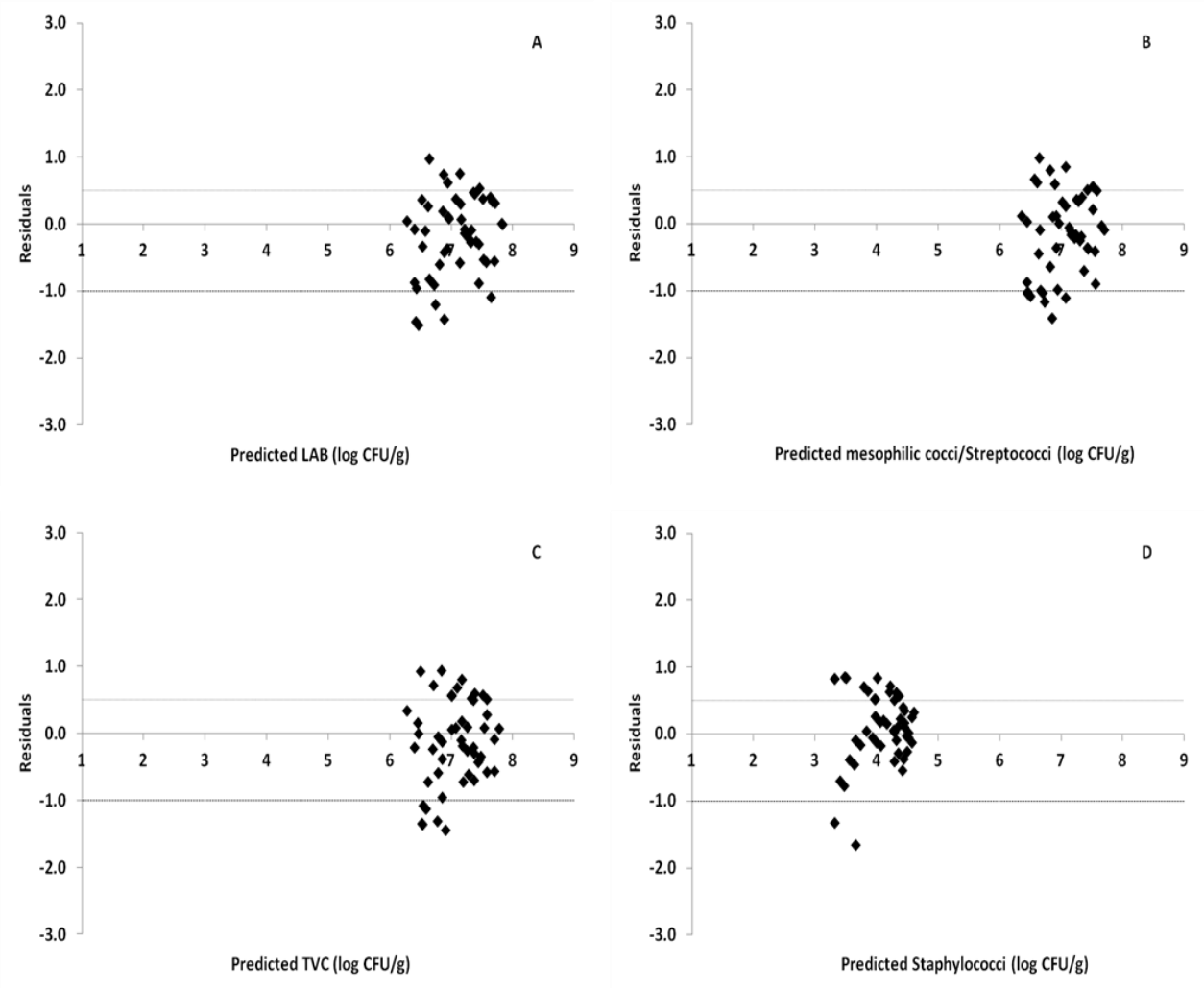

Figure 4: Residuals vs predicted values of LAB (A), mesophilic cocci/streptococci (B), TVC (C) and Staphylococci (D) of the test data set, as estimated by the PLS regression model based on FTIR spectral data of dry-fermented sausages samples. The horizontal lines represent the acceptable prediction zone (APZ).

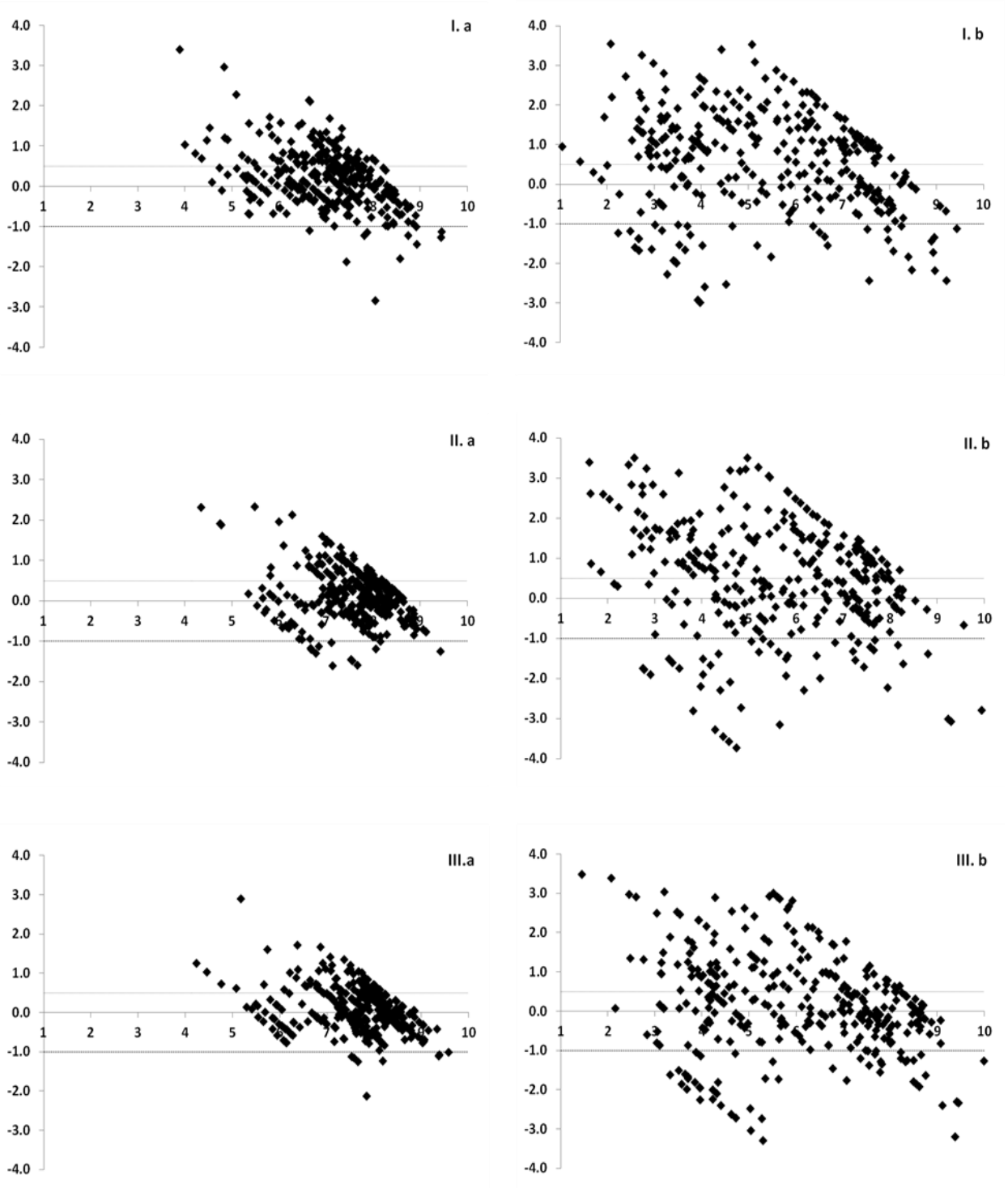
I. a
I. b
II. a
II. b
III.a
III. b

Figure 5. Residuals vs. predicted values of LAB (I) and lactococci/streptococci (II) and TVC (III) of the test data set, as estimated by the PLS regression model based on FTIR spectral data, for the cases of control for samples without (a) of after (b) HPP treatment. The horizontal lines represent the acceptable prediction zone (APZ).

Appendix II

Dissemination of work

Journal publications (Articles):

1. Pavli, F., Argyri, A., Papadopoulou, O., Nychas, G.-J.E., Chorianopoulos, N., Tassou, C. (2016). Probiotic potential of lactic acid bacteria from traditional fermented dairy and meat products: Assessment by *in vitro* tests and molecular characterization. Journal of Probiotics & Health 2016, 4:3.

2. Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Nychas, G.-J.E., Tassou, C., Chorianopoulos, N. (2017). Alginate-Based Edible Films Delivering Probiotic Bacteria to Sliced Ham Pretreated with High Pressure Processing. International Journal of Molecular Sciences, 18(9).

3. Pavli, F., Argyri, A., Nychas, G.-J. E., Tassou, C., Chorianopoulos, N. (2018). Use of Fourier transform infrared spectroscopy for monitoring the shelf life of ham slices packed with probiotic supplemented edible films after treatment with high pressure processing. Food Research International, 106, 1061-1068.

4. Pavli, F., Tassou, C., Nychas G-J. E, Chorianopoulos, N. (2018). Probiotic incorporation in edible films and coatings: Bioactive solution for functional foods. International Journal of Molecular Sciences, 19(1), 150.

5. Pavli, F., Argyri, A.A., Skandamis, P., Nychas, G-J.E., Tassou, C., Chorianopoulos, N. (2019). Antimicrobial Activity of Oregano Essential Oil Incorporated in Sodium Alginate Edible Films:

Control of *Listeria monocytogenes* and Spoilage in Ham Slices Treated with High Pressure Processing. Materials, 12, 3726.

6. Pavli F., Gkana E., Adebambo O., Karatzas K-A, Panagou E. and Nychas G-J. (2019). *In vitro* screening of γ-aminobutyric acid and autoinducer-2 signalling in lactic acid bacteria exhibiting probiotic potential from natural black Conservolea olives. Foods, 8 (12), 640.

7. Pavli, F., Argyri, A.A., Chorianopoulos, N.G., Nychas, G-J.E., Tassou, C.C. (2020). Effect of *Lactobacillus plantarum* L125 strain with probiotic potential on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages. LWT-Food Science and Technology, 118, 108810.

Conference contributions:

1. Pavli, F., Argyri, A., Chorianopoulos, N., Papadopoulou, O., Tassou, C. (2015). Probiotic potential of lactic acid bacteria isolated from traditional meat and dairy products. 29th EFFoST International Conference, 10-12/11/2015, Athens, Greece (Poster presentation).

2. Tassou, C., Pavli, F., Argyri, A., Chorianopoulos, N. (2015). Fermentation of traditional sausages with the use of three different strains of *Lactobacillus plantarum* with probiotic potential as co-starter cultures. 38th Somed Congress-Society for Microbial Ecology and Disease, Human Microbiome: from the Bench to Health Benefits 2015, 11-13 October 2015, Verona, Italy (Poster Presentation).

3. Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Chorianopoulos, N. and Tassou, C. (2016). Application of Edible Films Supplemented with Probiotic Bacteria in Ham Slices Packaged after High Pressure Processing. IAFP European Symposium on Food Safety, 11th -13th May Athens, Greece. (Poster presentation).

4. Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Chorianopoulos, N. and Tassou, C. (2016). Efficacy of the combined application of High Pressure Processing and oregano essential oil- based antimicrobial edible films for the control of *Listeria monocytogenes* on ham slices. IAFP European Symposium on Food Safety, 11th -13th May Athens, Greece. (Poster presentation).

5. Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Chorianopoulos, N. and Tassou, C. (2016). Alginate-based edible films as a vehicle for delivering probiotic bacteria to ham slices pretreated with high pressure processing. Food Micro Conference, 19th-22th July, Dublin, Ireland. (Poster presentation).

6. Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Tassou, C. and Chorianopoulos, N. (2016). Effect of the oregano essential-oil based edible films on spoilage of ham slices with or without prior high pressure processing. Food Micro Conference, 19th-22th July, Dublin, Ireland. (Poster presentation).

7. Pavli, F., Argyri, A., Kovaiou, I., Apostolakopoulou, G., Tasoula, V., Nychas, G.-J., Tassou, C., and Chorianopoulos, N. (2017). Use of Fourier Transform Infrared Spectroscopy for monitoring the shelf life of ham slices packed with probiotic supplemented edible films after treatment with High Pressure Processing. Q-Safe International Conference-Quantitative tools for Sustainable Food and Energy in the Food Chain. 10th-12th April, Greece. (Oral Presentation).

8. Pavli, F., Argyri, A., Kovaiou, I., Apostolakopoulou, G., Skandamis, P., Nychas, G.-J., Tassou, C. and Nikos Chorianopoulos (2017). Use of Fourier Transform Infrared Spectroscopy for rapid detection of microbial spoilage of ham slices packed with oregano essential oil edible films after treatment with High Pressure Processing. Q-Safe International Conference-Quantitative tools for Sustainable Food and Energy in the Food Chain. 10th-12th April, Greece. (Oral Presentation).

9. Pavli, F., Gkana, E., Karatzas, K-A., Nychas, G-J.E. (2019). Probiotic potential of lactic acid bacteria isolated from table olives using *in vitro* tests: Screening for their ability to produce autoinducer-2 signal molecule and γ-aminobutyric acid. Microbial Diversity. 25-27th September, Catania, Italy (Poster presentation).

10. Pavli, F., Komita, E., Doulgeraki, A, Panagou, E., Nychas G-J.E. (2019). Effect of different stress factors on the expression of the *lux*S gene and the production of Autoinducer-2 signal

molecules of *Lactobacillus* species that exhibit probiotic potential. Microbial Diversity. 25-27th September, Catania, Italy (Poster presentation).