AGRICULTURAL UNIVERSITY OF ATHENS
SCHOOL OF FOOD & NUTRITIONAL SCIENCES
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF FOOD MICROBIOLOGY & BIOTECHNOLOGY

PhD Thesis

Identification and Control of *Alicyclobacillus acidoterrestris* in fruit juices by Ultra High Pressure processing

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Department of Food Science and Human Nutrition
Laboratory of Food Microbiology and Biotechnology

Abstract

Fruit juices have an important place in humans' healthy diet. They are considered to be shelf stable products due to their low pH that prevents the growth of most bacteria. However thermo-acidophilic endospore forming bacteria of the genus *Alicyclobacillus* have the potential to cause spoilage of commercially pasteurized fruit juices. The flat sour type spoilage, with the presence of chemical taint compounds (mostly guaiacol) and the ability of *Alicyclobacillus* spores to survive after pasteurization and germinate under favorable conditions make them a major concern for the fruit juice industry worldwide. Their special characteristics and occurence in the fruit juice industry has made their early detection a challenge for the manufacturers in order to reduce economic loss. Furthermore, the development of control methods targeting the inactivation of *Alicyclobacillus* is of paramount importance, as well. High Hydrostatic Pressure has been highlighted as the most important nonthermal technology to ensure microbiological safety and nutritional quality of fruit juices. Moreover, with the combination of moderate to high temperatures it has been proved effective for the inactivation of bacterial spores.

Therefore, the objective of this thesis was to investigate the presence of *Alicyclobacillus* in fruit juices, the identification of isolated colonies with molecular techniques and the examination of their potential to produce guaiacol. Furthermore, HHP was employed in order to study the inactivation of *Alicyclobacillus acidoterrestris* spores and vegetative cells in orange and peach juice immediately after treatment and throughout storage at various temperatures.

Initially, the microbiological quality of commercially available orange and peach juices was undertaken with main focus on the occurrence of *Alicyclobacillus* species (C**hapter 2).** For this reason, refrigerated or pasteurized orange and peach juices obtained from the Greek market were analyzed physiochemically and microbiologically. The microbiological analysis aimed to enumerate the main spoilage microorganisms of fruit juices in terms of total viable counts (TVC), lactic acid bacteria (LAB), yeasts, molds and *Alicyclobacillus* spp., whereas physicochemical analysis included the pH and total soluble solids (TSS) measurement. Results showed low contamination level of the examined fruit juices from spoilage microorganisms, but confirmed the presence of *Alicyclobacillus*.

In **Chapter 3**, the isolated colonies of *Alicyclobacillus* that were confirmed according to the IFU method developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers, were analyzed with 16S rDNA PCR-RFLP with the use of three restriction endonucleases (*Hha*I, *RSa* I and *HiNF*I), for rapid identification. Furthermore, all isolated *Alicyclobacillus* colonies were checked for the ability to produce guaiacol. The results of PCR-RFLP grouped the isolates to 8 clusters and 7 of them were different from the used reference strain. Moreover, all isolates had the ability to produce guaiacol. In order to identify the isolates at species level a single enzyme PCR-RFLP method was utilized. A total of 78 Alicyclobacillus isolates were subjected to PCR-RFLP analysis using universal primers for the amplification of V1-V3 variable region of 16S rRNA gene and restriction endonuclease *Hha*I. Using these specific conditions of the PCR-RFLP assay, the *A. acidoterrestris* isolates were successfully differentiated from *A. acidocaldarius* and *A. hesperidum*.

The presence of *A. acidoterrestris* in fruit juices can lead to major spoilage problems. Therefore, the effectiveness of HHP for the inactivation of *A. acidoterrestris* was investigated in **Chapter 4**. In order to determine the HHP conditions that could inactivate the spores of the microorganisms, two *A. acidoterrestris* strains inoculated in orange juice were subjected to temperature-assisted HHP at 500 and 600 MPa in combination with different temperature regimes (25, 45, 60, and 70 °C) for pressurization time up to 30 min (1, 3, 5, 15 and 30 min). Furthermore, the inactivation kinetics of bacterial spores was described by means of the Weibull model. Results demonstrated that spore inactivation increased as high pressure and temperature levels increased. For both strains, complete spore inactivation was achieved during processing at the highest pressure (600 MPa) and temperature (70 °C). Moreover, the inactivation of *A. acidoterrestris* spores could be successfully described by the Weibull model for different pressurization levels and temperatures, although strain variability should be taken into consideration.
Even though the inactivation of *A. acidoterrestris* spores has been studied extensively during HHP treatment, however the dynamics of the spores and vegetative cells during storage (after HHP treatment) has not been substantiated thoroughly. Therefore, in **Chapter 5,** orange juice samples inoculated with the spores of the two different *A. acidoterrestris* strains used in the study were subjected to HHP treatment at 600 MPa/ 60 °C for 5 and 10 min. HHP treated and untreated samples were subsequently stored at 4, 12, and 25 °C for 60 days. Samples were analyzed every week in order to investigate the dynamic of the spore and vegetative cell population during storage. Results demonstrated that the germination of the surviving spores could be inhibited, while the remaining vegetative cells could be eliminated throughout storage at low temperatures, although strain variability should be taken into consideration. Furthermore, the spores of the reference strain *A. acidoterrestris* ATCC 49025 were inoculated in peach juice samples in order to examine the dynamic of the spore and vegetative cell population at higher storage temperatures. For this reason, samples were treated with HHP at 500 and 600 MPa, combined with 25, 45 and 60 °C for 10 min. HHP treated and control samples of peach juice were then stored at isothermal conditions (25, 35 and 45 °C) and analyzed microbiologically for up to 240 h. The outcome of this study was that HHP treatment at 600 MPa/ 60°C /10 min could eliminate the spore population during subsequent storage at 25 °C, whereas the vegetative cell population could be inhibited with the same treatment at the selected storage temperatures.

Scientific area: Food Microbiology

Keywords: *Alicyclobacillus, A. acidoterrestris,* PCR-RFLP, control, High Hydrostatic Pressure, spores, vegetative cells.

Ταυτοποίηση και έλεγχος του βακτηρίου *Alicyclobacillus acidoterrestris* σε χυμούς φρούτων με επεξεργασία Υπερυψηλής Υδροστατικής Πίεσης

Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου
Εργαστήριο Μικροβιολογίας και Βιοτεχνολογίας Τροφίμων

Περίληψη

Οι χυμοί φρούτων κατέχουν σημαντική θέση στην υγιεινή διατροφή του ανθρώπου. Θεωρούνται ασφαλή προϊόντα ραφιού λόγω της χαμηλής τιμής του pH που αποτρέπει την ανάπτυξη των περισσοτέρων βακτηρίων. Παρόλα αυτά θερμοανθεκτικά και οξυανθεκτικά σπορογόνα βακτήρια του γένους *Alicyclobacillus* είναι πιθανόν να προκαλέσουν την αλλοίωση των παστεριωμένων χυμών φρούτων. Η αλλοίωση χωρίς την εμφανή διόγκωση της συσκευασίας λόγω παραγωγής αερίων αλλά η παρουσία χημικών ενώσεων αλλοίωσης (κυρίως γουαϊακόλης) και η ικανότητα των σπορίων του βακτηρίου *Alicyclobacillus* να επιβιώνουν μετά την παστερίωση και να εκβλαστάνουν κάτω από ευνοϊκές συνθήκες, τα καθιστούν μείζων πρόβλημα για την βιομηχανία χυμών φρούτων παγκοσμίως. Τα ιδιαίτερα χαρακτηριστικά τους και η παρουσία τους στη βιομηχανία φρουτοχυμών, καθιστά την έγκαιρη ανίχνευση τους μεγάλη πρόκληση για την ελαχιστοποίηση οικονομικών απωλειών. Επιπρόσθετα πρωταρχικής σημασίας είναι η ανάπτυξη μεθόδων ελέγχου με στόχο την αδρανοποίηση των σπορίων και των βλαστικών μορφών του βακτηρίου *Alicyclobacillus*. Η υπερυψηλή υδροστατική πίεση έχει χαρακτηριστεί ως η σημαντικότερη τεχνολογία μη θερμικής επεξεργασίας , η οποία μπορεί να διασφαλίσει τη μικροβιακή ασφάλεια και τη θρεπτική αξία των χυμών φρούτων. Επιπλέον, σε συνδυασμό με μέτριες έως υψηλές θερμοκρασίες η υπερυψηλή πίεση έχει αποδειχθεί αποτελεσματική στην αδρανοποίηση βακτηριακών σπορίων.

Συνεπώς, ο σκοπός της εν λόγω διδακτορικής διατριβής ήταν αρχικά η διερεύνηση της παρουσίας του βακτηρίου *Alicyclobacillus* σε χυμούς φρούτων, η απομόνωση των αποικιών, η ταυτοποίηση τους με RFLP PCR και ο έλεγχος της δυνατότητας τους να παράγουν γουαϊακόλη. Επίσης μελετήθηκε η επίδραση της υπερυψηλής υδροστατικής πίεσης στην αδρανοποίηση των σπορίων και των βλαστικών κυττάρων του βακτηρίου *Alicyclobacillus acidoterrestris* σε εμπορικό δείγμα χυμού πορτοκαλιού και ροδάκινου αμέσως μετά την επεξεργασία και κατά τη διάρκεια της συντήρησης σε διαφορετικές θερμοκρασίες.

Αρχικά, πραγματοποιήθηκε έλεγχος σε εμπορικά δείγματα χυμών πορτοκαλιού και ροδάκινου από την ελληνική αγορά (**Κεφάλαιο 2**). Για το σκοπό αυτό, χυμοί ψυγείου και ραφιού από πορτοκάλι και ροδάκινο αναλύθηκαν φυσικοχημικά και μικροβιολογικά. Ο μικροβιολογικός έλεγχος είχε ως σκοπό την απαρίθμηση της ολικής αερόβιας μικροβιακής χλωρίδας, του πληθυσμού των οξυγαλακτικών βακτηρίων, των ζυμών και μυκήτων καθώς επίσης και του βακτηρίου *Alicyclobacillus* spp., ενώ ο φυσικοχημικός έλεγχος περιλάμβανε την μέτρηση της τιμής του pH και τη συγκέντρωση των ολικών διαλυτών στερεών. Τα αποτελέσματα έδειξαν χαμηλούς πληθυσμούς από τους εξεταζόμενους μικροοργανισμούς, αλλά επιβεβαίωσαν την παρουσία του βακτηρίου *Alicyclobacillus* spp. στους χυμούς.

Στο **Κεφάλαιο 3**, απομονώσεις του βακτηρίου *Alicyclobacillus* spp. από χυμό πορτοκάλι που επιβεβαιώθηκαν σύμφωνα με τη μέθοδο IFU που αναπτύχθηκε από την Ομάδα Εργασίας για την Μικροβιολογία της Διεθνούς Ομοσπονδίας Παραγωγών Χυμών Φρούτων, αναλύθηκαν με την χρήση της μοριακής τεχνικής 16S rDNA PCR RFLP με 3 ένζυμα περιορισμού (*Hha*I, *RSa* I and *HiNF*I), για ταχεία ταυτοποίηση. Επιπροσθέτως, όλες οι απομονώσεις του βακτηρίου *Alicyclobacillus* ελέγχθηκαν για τη δυνατότητα παραγωγής γουαϊακόλης. Τα αποτελέσματα έδειξαν ότι η τεχνική PCR RFLP ομαδοποίησε τις απομονώσεις σε 8 ομάδες, εκ των οποίων 7 ήταν διαφορετικές από το

χρησιμοποιούμενο στέλεχος αναφοράς. Επίσης, όλες οι απομονώσεις είχαν τη δυνατότητα παραγωγής γουαϊακόλης. Για την ταυτοποίηση των απομόνωσεων σε επίπεδο είδους χρησιμοποιήθηκε μονοενζυματική PCR RFLP. Για τον σκοπό αυτό, 78 απομονώσεις *Alicyclobacillus* υποβλήθηκαν σε ανάλυση PCR RFLP με τη χρήση εκκινητών για την ενίσχυση της V1-V3 μεταβλητής περιοχής του 16S rDNA γονιδίου και του περιοριστικού ενζύμου *Hha*I. Με την εφαρμογή της τεχνικής PCR RFLP οι απομονώσεις των *A. acidoterrestris* διαφοροποιήθηκαν επιτυχώς από εκείνες των *A. acidocaldarius* και *A. hesperidum*.

Η παρουσία του βακτηρίου *A. acidoterrestris* στους χυμούς φρούτων μπορεί να επιφέρει σοβαρά προβλήματα αλλοίωσης. Για τον λόγο αυτό, διερευνήθηκε η αποτελεσματικότητα της Υπερυψηλής Υδροστατικής Πίεσης στην αδρανοποίηση των σπορίων του βακτηρίου *A. acidoterrestris* (**Κεφάλαιο 4)**. Συγκεκριμένα, σπόρια από δύο στελέχη του εν λόγω βακτηρίου (ένα άγριο στέλεχος απομονωμένο από χυμό μήλου και ένα στέλεχος αναφοράς) εμβολιάστηκαν σε χυμό πορτοκάλι και υποβλήθηκαν σε υπερυψηλή υδροστατική πίεση σε 500 και 600 MPa σε συνδυασμό με ταυτόχρονη θέρμανση σε διαφορετικές θερμοκρασίες (25, 45, 60, and 70 °C) και διάρκεια έως 30 min (1, 3, 5, 15 και 30 min). Στη συνέχεια το πρωτογενές μοντέλο Weibull προσαρμόστηκε στα πειραματικά δεδομένα της αδρανοποίησης των δύο στελεχών. Τα αποτελέσματα έδειξαν ότι η αδρανοποίηση των σπορίων αυξάνονταν με την αύξηση των επιπέδων πίεσης και θερμοκρασίας. Πλήρης αδρανοποίηση των σπορίων και για τα δύο στελέχη επιτεύχθηκε στην υψηλότερη πίεση (600 MPa) και την υψηλότερη θερμοκρασία (70 °C). Επίσης παρατηρήθηκε ότι το μοντέλο Weibull μπόρεσε να περιγράψει ικανοποιητικά την αδρανοποίηση των σπορίων του βακτηρίου *A. acidoterrestris* για τα διαφορετικά επίπεδα πίεσης και θερμοκρασίας.

Παρότι η αδρανοποίηση του βακτηρίου *A. acidoterrestris* έχει μελετηθεί εκτενώς, η επιβίωση των σπορίων και των βλαστικών μορφών του μικροοργανισμού κατά τη διάρκεια της συντήρησης των χυμών φρούτων μετά την επεξεργασία με ΥΥΠ, δεν έχει τεκμηριωθεί επαρκώς. Για τον σκοπό αυτό, στο **Κεφάλαιο 5** δείγματα πορτοκαλοχυμού εμβολιάστηκαν με τα σπόρια δύο στελεχών του βακτηρίου *A. acidoterrestris* -που χρησιμοποιήθηκαν και στην μελέτη του Κεφαλαίου 4- και υποβλήθηκαν σε ΥΥΠ σε 600 MPa/60 °C για 5 και 10 min αντίστοιχα. Τόσο τα επεξεργασμένα όσο και τα μη επεξεργασμένα δείγματα (μάρτυρας) συντηρήθηκαν σε θερμοκρασίες 4, 12 και 25 °C για 60 ημέρες. Στα δείγματα πραγματοποιούνταν μικροβιολογικές αναλύσεις κάθε εβδομάδα ώστε να καταγραφεί η μεταβολή του πληθυσμού των σπορίων και των βλαστικών κυττάρων κατά τη διάρκεια της συντήρησης. Τα αποτελέσματα έδειξαν μείωση της εκβλάστησης των σπορίων που επιβίωσαν από την επεξεργασία, το μέγεθος της οποίας καθορίστηκε από το στέλεχος του βακτηρίου, την πίεση, τον χρόνο επεξεργασίας και την θερμοκρασία συντήρησης, ενώ τα εναπομείναντα βλαστικά κύτταρα δεν ανιχνεύθηκαν κατά την συντήρηση του χυμού σε χαμηλές θερμοκρασίες. Επίσης, με σκοπό την μελέτη της δυναμικής των σπορίων και των βλαστικών κυττάρων κατά την συντήρηση των χυμών σε υψηλότερες θερμοκρασίες, σπόρια του στελέχους αναφοράς *A. acidoterrestris* ATCC 49025 εμβολιάστηκαν σε δείγματα χυμού ροδάκινου. Τα δείγματα υποβλήθηκαν σε ΥΥΠ σε 500 και 600 MPa σε συνδυασμό με ταυτόχρονη θέρμανση (25, 45 και 60 °C) για 10 min. Επεξεργασμένα και μη επεξεργασμένα (μάρτυρας) δείγματα χυμού ροδάκινου συντηρήθηκαν σε θερμοκρασίες 25, 35 και 45 °C και αναλύθηκαν μικροβιολογικά για χρονικό διάστημα έως 240 h. Τα αποτελέσματα έδειξαν ότι η επεξεργασία με ΥΥΠ σε 600 MPa/60 °C/10 min μπόρεσε να εξουδετερώσει τα σπόρια του βακτηρίου κατά την συντήρηση του χυμού σε θερμοκρασία 25 °C, ενώ ο πληθυσμός των βλαστικών κυττάρων μπόρεσε να εξαλειφθεί με την ίδια επεξεργασία σε όλες τις εξεταζόμενες θερμοκρασίες συντήρησης του χυμού.

Επιστημονική Περιοχή: Μικροβιολογία τροφίμων

Λέξεις-Κλειδιά: *Alicyclobacillus*, *A. acidoterrestris*, PCR-RFLP, Υπερυψηλή Υδροστατική Πίεση, βακτηριακά σπόρια, βλαστικά κύτταρα, χυμοί φρούτων.

Ευχαριστίες

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Οφείλω να ευχαριστήσω θερμά την Δρ Ανθούλα Αργύρη, Εντεταλμένη Ερευνήτρια του ΙΤΑΠ που με παρότρυνε να ξεκινήσω αυτό το μεγάλο ταξίδι καθώς και για την στήριξη και καθοδήγηση της καθόλη τη διάρκεια της εκπόνησης της μελέτης. Ιδιαίτερες ευχαριστίες θα ήθελα να απευθύνω στην Δρ Αγάπη Δουλγεράκη Εντεταλμένη Ερευνήτρια του ΙΤΑΠ η οποία με μύησε στον μαγικό κόσμο της μοριακής μικροβιολογίας και με την υπομονή και την καθοδήγηση της ανταπεξήλθα σε αυτό το δελεαστικό κομμάτι της επιστήμης. Τις ευχαριστώ επίσης και τις δύο γιατί ήταν πάντα εκεί για να λύσουν απορίες και προβλήματα. Θερμές ευχαριστίες θα ήθελα να απευθύνω και στον Δρ Γιώργο Κατσαρό Εντεταλμένο Ερευνητή του ΙΤΑΠ για την τεχνική του υποστήριξη με την μονάδα της Υπερυψηλής πίεσης που πάντα είχε την λύση για την επισκευή και τη λειτουργία της.

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Όμως όλο αυτό δεν θα μπορούσα να το έχω ονειρευτεί και να το φέρω σε πέρας χωρίς τη συμβολή των γονιών μου. Μέσα από την εργατικότητα και την δημιουργικότητα τους με δίδαξαν να βάζω υψηλούς στόχους και να παλεύω για να τους πραγματοποιήσω, να αγωνίζομαι και να γίνομαι καλύτερη. Μαμά σε ευχαριστώ που είσαι πάντα εκεί και μπαμπά ελπίζω από εκεί ψηλά να είσαι υπερήφανος όπως ήσουν πάντα.

Το πιο μεγάλο ευχαριστώ όμως το οφείλω στον σύντροφο της ζωής μου Γιώργο που πίστεψε σε εμένα και με στήριξε με όλη την αγάπη και την δύναμη του για να τα καταφέρω. Ευχαριστώ εκείνον και τα παιδιά μου Γιάννη και Αθηνά, για την υπομονή τους, τις ώρες ησυχίας που χρειάστηκε να μου προσφέρουν και την κατανόηση που έδειξαν στη στέρηση ενασχόλησης μου κάποιες φορές με τις ανάγκες τους.

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BAT *Bacillus acidoterrestris* Thermophilic

BAM *Bacillus acidocaldarius* Medium

DGGE Denaturing Gradient Gel Electrophoresis

DNA Deoxyribonucleic Acid

DPA Dipicolonic Acid

ELISA Enzyme Linked Immunosorbent assay

EN Electronic Nose

EOs Essential Oils

FDA Food and Drug Administration

FTIR Fourier Transform Infrared Spectroscopy

GC Gas Chromatography

HACCP Hazard Analysis and Critical Control Points

HHP High Hydrostatic Pressure

HPH High Pressure Homogenization

HPLC High Performance Liquid Chromatography

HV Hyper Variable

IFU International Federation of Fruit Juice Producers

IMS Immunomagnetic Separation Method

LAB Lactic Acid Bacteria

MRS De Man Rogosa and Sharpe

OSA Orange Serum Agar

p shape parameter

PCA Plate Count Agar

PCR Polymerase Chain Reaction

PDA Potato Dextrose Agar

PECA Peroxidase Enzyme Colorimetric Assay

R^2 Coefficient of determination

RAPD Random Amplification of Polymorphic DNA

RBC	Rose Bengal Agar
RFLP	Restriction fragment length polymorphism
RMSE	Root Mean Squared Error
RNA	Ribonucleic Acid
RT	Reverse transcription
SPME	Solid Phase Microextraction
T	Temperature
TVC	Total aerobic Viable Counts
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UV-C	Ultraviolet
VIT	Vermicon Identification Technology
YSG	Yeast Starch Glucose
δ	scale parameter

CHAPTER 1

Fruit Juice Spoilage by *Alicyclobacillus*: Detection and Control Methods—A Comprehensive Review

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1.1. Introduction

Fruit juices are the most popular beverages, representing a significant market share within the food industry, and have an important role in human diet since their particular combination of physical and chemical characteristics render them natural and healthy [1]. They are low calorie foods rich in nutrients and bioactive compounds such as proteins, vitamins, carbohydrates, polyphenols, minerals, enzymes, fibers and antioxidants that can fit in today's busy life style [2]. The full definition for fruit juice is "the fermentable but unfermented product obtained from the edible part of fruit which is sound and ripe, fresh or preserved by chilling or freezing of one or more kinds mixed together having the characteristic color, flavor and taste typical of the juice of the fruit from which it comes" [3]. Fruit juices can be classified according to their composition as fruit juice, fruit juice from concentrate, concentrated fruit juice, water extracted fruit juice, dehydrated/powdered fruit juice and fruit nectar. Depending on their dispersion system composition they are divided into clear, opalescent, cloudy and pulp enriched juices. According to the preservation method employed in order to prevent spoilage (microbial, chemical and enzymatic), while retaining their quality and nutritional value, they are classified as freshly squeezed, chilled, frozen, pasteurized and concentrated [4,5]. Today consumers prefer fruit juices as an easy way to cover the five servings of fruits and vegetables recommended by the World Health Organization for a healthy diet. The variety of different juice products on the market in combination with the use of new preservation technologies make them even more attractive. Furthermore, due to their low pH value, fruit juices do not favor the survival of pathogenic and spoilage microorganisms, making them safer and therefore more attractive to consumers [6,7].

1.2. Spoilage and Safety Aspects of Fruit Juices

In recent years many outbreaks concerning fruit juice contamination have been reported and the fruit juice industry has suffered financial damage [8,9]. Pathogenic and spoilage microorganisms are a challenge for the fruit juice manufacturers. The type of microorganisms present in fruit juice can originate from the fruits before harvest, therefore fallen fruits or fruits wounded from insects should be avoided. Other sources of microbial contamination could be the added water, flavorings or other chemicals, and finally process machinery and filling lines with deficient hygiene protocols. The relevant microorganisms considered as threats for commercial fruit juices are yeasts,

molds and bacteria, while protozoa and viruses can also cause problems to a lesser extent [5,10].

Yeasts are the predominant spoilage microorganisms in fruit juices [11]. Their high acid tolerance and preference for anaerobic conditions, in combination with the sugar content and the refrigeration temperature during distribution and storage of the juice, favor spoilage incidents [12,13]. Contamination of fruit juices with yeasts results in carbon dioxide and alcohol production, increasing turbidity and flocculation, off-odors and changes in color [14]. It has been proved from previous researchers that representatives of *Saccharomyces*, *Candida*, *Zygosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Hanseniaspora*, *Pichia* and *Trichosporon* genera are most frequently encountered in fruit juices [5,14,15]. The occurrence of contamination from yeasts in the fruit juice industry could be attributed to highly contaminated raw materials, failure in the pasteurization process and poor hygiene practices [16].

Moulds are microorganisms frequently encountered in fruit juices [11]. They are aerobic microorganisms that prefer low pH and high sugar content for growth [13]. Depending on their response to thermal treatment, moulds can be classified into heat resistant and heat sensitive [14]. The dominant heat resistant molds that have appeared in fruit juices over the years are *Aspergillus ochraceus*, *Aspergillus tamarii*, *Aspergillus flavus*, *Byssochlamys nivea*, *Byssochlamys fulva*, *Paecilomyces variotii*, *Neosartorya fischeri*, *Eupenicillium brefeldianum*, *Phialophora mustea*, *Talaromyces flavus*, *Talaromyces trachyspermus*, *Thermoascus aurantiacum*, *Penicillium notatum*, *Penicillium roquefortii* and *Cladosporum* spp. [17–20]. These moulds can produce gas, form colonies and floating mycelia on the surface, and change the odor of the juice [13,16]. Furthermore, they can cause disintegration to the fruit juice since they have the ability to produce disintegrative and pectinolytic enzymes [21], such as amylases, cellulases, pectinases and proteinases [5]. The most frequent heat sensitive molds belong to the genera of *Aspergillus*, *Penicillium*, *Mucor*, *Alternaria*, *Cladosporium*, and *Botrytis* [16,22]. Although these moulds can be eliminated with the pasteurization process [23], their presence indicates high contamination in the raw material or insufficient hygiene conditions during the manufacturing process [14]. Moulds are also associated with the production of mycotoxins, which is a serious safety issue for the fruit juice industry. They are secondary metabolites produced by fungi growing on food matrices. A mould has the ability to produce different kinds of mycotoxins and on the other hand one mycotoxin can be produced from different kinds of moulds [24]. The

most dominant mycotoxins concerning the fruit juice industry are patulin and ochratoxin A. Patulin is mainly associated with apple juice and ochratoxin A with grape juice [25–27]. The maximum level for patulin in apple juice was established as 50 ppb [28], while the European regulation [29] recommends a maximum level of 25 ppb for solid apple products. The maximum concentration of ochratoxin A in grape juice and grape juice ingredients in other drinks has been defined as 2 ppb [30]. These mycotoxins could be a serious threat to human health worldwide due to their toxicity.

Bacteria are another group of microorganisms that has been associated with spoilage in fruit juices [7]. The acidic pH of most fruit juices favors the presence of lactic acid bacteria (LAB) and particularly the genera *Lactiplantibacillus* and *Leuconostoc* [7]. They produce off flavors similar to buttermilk and metabolic products such as lactic acid, formic acid, acetic acid, ethanol and carbon dioxide [16,31] that can change the juice flavor. Furthermore, their presence can cause haze and gas in the product [16]. Acetic acid bacteria are frequently found on many fruit surfaces and therefore associated with the spoilage of fruit juices, with *Acetobacter pasteurianus* and *Acetobacter aceti* being the predominant species [23]. These bacteria produce acetic acid from ethanol, sauerkraut and buttermilk off-flavors and can also cause browning of the juice [14]. Although both LAB and acetic acid bacteria are heat sensitive and can be destroyed with pasteurization [32], their presence indicates insufficient cleaning and sanitization of the equipment throughout the production line [14,33]. In order to avoid contamination, high standard hygiene protocols should be applied throughout processing [34]. Spore forming bacteria are a major issue for the fruit juice industry, since they can cause spoilage and cannot be controlled with standard pasteurization. The main problem from this group is due to *Alicyclobacillus* spp. and its predominant species *Alicyclobacillus acidoterrestris* [5]. Except from spoilage bacteria, pathogenic bacteria could be also considered as a threat to the fruit juice industry. Although fruit juices have been considered safe throughout the years, several foodborne outbreaks have been reported especially with unpasteurized fruit juices [35]. *Escherichia coli* O157:H7 [36–39], *Salmonella* [37,40–43] and *Staphylococcus aureus* [31] are considered to be implicated in many outbreaks of unpasteurized fruit juices including cider, apple juice and orange juice. Although *Listeria monocytogenes* has not been considered as a pathogen implicated directly in fruit juice outbreaks, it should be taken into consideration since it has the ability to survive throughout the production line of

fruit juices [44]. In order to avoid the presence of pathogenic bacteria, the industry must retain high standard protocols of hygiene throughout the production line [17].

Protozoa are another threat for the fruit juice industry. The parasites of concern are *Cryptosporidium parvum* and *Cyclospora cayetanensis* that cause diarrhea [7], and the protozoan *Trypanosoma cruzi*, which causes Chagas disease affecting the autonomous nervous system in the esophagus, the heart and the colon [45]. Unpasteurized apple juice and cider have been associated with outbreaks of cryptosporidiosis [46,47], while in Brazil *Trypanosoma cruzi* has been involved in outbreaks associated with consumption of bacaba juice [48] and acai palm fruit juice [49].

Viruses can also contaminate fruit juices. Norovirus and Hepatitis A have been associated with outbreaks involving fruit juices such as orange juice [50,51]. The transmission of the viruses passes through contaminated fruit or water that has come in contact with feces [14]. The presence of protozoa and viruses can be intercepted by good agricultural and manufacturing practices and implementation of HACCP [52].

1.2.1. Alicyclobacillus spp. General Characteristics

In recent years, *Alicyclobacillus* has become the most serious threat of the juice industry. The isolation of this bacterium from various acid thermal environments was reported for the first time in the USA by Darland and Brock [53] and in Italy by De Rosa et al. [54]. Based on a previous published work in Japan [55], the characteristics of these bacteria were very similar to thermo-acidophilic microbes containing unusual ω-cyclohexane fatty acids as a major component in their membranes. This microorganism was classified as a new species of the genus *Bacillus* and it was named *Bacillus acidocaldarius* [53]. In 1981, thermoacidophilic bacteria closely related to *Bacillus acidocaldarius* were isolated from neutral soils [56]. The first isolates from a non-thermal source of this species were reported by Cerny et al. [57] after a spoilage incident of pasteurized apple juice in Germany in 1982. These isolated strains were similar to those reported by Hippchen et al. [56] but differed from *Bacillus acidocaldarius* in the use of carbon sources. Thus Deinhard et al. [58] proposed to name the new species *Bacillus acidoterrestris*. Poralla and König [59] identified another ω-alicyclic fatty acid microorganism that contained mainly ω-cycloheptane and named this bacterium *Bacillus cycloheptanicus*. However, after sequence analysis on the 16S ribosomal RNA genes of the three species (*B. acidocaldarius*, *B acidoterrestris* and *B.*

cycloheptanicus), results indicated that they were very similar to each other but distinct from any other *Bacillus* species. Consequently, it was proposed that these three species should be reclassified and the new genus was named *Alicyclobacillus,* in favor of the *ω*-alicyclic fatty acids in their membranes [60].

Throughout the years more *Alicyclobacillus* species have been described (Table 1.1), but according to many researchers the predominant spoilage species is *A. acidoterrestris*. *Alicyclobacillus* species are Gram positive, except for *A. sendaiensis* [61], non-pathogenic, thermo-acidophilic rod-shaped endospore forming bacteria [62,63]. They have the ability to grow in a temperature range of 20–70 °C, with the optimum between 40–60 °C, and in a wide pH range (2.0–6.0), with an optimum between 3.5 and 4.5 [64]. Although all species are anaerobic and the presence of oxygen is expected to influence the growth of the microorganism, there is no agreement in the literature about the effect of oxygen on bacterial growth. Cerny et al. [65] reported that the presence or absence of the headspace in the container made no essential difference in the growth of *A. acidoterrestris* and no spoilage was observed in either case. On the contrary, Walker and Philips [66] demonstrated that containers of apple juice without headspace showed significantly lower growth levels in comparison to those containing headspace. Siegmund and Pöllinger-Zierler [67] also verified that the presence of limited oxygen decelerated *A. acidoterrestris* growth in apple juice without preventing high cell concentrations.

The presence of *ω*-alicyclic fatty acids in the membranes of *Alicyclobacillus* species is the dominant characteristic that distinguishes them from other spore forming bacteria. Researchers have claimed that *ω*-cyclohexane and *ω*-cycloheptane rings in fatty acids contribute to the strong heat and acid resistance of *Alicyclobacillus* [68]. It has been also stated that the presence of cyclohexane rings in membranes increased the acyl chain density, resulting in a denser packing of the lipids in the membrane core, structural stabilization and lower fluidity and permeability of the membrane. This probably justifies the maintenance of the barrier function of the membrane, thus protecting the microorganisms in acidic and high temperature environments by forming a protective coating with strong hydrophobic bonds [60,68,69]. Another characteristic that may contribute to the resistance to extreme environments is the presence of hopanoids in the cells of most *Alicyclobacillus* strains [56,70]. The hopane glycolipids are structurally similar to cholesterol, and they affect the membrane lipid organization

due to a decreased mobility of the acyl chain lipids. Furthermore, this action is more advantageous at low pH values [70].

The heat resistance of *Alicyclobacillus* endospores has been associated with several other factors including temperature, pH and water activity. Specifically, the temperature of thermal treatment exerts the greatest influence on the heat resistance of endospores, since the *D*-value decreases with increasing temperature. In addition, pH and Total Soluble Solids (TSS) also affect the heat resistance with a linear decrease in *D*-value with decreasing pH, and a linear increase in *D*-value when the content of TSS increases. Water activity also has an impact, since it has been shown that bacterial spores become more resistant as the values of a_w decrease [71]. Moreover, endospore resistance to heat can also be influenced by the presence of heat stable proteins and enzymes and the mineralization of dipicolinic acid (DPA) with divalent cations of calcium or manganese [68,72]. It needs to be noted that different strains even of the same species of *Alicyclobacillus* may have different *D*-values [73]. Furthermore, the cell number, the cell age, the sporulation temperature and the state of the endospore protoplast cortex can influence the heat resistance of the endospores [72,74,75].

Throughout the years, *Alicyclobacillus* species have been isolated from various environments, such as hot springs [60] and soils [76,77], as well as beverages, fruit concentrates and fruit juices [78–82]. The contamination of fruit juices by *Alicyclobacillus* species is most likely caused by soil, during harvest, as well as by fallen and unwashed or poorly washed fruits [68]. Employees can also transfer spores from the soil in the manufacturing facilities. Researchers have reported that water can also be a source of contamination [83–87] in the processing environment. Spoilage incidents of fruit juices by *Alicyclobacillus* species have increased considerably in the last years [78,79,81,88–90] including concentrated orange juice [6,91,92], apple juice [85,93], mango juice [94], passion fruit juice [95], pear juice [84,86], banana and watermelon juice [75], grapefruit and blueberry juice [96], and lemon juice [62].

The fact that spoilage due to the presence of *Alicyclobacillus* is difficult to detect makes this microorganism a serious problem for the fruit juice industry. Since there is no gas production or swelling of the container, contamination cannot be perceived until the consumer complains [68]. The evident sign of spoilage after consumption is an off-flavor described as medicinal, phenolic and antiseptic [69,97] associated mainly with the production of guaiacol (2-methoxyphenol), but also with the halophenols 2,6 dibromothenol and 2,6 dichlorophenol [63,98]. Guaiacol, which is the major metabolite

Table 1.1. *Alicyclobacillus* species isolated from various sources

***Alicyclobacillus* Species**	**Source**	**Reference**
A. acidiphilus	Acidic beverage	[78]
A. acidocaldarius	Thermal acid waters	[53,55,60]
A. acidocaldarius subsp. *acidocaldarius*	Fruit juice or soft drink	[101]
A. acidocaldarius subsp. *rittmannii*	Geothermal soil of Mount Rittmann, Antarctica	[102]
A. acidoterrestris	Soil/apple juice	[56,58,60,87]
A. aeris	Copper mine, China	[103]
A. cellulocilyticus	Steamed Japanese cedar chips	[104]
A. consociatus	Human clinical specimen	[105]
A. contaminans	Soil, Fuji city Japan	[76]
A. cycloheptanicus	Soil	[58–60]
A. dauci	Mixed vegetable/fruit juices	[106]
A. disulffidooxidans	Water sludge, Canada	[107,108]
A. fastidiosus	Apple juice	[76]
A. ferrooxydans	Solfataric soil	[109]
A. fodiniaquatilis	Acid mine water, China	[110]
A. herbarius	Hibiscus herbal tea	[111]
A. hesperidum	Solfataric soil	[112]
A. kakegawensis	Soil, Japan	[76]
A. macrosporangiidus	Soil, Japan	[76]
A. montanus	Hot spring	[113]
A. pohliae	Geothermal soil, Antarctica	[114]
A. pomorum	Mixed fruit juice	[115]
A. sacchari	Liquid sugar	[76]
A. sendaiensis	Soil, Japan	[61]
A. shizuokensis	Soil in crop fields, Japan	[76]
A. tengchongensis	Soil in hot spring, China	[116]
A. tolerans	Oxidizable lead-zink ores	[108]
A. vulcanalis	Hot spring, United States	[117]

associated with off-flavors in fruit juices, can be a product of microbial metabolism [68]. It is a taint compound produced during ferulic acid metabolism, from a non-oxidative decarboxylation of vanillic acid, catalyzed by vanillate decarboxylase [99,100].

1.2.2 Alicyclobacillus acidoterrestris

Since its first association with spoilage in fruit juices in 1984 [57], *Alicyclobacillus acidoterrestris* has been considered as a challenge for the fruit juice industry worldwide [14]. It is the most important representative of the genus due to the number of reported spoilage incidents [63]. *A. acidoterrestris* has been isolated from a variety of juices and concentrates including apple, orange, lemon, mango, grapefruit, pear, tomato, white grape, pineapple, passion fruit, blueberry, pomegranate, cherry, strawberry, chokeberry, raspberry, watermelon, blackcurrant, kiwi and banana [82,92,95,118–123]. It is a spore forming bacterium that can survive thermal treatment during pasteurization, grow at low pH, germinate, and spoil the juice [124]. Therefore, it has been proposed as a target microorganism to control the effectiveness of the pasteurization process in acid fruit juices. The maximum accepted concentration of *A. acidoterrestris* spores as defined by the fruit juice industry is 100 CFU/mL of raw material [125]. *A. acidoterrestris* is an aerobic, Gram-positive, rod-shaped endospore-forming spoilage microorganism [62,63]. It can grow in a wide pH range (2.0–7.0) with the optimum between 3.5 and 4.0, and in a temperature range of 25–60 °C with the optimum between 40 and 45 °C [126–128]. *A. acidoterrestris* spores are very heat resistant and, depending on the conditions of thermal treatment and bacterial strain, $D_{90°C}$ ranges between 5.95 and 23 min [129] and $D_{95°C}$ between 0.06 and 8.55 min [130]. The main characteristic of *A. acidoterrestris* strains that make them so tolerant to heat is the presence of ω-cyclohexane fatty acids in their membranes, as previously reported [60,87].

A. acidoterrestris spores have a slow growth cycle of ca. 5 days [62]. Spoilage is not visible during storage or retail since there is no gas production and swelling of the juice container (flat-sour type spoilage). Only after consumption, flavors described as “smoky”, “antiseptic” or “disinfectant” and possible increased turbidity and sediment formation can lead to the conclusion of spoilage of the juice [87,131,132]. The predominant taint compound responsible for this is guaiacol [131]. Although the contamination pathway with guaiacol from *A. acidoterrestris* has not been clearly

elucidated, the most accepted assumption is that it is produced during ferulic acid metabolism [69,133]. Microorganisms usually decarboxylate ferulic acid to 4-vinylguaiacol [134] causing a "rotten" flavor especially in orange juice [135]. However, it can also be directly metabolized to vanillin [136] or vanillic acid [137]. *A. acidoterrestris* is capable of producing guaiacol from vanillin [138] and vanillic acid [139]. Although tyrosin and lignin have been suggested as precursors for the production of guaiacol from *A. acidoterrestris*, the pathway has not been studied extensively [69,133]. It has been proved that when the concentration of *A. acidoterrestris* cells ranges between 10^5 and 10^6 CFU/mL it produces enough guaiacol to spoil the juice [75,131]. Considering the substantial economic losses in the fruit juice industry due to the growth of *A. acidoterrestris* spores, the factors that induce spoilage should be seriously taken into account, specifically since spoilage is not apparent before consumption. The cell concentration of the microorganism, the heat shock treatment, the incubation temperature, the oxygen availability and the growth medium are among the factors influencing spoilage [68]. It must be noted that spore germination and growth is inhibited under 20 °C [140] and even at low oxygen concentration, contamination cannot be completely suppressed [67]. Furthermore, the growth behavior of *A. acidoterrestris* strains depends on the type of the juice and the isolation source of the strain [76]. However, the presence of *A. acidoterrestris* in juice is not a threat for human health, since neither the microorganism nor its metabolites have ever been associated with illness from the consumption of contaminated juice [141]. Although *A. acidoterrestris* is a non-pathogenic bacterium, spoilage incidents are a major concern for the fruit juice industry mostly because of the difficult detection of the bacterium, due to the absence of visible deterioration of the containers and associated spoilage.

1.3. Isolation and Identification of *Alicyclobacillus* spp.

Since spoilage from *Alicyclobacillus* has become a major issue for the fruit juice industry resulting in high economic losses, the need for developing rapid, accurate and sensitive methods for the early detection of the bacterium is of paramount importance. Initially researchers used mainly direct plating and spoilage detection methods, but nowadays detection methods based on instrumental analysis, immunodetection and molecular analysis are becoming more popular. Detection can be separated into three strategies, namely (a) targeting the cell/spore detection, (b) nucleic acid analyses, and

(c) metabolites measurement [142]. An overview of the detection and identification methods for *Alicyclobacillus* is displayed in Table 1.2.

Plating methods are simple and reliable but cannot detect low populations of the bacterium. Since research findings indicate that *A. acidoterrestris* spores do not grow on acidified agar such as Brain Heart Infusion agar, Nutrient agar, Standard Plate Count agar, Tryptone Soy agar and Veal Infusion agar, new media have been developed in order to isolate and successfully enumerate these spores [69,171]. Thus, the media that favor the growth of *Alicyclobacillus* after being acidified to pH 3.5–5.6 by HCl, H_2SO_4 and malic acid after autoclaving [68,75] are *Bacillus acidocaldarius* medium (BAM) and *Bacillus acidoterrestris* thermophilic (BAT) agar [58,92,187,188], Yeast Starch Glucose Agar (YSG) [78,111,189], Orange Serum Agar (OSA) [190], K agar [85], Potato Dextrose Agar (PDA) [91,96,118] and SK agar [191]. It has also been suggested that spread plating is more effective than pour plating for bacterial growth [171,188,192], but Yokota et al. [64] reported the opposite on YSG agar. Although several traditional microbiological methods have been employed for the detection of *Alicyclobacillus* strains, the IFU Method No 12 developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (IFU) is considered to be the most effective [193]. This method can distinguish spoilage and non-spoilage species but it is time-consuming. The membrane filtration technique is another method used in combination with the plating method, mainly when high populations of the bacterium must be detected [189]. IFU Method No 12 recommends the use of 0.45 μm filters, while the European Fruit Juice Association (AIJN) recommends 0.2 μm filters. Although this technique is more sensitive and has a lower detection limit [194], it cannot be used for all products [195]. Several isolation methods including the IFU Method No 12, apply heat shock treatment to the endospores of the microorganism in order to destroy the existing vegetative cells and induce the germination of predominant spores [64]. Many heat treatment schemes have been suggested but the predominant include thermal treatment at 80 °C for 10 min (recommended by the IFU) and at 70 °C for 20 min (recommended by the JFJA) (Japan Fruit Juice Association) [69,75,133]. Although these methods have been widely employed for routine analysis by the industry due to their low cost, they are time consuming and demanding, thus novel rapid detection and identification techniques are needed.

Table 1.2. Detection methods of *Alicyclobacillus* species

Detection Method	Isolation Source/Medium	Reference
Cell/Spore-Based Methods		
ELISA	Apple juice	[143–146]
	Apple juice concentrate	[147,148]
	Orange, clear apple, unfiltered apple, pear, tomato, pink grapefruit, and white grape	[149]
Flow Cytometry	Apple juice concentrate	[141]
Molecular Methods		
PCR	Isotonic water, lemonade, fruit juice blend, fruit carrot juice blend	[81]
	Orchard soil	[77]
	Mango juice	[94]
	Fruit concentrates and soils	[122]
	Various food and soil	[150]
PCR/RAPD-PCR	Orchard soil, soil on the fruit (pear, peach, apricot and apples) and samples of water and materials through the production line	[86]
	Soil from lemon orchard	[151]
	Soil of Foggia and pear juice	[152]
RAPD-PCR	Passion fruit juice	[95]
	N/A*	[149]
PCR/ERIC-PCR	Orchard soil	[153]
PCR/PCR-DGGE	Fruit juices and fruit juice blends from Ghana and Nigeria	[80]
PCR/RT-PCR	Apple juice and saline	[154]
PCR-RFLP	Various juices and concentrates, drinks and intermediates	[155]
	Concentrated apple juice	[156]
	Concentrated apple juice and processing environment	[85]
	Orange juice	[157]
RT-PCR	Orange juice	[158,159]
	Orange juice, sports drink, lemonade and NaCl solution	[160]
	Apple juice	[161]
	Flavored non-carbonated drinks	[162]
	Acid buffer	[163]
IMS-PCR	Apple juice	[144]
	Sterile water, apple juice and kiwi juice	[164]
IMS-RT-PCR	Apple and kiwi fruit orchard and fruit juice production line	[165]
qPCR(quantitative)	Apple juice	[166]

16S rRNA sequencing	N/A*	[60]
	Soil and water	[111]
	Various orchards	[76]
	Kiwi juice, fruit, soil and air of orchards and fresh cut and frozen fruit	[82]
Analytical Methods		
HPLC	Apple juice	[138,167,168]
	Flavored non-carbonated drinks	[162]
	Pear concentrate	[169]
	Fruit juices and fruit juice blends from Ghana and Nigeria	[80]
	Soil from lemon orchard	[151]
	Tomato puree	[170]
GC	Mixed fruit drink	[140]
GC-MS	Apple drinks, apple juice concentrate and orange juice	[171]
	Apple, orange and peach juice	[172]
	Fruit concentrate, flume water and vinegar flies	[173]
	Kiwi juice and fruit	[110]
	Apple juice	[174]
GC-MS/GC-Olfactometry	Orange juice	[131]
	Fruit concentrates and soils	[122]
GC-MS/SPME	Apple juice	[67,161,175–178]
	Apple, pear and orange juice	[179]
	Orange juice	[180,181]
Electronic Nose	Apple, pear and orange juice	[179]
	Flavored non-carbonated drinks	[162]
	Apple, orange and peach juice	[172]
	Apple and orange juice	[98]
	Apple juice	[178,182]
	Concentrated apple juice	[174]
	Mixed fruit juice beverage	[183]
	Orange juice	[181]
Fourier Transform Infrared Spectroscopy (FTIR)	Apple juice	[184–186]

* N/A: Not Available

Enzyme-linked immunosorbent assay (ELISA) is a biochemistry assay that has been applied for the detection of *Alicyclobacillus* mostly in apple juice, using a specific polyclonal anti-*Alicyclobacillus* antibody [143,144]. This method reduces the detection time to 6–7 h but detects populations higher than 10^5 CFU/mL. The above procedure has been improved by adding immunomagnetic separation that has shortened the time of analysis to 3 h and the detection limit to 10^3 CFU/mL [196]. Even though ELISA is rapid and reliable, it has high cost of analysis and legal limitations of animal use for the antibody production [159].

Flow cytometry is another cell detection method based on laser light that scatters samples in order to obtain cell size and corresponding light patterns of DNA density. This method detects cell concentration higher than 10^3 CFU/mL for *Alicyclobacillus* strains in fruit juice concentrates within 10 h [141]. Although the detection is achieved within limited time, flow cytometry can only be used for fluid samples [133].

Over the years methods based on Polymerase Chain Reaction (PCR) have been widely employed in research for the rapid identification of microorganisms and were also successfully applied to *Alicyclobacillus*. Reverse transcription polymerase chain reaction (RT-PCR) was first used by Yamazaki et al. [197]. Based on *shc* (squalene-hopene cyclase) gene, a key enzyme in the biosynthesis of hopanoids, researchers managed to detect *A. acidoterrestris* and *A. acidocaldarius* with a detection level of 1–2 CFU/mL after 15 h of enrichment. In 2004, Luo et al. [154] developed a Taqman® RT-PCR method also based on *sch* gene with a detection level less than 100 CFU/mL within 3–5 h for both species. A Taqman® PCR targeting the 16S rRNA gene was able to detect more species of *Alicyclobacillus* within 5 h and with a detection limit lower than 100 CFU/mL [160]. Random Amplification of Polymorphic DNA (RAPD) PCR has also been selected as a rapid method in order to distinguish *Alicyclobacillus* strains [77,82,95,151,152,198,199]. The selected primer and the lysed DNA of the microorganism are mixed with *Taq* polymerase and after PCR and electrophoresis the bands that appear are further analyzed [200]. Yamazaki et al. [198] reported the identification of *A. acidoterrestris* from acidic juice by applying RAPD PCR within 6 h. Restriction fragment length polymorphism (RFLP) PCR is another rapid and low-cost method that differentiates homologous DNA sequences, which are detected by different length fragments after DNA digestion and are then cut by restriction endonuclease. After gel electrophoresis, a unique fingerprint is received. Analysis of

16 S rRNA RFLP has been used for the characterization of *Alicyclobacillus* strains from concentrated apple juice [85] and orange juice [157].

16 S rRNA sequence analysis has been used widely for identification, because this gene deviates among the closely related *Alicyclobacillus* bacterial species. Furthermore, the 5′-end hyper-variable region of the gene varies among *Alicyclobacillus* species and makes it sufficient for discrimination among species [201]. Moreover, the immunomagnetic separation method (IMS), which is based on magnetic beads that capture the microorganism cells improved the sensitivity of PCR and RT-PCR when cooperating with the 16 S rRNA gene for the detection of *Alicyclobacillus* [164,165,196].

Denaturing gradient gel electrophoresis (DGGE) has been also proved to be effective not only for the detection of *Alicyclobacillus* but also for the distinction of guaiacol producing and non-producing species by adding an *Alicyclobacillus* DNA sequence ladder mix on the DGGE gel [80]. Vermicon Identification Technology (VIT), which is based on fluorescent labelled probes, has been shown to have a detection limit of 1 CFU/mL within 3 h of isolation and can be applied directly to fruit juice concentrate. This method is also capable of differentiating *Alicyclobacillus acidoterrestris* from other *Alicyclobacillus* species since they glow in different colors [202].

Indirect detection of *Alicyclobacillus* spoilage can be determined by measuring the metabolites, mainly guaiacol. The determination can be accomplished using sensory, analytical, or chemical methods.

Sensory methods are mostly used for screening the sample for the presence or absence of taint compounds. A trained panel is usually asked to describe the taste, aroma, sourness, color, and finally the acceptability of the sample when compared to a control (unspoiled) sample [174]. The published studies concerning the detection of guaiacol by a sensory panel showed that the detection is highly dependent on the sensitivity of the panelists and on the sample matrix, due to the variation of the components in fruit juices [67,171,174,175,180]. Although analytical methods are considered to be more sensitive to the detection limit of guaiacol, other researchers reported that sensory analysis presented greater sensitivity [171,203].

Analytical methods are used for both qualitative and quantitative detection and the most frequently used are chromatographic analysis such as Gas-Chromatography (GC) and High-Performance Liquid Chromatography (HPLC). These methods include three steps: extraction, separation and identification [68,69]. After collecting an adequate

quantity of the sample, heat desorption or solvent extraction follows for GC or HPLC, respectively. The separation of the compounds with the use of specific columns depends on their molecular weight, solubility, ion exchange capacity and polarity, which emerge at different retention times. With the use of standards, the method can detect the chemical compound and its quantity [68]. The GC-MS (mass spectrometry) is widely employed due to the sensitivity of the detector [131,171,180] together with GC-O (olfactometry) [131]. Solid Phase Microextraction (SPME) has also been successfully combined with GC for the determination of volatile compounds [175,176,180,181]. Apart from GC, the use of HPLC for the detection of *Alicyclobacillus* spoilage has also been reported [138,167]. Although the former mentioned analytical techniques have been shown to be accurate, they are expensive, time consuming, require skilled personnel for operation and analysis of the results and cannot be adapted easily in the production line [133,142].

Electronic nose (EN) is an artificial sensing system, based on a chemical sensor array of semi selective gas sensors combined with pattern recognition algorithms. With the proper data analysis tools, EN could result in the early detection of *Alicyclobacillus* contamination. Gobbi et al. [172] detected *Alicyclobacillus* spp. in peach, orange and apple juice after 24 h from inoculation, while Cagnasso et al. [179] identified spoilage from *A. acidoterrestris* in orange and pear juice at the same time period. Concina et al. [162] identified the contamination of *Alicyclobacillus* spp. in commercial flavored drinks at the early stage of growth and Huang et al. [182] reported that EN could perceive a contaminated apple juice beverage after 4 h when coupled to linear discriminant analysis. It is a promising method because it is simple, quick, reliable and of low cost, and can be easily used in the production line [181].

Another rapid method that is widely used for the detection and identification of bacteria is Fourier Transform Infrared Spectroscopy (FTIR). FTIR is based on measuring distinct biochemical characteristics of the cytoplasm and the cell wall components, presenting them as different spectral features at 400–4000 cm^{-1}. The detection limit of this method is 10^3–10^4 CFU/mL and it can distinguish different species of *Alicyclobacillus* and classify them as guaiacol and non-guaiacol producing strains [184,185]. Some drawbacks of this method include the cost of the equipment and an essential extension with comprehensive spectral reference database in order to limit detection time for unclassified *Alicyclobacillus* strains [133,142].

The chemical method that has been broadly used for the detection of guaiacol is Peroxidase enzyme colorimetric assay (PECA), which can detect and quantify the presence of guaiacol. PECA is based on the oxidation of guaiacol by peroxidase enzymes in the presence of H_2O_2 with the formation of a brown compound, identified as 3,3′-dimethoxy-4,4′-biphenoquinone [204], which can be measured by spectrophotometry at 420 nm [138,167] or 470 nm [204–206]. The guaiacol detection kits that are available in the market are based on this method and besides detecting guaiacol they can also quantify it by using standard concentration curves. Although this method is simple, less time consuming and of low cost, it is imprecise and most frequently used only for the detection of the presence of guaiacol and not for quantification.

1.4. Control of *Alicyclobacillus* spp.

The spoilage of fruit juices from *Alicyclobacillus* spp. has been shown to start from the beginning of the supply chain, since contaminated fruits at harvest can intrude into the production line and cause problems that will appear only after consumption. This observation necessitates the implementation of highly effective measurements in order to avoid spoilage from the beginning and therefore economic loss for the fruit juice industry. Good Manufacturing Practices and systematic use of Hazard Analysis and Critical Control Points (HACCP) rules throughout the whole supply chain can control contamination from *Alicyclobacillus*. In order to ensure fruit juices with high safety and extended shelf life, chemical, physical, and combined methods have been developed.

1.4.1 Chemical treatments

The first step to control the contamination from this microorganism is to avoid harvesting fallen fruit or at least wash the surface of the fruit properly with the use of disinfectants. The oxidants that are usually diluted in water are sodium chlorite ($NaClO_2$), chlorus acid ($HClO_2$) and chlorine dioxide (ClO_2) [175]. Since 1998, the use of ClO_2 has been allowed by the Food and Drug Administration [207] as an antimicrobial chemical and therefore it is widely used for the disinfection of fruit, containers, and processing equipment. The effectiveness of this sanitizer on the inactivation of *Alicyclobacillus* spores is possibly due to the injury of the inner membrane of the spore resulting in germination and outgrowth [208]. Bevilacqua et al.

[209] also implied that this oxidizing compound aimed to damage the inner membrane *of A. acidoterrestris* spores. These disinfectants can also be used as preservatives in the fruit juice processing line [133].

Ozone (O_3) is another oxidant also recognized as safe from the FDA that can be used in fruit juices. It also has the potential to eliminate *Alicyclobacillus* spores, since it has been shown that as the concentration and the treatment time of O_3 increases, the inactivation of *A. acidoterrestris* also increases [210].

The growth of *Alicyclobacillus* can also be controlled with the use of some organic acids. The effectiveness of acids on bacterial cells, but not on spores, in ascending order was benzoic, butyric-caprylic, acetic, citric-malic-lactic, and tartaric acids [211]. Chemical preservatives like sodium benzoate and potassium sorbate have been allowed to be added in beverages with a limit of 1500 mg/L [212]. It has been reported that they can control *A. acidoterrestris* growth [103] with the need for higher concentrations for vegetative cells than for spores [213].

The increasing demand of consumers for natural additives in food products has led to the use of natural compounds in fruit juices for the control of *Alicyclobacillus*. Natural antimicrobials of microbial origin, animal origin and plant origin have been successfully used for the inhibition of the microorganism [133].

1.4.1.1. Natural compounds of microbial origin

Bacteriocins are antimicrobial peptides or proteins that are produced from various bacteria, which present antimicrobial activity against closely related species [214]. Nisin is a non-toxic polypeptide used in many countries as a safe food preservative [215,216]. It is obtained from *Lactococcus lactis* subsp. *lactis* and has a significant effect especially on the spores of *A. acidoterrestris* [89,129,217–220]. Nisin is the only bacteriocin used for the control of *A. acidoterrestris* in the fruit juice industry at present [214], added either directly in the juice [129,217] or integrated into the biodegradable polylactic and polymer film of the container [221,222]. Studies have revealed more bacteriocins to be effective against *Alicyclobacillus* including enterocin AS-48 produced from *Enterococcus faecalis* [223], bificin C6165 from *Bifidobacterium animalis* subsp. *animalis* [224], biovicin HC5 purified from *Streptococcus bovis* [225], warnericin RB4 from *Staphylococcus warneri* [226], paracin C from *Lactiplantibacillus paracasei* [227,228] and cyclin A from *Lactiplantibacillus*

plantarum [229]. Although all of them have high potential in inhibiting *Alicyclobacillus* strains they have limited application in the industry due to the high cost of extraction and purification [133].

1.4.1.2. Natural compounds of animal origin

Lysozyme is an enzyme present in various biological tissues and fluids like tears, saliva, eggs and milk, often used to inhibit Gram positive bacteria and especially thermophilic spore forming bacteria at a concentration of 20 μg/mL [230,231]. Lysozyme is also considered as a safe preservative [232] and has been applied directly in fruit juices [233] or through the polymeric matrix film in packaging in order to control *Alicyclobacillus* [234,235]. The efficiency of lysozyme depends on the concentration, the strain of the bacterium and the external conditions applied [233,236–238].

Chitosan, the only basic polysaccharide in nature is a derivative of chitin, extracted from the shell of shrimps, crabs and crawfishes; it has the ability to control bacteria, yeasts, and molds [239,240]. When combined with thermal processing it can inhibit *A. accidoterrestris* spores from germinating at a concentration level of 1.4 g/L [241].

1.4.1.3 Natural compounds of plant origin

Essential oils (EOs) are aromatic liquids obtained by extraction, distillation, fermentation or enfleurage from plant materials, mostly herbs and spices that are used in the fruit juice industry as food flavorings [133,242,243]. The antimicrobial activity of cinnamaldehyde, eugenol and carvacrol has been reported to be efficient against *A. accidoterrestris* spores [90,244]. Lemon essential oil and extracts of *Eycalyptus maculata* also controlled the germination of *A. accidoterrestris* spores as reported by Maldonado et al. [245] and Takahashi et al. [244]. *Fatty acids and esters* have also been reported to have antibacterial activity against *A. accidoterrestris* spores. Manolaurin, which is recognized as a safe compound from the FDA, was effective against the vegetative cells of the microorganism [246]. Sucrose palmitate, sucrose stearates and sucrose laurates have also been reported as efficient antimicrobials against *Alicyclobacillus* spores [247]. Other plant extracts have been reported to have antimicrobial effectiveness against *Alicyclobacillus*. Saponin that was extracted from *Sapindus saponaria* fruits inhibited *A. acidoterrestris* spore germination, but affected the sensory quality and resulted in foam production [248]. Papain and bromelain

enzymes extracted from *Carica papaya* and *Ananas comosus*, respectively, also controlled *A. acidoterrestris* spore germination [249], whereas two formulations of *Rosmarinus officinalis* were effective against *A. acidoterrestris*, *A. hesperidum* and *A. cycloheptanicus* vegetative cells [250]. An overview of chemical treatments applied for the inactivation of *Alicyclobacillus* species is presented in Table 1.3.

Table 1.3. Overview of chemical treatments applied for the inactivation of *Alicyclobacillus* species

Chemical Treatments	Compounds	References
Oxidants	ClO_2	[133,207–209]
	Ozone	[210]
	Sodium benzoate, Potassium sorbate	[103]
Natural compounds of microbial origin (bacteriocins)	Nisin	[89,129,217–220]
	Enterocin A5-48	[223]
	Bificin C6165	[224]
	Biovicin HC5	[225]
	Warnericin RB4	[226]
	Paracin C	[227,228]
	Cyclin A	[229]
Natural compounds of animal origin	Lysozyme	[234,235]
	Chitosan	[241]
Natural compounds of plant origin	Essential Oils	[90,244,245]
	Fatty acids and esters	[246,247]
	Plant extracts	[248–250]

1.4.2 Physical treatments

Thermal pasteurization is a heat treatment successfully employed by the fruit juice industry in order to extend the shelf life of processed fruit juices. It manages to inactivate heat sensitive microorganisms and enzymes that can degrade the quality without influencing the sensory characteristics of the fruit juice. The conventional heat treatment (88–96 °C for 2 min) however is not sufficient against *Alicyclobacillus*, since its spores can survive pasteurization treatments and germinate under favorable conditions during storage [68,71]. Provided that refrigerated temperatures are ensured throughout the whole supply chain, *Alicyclobacillus* spores would not grow since germination is inhibited at temperatures below 20 °C. Distribution though does not always take place under refrigerated conditions, due to high cost, therefore spores may induce spoilage during warmer months [68]. In order to control the presence of *Alicyclobacillus*, the temperature of thermal treatment should be increased, but this would result in quality (vitamin and nutrient loss) and sensory (nonenzymatic browning and flavor compounds) deterioration [251–253]. Consequently, the development of

nonthermal methods is necessary to retain both safety and quality attributes of fruit juices.

1.4.3. Nonthermal treatments

High hydrostatic pressure (HHP) is based on two principles, namely the isostatic and the *Le Chatelier*. The first one secures the homogeneous and instant distribution of the pressure applied equally in all directions of the sample and the second one states that any occurrence of chemical or biochemical reaction, molecular configuration or phase transition that can lead to volume reduction is improved by pressure [254,255]. The food industry uses HHP with pressure ranging from 100 to 800 MPa, duration from milliseconds to more than 20 min, and treatment temperatures from 0 to 90 °C. The main mechanism of HHP that causes the inactivation of *Alicyclobacillus* is the damage to the noncovalent bonds that are present in lipids, proteins, nucleic acids and polysaccharides. In this way, HHP affects the cell membrane constituents such as proteins, enzymes and ribosomes and therefore damages the genetic material of the microorganism, since it causes denaturation of cell components resulting in the injury and death of the microorganism [256]. HHP has been used extensively for the inactivation of *Alicyclobacillus* because when combined with heat treatment it is very effective against spores that are very resistant to inactivation. HHP can cause the germination of spores, which are less resistant to dormant spores that will be subsequently killed with the simultaneous heat treatment [257,258]. The germination of spores at lower pressures (50 to 300 Mpa) proceeds via activation of nutrient receptors (D-sugars, L-amino and purine nucleosides), while at higher pressures (400–800 Mpa) germination is triggered by the direct release of Ca-DPA (dipicolinic acid) [259,260]. It has been proved that cycle pressure treatments can enhance the inactivation of the spores for an equivalent duration of a single pressure application. Treatment with the low-pressure exposure results in spore germination and the higher pressure inactivates spores and vegetative cells [261]. Relevant research concerning the application of HHP for the inactivation of *Alicyclobacillus* is summarized in Table 1.4. Although HHP is very effective, some dormant spores have the ability to remain immutable, a fact that must be taken into serious consideration by the fruit juice industry when applying HHP treatments [262]. Juices treated with HHP often exhibit superior quality compared to those treated with thermal processing since HHP has minimum

impact on color, flavor and taste while retaining nutrients, vitamins, amino acids and functional properties [263]. Taking into account consumers' demands for minimally processed products, HPP has many industrial applications and therefore it has been the most popular non-thermal treatment since the late 1980s.

High pressure homogenization (HPH) (150–200 MPa) or *Ultra High pressure homogenization* (350–400 MPa) is a food processing technology that is based on the principles of conventional homogenization with higher pressures [277] and can be applied only to fluid products. The shear stress distribution across the treated product is responsible for the changes occurring in microorganisms resulting in inactivation [5]. Bevilacqua et al. [126] reported reduction of *A. acidoterrestris* population with cells being more sensitive than spores when applying HPH (500, 800, 1100, 1400 and 1700 bar) for 2 ms to three different strains inoculated in malt extract broth. The susceptibility was proved to be strain dependent. This treatment has limited industrial applicability because of the need for refrigeration in order to guarantee the safety of final products.

Ultrasound or ultrasonic waves are electromagnetic waves with frequency above 20 kHz that can create cavitation in the cell wall of the microorganism and thus destroy it [278]. *A. acidoterrestris* vegetative cells in apple juice were inactivated with ultrasonic treatment that seemed to be more effective as the power level and the processing time increased [279]. Wang et al. [280] also reported that ultrasonic waves inhibited *Alicyclobacillus* vegetative cells and that the effectiveness of the method depended on the matrix, the strain, the power level, and the exposure time. Ultrasound treatment has been proved to be more effective when combined with other processes, in particular high pressure and heat. Although this treatment is considered to improve the quality of many products including fruit juices [281], it affects the sensory characteristics of the fruit juice and thus may not meet consumer's demand [279].

Microwaves are also electromagnetic waves that have the ability to change the cell membrane permeability, break the hydrogen bonds of RNA and DNA and thus inhibit the cell growth [133]. Microwave sterilization has been employed as a nonthermal treatment since it heats the product faster without influencing the texture and the taste and does not lead to cell cortex swelling like conventional sterilization [133,282].

Table 1.4. Overview of HHP conditions applied for the inactivation of *Alicyclobacillus* species

***Alicyclobacillus* Species**	**Medium**	**Experimental Conditions**	**Reference**
A. acidoterrestris ATCC49025 *A. acidoterrestris* NFPA 1013 (apple juice isolate)	Apple juice	207, 414 and 621 Mpa/1, 5 and 10 min/22, 45, 71 and 90 °C	[264]
A. acidoterrestris DSMZ 2492	BAM broth	Broth	[265]
	Orange juice	350 and 450 Mpa/5, 10 and 20 min/35, 45 and 50 °C	
	Tomato juice	Juices	
	Apple juice	350 Mpa/20 min/50 °C and storage 3 weeks/30 °C	
A. acidoterrestris DSMZ 2492	BAM broth	350 and 450 Mpa/35, 45 and 50 °C	[266]
A. acidoterrestris NFPA 1101 (apple juice isolate) *A. acidoterrestris* NFPA 1013 (apple juice isolate)	Apple Juice concentrate	207, 414 and 621 Mpa/1, 5 and 10 min/22, 45, 71 and 90 °C Various concentrations of juice (17.5, 35 and 70 °Brix)	[267]
A. acidoterrestris LMG 16906	Citric acid buffer (Ph 4.0 and 5.0) Potassium phosphate buffer (Ph 7.0) Tomato sauce (Ph 4.2 and 5.0)	HHP and combined treatment HHP + heat Buffers 100, 200, 300, 400, 500 and 600 Mpa/40 °C/10 min + heat 80 °C/10 min Tomato sauce 100, 200, 300, 400, 500 and 600 Mpa/25, 40 and 60 °C/10 min + heat 80 °C/10 min	[268]

A. acidoterrestris TO-29/4/02 (apple juice isolate)	Apple juice	• 200, 300 and 500 Mpa/30 min/50 °C continuously • 100, 200, 300 and 500 Mpa/2, 4 and 6 cycles of 5 min with 5 min pause/50 °C • 100, 200 Mpa × 6 cycles and 200 Mpa x 4 cycles/5 min with 5 min pause/50 °C incubation 60 min at 50 °C/pressure 500 Mpa/30 min 50 °C • Combined treatment of HHP + lysozyme: 300 Mpa/5, 10, 15 and 30 min/50 °C + 0.05 and 0.1 mg/Ml lysozyme • Combined treatment of HHP +nisin: o 300 Mpa/5, 10, 15 and 30 min/50 °C + 500, 750 and 1000 IU/Ml nisin o 200 Mpa/5, 10, 15 and 20 min/50 °C + 250 IU/Ml nisin	[237]
A. acidoterrestris NZRM 4098	Orange juice	200 and 600 Mpa/1–15 min/45, 55 and 65 °C	[263]
A. acidoterrestris DSMZ 2498	Apple juice Orange juice	200, 400 and 600 Mpa/10 min/20, 50 and 60 °C and storage for 28 days	[98]
A. acidoterrestris TO-29/4/02 (apple juice isolate) *A. acidoterrestris* TO-117/02 (apple juice isolate)	Apple juice (11.2, 23.6, 35.7 and 71.1 °Brix)	200 Mpa/5, 10, 15, 30 and 45 min/50 °C 200 Mpa/5, 10, 15 and 30 min/50 °C for 11 days/11 and 16 months spores of TO-29/4/02 200 Mpa/5, 10, 15 and 30 min/50 °C for 10 days/2, 10, 11 and 23 months spores of 117/02 200 Mpa/1, 5, 10, 15 and 30 min/50 °C × 3 subsequent treatments	[269]

A. acidoterrestris TO-117/02 (apple juice isolate)	Mcllvain buffer (Ph 4.0 and 7.0) Apple juice	Germination and Inactivation • 100, 200, 300, 400 and 500 Mpa/20 min/50 °C (buffers and juice) • 200 Mpa/5, 10, 15 and 30 min/20, 50 and 70 °C (juice) • 200 Mpa/5, 10, 15 and 30 min/11.3, 23.7, 35.5 and 70.7 °Brix • 200 and 500 Mpa/20 min/50 and 70 °C (juice) • 200 Mpa/5, 10, 15 and 30 min/50 °C (buffers and juice) • 200 Mpa/50 °C for 2, 4 and 6 cycles/5 min with 5 min pause	[270]
A. acidoterrestris TO-117/02 (apple juice isolate)	Apple juice Buffer (Ph 4.0)	200, 300, 400 and 500 Mpa/15 min/4, 20 and 50 °C Determination with optical density	[271]
A. acidoterrestris NZRM 4447	Orange juice	200 and 600 Mpa/15 min/39 °C + thermosonication 20.2 W/Ml/78 °C	[127]
A. acidoterrestris NZRM 4447	Malt Extract Broth (10, 20 and 30 °Brix)	600 Mpa/up to 45 min/35, 45, 55 and 65 °C 600 Mpa/up to 45 min/45 °C Validation for apple juice, lime juice concentrate and Blackcurrant juice concentrate	[97]
A. acidoterrestris CCT 7547	Acai pulp	600 Mpa/5, 10, 15, 20 and 25 min/65 °C	[272]
A. acidoterrestris CCT 7547	Deionized Water	300 and 600 Mpa/5 min/25 and 70 °C + heat shock	[273]
A. acidoterrestris AJA 66 (apple juice isolate) *A. acidoterrestris* ATCC 49025	Apple juice Potassium Phosphate Buffer (Ph 3.7 and 7.0)	600 Mpa/1, 3 and 5 min/70, 80 and 90 °C	[274]

A. acidoterrestris (apple juice isolate) *A. acidoterrestris* DSMZ 2498	Orange juice	500 and 600 Mpa/1, 3, 5, 15 and 30 min/25, 45, 60 and 70 °C	[275]
A. acidoterrestris CCT 7547	Mango pulp	600 Mpa • 0, 5, 10, 15, 20 and 25 min/65 °C • 0, 4, 8, 12, 16 and 20 min/70 °C • 0, 2, 4, 6, 8 and 10 min/75 °C • 0, 1.5, 3, 4.5, 6 and 7.5 min/80 °C • 0, 1, 2, 3, 4 and 5 min/90 °C	[276]
Alicyclobacillus spp. N1089 (canned tomatoes isolate) *Alicyclobacillus* spp. N1098 (apple juice isolate)	Tomato juice Apple juice	Combined treatment of HHP + Sucrose laurate L1695 392 Mpa/10 min/45 °C + 0.005% and 0.01% for N1089 392 Mpa/10 min/45 °C + 0.025, 0.04 and 0045% for N1098	[247]

Ultraviolet (UV-C) light is a form of electromagnetic radiation ranging from 200 to 280 nm [283] that has the ability to damage the DNA of the microorganism and therefore eliminate it [284]. The treatment has been proved effective against *A. acidoterrestris* spores in grape and apple juice [285,286]. The low energy consumption and the absence of toxic byproducts in the final product makes UV-C light a promising control treatment and for this reason FDA approved its use in order to clear fruit juices (FDA, 2000).

Irradiation treatment uses gamma rays, electrons, or X rays [253,287] targeting the chromosome in order to split the double helix of DNA and thus damage the cell of the microorganism [253]. The use of gamma rays and electrons were reported to be effective against *A. acidoterrestris* spores in citrus juice in combination with heat treatment (85–95 °C) [288]. In addition, Lee et al. [289] reported the inactivation of *A. acidoterrestris* spores in apple and orange juice with the use of gamma rays. Irradiation has limited applicability in the fruit juice industry today due to its association with radioactivity that is unacceptable from the consumers' point of view [277].

Ohmic heating uses an electrical current to generate heat instantly inside the food in order to kill microorganisms [73]. *A. acidoterrestris* vegetative cells were inactivated in apple juice with the use of an ohmic heating system. When the temperature was above 70 °C the death rate was close to 100% [290]. Moreover, the inactivation of *A. acidoterrestris* spores was reported to be higher with the use of ohmic heating than with conventional heating in orange and apple juice [291,292]. However, additional studies should be undertaken to verify the effectiveness of this method in the fruit juice industry.

Pulsed electric field generates pulse waves that have enough intensity to cause cell membrane damage that leads to cell destruction [293]. Uemura et al. [294] reported the reduction in the population of *A. acidoterrestris* in orange juice in a very short time (0.9 s) at 125 °C without influencing the nutritional quality of the juices. This technology has the ability to improve the microbiological quality and preserve the physicochemical and nutritional attributes of juices, but concerning *Alicyclobacillus* spores, more studies, including sensory assessment, should be performed in order to elucidate the effects of temperature assistance on the organoleptic traits of fruit juices.

1.5. Conclusions

Fruit juices have gained popularity due to their health benefits resulting in the expansion of the global juice market. Therefore, spoilage incidents can cause significant financial losses to the industry. *Alicyclobacillus* and *Alicyclobacillus acidoterrestris* in particular are thermo-acidophilic spore forming bacteria responsible for spoilage that cannot be detected until consumption of the juice, making them a major hazard for the fruit juice industry. The quality of the raw material and the hygiene processing conditions should be taken under consideration to avoid spoilage. Subsequently, various control and prevention methods have been established to inactivate *Alicyclobacillus* spores and preserve the quality and the shelf life of the fruit juice. The early detection of spoilage using rapid methods is also a requirement of the industry. Consequently, future studies should focus on the improvement of the existing techniques and the development of new methods to ensure the rapid and early detection of *Alicyclobacillus* and preserve the quality of fruit juices.

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CHAPTER 2

Detection of spoilage microorganisms in commercial Greek orange and peach juice.

2.1. Introduction

Throughout the years fruit juices have become very popular and therefore the fruit juice industry has expanded globally. In recent years consumers have cultivated a healthy and fitness profile and demand fresh-like, low caloric food products that fit to their busy life style and provide them with beneficial nutrients. Since fruit juices fulfill these requirements they have become important in human diet. The increasing consumption provides a challenge to the fruit juice industry in order to produce new formulations of fruit juices that are free from spoilage and pathogenic microorganisms and thus minimize the risk of health issues to consumers. Although commercial fruit juices have low pH that inhibits most microorganisms and they are also subjected to pasteurization (88-90 ℃ for 2 min or 90-95 ℃ for 30-60 sec), some microorganisms have the potential to survive and limit the shelf life of these products [1-3]. Contamination of fruit juices is related to pathogenic and spoilage microorganisms, but the main threat for the fruit juice industry are yeasts, molds and bacteria [4,5]. Yeasts are the predominant spoilage microorganisms [6] whereas molds which are associated with the production of mycotoxins such as patulin and ochratoxin A, can be a threat for human health due to the toxicity of these compounds [7,8]. Fruit juices are also correlated with deterioration from lactic acid and acetic acid bacteria, but the major problem is due to the presence of spore forming bacteria. *Alicyclobacillus* spp. and its predominant species *Alicyclobacillus acidoterrestris* can cause spoilage to fruit juices and their control cannot be achieved by standard pasteurization procedures. Furthermore, since there is no indication of gas production or swelling of the container due to the contamination from *Alicyclobacillus*, spoilage cannot be detected until consumption by consumers who complain of a medicinal, antiseptic and phenolic off-odor. The predominant metabolite associated with the presence of off-flavor is guaiacol [9-11]. Therefore, the objective of this study was to perform a survey from the Greek fruit juice retail market to determinate the presence of the spoilage microorganisms in orange and peach juice.

2.2. Material and methods

2.2.1. Juice samples

Orange (58 samples) and peach (42 samples) juices were obtained from different market places, including supermarkets (83 samples), mini markets (13 samples) and street

markets (4 samples). The selected juices were either refrigerated (short shelf-life) (19 samples) or pasteurized (long shelf-life) (81 samples). Special attention was given to obtain fruit juices from different batches from the same manufacturer to include as much variability as possible. The obtained juices were subjected to microbiological and physicochemical analyses as detailed below.

2.2.2. Microbiological and physicochemical analyses

For the microbiological analysis 1.0 mL of each fruit juice was aseptically transferred to 9.0 mL quarter strength Ringer's solution (LABM, Lancashire, UK) and homogenized by vortexing for 60 s at room temperature. Serial tenfold decimal dilutions were prepared and 0.1 or 1.0 mL of the appropriate dilution was spread or poured in duplicate on the following agar media: i) Plate Count Agar (PCA, 4021452, Biolife, Milano, Italy) for the enumeration of Total Aerobic Viable Counts (TVC) incubated at 25 ℃ for 48-72 h, ii) De Man Rogosa and Sharpe Agar (MRS agar, MERCK, Darmstadt, Germany) incubated at 30 ℃ for 48-72 h for the enumeration of lactic acid bacteria (LAB), iii) Rose Bengal Chloramphenicol Agar (RBC Agar, MERCK, Darmstadt, Germany) for the enumeration of molds and yeasts incubated at 25 ℃ for 48-120 h and iv) *Bacillus acidoterrestris* medium (BAT) agar (adjusted to pH 3.7 after sterilization with 1N H_2SO_4) (BTA20500, Biolab Budapest, Hungary) incubated at 45 °C for 48-120 h for the enumeration of *Alicyclobacillus spp*. To reduce the detection limit of the enumeration method (spread plating), 1.0 mL of the first decimal dilution was spread equally on three BAT agar plates.

The pH value of each fruit juice was measured after the end of the microbiological analysis with a digital pH meter (HI2211, pH/ORPMeter, HANNA instruments, Woonsocket, RI, USA). Furthermore, the determination of total soluble solids of peach juice was measured and expressed in °Brix using a refractometer (Index Instruments, model GPR12-70, series SN 26-006). Duplicate fruit juices were analyzed on each sampling occasion and the results are expressed as mean values ± standard deviation (n=2).

2.3. Results and Discussion

The results of the microbiological and physicochemical analyses of orange and peach juice samples are presented in Table 2.1 and 2.2, respectively. For the analyzed orange

juice samples, there was no contamination from lactic acid bacteria since the counts were below the detection limit of the enumeration method (0.00 log CFU/mL), except for two samples (L3 and K5) that presented low populations of LAB, namely 1.00 and 1.30 log cfu/mL, respectively. Molds and yeasts were also below the detection limit of the enumeration method (1 log cfu/mL) in most samples with the exception of 10 samples with counts ranging between 1.00 and 2.04 log CFU/mL. Total Viable Aerobic counts varied from 1.00 to 2.23 log CFU/mL and in many samples they were below the detection limit (1.00 log CFU/mL). *Alicyclobacillus* spp. was detected in 10 out of 58 samples with counts ranging from 0.30 to 3.53 log CFU/mL. The pH values for the commercial orange juices were 3.77± 0.12 on average, with minimum and maximum of 3.59 and 4.07 for samples V2 and A15, respectively.

Table 2.1. Microbiological and physicochemical analyses of commercial orange juices. Different capital letters indicate samples from different manufacturers, whereas different numbers within the same letter indicate different batches of orange juice

CODE	Total Viable Counts (log CFU/mL)	Lactic Acid Bacteria (log CFU/mL)	Yeasts/ Molds (log CFU/mL)	*Alicyclobacillus* spp. (log CFU/mL)	pH	Collection point super market, mini market, street market)
Long shelf-life juices						
A1	1.00	0.00	1.00	0.00	4.01	super market
A2	1.00	0.00	1.00	0.00	3.94	super market
A3	1.00	0.00	1.00	0.00	3.90	super market
A4	1.00	0.00	1.00	0.00	3.84	super market
A5	1.00	0.00	1.00	0.69	3.90	super market
A6	1.17	0.00	1.00	0.00	3.86	super market
A7	1.00	0.00	1.00	0.00	3.89	super market
A11	1.00	0.00	1.00	0.48	3.91	street market
A12	1.00	0.00	1.00	0.30	3.96	street market
A13	1.00	0.00	1.00	0.00	3.90	super market
A15	1.00	0.00	1.90	0.00	4.07	super market
L1	2.23	0.00	1.00	0.00	3.80	super market
L2	1.00	0.00	1.30	0.00	3.74	super market
L3	1.00	1.30	1.00	0.48	3.75	super market
L4	1.00	0.00	1.00	0.00	3.75	super market
X1	1.90	0.00	1.00	0.00	3.81	super market
X2	1.00	0.00	1.00	0.00	3.64	super market
X3	1.00	0.00	1.00	0.00	3.64	super market
K1	1.00	0.00	1.00	0.00	3.78	super market
K2	1.00	0.00	1.00	0.00	3.78	super market
K3	1.00	0.00	1.00	3.53	3.74	super market

K4	1.00	0.00	1.00	0.00	3.64	super market
K5	1.00	1.00	1.00	0.00	3.71	super market
K6	1.30	0.00	1.00	0.00	3.66	super market
K7	1.00	0.00	1.00	0.00	3.64	super market
H1	1.00	0.00	1.00	0.00	3.80	super market
H2	1.00	0.00	1.00	0.00	3.81	super market
H3	1.00	0.00	1.84	0.00	3.73	super market
V1	1.00	0.00	1.00	0.00	3.63	super market
V2	1.23	0.00	1.00	0.00	3.59	super market
B1	1.00	0.00	1.00	0.00	3.76	super market
B2	1.00	0.00	1.00	0.00	3.71	super market
B3	1.00	0.00	1.00	0.00	3.75	super market
R1	1.00	0.00	1.00	0.00	3.67	super market
A8	1.04	0.00	1.00	0.48	3.86	mini market
A9	1.00	0.00	1.47	0.00	3.87	mini market
A10	1.00	0.00	1.00	0.00	3.87	mini market
A14	1.00	0.00	1.00	0.30	3.86	mini market
L5	1.00	0.00	1.00	0.00	3.71	mini market
A11	1.00	0.00	1.00	0.48	3.91	street market
A12	1.00	0.00	1.00	0.30	3.96	street market
Short shelf-life juices						
O1	1.00	0.00	1.00	0.00	3.73	super market
O2	1.00	0.00	2.04	0.00	3.69	super market
O4	1.00	0.00	1.00	0.00	3.65	super market
O5	1.00	0.00	1.00	0.00	3.69	super market
O6	1.00	0.00	1.00	0.00	3.42	super market
O7	1.00	0.00	1.00	0.00	3.75	super market
E1	1.00	0.00	1.00	0.00	3.80	super market
E2	1.00	0.00	1.00	0.00	3.76	super market
E3	1.00	0.00	1.00	0.00	3.57	super market
C1	1.00	0.00	1.00	0.30	3.53	super market
C2	1.00	0.00	1.00	0.00	3.56	super market
C3	1.00	0.00	1.00	0.00	3.59	super market
LR1	1.18	0.00	1.00	0.00	3.77	super market
LR2	1.40	0.00	1.00	0.00	3.76	super market
F1	1.00	0.00	1.00	0.48	3.93	super market
F4	1.00	0.00	1.00	0.00	3.88	super market
O3	2.20	0.00	1.00	0.00	3.81	mini market
F2	1.08	0.00	1.00	0.48	3.90	mini market
F3	1.00	0.00	1.00	0.00	3.85	mini market

Detection limit of the enumeration method 0.00 log CFU/mL for Lactic Acid Bacteria and *Alicyclobacillus* spp. and 1.00 log CFU/mL for Total Viable Counts and Yeasts/Molds

With regard to peach juice samples (Table 2.2), there was no contamination from lactic acid bacteria, considering that counts were below the detection limit of the enumeration method (0 log cfu/mL). Concerning molds and yeasts, they were detected in 3 samples only, namely B2 (1.30 log CFU/mL)-B3 (2.00 log CFU/mL) and G2 (3.90 log CFU/mL), whereas the remaining 39 samples showed no presence of yeasts/molds (≤1 log CFU/mL). The Total Viable Aerobic counts were below the detection limit of the

Table 2.2. Microbiological and physicochemical analyses of commercial peach juices. Different capital letters indicate samples from different manufacturers, whereas different numbers within the same letter indicate different batches of orange juice

CODE	Total Viable Counts (log CFU/mL)	Lactic Acid Bacteria (log CFU/mL)	Yeasts/ Molds (log CFU/mL)	*Alicyclobacillus* spp. (log CFU/mL)	pH	°Brix	Collection point (super market mini market street market)
Long shelf-life juices							
A1	2.60	0.00	1.00	0.00	3.05	11.6	super market
A2	1.00	0.00	1.00	0.00	3.05	11.6	super market
A3	1.00	0.00	1.00	0.00	3.11	13.0	super market
H1	1.00	0.00	1.00	0.00	3.18	11.9	super market
H2	1.00	0.00	1.00	0.00	3.15	12.0	super market
H3	1.00	0.00	1.00	0.00	3.30	13.2	super market
H4	1.00	0.00	1.00	0.00	3.14	13.1	super market
H5	1.00	0.00	1.00	0.00	3.23	13.2	super market
H7	1.00	0.00	1.00	0.00	3.47	13.1	super market
H8	1.00	0.00	1.00	0.00	3.44	13.9	super market
P1	1.00	0.00	1.00	0.00	3.17	11.0	super market
P2	1.00	0.00	1.00	0.00	3.18	11.0	super market
P3	1.00	0.00	1.00	0.00	3.16	12.3	super market
P4	1.00	0.00	1.00	0.00	3.30	12.6	super market
P5	2.00	0.00	1.00	0.00	3.30	12.6	super market
B1	1.00	0.00	1.00	0.00	2.67	11.8	super market
B2	1.00	0.00	1.30	0.00	2.72	11.5	super market
B3	1.00	0.00	2.00	0.00	3.44	12.3	super market
B4	1.00	0.00	100	0.00	2.98	12.3	super market
B5	1.00	0.00	1.00	0.00	3.11	12.3	super market
G1	1.00	0.00	1.00	0.00	3.65	11.8	super market
G2	1.00	0.00	3.90	0.00	3.32	11.7	super market
G3	1.00	0.00	1.00	0.00	3.52	12.1	super market
G4	1.00	0.00	1.00	0.00	3.70	11.8	super market
D1	1.00	0.00	1.00	0.00	3.05	11.9	super market
D2	1.00	0.00	1.00	0.00	3.06	12.0	super market
E1	1.00	0.00	1.00	0.00	3.22	12.4	super market
E2	1.00	0.00	1.00	0.00	3.00	13.1	super market

E3	1.00	0.00	1.00	0.00	3.66	12.7	super market
E4	1.00	0.00	1.00	0.00	3.70	13.0	super market
E5	1.00	0.00	1.00	0.00	3.42	12.8	super market
Z1	1.00	0.00	1.00	0.00	3.20	11.5	super market
Z2	1.00	0.00	1.00	0.00	3.56	12.0	super market
L1	1.00	0.00	1.00	0.00	3.64	13.1	super market
L2	4.30	0.00	1.00	1.33	3.43	12.7	super market
A4	1.00	0.00	1.00	0.00	3.15	12.8	mini market
A5	1.00	0.00	1.00	0.00	3.26	13.0	mini market
H6	2.00	0.00	1.00	0.00	3.45	13.7	mini market
D3	1.00	0.00	1.00	0.00	3.32	12.4	mini market
D4	1.00	0.00	1.00	0.00	3.30	11.0	mini market
D5	1.00	0.00	1.00	0.00	3.34	12.5	street market
X1	1.00	0.00	1.00	0.00	3.56	12.2	street market

Detection limit of the enumeration method: 0.00 log CFU/mL for Lactic Acid Bacteria and *Alicyclobacillus* spp. and 1.00 log CFU/mL for Total Viable Counts and Yeasts/Molds

plating method (1.00 log CFU/mL) in most peach juice samples, with the exception of samples H6 and P5 (2.00 log CFU/mL and A1 (2.60 log CFU/mL), while the highest TVC value was measured in for sample L2 (4.28 log CFU/mL). *Alicyclobacillus* spp. was detected in only one sample (1.30 log CFU/mL). The pH of the peach juice samples was 3.28 ± 0.24 on average, with minimum value of 2.67 for sample B1 and maximum 3.70 for sample G4. With regard to total soluble solids, the mean value was 12.35 ± 0.70 °Brix, with minimum 11.0 °Brix for samples P1, P2 and D4 and maximum 13.9 °Brix for sample H8. Results revealed that the overall microbiological quality of commercially available peach juices was better compared to orange juices. In addition, lactic acid bacteria and yeasts/molds were detected in low populations in few juice samples and could not thus become a serious spoilage threat for the surveyed fruit juices. On the other hand, the presence of the spore forming bacteria *Alicyclobacillus* spp. could potentially result in spoilage of the samples tested. In particular, *Alicyclobacillus acidoterrestris* that has been proposed as the target microorganism for the control of the pasteurization process, with spore concentration limit of 100 CFU/mL of raw material by the fruit juice industry [12], increases the potential of spoilage through storage in some samples. Moreover, it was indicated that orange juice was found to have more contaminated samples than peach juice. In addition, no clear association could be established between the microbiological status and the selling points of the collected juices.

2.4. Conclusions

The results of this survey indicated that *Alicyclobacillus* spp. was present in commercial fruit juices of the Greek market. This observation leads to the conclusion that further investigation of juices concerning the isolation and identification of *Alicyclobacillus* spp., should be taken into consideration. Since *Alicyclobacillus acidoterrestris* is the main spoilage representative of this microbial group, due to the increased number of reported incidents, it is important to isolate and identify its presence in fruit juices.

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CHAPTER 3

A. Isolation and Identification of guaiacol producing *Alicyclobacillus* spp. from orange juice
B. A single enzyme PCR-RFLP assay targeting V1-V3 region of 16S rRNA gene for direct identification of *Alicyclobacillus acidoterrestris* from other *Alicyclobacillus* species

3B published in *Journal of Applied Genetics* **2019**, *60*, 225–229.

3.A. Isolation and Identification of guaiacol producing *Alicyclobacillus* spp. from orange juice

3.A.1. Introduction

The presence of *Alicyclobacillus* in fruit juices has become a major issue for the fruit juice industry. Throughout the years spoilage incidents have increased considerably in a variety of fruit juices, including orange juice [1-4]. *Alicyclobacillus* spp. are thermoacidophilic, non-pathogenic spore forming bacteria that can survive juice pasteurization temperatures and subsequently germinate under favourable conditions during storage [5-7]. Spoilage due to the presence of *Alicyclobacillus* is difficult to be detected since there is neither gas production nor swelling of the container. Contamination can be perceived only after consumption by consumers who complain for the presence of an off-odor described as medicinal, antiseptic and phenolic [8-10], mainly due to the production of guaiacol (2-methoxyphenol), but also halophenols 2,6 dichlorophenol and 2,6 dibromophenol [6,11]. Not all *Alicyclobacillus* species have the potential to produce guaiacol, but *Alicyclobacillus acidoterrestris* has been implicated in most spoilage incidents [6]. Therefore, the fruit juice industry considers this bacterium to be the target microorganism in order to control the effectiveness of the pasteurization process [12,13]. A survey on commercial orange juices purchased from the Greek market was carried out with the use of the IFU method No 12 (IFU no. 12 September 2004/March 2007) [14], that is considered to be the most effective microbiological method for the detection of *Alicyclobacillus* [15]. The purpose of this survey was not only to investigate the presence of the bacterium in orange juice, to differentiate the *Alicyclobacillus* spp. isolates, to compare the relative presence of *Alicyclobacillus acidoterrestris* against other *Alicyclobacillus* species but also to detect whether the isolates could produce guaiacol and therefore cause spoilage problems. Over the years many Polymerase Chain Reaction (PCR) methods have been successfully applied for the differentiation and/or identification of *Alicyclobacillus* and among them PCR-RFLP analysis of 16S rDNA or relevant genes [16-18]. In order to differentiate and identify the *Alicyclobacillus* isolates, several restriction enzymes were used in PCR-RFLP. The differentiation of *Alicyclobacillus* isolates in clusters and their correlation with guaiacol production is of great interest and importance for the juice industry in order to avoid spoilage incidents.

3.A.2. Material and methods

3.A.2.1 Bacterial strains and isolation

A total of 72 *Alicyclobacillus* isolates were recovered from four brands of commercial orange juice obtained from the Greek market (JA, JL, JH and JP). All isolates were recovered according to the IFU method No 12 developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (IFU no. 12 September 2004/March 2007) [15]. *A. acidoterrestris* DSMZ 2498 obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkuturen, Braunschweig, Germany) culture collection was used as reference strain.

3.A.2.2 DNA extraction

Bacterial cultures were inoculated in Yeast Extract Starch Glucose (YSG) broth [2 g/L yeast extract (Biolife Italiana); 1 g/L glucose (LAB M Limited); 2 g/L soluble starch (neoLab Migge Laborbedarf-Vertiebs GmbH), pH adjusted to 3.7 with HCl 1N] and incubated at 45 °C for 48 h [15]. Further on, 10 mL of bacterial cells at the exponential phase (10^7 CFU/mL) were collected and centrifuged at 8000 rpm for 5 min at 4 °C. The pellet was treated with 20 mg/mL lysozyme in lysis buffer (20 mmol/L Tris-HCl, pH = 8.0, 2 mmol/L EDTA, 1% Triton X100) for 60 min at 37 °C to achieve lysis of *Alicyclobacillus* cells. Genomic DNA was extracted from *Alicyclobacillus* cultures using a commercial DNA extraction kit according to the manufacturer's instructions (Nucleospin Tissue-Macherey-Nagel). The DNA concentrations were measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) at 260/280 nm.

3.A.2.3 16S rDNA Amplifcation and PCR-RFLP

The 16S rDNA gene was amplified using the universal primers P1: 5′-AGCAGTAGGGAATCTTCCA-3′ and P2: 5′- TTCCCCACGCGTTACTCACC- 3′. PCR amplifications were performed in a total volume of 25 μL containing:1.25 U of thermostable (Taq) DNA polymerase (Kapa Biosystems), 2.5 μL Taq buffer, 0.8 mM dNTP's, 0.2 μM of each primer, and 2 mM total $MgCl_2$ in a Bio Rad thermocycler (T100TM Thermal Cycler, Bio Rad Laboratories, Emeryville, CA, USA). PCR cycling conditions were determined as following: initial denaturation at 94 °C for 5 min, followed by 35 cycles of (denaturation at 94 °C for 30 sec, primer annealing at 56 °C

for 30 sec, primer extension at 72 °C for 1 min) and a final extension at 72°C for 10 min. Five μL of each PCR product were digested with 5 U of the restriction endonucleases *Hha*I, *RSa* I and *HiNF*I (NEB) and incubated at 37 °C for 2 h. The restriction profiles were separated by gel electrophoresis on 3% (w/v) agarose for 2 h and after staining with ethidium bromide, the restriction fragments were detected under UV light (Gel Doc, Biorad). The size of the fragments was estimated with the use of a 50 bp molecular weight marker (NEB). Normalization and dendrogram construction were performed with the use of Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (Optimization 0.5 and Tolerance 1.0), via the BioNumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

3.A.2.4 Test of the guaiacol producing potential of Alicyclobacillus strains

The isolates of *Alicyclobacillus* were also checked for the ability to produce guaiacol. Guaiacol production was determined with the Peroxidase Enzyme Colorimetric Assay (PECA) method, in particular with the use of the commercial Guaiacol detection kit (Dohler DMD, Microsafety Design) according to the manufacturer's instructions.

3.A.3. Results and Discussion

Spoilage from *Alicyclobacillus* has been reported extensively in fruit juices including orange juice [19,20]. In addition, isolation from other fruit juices has been reported including mango [21], passion [22] and kiwi [23]. Yamazaki et al. [24] also isolated *Alicyclobacillus* strains from fruit juice blends and Luong et al. [25] from various fruit juices. In the present study 4 types of commercial orange juices were purchased from the Greek market and the isolations for each type of juice are displayed in Table 3.A.1. All isolates were amplified by PCR-RFLP and were restricted with 3 endonucleases *Hha*I, *RSa*I and *HiNF*I. The dendrogram obtained after cluster analysis of restriction patterns is shown in Figure 3.A.1, where their grouping resulted in 8 and 16 clusters at a similarity level of 80 and 90 %, respectively. At the similarity level of 80 %, 16 out of 72 isolates from orange juice (A14, A26, A28, A29, A30, A31, B1, B24, B26, B27, B32, B34, B35, B38, B42, B43) (Group V) had similar restriction patterns with the reference strain *A. acidoterrestis* DSMZ 2498 (Figure 3.A.1). This group together with

Table 3.A.1. *Alicyclobacillus* isolates from 4 types of commercial orange juices

Juice type	*Alicyclobacillus* isolates
JA	A1-A31
JL	B1-B10, B21-B38 and B42-B44
JH	B11-B16
JP	B17-B20

Group III that included 15 isolates (A1, A2, A3, A4, A5, A6, A9, A12, A13, A15, A17, A18, A19, B22, B44) as shown on the dendrogram were the larger clusters. The other groups consisted of 12 isolates (A8, A10, A11, A22, A23, A25, B3, B4, B5, B8, B9, B13) (Group I), 11 isolates (A7, A16, A20, B6, B7, B14, B15, B16, B20, B25, B37) (Group IV), 8 isolates (B2, B10, B11, B18, B28, B29, B30, B31) (Group II), 6 isolates (A21, A24, A27, B23, B33, B36) (Group VII) and 3 isolates (B17, B19, B21) (Group VIII). Group III consisted of a unique isolate (B12) that had a distinct pattern. The distribution of the *Alicyclobacillus* isolates to 8 different groups according to their restriction patterns and depending on the orange juice type from which they were isolated, is summarized in Table 3.A.2.

Table 3.A.2. Distribution of *Alicyclobacillus* isolates from different orange juices

Juice	Total isolates	Groups[a]							
		I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
JA	31	6	13	6	3		3		
JL	31	10	2	5	4	6	3	1	
JH	6			1	3	1			1
JP	4				1	1		2	
Total	72	16	15	12	11	8	6	3	1

[a] According to Dendrogram (Figure 1)

According to the results presented in Table 3.A.2, it was shown that *Alicyclobacillus* isolates from each juice were allocated in different groups, suggesting that isolates from the same juice may differ. The dendrogram also showed that all groups did not appear

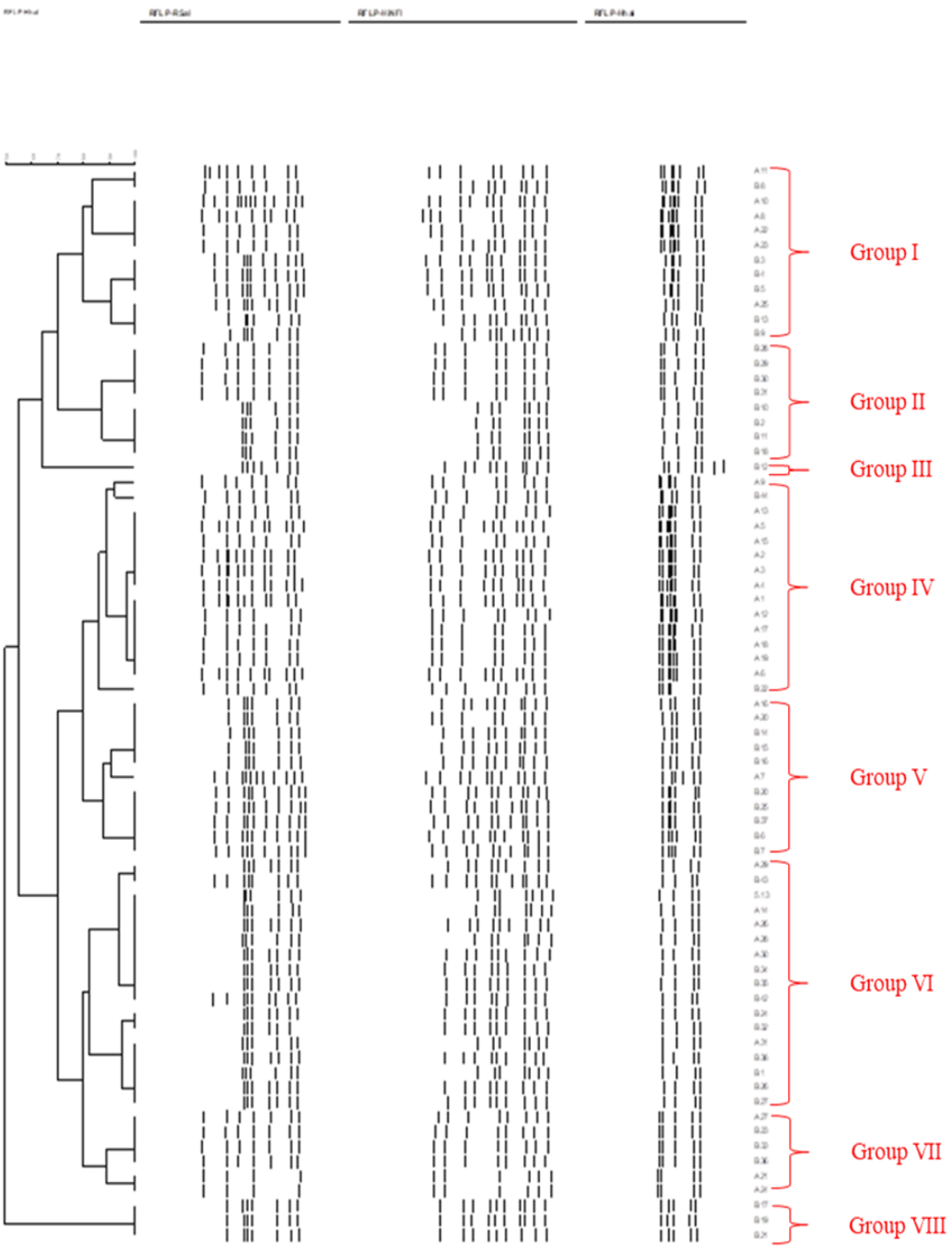

Figure 3.A.1. Dendrogram of 16S rDNA PCR-RFLP with restriction endonucleases *Hha*I, *RSa*I and *HiNF*I of *Alicyclobacillus* isolates from commercial orange juices

in all juices, even the isolates clustered in the largest groups (III and V) were present only in the juices with many recovered colonies (JA and JL). Furthermore, it needs to be noted that juices from which fewer colonies were recovered (JH and JP) contained isolates that were distributed in different groups.

Moreover, guaiacol production was detected in all isolates with the PECA method, which is based on the oxidation of guaiacol by peroxidase enzymes in the presence of H_2O_2 with the formation of a brown compound, identified as 3,3′-dimethoxy-4,4′-biphenoquinone [26]. Following the instructions of the commercial detection kit, all isolates were tested for color change, with brown color being the indication of guaiacol production. Results displayed that all isolates could produce guaiacol. As displayed in Image 3.A.1, the negative and positive control samples are located on the left and right side, respectively, and the isolates have been placed with increasing intensity of brown coloration.

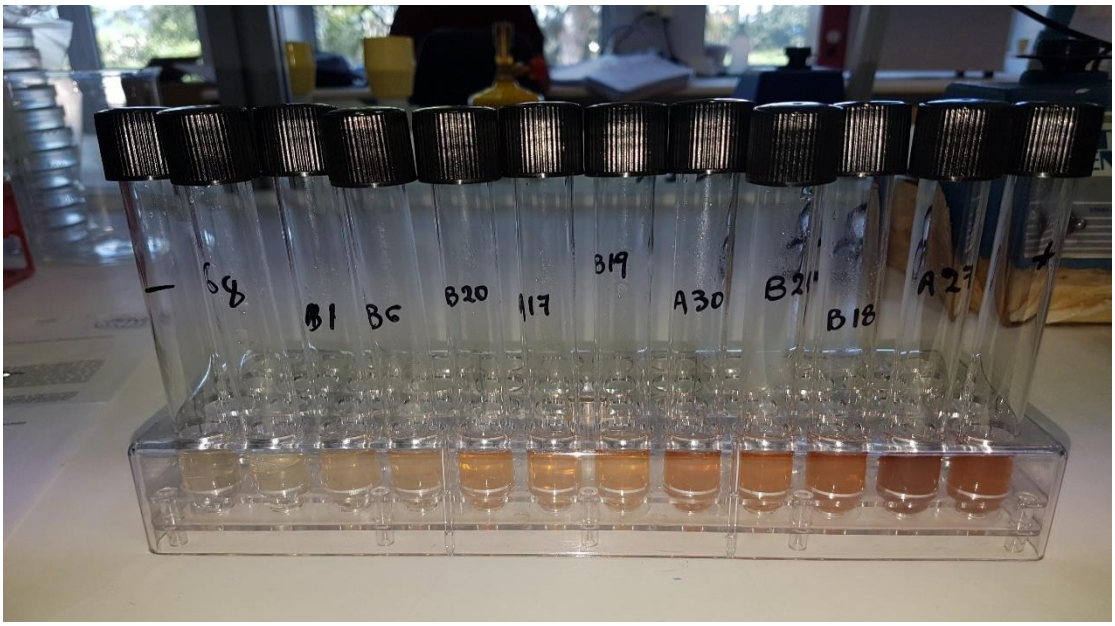

Image 3.A.1. Colour gradient of *Alicyclobacillus* isolates after incubation for the detection of guaiacol production with Dohler DMD kit

Representative isolates are illustrated in Images 3.A.2 and 3.A.3, indicating the ability to produce guaiacol based on brown color development. The isolates were grouped visually, depending on the intensity of the brown color into 6 clusters. After correlating the groups that were separated with the PECA method with those from the dendrogram

(Figure 3.A.1) there seemed to be no association between the restriction patterns and the color intensity resulting from the guaiacol test.

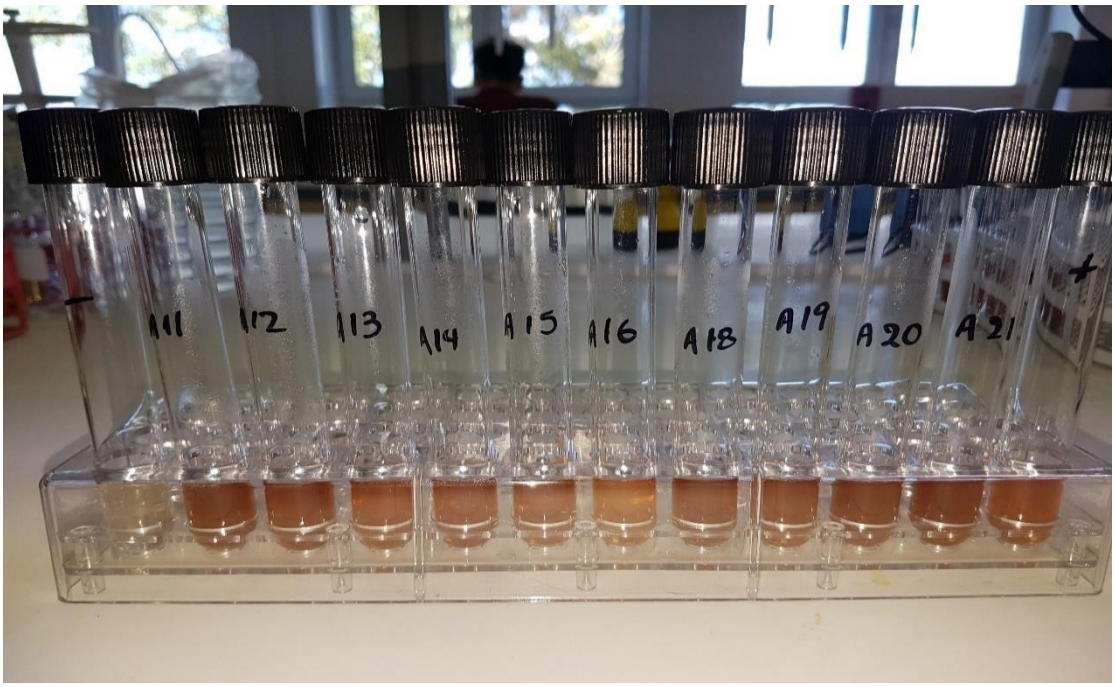

Image 3.A.2. Representative isolates of *Alicyclobacillus* for the detection of guaiacol production with the Dohler DMD kit

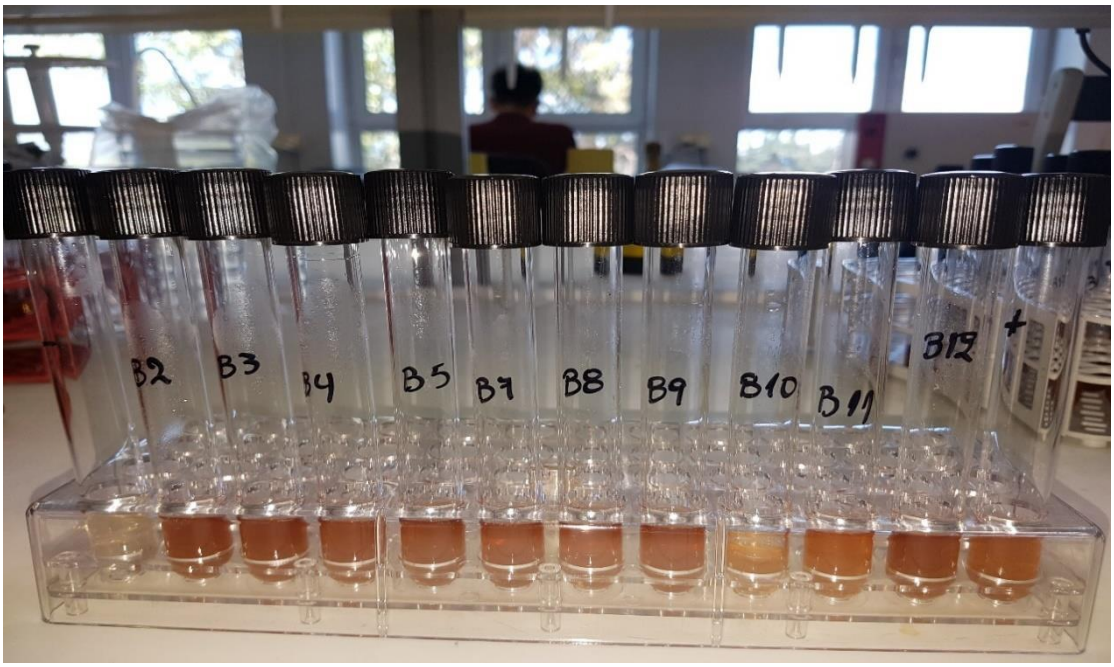

Image 3.A.3. Representative isolates of *Alicyclobacillus* for the detection of guaiacol production with the Dohler DMD kit

In conclusion *Alicyclobacillus* isolates from different commercial orange juices presented different patterns, even for those originating from the same juice. Application

of PCR-RFLP with the restriction endonucleases *Hha*I, *RSa*I and *HiNF*I was used successfully to cluster the isolates in 8 groups at a similarity level of 80 %. The method used to differentiate the isolates revealed that there were many different isolates indicating that the orange juice may contain various strains or subgroups or even to different species. Moreover, all isolates had the ability to produce guaiacol and therefore spoil the juice under favorable conditions, consequently the presence of *Alicyclobacillus* has become a major issue for the fruit juice industry. Further on, it is important to identify if the isolates belong to *Alicyclobacillus acidoterrestris*, since it is considered to be the main spoiler of fruit juices that produce guaiacol.

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3.B. A single enzyme PCR-RFLP assay targeting V1-V3 region of 16S rRNA gene for direct identification of *Alicyclobacillus acidoterrestris* from other *Alicyclobacillus* species

Abstract

Alicyclobacillus acidoterrestris is considered to be one of the most important target microorganisms in quality control of heat-processed acidic foods. This species can cause spoilage by forming spores with very high heat resistance; however, no gas production or visible changes occur in the contaminated product during storage. Thus, the differentiation of *A. acidoterrestris* from other *Alicyclobacillus* species is of great importance. The present study aims to find a rapid method for the identification of *A. acidoterrestris*. To achieve this, 78 *Alicyclobacillus* isolates were subjected to PCR-RFLP (Restriction Fragment Length Polymorphism). Specifically, PCR products of amplified V1-V3 region of 16S rRNA gene were digested with restriction endonuclease *Hha*I. According to the obtained results, all *A. acidoterrestris* isolates showed similar restriction fragments of 16S rRNA gene and different from *A. acidocaldarius* and *A. hesperidum*. In conclusion, a single enzyme PCR-RFLP assay was developed and showed rapid, inexpensive and direct identification of *Alicyclobacillus* isolates. The application of this method will be useful to identify this contaminant in fruit juices.

3.B.1. Introduction

In the early 1980s, spoilage incidents attributed to bacteria of *Alicyclobacillus* genus appeared in the fruit juice industry [1,2,3]. *Alicyclobacillus* spp. are non-pathogenic, Gram positive, rod shaped, thermoacidophilic spore forming bacteria, able to grow at a temperature range of 25-60°C and at a wide pH range (2.0-7.0) [3,4,5]. The species most recorded in juice spoilage is *A. acidoterrestris* [4], while *A. acidocaldarius*, *A. hesperidum* and *A. cycloheptanicus*, have been also sporadically reported [6]. Spores represent the most resistant form of *A. acidoterrestris*, and have a very different structure from their vegetative cells [7]. These spores, which are very high heat-resistant can survive under the conditions of pasteurisation and germinate during storage, causing spoilage [8,9]. The spoilage appears without gas production and no occurrence of swelling [8] and thus, it is usually difficult to be detected since the characteristic of the contamination is an off odour and medicinal flavour caused by guaiacol [4]. Since

A. acidoterrestris is the most recovered species in the spoilage incidents of fruit juice, it has become an industrial issue [10] and its identification is of great importance. According to IFU method [11], the isolation procedure of *Alicyclobacillus* from juices last about 10 days, thus it is crucial to minimize the time needed for the identification of the *A. acidoterrestris.* In recent years, several gene-based methods have been proposed for the identification of *A. acidoterrestris*. In brief, in a recent review, most of these methods were summarized, whereas it was noted that beyond their effectiveness and sensitivity, it is not easy to be adopted to routine inspection as skilled personnel is required [5]. Different real time PCR approaches have been proposed for specific differentiation of *A. acidoterrestris* from *A. acidocaldarius* [12,13,14,15]. However, they are also time-consuming and expensive since additional steps are required particularly for sequencing for the characterization of *A. acidoterrestris* after the application of RAPD (Random Amplification of Polymorphic DNA) [16,17], RFLP [18,19], hyper – variable (HV) region in 16s rRNA gene sequence [20,21] and genus specific PCR [22]. Therefore, the detection of *A. acidoterrestris* can be expensive for the manufacturer and there is a need of new characterization methods that will be of low cost and with no need of special expertise [23,24]. In two studies even though, the application of RAPD succeeded to differentiate the *A. acidoterrestris* from other *Alicyclobacillus* isolates, a unique profile for all *A. acidoterrestris* isolates was not obtained [25,26]. The present study aims to develop a rapid and cost-effective method for identification of *A. acidoterrestris* from other *Alicyclobacillus* species.

3.B.2. Materials and Methods

3.B.2.1 Bacterial strains and growth conditions

A total of 74 *A. acidoterrestris* cultures previously isolated from commercially processed orange juices were used in this study. In brief, the isolates were recovered from orange juices samples sporadically analysed for support services in the microbiological laboratory of Institute of Technology of Agricultural Products (ITAP) following IFU method [11]. In addition, *A. acidoterrestris* ATCC 49025, *A. acidoterrestris* DSMZ 2498, *A. acidocaldarius* DSMZ 446 and *A. hesperidum* FMCC B-462 (Food Microbiology Culture Collection, Agricultural University of Athens) were tested as reference strains. All cultures used in this study, belong to the culture

collection of the ITAP, Hellenic Agricultural Organization-DEMETER. The bacterial cultures were routinely incubated on YSG (Yeast extract Starch Glucose) broth (2 gL^{-1} Yeast Extract (Biolife Italiana); 1gL^{-1} Glucose (LAB M Limited); 2 gL^{-1} Soluble Starch (neoLab Migge Laborbedarf- Vertiebs GmbH), pH 3.7) at 45 °C for 48 h (IFU) [11].

3.B.2.2 DNA extraction from pure cultures

Bacterial cells (10^7) at the exponential phase (48 h) were collected by centrifugation (5000 g for 10 min at 4 °C) and washed twice with 1/4 Ringer solution. Genomic DNA was extracted from Alicyclobacillus cultures using Nucleospin Tissue (Macherey-Nagel) according to manufacturer instructions. DNA concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

3.B.2.3 PCR-RFLP

Fifty nanograms of genomic DNA were subjected to PCR using the universal primers (P1: GCGGCGTGCCTAATACATGC and P4: ATCTACGCATTTCACCGCTAC) for the amplification of V1-V3 variable region of 16S rRNA gene (position 31-50 and 688-668, respectively of *A. acidoterrestris* ATCC 49025) [27]. PCR amplification was performed in a final volume of 25 μL containing: 1.25 U of thermostable (*Taq*) DNA polymerase (Kapa Biosystems), 2.5 μL *Taq* buffer, 0.8 mM dNTP's, 0.2 μM of each primer and 2mM total $MgCl_2$. Amplification was performed in a Bio-Rad thermocycler where the PCR conditions were determined as follows: initial denaturation at 94 °C for 10 min followed by 35 cycles (denaturation at 94 °C, 1 min, primer annealing at 42 °C, 1 min, primer extension at 72 °C, 2 min) and a final extension step at 72 °C for 10 min. The restriction profiles of isolates, after digestion of the ~700 bp PCR amplicons with the restriction endonuclease *Hha*I (5 U) (NEB) for 1h at 37°C, were separated by gel electrophoresis in 3% (w/v) agarose for 2 h. The restriction fragments after staining with ethidium bromide were detected under UV (Gel Doc, Biorad). A 50bp standard molecular weight marker (NEB) was used to estimate the sizes of fragments.

3.B.3 Results and Discussion

Bacteria of *Alicyclobacillus* genus can survive in low acid and heat-processed fruit juices and seem to contribute most to spoilage of shelf stable products [18,28]. In brief, *A. acidoterrestris* along with *A. acidocaldarius* is found to be able to cause off-flavor and cloudiness spoilage of fruit juices, while *A. acidoterrestris* is the most common detected species [4,18]. *A. acidoterrestris* is able to produce guaiacol, which has been associated with a phenolic off-flavor [10]. This type of spoilage is slow and often hard to notice. Consequently, the early detection of the species or its metabolic compounds is essential for the juice industries [5]. A simple, directed and rapid identification of such thermo-acidophilic bacteria in fruit juices and relevant environments seems to be of great importance for the quality control of juice processing environments.

In this study, 78 *Alicyclobacillus* isolates were subjected to RFLP – PCR analysis using universal primers for the amplification of V1-V3 variable region of 16S rRNA gene and restriction endonuclease *Hha*I. Using these specific conditions of the PCR-RFLP assay, the *A. acidoterrestris* isolates were successfully differentiated from *A. acidocaldarius* and *A. hesperidum* (Figure 3.B.1) within 8 hours (one day protocol). However, it is needed to clarify that the proposed rapid protocol refers to the identification of *A. acidoterrestris* after the isolation of *Alicyclobacillus* colonies according to IFU method [11]. Specifically, after digestion of V1-V3 variable region of 16S rRNA gene using restriction endonuclease *Hha*I, 3 fragments were generated and were assigned to 140,190 and 340 bp. The specificity of the assay was confirmed with 74 *A. acidoterrestris* isolates. On the other hand, 4 fragments were generated from *A. acidocaldarius* which were assigned to 85, 125, 140 and 340 bp and 2 fragments assigned to 170 and 450 bp for *A. hesperidum*. Goto et al. [21] suggested that sequencing of HV region of 16S rRNA was also able to differentiate sufficiently *Alicyclobacillus* species. In an earlier study, a 16s rRNA gene PCR-RFLP assay, using primers and restriction enzymes different from the ones used in this study, was applied successfully for the clustering of isolates in different species after digestion of amplicons with 4 restriction enzymes [18]. However further analysis e.g. DNA sequencing was required to identify the isolates. In a similar study, the analysis of RFLP patterns of two genes i.e *rpoB* and *vdc* divided the *A. acidoterrestris* isolates in two major types [19]. With a RAPD-PCR also applied previously for the differentiation of *A. acidoterrestris* among other *Alicyclobacillus* species, different well distinguished

subgroups of *A. acidoterrestris* were detected [17,25]. Using three different primers, RAPD assay was able to distinguish *A. acidoterrestris* within 6 hours, where two different groups of *A. acidoterrestris* were detected [26].

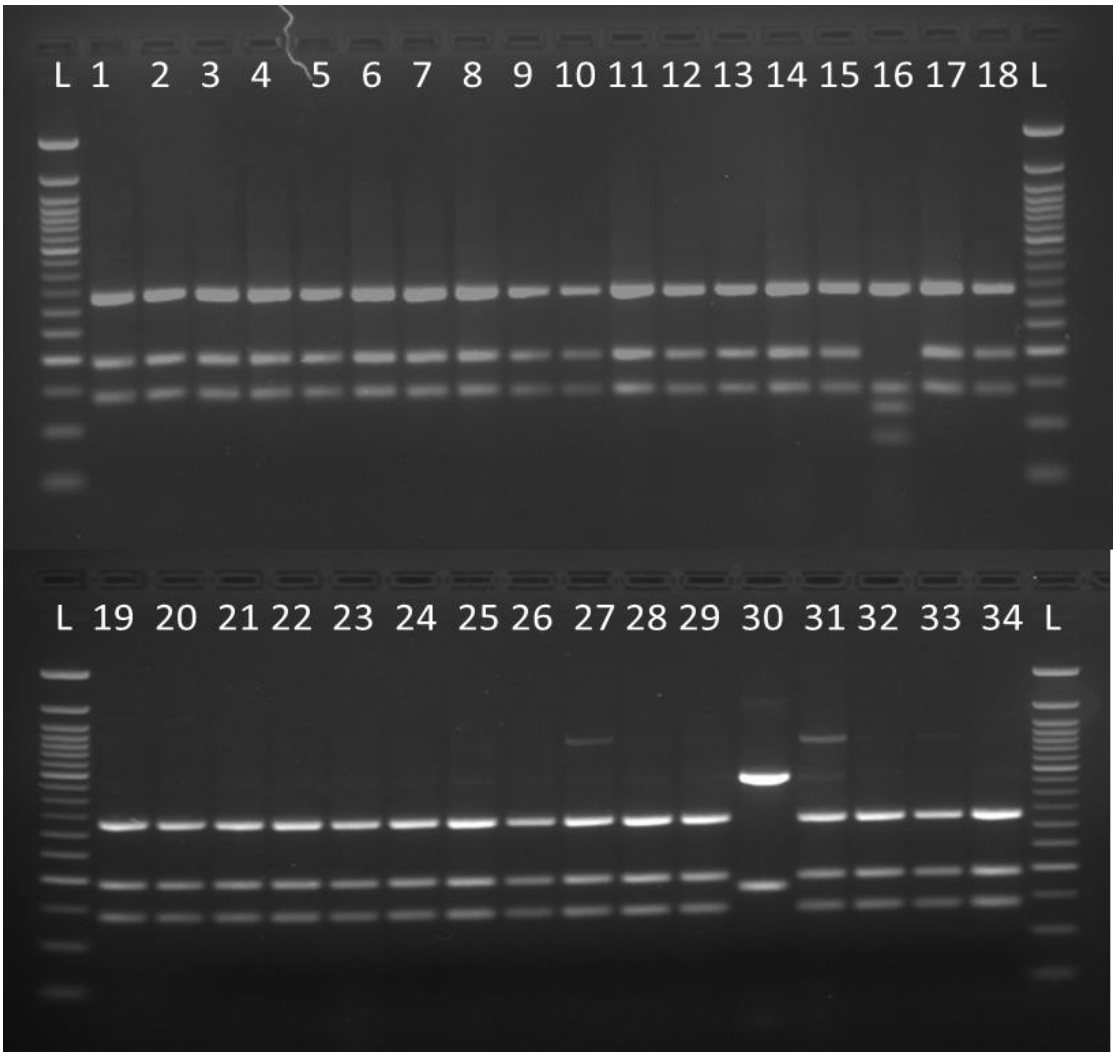

Figure 3.B.1. Representative restriction patterns of the 16S rRNA gene – V1-V3 region of *Alicyclobacillus* isolates obtained with *Hha*I. L: 50 bp DNA ladder; 1-14 and 19-29 and 32-34 *A. acidoterrestris*; 15: *A. acidoterrestris* DSMZ 2498; 16: *A. acidocaldarius* DSMZ 446; 30: *A. hesperidum* FMCC B-462; 31: *A. acidoterrestris* ATCC 49025

In a similar study, a great variability in banding pattern was detected after the application of RAPD-PCR for the investigation genotypic heterogenicity of 25 *A. acidorerrestris* isolates, resulting into three different groups with appreciated genetic similarity between the strains of the same group [16]. Moreover, the phylogenetic analysis of 16S rRNA gene of different *Alicyclobacillus* isolates divided the species and strains into different groups, where *A. acidoterrestris* isolates were assigned in three distinct groups [29].

The advantages of the protocol proposed in this study for the identification of *A. acidoterrestris*, compared to the already existing methods, mentioned in the previous paragraphs, could be summarized: (a) the rapid and direct differentiation of *A. acidoterrestris* from other *Alicyclobacillus* species (i.e. *A. acidocaldarius*, *A. hesperidum*) at species level, (b) the reduction of consumables e.g. use of only one restriction enzyme and, set of primers etc, (c) no need of additional steps to successfully identify the target microorganism e.g. sequencing etc, (d) the use of universal primers which can be used for the analysis of other microorganisms and (e) less skilled personel is required. However, it has to be noted that the proposed method is species specific, therefore for the differentiation of *A. acidoterrestris* at strain level another method has to be applied.

In conclusion, a PCR- RFLP assay that specifically differentiates *A. acidoterrestris* from *A. acidocaldarius* and *A. hesperidum* isolates was developed. Using these universal primers and the specified restriction endonuclease, the proposed single enzyme PCR-RFLP assay will be a useful and easy to use method for the rapid, direct and inexpensive identification of *Alicyclobacillus* isolates that could be useful for the identification of this contaminant at heat-processed acidic foods.

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CHAPTER 4

Alicyclobacillus acidoterrestris Strain Variability in the Inactivation Kinetics of Spores in Orange Juice by Temperature-Assisted High Hydrostatic Pressure

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Abstract

In this work, the inactivation kinetics of *Alicyclobacillus acidoterrestris* spores by temperature-assisted high hydrostatic pressure was assessed by means of the Weibull model. Spores from two *A. acidoterrestris* strains (a wild-type strain and a reference strain) were inoculated in commercial orange juice and subjected to high pressure levels (500 and 600 MPa) combined with four temperature regimes (25, 45, 60 and 70 °C) for time up to 30 min. Results showed that for a given high-pressure level spore inactivation was higher as temperature progressively increased. Furthermore, the Weibull model consistently produced satisfactory fit to the inactivation data based on the values of the root mean squared error (RMSE < 0.54 log colony-forming units (CFU)/mL) and the coefficient of determination ($R^2 > 0.90$ in most cases). The shape of inactivation curves was concave upward ($p < 1$) for all temperature/high pressure levels tested, indicating rapid inactivation of the sensitive cells of the bacterium whereas the remaining ones adapted to high hydrostatic pressure (HHP) treatment. The values of the shape (p) and scale (δ) parameters of the Weibull model were dependent on the applied temperature for a given high pressure level and they were further described in a secondary model using first-order fitting curves to provide predictions of the surviving spore population at 55 and 65 °C. Results revealed a systematic over-prediction for the wild-type strain regardless of temperature and high pressure applied, whereas for the reference strain under-prediction was evident after 3 log-cycles reduction of the surviving bacteria spores. Overall, the results obtained indicate that the effectiveness of high hydrostatic pressure against *A. acidoterrestris* spores is strain-dependent and also underline the need for temperature-assisted HPP for effective spore inactivation during orange juice processing.

4.1. Introduction

In the early 1980s the fruit juice industry had to deal with spoilage incidents that were caused by a bacterium later on named *Alicyclobacillus* [1–3]. This bacterium is difficult to be detected since no acid or gas production is apparent in the product when spoiled. Only after consumption an off-flavour described as “medicinal, phenolic and antiseptic” is the evident sign of spoilage [4,5] due to the production of the compounds 2-methoxyphenol (guaiacol), 2, 6 dibromophenol and 2,6 dichlorophenol [3,6–8].

Alicyclobacillus acidoterrestris is an aerobic, rod shaped Gram-positive, endospore-forming and non-pathogenic spoilage microorganism [2,8]. It has the ability to grow in a wide pH range (2.0–7.0) with optimal pH values between 3.5–4.0 and at a temperature range of 25–60 °C with optimal between 40–45 °C [9–11]. *A. acidoterrestris* spores have the ability to survive pasteurization procedures of fruit juice and because of their acidophilic nature they can germinate and result in spoilage after favourable conditions [4,12]. The maximum concentration of *Alicyclobacillus* spores that is accepted in the raw material by the fruit industry is 100 colony-forming units (CFU)/mL [13]. Part of the spore resistance is due to the presence of an external protein coat with strong hydrophobic bonds that stabilizes and reduces membrane permeability in extreme acidic and high-temperature environments [14].

Acidothermophilic bacteria like *A. acidoterrestris* are considered to be important target microorganisms in quality control of heat-processed acidic foods. Therefore, it has been suggested as a reference microorganism for designing pasteurization processes in acidic fruit products [15,16]. Results concerning thermal inactivation of *A. acidoterrestris* spores in orange juice showed a D-value at 90 °C between 10 and 23 min and at 95 °C between 2.5 and 8.7 min, depending on bacterial strain and the conditions of the experiment [17,18]. Consequently, typical juice pasteurization processes employed by the fruit juice industry today (i.e., 86 to 96 °C for 2 min) cannot ensure spore inactivation especially for products with longer shelf life [19]. The inactivation of *A. acidoterrestris* has been investigated by a number of challenging non thermal-based technologies, such as electric fields [20], ultrasounds [21], pulsed light [22], hyperbaric storage [23], ultraviolet radiation [24] natural antimicrobials [25], high hydrostatic pressure (HHP) combined with mild heat treatment [5], supercritical carbon dioxide assisted HHP [26].

HHP is a modern method of non-thermal food pasteurization, commercially employed by many food industries with an increasing number of HHP-treated foods placed in the market. It relies on the application of high pressures (400–600 MPa) on food and beverages so as to inactivate spoilage or pathogenic microorganisms and therefore extend the shelf life of the product [27,28]. It needs to be noted however that bacterial spores are resistant to HHP, so it has been proposed that this process must be combined with mild heat treatment for effective spore inactivation [29–33]. HHP processing is a challenging alternative to thermal processing in orange juice production. With HHP treatment flavour, colour and taste can be hardly affected since most of the molecules

such as amino acids, vitamins, nutrients and functional properties are retained [18]. HHP can inactivate microorganisms at lower temperatures compared to conventional pasteurization while maintaining a maximum degree of sensory and nutritional quality [34,35].

The objectives of this study were: (a) to investigate the effect of temperature-assisted high pressure processing on the inactivation of the spores of two strains of *A. acidoterrestris* in orange juice and (b) to describe the inactivation kinetics by means of the Weibull model.

4.2. Materials and Methods

4.2.1. Bacterial strains and spore suspension

Two strains of *A. acidoterrestris* were used in this study: a wild-type strain isolated previously from apple juice that was kindly provided by the Laboratory of Food Microbiology and Hygiene of the Aristotle University of Thessaloniki (denoted strain A). The GenBank accession number of 16S rRNA sequence of strain A is MW142406**.** A reference strain of *A. acidoterrestris* DSMZ2498 (denoted strain B) obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkuturen, Braunschweig, Germany) culture collection. Both strains were maintained at −80 °C in Yeast Extract Starch Glucose (YSG) broth (Yeast Extract 2.0 g; Glucose 1.0 g; Soluble starch 2.0 g; 1000 mL H20) with pH adjusted to 3.7 using 1N HCl and supplemented with 20% (*v*/*v*) glycerol (APPLICHEM, Darmstadt, Germany). The strains were pre-cultured in the same medium at 45 °C for 48 h to obtain a stock culture.

The cells from the culture were inoculated on acidified *Bacillus acidoterrestris* medium (BAT) agar plates (pH 3.7) (BTA20500, Biolab, Budapest, Hungary) and incubated at 45 °C for 7 days to sporulate. The sporulation of the cells was confirmed by phase contrast microscopy. When at least 80% of the cells had sporulated, the spores were harvested by adding 2.5 mL of cold sterile distilled water to the plates and scraping gently the surface with a sterile glass rod. The process was repeated twice. The spores obtained were centrifuged at 5000 rpm for 20 min at 4 °C. The supernatant was then discarded and the spores were washed three times by centrifugation (5000 rpm for 20 min at 4 °C) using cold sterile distilled water. The spores of 15 plates were re-suspended in 10 mL of sterile phosphate buffer (pH 7.2) and stored at 4 °C until use. The

concentration of the spore suspension was determined by plating and it was ca. 10^7 spores/mL (see § 2.2) [18,19].

4.2.2. Enumeration of Alicyclobacillus acidoterrestris spores

For the determination of spore concentration of *A. acidoterrestris* in non-treated and HPP-treated orange juice samples, a volume of 2 mL of each sample was heat shocked at 80 °C for 10 min [18]. Colony counting for each sample was performed from the appropriate decimal dilutions followed by spread plating on duplicate acidified BAT agar plates (pH 3.7) after incubation of the plates at 45 °C for 3 days and the results are expressed as log CFU/mL. Moreover, in order to estimate the difference in the amount of spores and vegetative cells, non-heat-shocked juice was also evaluated following serial dilutions and spread plating as described above (data not shown).

4.2.3. Orange juice samples

Experiments were undertaken using commercially available pasteurized orange juice (pH 3.7; 11.45 °Brix) purchased from a local supermarket, with no initial *A. acidoterrestris* contamination (assessed experimentally, (see Section 2.2.). A volume of 0.4 mL of spore suspension was added to 4 mL of orange juice in plastic film pouches (45 mm wide × 95 mm long × 90 μm thickness) (Flexo-Pack SA., Athens, Greece) to achieve a final spore concentration of ca. 10^6 spores/mL [18,19]. The pouches were heat-sealed using a HenkoVac 1700 machine (Howden Food Equipment B.V., The Netherlands) taking care to expel most of the air.

4.2.4. High Pressure treatment of orange juice samples

The inactivation of *A. acidoterresris* spores for both strains A and B was undertaken at 500 and 600 MPa in combination with different temperature regimes (25, 45, 60, and 70 °C) for pressurization time up to 30 min (1, 3, 5, 15, 30 min). The HHP processing was carried out with a Food Pressure Unit (FPU) 1.01 (Resato International BV, Roden, Holland). The system consists of a high-pressure intensifier for the buildup of pressure, an electric motor to drive a hydraulic pump and a block of 6 small vessels (42 mL) measuring 2.5 cm in diameter and 10 cm in length each. The vessels are closed with a unique Resato thread connection on the top of the vessel. The pressure transmitting fluid is polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, Holland) and the maximum operating pressure and temperature of the system is 1000

MPa and 90 °C, respectively, with pressure adjustable in steps of 20 MPa. Pressure transducers are used to monitor the pressure and temperature transmitters are mounted in each vessel to monitor the temperature. The come-up rate was approximately 100 MPa per 7 s and the pressure release time was less than 3 s. Pressurization time reported in this work does not include the pressure come-up and release times. Right after high-pressure treatment, the pouches were immediately cooled in an ice bath. Overall, the experiment was repeated twice with duplicate samples from different pouches analyzed for each combination of pressure, temperature and time.

4.2.5. Determination of inactivation kinetics

Log-survival data of *A. acidoterresris* spores from strains A and B obtained in temperature-assisted high-pressure processing were described by the Weibull model [36]:

$$log(N_t) = log(N_0) - \left(\frac{t}{\delta}\right)^p \quad (1)$$

where N_t is the surviving load of spores (CFU/mL) at a given treatment time (min), N_0 is the initial load of spores in the juice (CFU/mL), p is the shape parameter of the curve (dimensionless) showing upward ($p < 1$) and downward ($p > 1$) concavity and δ is the scale parameter (min) corresponding to the time of the first decimal reduction (1D). The Weibull model was fitted to the experimental data using the GInaFiT ver. 1.7 software [37] and the goodness-of-fit of survivor curves was assessed using the coefficient of determination (R^2) and the root mean squared error (RMSE). In addition, the z values were determined at each high pressure level separately (500 and 600 MPa) and strain type of the bacterium by calculating the reciprocal of the slope of the straight line between log(δ) and temperature to compare with previous results.

The Weibull model was further validated with independent experiments to find out whether survival spore populations from both strains A and B obtained from different temperatures at the same pressurization levels could be effectively predicted. For this purpose, two additional temperatures (55 and 65 °C) within the range used to develop the model were selected for the same pressure levels (500 and 600 MPa). Additional orange juice samples were prepared, inoculated, pressurized (1, 3, 5, 15, and 30 min) and enumerated as mentioned above. The performance of validation procedure was assessed graphically by plotting the observed vs. predicted values of *A. acidoterrestris* for each strain individually.

4.3. Results – Discussion

Results of *A. acidoterrestris* spores' inactivation of strains A and B in orange juice pressurized at 500 and 600 MPa in combination with different temperature regimes (45, 60, and 70 °C) are illustrated in Figures 4.1 and 4.2, respectively. To prevent the effect of the initial load of spores (N_0), data are presented in terms of log (N/ N_0), where N_0 was ca. 10^6 CFU/mL in all experiments. Inactivation kinetics did not follow a first-order pattern but an upward concavity was noticeable at all temperatures assayed. A possible explanation for this inactivation pattern is that the microorganism population is composed by several subpopulations (spores and vegetative cells in different physiological state), each one presenting a distinct inactivation pattern, which causes the non-linear curves [37,38]. In other words, the presence of subpopulations has symmetric or asymmetric heat resistance distributions [35,39]. Thus, upward concavity could be considered as evidence of quick inactivation of the sensitive cells of the population, whereas the remaining survivors were more resistant to the lethal agent. Similar upward inactivation curves were obtained for *A. acidoterrestris* during temperature-assisted HHP processing of fruit juices with different soluble solids content (up to 20 °Brix) [5], as well as in orange juice where high pressure processing was combined with mild heat treatment (45–65 °C), although inactivation kinetics were simulated by the Bigelow model (first order) despite the fact that deviation from linearity was evident. The results obtained in this work demonstrated that temperature was a major parameter in the inactivation of *A. acidoterrestris* spores. Thus for a given high pressure level, the higher the temperature the higher the spore inactivation. It needs to be noted that when high-pressure processing was undertaken at 500 MPa/25 °C and 600 MPa/25 °C the reduction in spore population was less than 1.0–1.5 log cycles throughout the process for both strains of the bacterium. These results are in agreement with previous researchers [19,28,32] who reported little or no inactivation of *A. acidoterrestris* spores when HHP was applied at ambient temperature.

For the wild-type strain A of *A. acidoterrestris*, results indicated that at 45 °C there was ca. 3 log-cycles reduction of spore counts after 30 min processing time at both 500 and 600 MPa, whereas at 60 °C the same reduction magnitude was achieved in 1–2 min in both high pressures employed. For complete inactivation of the spores the required time was 20 min at 600 MPa/70 °C and 30 min at 500 MPa/70 °C. It is worth noting that for

strain A an initial decrease of ca. 2.5 log-cycles was observed within the first minute of processing at 500 MPa followed by an increase thereafter until 5 min (Figures 4.1 and 4.2).

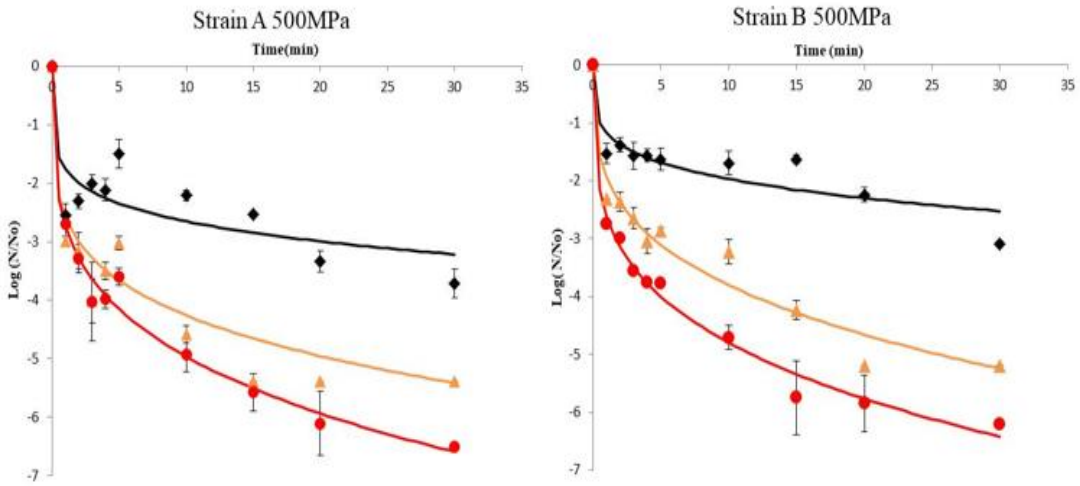

Figure 4.1. Inactivation curves of *A. acidoterrestris* spores at 500 MPa and 45 °C (◆), 60 °C (▲) and 70 °C (●). Solid lines represent data fitting with the Weibull model. Data are mean values of two replications ± standard deviation

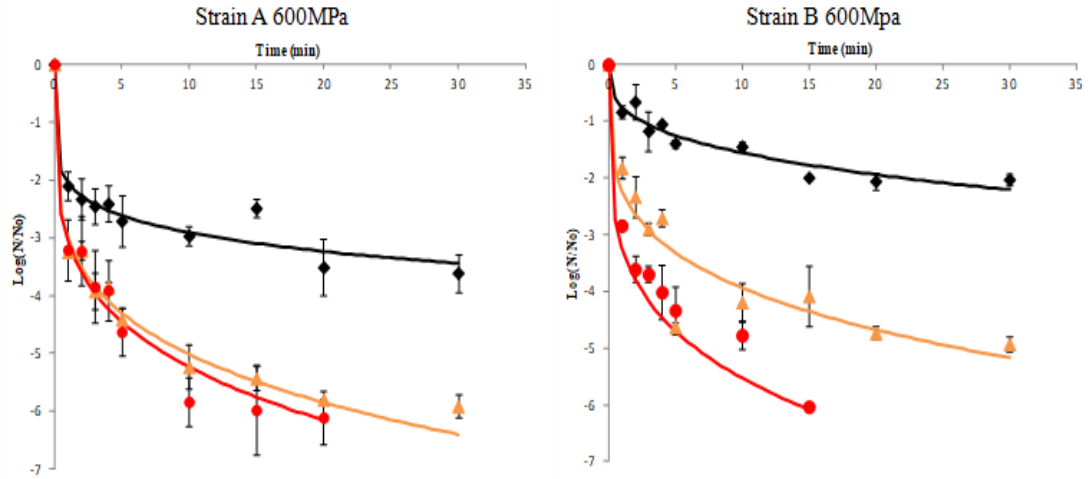

Figure 4.2. Inactivation curves of *A. acidoterrestris* spores at 600 MPa and 45 °C (◆), 60 °C (▲) and 70 °C (●). Solid lines represent data fitting with the Weibull model. Data are mean values of two replications ± standard deviation

This could be attributed to the dormancy of spores that was interrupted by the implementation of HHP treatment, since HHP has proven to induce the germination of spores [40]. A two-step HHP treatment has been proposed to inactivate alicyclobacilli

spores. In the first step, high pressure induces spore germination whereas in the second step high pressure causes the inactivation of germinated spores [27].

For the reference strain B of *A. acidoterrestris*, results revealed ca. 1.5 log-cycles reduction after treatment at 500 MPa/45 °C for 10 min and ca. 1.3 log-cycles reduction under 600 MPa/45 °C for the same time (Figures 1 and 2). This is in agreement with Vercammen et al. [28] who inoculated *A. acidoterrestris* spores in tomato sauce at various pH values and applied high-pressure treatment at 600 MPa/40 °C for 10 min and reported spores' reduction of ca. 1.5 log-cycles for tomato sauce at pH 4.2. Moreover, other researchers [5] applied high-pressure treatment at 600 MPa/45 °C for 10 min using a 10 °Brix broth medium (close to 11.45 °Brix of the orange juice employed in this study) and reported 1.2 log-cycles reduction of *A. acidoterrestris* spores. Complete spore inactivation was achieved at 600 MPa/70 °C and 500 MPa/70 °C after 15 and 30 min, respectively (Figures 4.1 and 4.2).

Comparing the two strains, it can be underlined that the wild-type strain A was less resistant to high-pressure treatment compared to the reference strain B at all temperatures assayed (45 and 60 °C) with the exception of 70 °C where stain A was proved more resistant (Figures 4.1 and 4.2).

Specifically, strain A presented 5 log-cycles' reduction when treated at 500 MPa/60 °C and 600 MPa/60 °C for 15 min, whereas the respective reduction for strain B under the same conditions was 3 log-cycles. For complete spore inactivation, a treatment at 600 MPa/70 °C for 15 and 20 min was necessary for strains B and A, respectively (Figures 4.1 and 4.2). This difference in high-pressure tolerance could be attributed to the fact that the effectiveness of HHP against *A. acidoterrestris* spores is strain-dependent [9,41], possibly due to the different distribution of fatty acids in the cytoplasmatic membrane of the bacterium [9,42]. Furthermore, other factors that may affect HPP thermal resistance include the number and age of spores, protoplast dehydration and sporulation temperature [43].

The inactivation curves of both strains A and B of *A. acidoterrestris* spores fitted with the Weibull model are also depicted in Figures 1 and 2, respectively. The model provided a good fit enabling the study of inactivation of *A. acidoterrestris* spores through the variation of δ and p parameters (Table 1). Concave upward ($p < 1$) inactivation curves were observed at all temperature/high-pressure combinations tested (Table 4.1). From this type of concavity, we can assume that spores presented a mixed

resistance to inactivation treatment [38,39] and the remaining spores were more resistant or maybe they could adapt better to stressful conditions and therefore have higher possibility to survive. The fitting capacity of the model was evaluated by estimating the RMSE and the R^2 values. The mean values of RMSE were 0.421 log CFU/mL and 0.281 log CFU/mL for strain A at 500 and 600 MPa, respectively, whereas for strain B the RMSE mean values were 0.281 log CFU/mL and 0.418 log CFU/mL at the same high-pressure levels. In addition, the R^2 values for most curves were higher than 0.940 indicating that the Weibull model fitted the experimental data closely, justifying thus its use to describe the inactivation kinetics of *A. acidoterrestris* (Table 4.1). The Weibull model has been extensively used in previous works to model the inactivation of *A. acidoterrestris* due to its simplicity and flexibility. Specifically, Uchida and Silva [5] reported the successful use of the Weibull model during HHP treatment at 600 MPa combined with different temperatures (35, 45, 55, and 65 °C) in malt extract broth adjusted to 10, 20 and 30 °Brix for the inactivation of *A. acidoterrestris* spores. In addition, it has been successfully employed to model the inactivation kinetics of *A. acidoterrestris* in BAM broth when treated at 350 and 450 MPa combined with different temperatures (35, 45, and 50 °C) [35]. Moreover, apart from HHP treatment, the Weibull model could effectively describe the inactivation of *A. acidoterrestris* vegetative cells and spores by other emerging technologies such as ultrasound [21], UV-C light irradiation [44] and pulsed light [22].

Table 4.1. Weibull model-estimated kinetic parameters and goodness-of-fit indices for the inactivation of *A. acidoterrestris* spores for Strains A and B using temperature-assisted high-pressure processing

Strain A	**T (°C)**	**δ (min)**	***p***	**RMSE**	**R^2**
500 MPa	45	0.039	0.176	0.517	0.769
	60	0.013	0.218	0.496	0.940
	70	0.020	0.258	0.249	0.985
600 MPa	45	0.009	0.153	0.251	0.944
	60	0.007	0.222	0.185	0.991
	70	0.004	0.286	0.407	0.962
Strain B	**T (**°C)	**δ (min)**	***p***	**RMSE**	**R^2**
500 MPa	45	0.492	0.225	0.320	0.844
	60	0.104	0.293	0.323	0.961
	70	0.027	0.264	0.200	0.990
600 MPa	45	2.493	0.318	0.172	0.407
	60	0.040	0.248	0.544	0.894
	70	0.007	0.235	0.538	0.981

An increasing trend in the shape factor (p) with the HHP treatment was observed at all temperatures assayed with the exception of 600 MPa for strain B where a decrease in the values of p was evident with increasing temperature level (Table 4.1; Figure 4.3). This trend in shape factor values is in agreement with previous researchers [35,45] who reported that the shape parameter was dependent on temperature at certain pressures. However, other reports noted that the shape factor indicates the kinetic pattern that describes the inactivation process and thus it should be independent of the external factor (i.e., temperature) [5,46], whereas van Boekel [38] reported that in only 7 out of 55 studies the shape factor seemed to be dependent from temperature. The scale parameter δ in this model equals the first decimal reduction time (1D, min) that results in 1-log CFU/mL reduction of the surviving spores' population and could be used as an indication of how rapidly the spores are inactivated [47]. Based on the estimated values of this parameter (δ), the longest spore survival in orange juice was observed for the reference strain B at 600 MPa/45 °C, whereas the wild-type strain A presented lower survival at the same temperature (Figure 4.4). The differences for the 1-log CFU/mL reduction in the surviving population of spores between the two strains were practically diminished as processing temperature increased. It could thus be suggested that at the selected high pressure levels, the temperature should be higher than 60 °C for effective spore inactivation. Regarding the scale parameter (δ), a decrease of log(δ) with temperature was noted at all high pressure levels applied (Figure 4.3).

The data points were fitted with a linear function as shown in Figure 3 providing information on the temperature sensitivity of δ values for a given high pressure level. Thus, for the wild strain A the z values were 76 and 74 °C for HPP treatment at 500 and 600 MPa, respectively, indicating that temperature changes have minor effect on δ values for this strain. However, the respective z values for strain B were 20 and 10 °C for the same pressurization levels, indicating a larger effect of temperature changes on δ values for this strain of the bacterium. The results obtained in this work for the z values of the reference strain B are comparable with previous researchers [5] who reported z values between 20.07 and 21.43 °C for *A. acidoterrestris* spore inactivation at 600 MPa combined with mild heat in malt extract broth adjusted to 10, 20 and 30 °Brix. In another work [18], a z value of 34.4 °C was reported for spore inactivation of *A. acidoterrestris* in orange juice during HPP processing at 600 MPa in the temperature range of 45–65 °C.

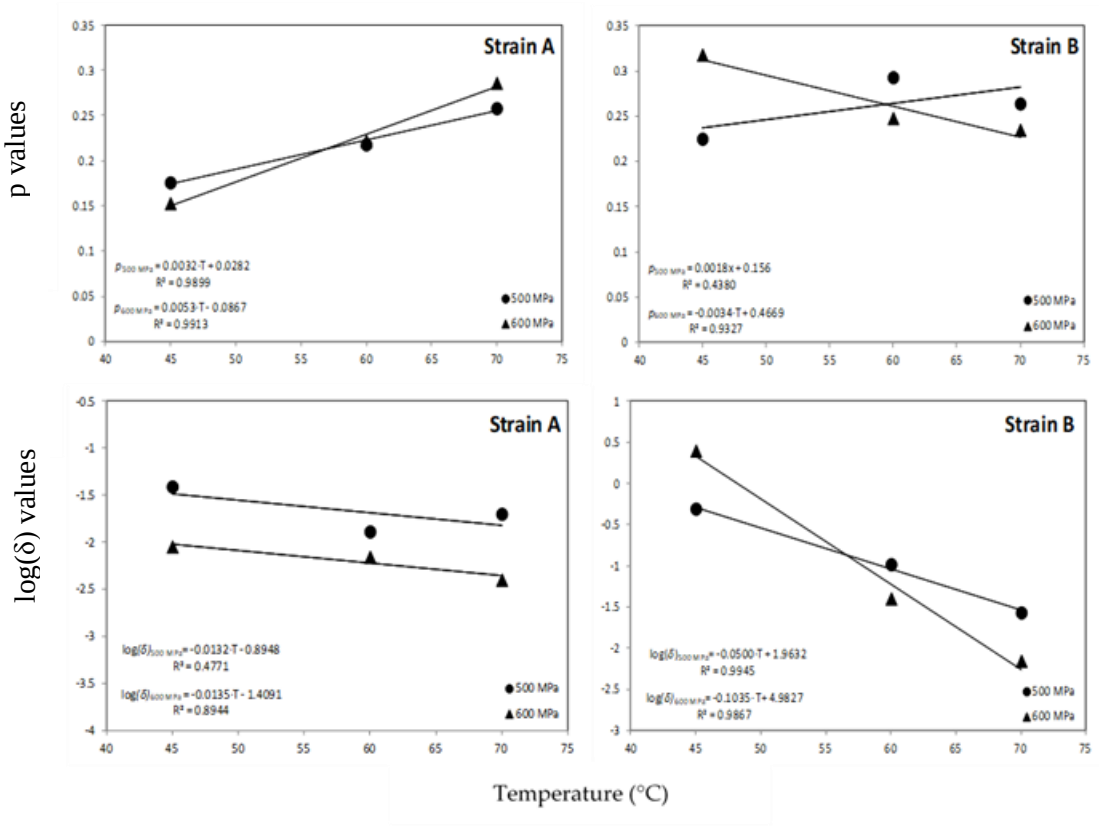

Figure 4.3. Effect of HPP temperature on the Weibull log (δ) and p values for *A. acidoterrestris* spore inactivation at 500 and 600 MPa

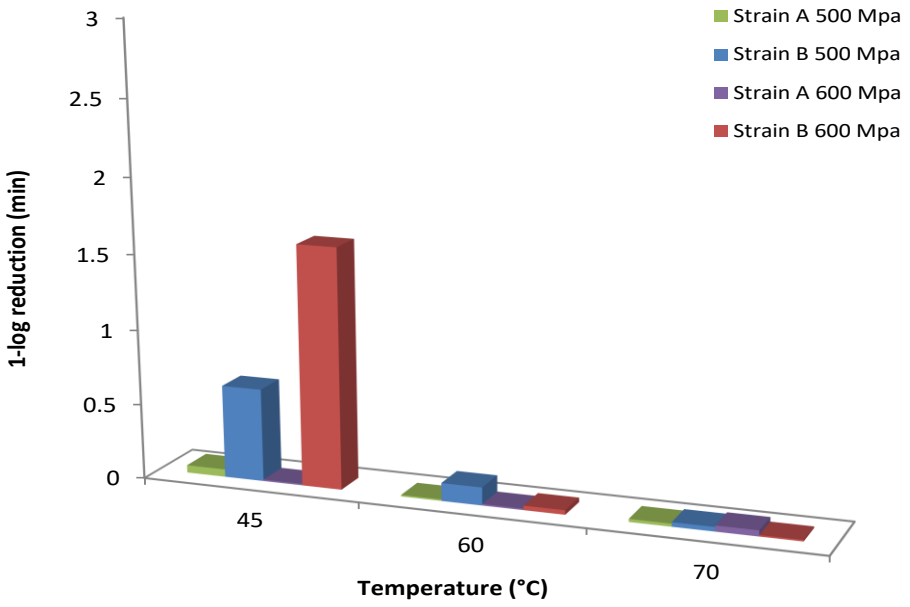

Figure 4.4. Model based comparison of 1 log reduction (1D value) time of the spores of *A. acidoterrestris* strains A and B when treated with high pressure levels of 500 and 600 MPa at 45, 60 and 70 °C

In model validation, two different temperatures were assayed (55 and 65 °C) for each HHP treatment (500 and 600 MPa) for both strains of *A. acidoterrestris*. The results are shown in Figure 5 in terms of observed vs. predicted log (N/N_0) of surviving spores of the bacterium. For the wild-type strain A, over-prediction was observed (i.e., the predicted surviving population of the spores was higher than the experimentally measured) regardless of temperature and pressure level applied. However, for the reference strain B satisfactory performance of the model was observed until −3 log reduction cycles, but after this value a systematic under-prediction was evident (Figure 5). No data could be found in the literature to compare our results because the majority of published reports on *A. acidoterrestris* spore inactivation do not include external validation of the developed models. In a recent work [5], validation results were presented for the inactivation of *A. acidoterrestris* spores by high pressure (600 MPa) combined with mild heat (45 °C) in fruit concentrates adjusted to different soluble solids concentrations (10, 20 and 30 °Brix) compared to malt extract broth. The authors also used the Weibull model to describe inactivation kinetics and reported close estimates of δ and p values between the laboratory medium and the fruit concentrates.

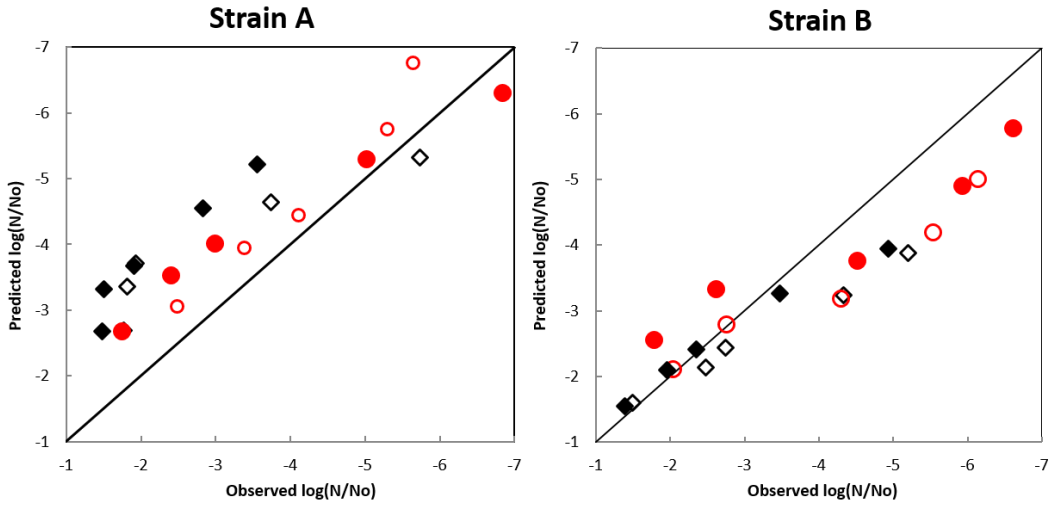

Figure 4.5. External validation for Weibull model correlating the observed and predicted values of *Alicyclobacillus acidoterrestris* spores when treated with the combination of 500 MPa at 55 °C (◇) and 65 °C (◆) and 600 MPa at 55 °C (○) and 65 °C (●)

4.4. Conclusions

Results obtained in this study revealed increased inactivation of the spores by increasing high pressure and temperature levels. Complete spore inactivation was achieved at

treatments with the highest pressure (600 MPa) and temperature (70 °C) for both strains. Therefore, HHP presents promising perspectives for the juice industry to be employed for the inactivation of *A. acidoterrestris* spores and thus increase the shelf life of fruit juices. Furthermore, the Weibull model could be successfully used to describe the inactivation of *A. acidoterrestris* spores when treated at different temperature and high pressure levels. However, strain variability is an important factor affecting the performance of the model and thus future work should be undertaken to include more strains and processing conditions.

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CHAPTER 5

A. The Effect of Temperature Assisted High Hydrostatic Pressure on the Survival of *Alicyclobacillus acidoterrestris* Inoculated in Orange Juice Throughout Storage at Different Isothermal conditions

B. The effect of temperature assisted High Hydrostatic Pressure on the survival of *Alicyclobacillus acidoterrestris* in peach juice throughout storage at different isothermal conditions

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5.A. The Effect of Temperature Assisted High Hydrostatic Pressure on the Survival of *Alicyclobacillus acidoterrestris* Inoculated in Orange Juice Throughout Storage at Different Isothermal conditions

Abstract

The purpose of this work was to investigate the population dynamics of the spores and vegetative cells of *A. acidoterrestris* in orange juice treated with temperature-assisted HHP and stored in different isothermal conditions. For this reason, the spores of two *A. acidoterrestris* strains were inoculated in commercial orange juice and subjected to HHP treatment at 600 MPa/60 °C for 5 and 10 min. Inoculated samples were subsequently stored at 4, 12 and 25 °C for 60 days. During storage, the population of *A. acidoterrestris* was determined before and after heat shock at 80 °C for 10 min in order to estimate the quantity of spores and any remaining vegetative cells on the *Bacillus acidoterrestris* medium agar. Results showed that spore populations decreased by 3.0–3.5 log cycles directly after HHP treatment. Subsequently, no significant changes were observed throughout storage regardless of temperature and bacterial strain. However, at 25 °C, an increase of 0.5–1.0 log cycles was noticed. For the remaining vegetative cells, the results illustrated that HHP treatment could eliminate them during storage at 4 and 12 °C, whereas at 25 °C inactivation was strain-dependent. Therefore, temperature-assisted HHP treatment could effectively inactivate *A. acidoterrestris* spores in orange juice and ensure that the inhibitory effect could be maintained throughout storage at low temperatures.

5.A.1. Introduction

Nowadays, consumers demand healthier, minimally processed and fresh-like products; therefore, fruit juices which comply with the above requirements have an important place in the human diet [1,2], with orange juice being undoubtedly the most popular fruit beverage worldwide. Although fruit juices are considered to have a prolonged shelf life, the survival of several pathogenic and spoilage microorganisms could limit their marketability and pose serious health issues to consumers [3–5].

The species of *Alicyclobacillus* spp. are the most dominant spore-forming bacteria in the fruit juice industry. *Alicyclobacillus acidoterrestris* in particular is the most important, since it has been considered as a reference microorganism in fruit juice

pasteurization due to its high isolation incidence from spoiled products [4,6–9]. *A. acidoterrestris* is a Gram-positive, thermo-acidophilic, non-pathogenic and endospore-forming bacterium. It can grow in a temperature range of 25–60 °C and in a wide pH range from 2.0 to 7.0 [10,11]. The spoilage by *A. acidoterrestris* is characterized by off-flavors described as phenolic, medicinal and antiseptic due to the production of the chemical spoilage compounds 2-methoxyphenol (guaiacol), 2,6 dibromophenol and 2,6 dihlorophenol [12–15]. The predominant compound indicative of juice spoilage is guaiacol, which is produced by the non-oxidative decarboxylation of vanillic acid and other natural fruit juice components [16]. Spoilage detection is difficult since there is no appearance of acid or gas production leading to swelling of the containers [17].

The fruit juice industry usually controls microbiological growth with pasteurization processes (88–90 °C for 2 min or 90–95 °C for 30–60 s). However, the inactivation of *A. acidoterrestris* spores by thermal processing in orange juice depends on the intensity of the treatment and the bacterial strain, presenting D values from 10 to 23 min and from 2.5 to 8.7 min at 90 and 95 °C, respectively. Therefore, the spores are resistant and can germinate and grow during storage at favorable conditions [3,17–20]. The resistance of *A. acidoterrestris* spores to high temperatures and acidic environments is due to a protective external protein coat composed of ω-alicyclic fatty acids in their membranes [21,22]. Since the inactivation of *A. acidoterrestris* spores cannot be achieved by traditional thermal processing without affecting the quality properties of the juice [23], alternative methods should be employed in order to prolong the shelf life of fruit juices and also fulfill consumer demands for minimally processed, healthy and nutritious food commodities. Alternative methods that have been used successfully to inactivate the spores of *A. acidoterrestris* include chemical treatments (e.g., ozone, chlorine dioxide, organic acids, potassium sorbate, sodium benzoate, poly dimethyl ammonium chloride); natural antimicrobial compounds of microbial origin (e.g., nisin, enterocin AS-48, Bificin C6165); natural antimicrobials of animal and plant origin (e.g., lysozyme, chitosan, essential oils); and non-thermal methods such as high hydrostatic pressure (HHP) combined with mild heat treatment, ultra-high pressure homogenization, supercritical carbon-dioxide-assisted HHP, UV-C light inactivation, irradiation, microwaves, ultrasonic waves and ohmic heating techniques [22,24,25].

High hydrostatic pressure (HPP) is an innovative processing method commercially used by many food industries, with a constantly increasing number of HHP-treated foods available on the market. It does not deteriorate food quality in terms of amino acids,

vitamins, nutrients and functional properties; therefore, HHP-treated juices are of a superior quality compared with thermally processed ones [20,22]. During HHP treatment, the juice is exposed to high pressures (100–800 MPa) in order to inactivate spoilage or pathogenic microorganisms and therefore extend the shelf life of the product [12]. *A. acidoterrestris* spores are extremely resistant to HHP and the treatment must be combined with moderate heating of the juice in order to increase the effectiveness of the process [26–31].Although the inactivation of *A. acdoterrestris* spores in fruit juices by temperature-assisted high pressure has been explored, there is little information in the literature concerning the survival of spores during storage after HHP treatment. Therefore, the objective of this study was to investigate the population dynamics of spores and vegetative cells of two *A. acidoterrestris* strains inoculated in orange juice during temperature-assisted high-pressure processing (600 MPa/60 °C for 5 and 10 min) followed by storage of the orange juice at isothermal conditions (4, 12 and 25 °C) for 2 months.

5.A.2. Materials and methods

5.A.2.1. Bacterial strains

Two strains of *A. acidoterrestris* were employed in this study, namely a wild-type strain (GenBank accession number MW142406) [9], kindly provided by the Laboratory of Food Microbiology and Hygiene of the Aristotle University of Thessaloniki, that was isolated previously from apple juice (denoted herewith as strain A) and a reference strain DSMZ 2498 (denoted herewith as strain B) obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkuturen, Braunschweig, Germany) culture collection. Cultures of both strains were maintained at −80 °C in yeast extract starch glucose (YSG) broth (yeast extract, 2.0 g; glucose, 1.0 g; soluble starch, 2.0 g; 1000 mL H_2O) with pH adjusted to 3.7 using HCl (1N), supplemented with 20% (*v*/*v*) glycerol (APPLICHEM, Darmstadt, Germany). To obtain the stock cultures both strains were pre-cultured in YSG broth for 48 h at 45 °C.

5.A.2.2. Spore production

Fresh cultures were spread on acidified *Bacillus acidoterrestris* medium (BAT) agar plates (adjusted to pH 3.7 after sterilization with 1N H_2SO_4) (BTA20500, Biolab,

Budapest, Hungary) and incubated at 45 °C for 7 days to sporulate. The sporulation of the cells was monitored by phase contrast microscopy and the spores were harvested when at least 80% of the cells had sporulated. Specifically, 2.5 mL of cold, sterile, distilled water was added to the BAT agar plates and the culture was gently removed from the surface by means of a sterile glass rod. The process was repeated twice and the suspensions from 15 plates were centrifuged at 5000 rpm for 20 min at 4 °C. The pellet was washed three times using cold sterile distilled water by centrifugation (5000 rpm for 20 min at 4 °C). Finally, the spores were re-suspended in 10 mL sterile phosphate buffer (pH 7.2) and stored at 4 °C until use.

5.A.2.3. Spore enumeration

Volumes of 2 mL of HHP-treated (see Section 2.5) and non-treated samples were initially heat shocked at 80 °C for 10 min and then cooled in ice water in order to destroy the vegetative cells. The spore concentrations of the orange juice samples were determined from the appropriate decimal dilution after spread plating on acidified BAT agar plates (pH 3.7), incubated at 45 °C for 3 days. After incubation, plates containing 30 to 300 colonies were used for enumeration and the results were log transformed and expressed as log CFU/mL. In order to lower the detection limit of the enumeration method to 0 log CFU/mL, 1 mL of the sample was spread plated to three Petri dishes of BAT agar. It needs to be noted that microbiological analyses were undertaken before and after the heat shock treatment in order to determine the population of vegetative cells of the bacterium, by subtracting the counts before (spores and vegetative cells) and after (spores only) the heat shock treatment for HHP-treated and untreated (control) samples.

5.A.2.4. Orange juice samples

Commercially pasteurized orange juice (pH 3.7, 11.45 °Brix) was purchased from the local market. The juice was subjected to microbiological analysis to detect the presence of *A. acidoterrestris* and the results indicated absence of the bacterium from the commercial sample of orange juice. Plastic film pouches (45 mm wide × 95 mm long × 90 μm thickness) with O_2 permeability of 75 mL/m^2/24 h/1 atm at 23 °C and 75% relative humidity (Flexo-Pack SA., Athens, Greece) were filled with 4 mL of orange juice and 0.4 mL of spore suspension in order to obtain final spore concentration of ca.10^7 spores/mL. The pouches were heat-sealed with the use of a HenkoVac 1700

machine (Howden Food Equipment B.V., Hertogenbosch, The Netherlands) taking care to expel most of the air.

5.A.2.5. HHP thermal processing of orange juices

The pouches were subjected to temperature-assisted high-pressure treatment at 600 MPa/60 °C for pressurization times of 5 and 10 min, respectively. The HHP treatments were conducted with a Food Pressure Unit (FPU) 1.01 (Resato International BV, Roden, The Netherlands). The system comprised a pressure intensifier and a 1.5 L vessel (7 cm diameter and 40 cm length), operating at a maximum pressure of 1000 MPa and temperature up to 90 °C, with pressure adjustable in steps of 20 MPa. The pressure transmitting fluid was polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, The Netherlands). Further details of the HHP equipment can be found elsewhere [32]. The come-up rate was approximately 100 MPa per 7 s and the pressure release time was less than 3 s. Pressure and temperature were constantly monitored and recorded during the process with the use of pressure transducers and temperature transmitters. The pressure come-up and release times were not included in the reported treatment times. After HHP treatment, the pouches were stored in isothermal conditions (4, 12 and 25 °C) for two months and the population of *A. acidoterrestris* spores was determined every week. Overall, the experiment was repeated twice with duplicate pouches analyzed for each combination of HHP treatment, bacterial strain, storage time and storage temperature.

5.A.3. Results and discussion

Temperature-assisted high-pressure processing can achieve inactivation of *A. acidoterrestris* spores in fruit juices and, as temperature increases, the rate of microbial inactivation also increases [26,33,34]. Specifically, studies with HHP treatments at 600 MPa report microbial reduction in spore population of the microorganism depending on temperature and pressurization time. The effectiveness of HHP treatment at 600 MPa combined with a temperature of 60 °C for 5 and 10 min on the survival rate of *A. acidoterrestris* spores is illustrated in Figures 1 and 2, respectively. Results indicated an initial decrease of ca. 3 log cycles instantly after treatment of the wild-type strain A regardless of pressurization time, whereas the respective reduction for the reference strain B was ca. 3 and 3.5 log cycles for 5 and 10 min pressurization times, respectively

(Figure S1 and Figure S2). These results are in agreement with Hartyani et al. (2013) [12] who inoculated *A. acidoterrestris* spores in orange juice and applied high-pressure treatment at 600 MPa/60 °C for 10 min and reported a reduction of 3 log cycles in the spore population immediately after pressurization. Moreover, Vercamenn et al. (2012) [34] applied high-pressure treatment at 600 MPa/60 °C for 10 min using tomato sauce (pH 4.2) and reported a reduction of 3.5 log cycles in *A. acidoterrestris* spores. Moreover, the spore population of the wild strain A presented a change of less than 0.5 log cycles throughout storage at 4 °C for the orange juice treated at 600 MPa/60 °C for 5 min (Figure 1), while for the respective treatment for 10 min, the population showed a gradual decrease of ca. 1 log cycle up to 21 days of storage ($p < 0.05$) followed by fluctuations of ca. 0.5 log cycles. For storage at 12 °C, the spore population after the 5 min HHP treatment showed fluctuations that ranged between 0.5–1 log cycles, with the exception of 35 days where a 1.5 log cycle reduction was noticeable. For the 10 min HHP treatment, fluctuations ranged between 0.7 and 1.3 log cycles within the first 35 days and subsequently increased gradually throughout storage, exceeding the initial population by ca. 0.5 log cycles. In the case of orange juice samples stored at 25 °C, both HHP treatments (5 and 10 min) resulted in a 1 log cycle increase in spores at the end of storage. The spore population of the reference strain B (Figure 2), when stored at 4 °C, also showed fluctuations and a decrease of 1.5 log cycles below the initial counts after 60 days, regardless of treatment time. Storage at 12 °C presented different trends for the two treatments. Specifically, for the 5 min treatment a decrease of 1.5 log cycles was observed up to day 35, followed by an increase without reaching the initial counts. On the contrary, for the 10 min treatment, the population oscillated between 0.6 and 0.8 log cycles without presenting a particular trend. At 25 °C, both treatments presented ca. 1 log cycle reduction in spore counts after 1 week and subsequent fluctuations for the remaining time without reaching the initial population at the end of storage. Overall, no remarkable changes in the spore population of the wild strain A were observed after 60 days of storage of the orange juice at 4 and 12 °C, whereas an increase of 1 log cycle in *A. acidoterrestris* spores was observed after 60 days storage at 25 °C, for both HHP treatments. For the reference strain B, results also did not reveal remarkable differences throughout storage at all temperatures assayed after HHP treatment for 5 min, whereas for 10 min processing, spore population increased by ca. 1 log cycle after 60 days of storage at 12 °C, but no differences were established throughout storage at 4 and 25 °C. Hartyani et al. (2013) [12] inoculated *A.*

acidoterrestris spores of the same reference strain (DSMZ 2498) in orange juice and applied HHP at 600 MPa/60 °C for 10 min followed by juice storage at 4 °C for 28 days and reported ca. 1 log cycle reduction in spore population after 28 days of storage, which is in good agreement with the results obtained in this work for the same strain (Figure 2). In order to determine the presence of vegetative cells after the HHP treatment and elucidate their behavior throughout storage at all temperatures (4, 12 and 25 °C) for both *A. acidoterrestris* strains, counts were considered before and after the heat shock treatment. The difference represents the population of vegetative cells as shown in Figures 3 and 4 for strain A and B, respectively. The population of vegetative cells after the HHP treatment for both strains was 1.5 log CFU/mL and ca. 1.0 log CFU/mL for 5 and 10 min pressurization times, respectively. Vercamenn et al. (2012) [34] reported a 0.5 log CFU/mL population of vegetative cells when applying HHP and heat treatment under the same conditions for a different strain of *A. acidoterrestris* in tomato sauce at pH 4.2, while Hartyani et al. (2013) [12] reported a 1.0 log CFU/mL population of vegetative cells for the same treatment with the same reference strain (DSMZ 2498) in orange juice. In addition, Riberio and Cristianini (2020) [35] reported a 1.24 log CFU/mL population of vegetative cells after HHP treatment at 600 MPa/70 °C/5 min in phosphate buffer medium (pH 7.2).

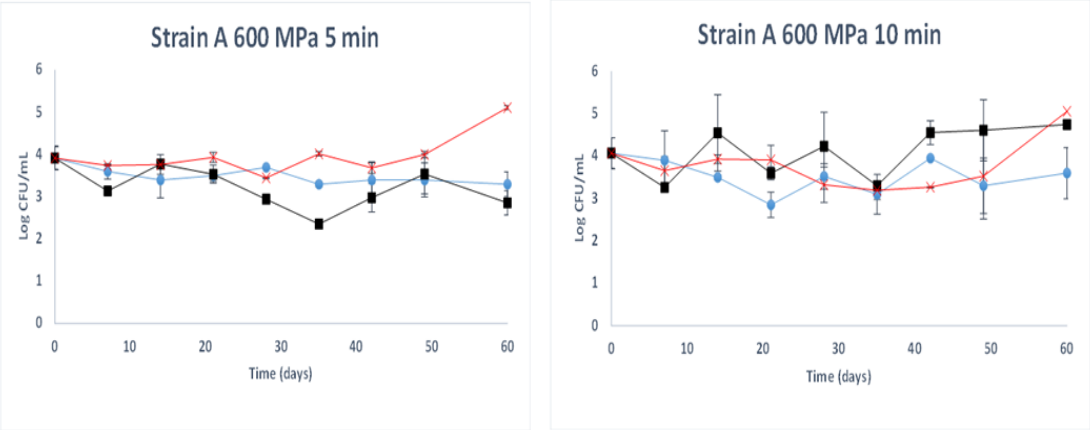

Figure 5.A.1. Survival curves of *A. acidoterrestris* (Strain A) spores in orange juice treated at 600 MPa/60 °C for 5 and 10 min and stored at 4 °C (●), 12 °C (■) and 25 °C (x). Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

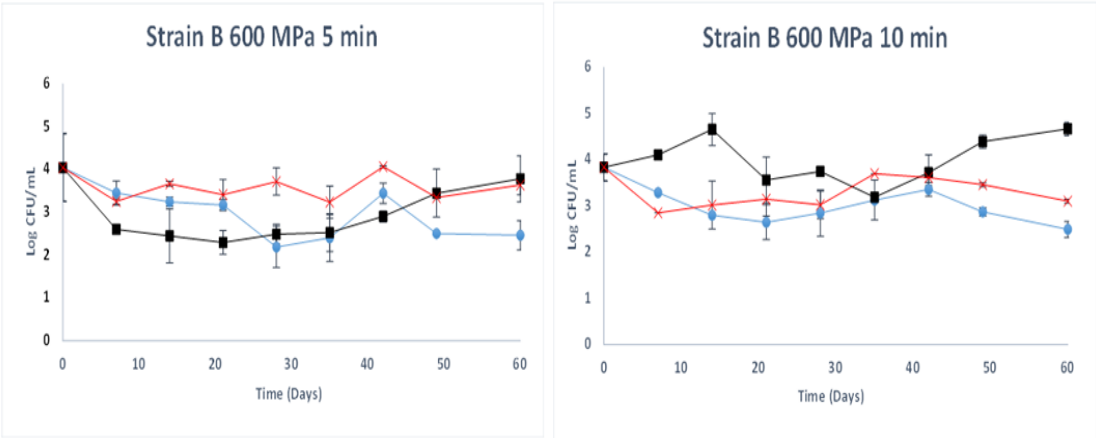

Figure 5.A.2. Survival curves of *A. acidoterrestris* (Strain B) spores in orange juice treated at 600 MPa/60 °C for 5 and 10 min and stored at 4 °C (●), 12 °C (■) and 25 °C (x). Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

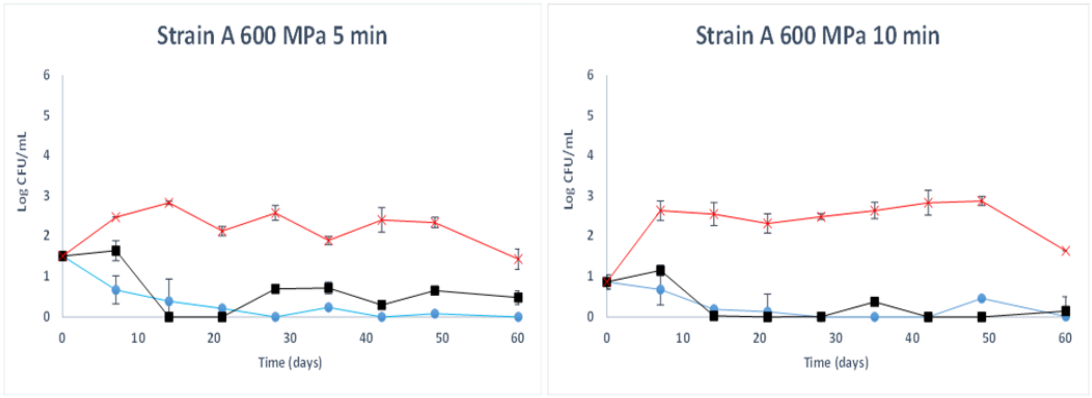

Figure 5.A.3. Survival curves of *A. acidoterrestris* (Strain A) vegetative cells in orange juice treated at 600 MPa/60 °C for 5 and 10 min and stored at 4 °C (●), 12 °C (■) and 25 °C (x). Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

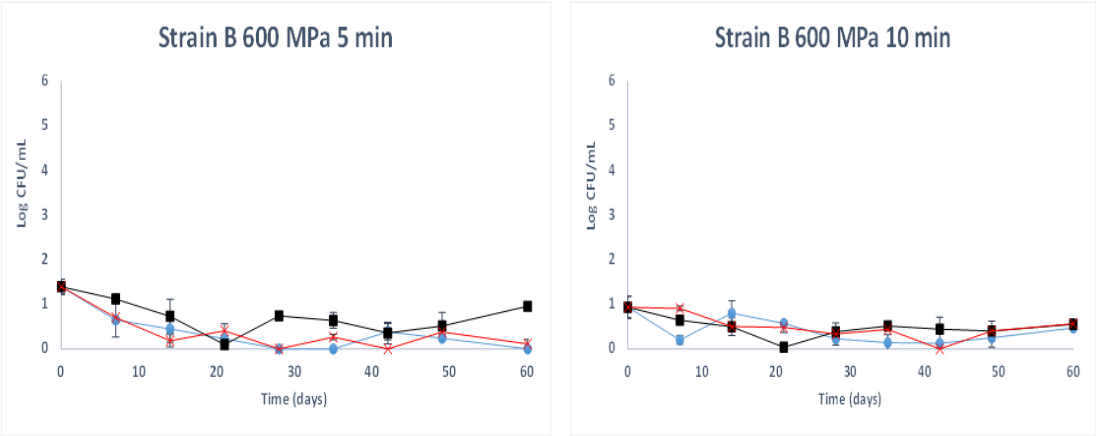

Figure 5.A.4. Survival curves of *A. acidoterrestris* (Strain B) vegetative cells in orange juice treated at 600 MPa/60 °C for 5 and 10 min and stored at 4 °C (●), 12 °C (■) and 25 °C (x). Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

However, at 12 °C, the population of vegetative cells was reduced after day 7, whereas at 4 °C their population reduced throughout storage and was found near the detection limit after day 21 for both HHP treatments of 5 and 10 min. Consequently, vegetative cells can be eliminated with HHP treatment during storage at low temperatures, which is in agreement with Hartyani et al. (2013) [12], who reported that the population of vegetative cells of *A. acidoterrestris* treated with HHP at three different pressure levels (200, 400 and 600 MPa) at 60 °C for 10 min in orange and apple juice was decreased to the detection limit of the plating method throughout storage at 4 °C for 28 days. As shown in Figure 3, the counts of vegetative cells of strain A were increased and maintained above the initial population throughout storage at 25 °C for 60 days. On the contrary, the vegetative cells of strain B did not follow the same trend, since the population decreased even at 25 °C. This difference between the wild-type strain A and the reference strain B could be attributed to the fact that the effectiveness of the HHP treatment against *A. acidoterrestris* is strain-dependent [36], presumably due to the different distribution of fatty acids in the membranes of the bacteria [37], although other factors could be cell age, cell population and nutrient availability [38,39].

5.A.4. Conclusions

In conclusion, the results from this study indicated that HHP could induce the inactivation of *A. acidoterrestris* spores in orange juice when combined with mild heat treatment and ensure the inhibition of surviving spore germination during the shelf life of the final product at refrigerated temperatures. However, during storage at different isothermal conditions for two months, the changes in spore population were strain-dependent and did not present a consistent pattern. It was also demonstrated that the remaining vegetative cells could be eliminated throughout storage at low temperatures, although strain variability should be taken into consideration. Therefore, heat-assisted HHP treatment could offer promising perspectives for reducing spoilage and extending the shelf life of orange juice.

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5B. The effect of temperature assisted High Hydrostatic Pressure on the survival of *Alicyclobacillus acidoterrestris* in peach juice throughout storage at different isothermal conditions

5.B.1. Introduction

The purpose of this work was to investigate the population dynamics of the spores and vegetative cells of *A. acidoterrestris* in peach juice treated with temperature assisted HHP and stored at different isothermal conditions. Since there is little information in the literature on the storage of High Hydrostatic Pressure (HPP) treated juices at isothermal conditions, it was deemed necessary after studying the survival of *A. acidoterrestris* at refrigerated (4 and 12 ℃) and shelf temperatures (25 ℃) in orange juice, to investigate the dynamics of the spore and vegetative cells population at higher temperatures. For this reason, the spores of *A. acidoterrestris* were inoculated in commercial peach juice and subjected to HHP treatment at 500 and 600 MPa combined with heat treatment at 25, 45 and 60 ℃ for 10 min. Inoculated samples were subsequently stored at 25, 35 and 45 ℃. During storage the population of *A. acidoterrestris* was determined before and after heat shock treatment at 80 ℃ for 10 min in order to estimate separately the spores and the possible remaining vegetative cells on K medium agar. Results indicated that the effectiveness of HHP against *A. acidoterrestris* was more effective as pressurization level and temperature increased. Furthermore, storage of peach juice samples at higher temperatures could be prolonged with effective HHP treatment.

5.B.2. Material and methods

5.B.2.1. Bacterial strains

The strain *Alicyclobacillus acidoterrestris* ATCC 49025 (NCBI: txid1356854) used in this survey was obtained from NCBI culture collection. Cultures of the strain were maintained at -80 °C in Yeast Extract Starch Glucose (YSG) broth (yeast extract, 2.0 g; glucose, 1.0 g; soluble starch, 2.0 g; 1000 mL H_2O) with pH adjusted to 3.7 using HCl (1N), supplemented with 20% (*v/v*) glycerol (APPLICHEM, Darmstadt, Germany). The strain was pre-cultured in YSG broth for 48 h at 45 °C in order to obtain a fresh culture.

5.B.2.2. Spore production

Spore production was performed as detailed in paragraph2.2. of chapter 5.A. for orange juice.

5.B.2.3. Spore enumeration

A volume of 2 mL of HHP treated and non-treated samples was initially heat shocked at 80 °C for 10 min and cooled in ice water in order to destroy vegetative cells. The spore concentration of the peach juice samples was determined from the appropriate decimal dilution after spread plating on acidified K agar (yeast extract, 2.5 g; peptone, 5.0 g; tween 80, 1.0 g; agar, 15.0 g; 1000 mL H_2O; pH adjusted to 3.7 with 25% malic acid), incubated at 45 °C for 3 days. After incubation, plates containing 30 to 300 colonies were used for enumeration and the results were log transformed and expressed as log CFU/mL. It needs to be noted that microbiological analyses were undertaken before and after the heat shock treatment in order to determine the population of vegetative cells of the bacterium, by subtracting the counts before (spores and vegetative cells) and after (spores only) the heat shock treatment.

5.B.2.4. Peach juice samples

Commercially pasteurized peach juice (pH 3.25, 12.10 °Brix) was purchased from the local market. The juice was initially subjected to microbiological analysis to detect the presence of *A. acidoterrestris* and the results indicated absence of the bacterium from the commercial samples of peach juice. Plastic film pouches (45 mm wide x 95 mm long x 90 μm thickness) with O_2 permeability of 75 mL/m^2/24h/1 atm at 23 °C and 75% relative humidity (Flexo-Pack SA., Athens, Greece) were filled with 4 mL of orange juice and 0.4 mL of spore suspension in order to obtain final spore concentration of *ca.*10^6 spores/mL. The pouches were heat-sealed with the use of a HenkoVac 1700 machine (Howden Food Equipment B.V., The Netherlands) taking care to expel most of the air.

5.B.2.5. HPP thermal processing of peach juice samples

The pouches were subjected to temperature-assisted high pressure treatment at 500 and 600 MPa in combination with different temperature regimes (25, 45 and 60 °C) for 10 min pressurization time, respectively. The HHP treatments were conducted with a Food Pressure Unit (FPU) 1.01 (Resato International BV, Roden, Holland). The details and operating conditions of the HHP system are described in paragraph 2.5 of Chapter 5A

for orange juice. Further on, treated and untreated pouches were stored at isothermal conditions (25, 35 and 45 °C) for up to 240 h, until spoilage was pronounced (presence of off-odors and discoloration). The population of *A. acidoterrestris* spores and vegetative cells was determined at appropriate time intervals depending on storage temperature and treatment conditions. The experiment was repeated twice with duplicate pouches analyzed for each combination of HHP treatment, storage time and storage temperature.

5.B.3. Results and Discussion

The survival curves of untreated and treated *A. acidoterrestris* spores in peach juice at 500 and 600 MPa for 10 min in combination with different temperatures (25, 45 and 60 °C) during storage at 25, 35 and 45 °C are presented in Figures 5.B.1 and 5.B.2, respectively. Immediately after HHP treatment at 500 MPa for 10 min, an initial decrease of *ca*. 1.0, 3.0 and 3.5 log cycles was observed with heat treatment of the samples at 25, 45 and 60 °C, respectively. Similarly, HHP treatment at 600 MPa/10 min presented *ca*. 1.0, 3.4 and 3.6 log cycles reduction at the respective storage temperatures. Our results are comparable with Lee et al. [1] who inoculated *A. acidoterrestris* spores in apple juice and reported 3.5 log cycles reduction after pressurization at 621 MPa / 45 °C for 10 min. Hartyani et al. [2] also stated 3.0 and 2.0 log cycles reduction of *A. acidoterrestris* spores inoculated in apple and orange juice, respectively for HHP treatment at 600MPa/50 °C for 10 min, while for the same treatment with applied temperature at 60 °C spore population was reduced by 3.8 and 3.0 log cycles for apple and orange juice correspondingly. Moreover Vercamenn et al. [3] applied high pressure treatment at 500 and 600 MPa for 10 min in combination with 40 and 60 °C using tomato sauce (pH 4.2) and reported 3.0 and 3.5 log cycles reduction of *A. acidoterrestris* spores for treatments at 500 and 600 MPa, respectively regardless the temperature. The reduction of 1.0 log cycle for both high pressure treatments (500 and 600 MPa for 10 min) at 25 °C is in agreement with previous results in this thesis and also with previous studies, reporting little or no inactivation when HHP was combined with ambient temperature [1,3,4]. As shown in Figures 5.B.1 and 5.B.2, the spore population of the control samples did not present remarkable differences throughout storage at all temperatures assayed for all HHP treatments. Throughout storage at 25 °C results showed no remarkable changes for all HHP treatments, since

the spore population showed small fluctuations without increasing above the initial counts. Treatment at 600 MPa/60 °C managed to decrease the spore population to the detection limit of the enumeration method (< 1.0 log CFU/mL) during storage. In the case of peach samples stored at 35 °C, the spore population presented the same trend for both treatments (500 and 600 MPa) at all applied temperatures (25, 45 and 60 °C). The counts throughout storage at 35 °C were higher compared with 25 °C, with the exception of HHP treatments at 500 MPa/60 °C and 600 MPa/60 °C that presented a decrease in the spore population to the detection limit of the enumeration method after 96 h. For incubation at 45 °C, the spore population presented the same trend with the HHP applied temperature of 25 °C for both 500 and 600 MPa treatments, while treatments combined with 45 °C presented differences. Specifically, after treatment at 500 MPa/45 °C counts were below the initial population for 168 h, whereas the spore population after treatment at 600 MPa/45 °C exceeded the initial population throughout storage. In the case of treatment at 500 and 600 MPa/60 °C, the spore population was below the detection limit of the method for 120 h of storage and then the counts increased 1.5 (500 MPa) and 2.5 (600 MPa) log cycles above the initial population throughout storage.

Kakagianni et al. [5] reported that untreated spores of the same strain of *A. acidoterrestris* when inoculated in peach juice and stored at 35 °C presented 5 log cycles increase after 90 h. However, treatment of peach juice at 500 MPa/45 °C for 10 min, followed by storage at 35 °C, resulted in 2 log cycles increase in spore population for the same time period (90 h). On the contrary, reduction in spore population close to the detection limit of the plating method was observed when the peach juice was treated at 600 MPa/60 °C for 10 min and stored at 35 °C for the same time span. From the above observations it can be concluded that spore inactivation increased as the temperature level of the HHP treatment also increased, which is in agreement with previous studies [2,3,6-8]. There are few reports in the literature concerning the evolution of HHP treated spore population during storage [2]. The results obtained in this study indicated that for both heat-assisted HHP treatments at 25 °C, the population of spores increased with increasing storage temperature. However, for heat-assisted HHP treatments at 45 and 60 °C, no clear trend could be established for the changes in spore population throughout storage at all temperatures. It could also be inferred that heat-assisted HHP treatment at 500 MPa/45 and 60 °C was more effective to control the spores of the bacterium throughout storage of peach juice samples at 45 °C.

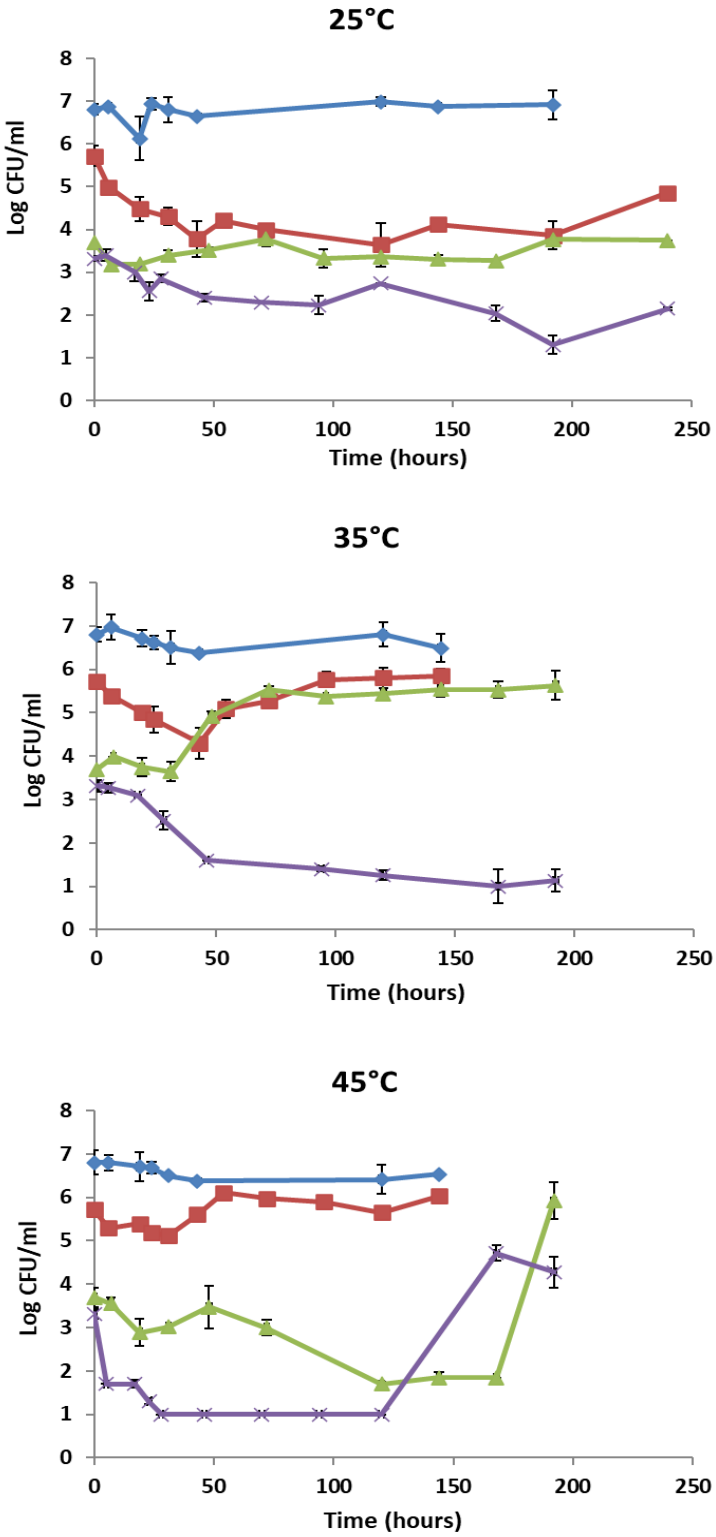

Figure 5.B.1. Survival curves of unpressurized (♦) *A. acidoterrestris* spores and pressurized at 500 MPa for 10 min combined with temperature at 25 ℃ (■), 45 ℃ (▲) and 60 ℃ (x) during storage of peach juice at 25 ℃, 35 ℃ and 45 ℃. Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

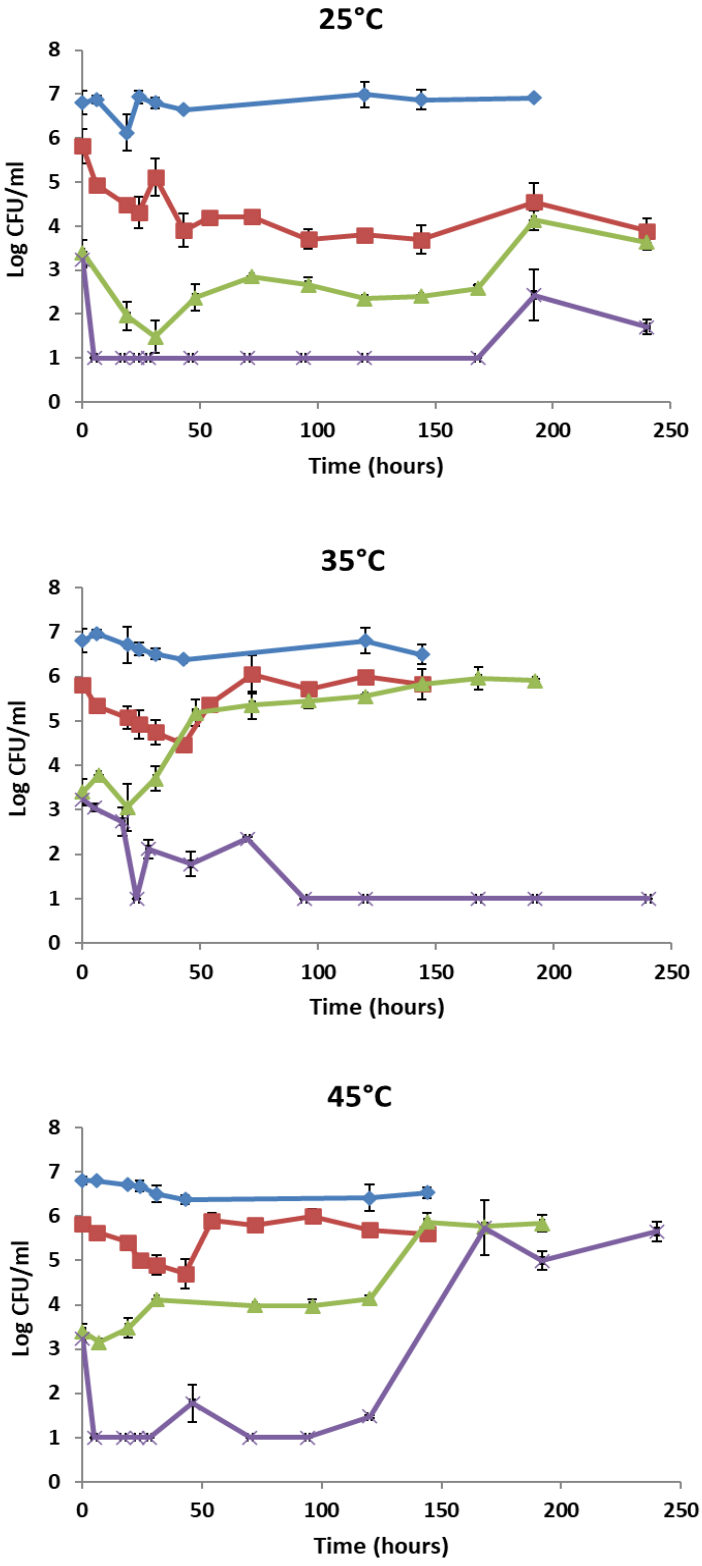

Figure 5.B.2. Survival curves of unpressurized (♦) A. acidoterrestris spores and pressurized at 600 MPa for 10 min combined with temperature at 25 °C (■), 45 °C (▲) and 60 °C (x) during storage of peach juice at 25 °C, 35 °C and 45 °C. Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

Another aim of this research was to determine the presence and the trend of untreated and HHP treated vegetative cells of *A. acidoterrestris* during storage at the same isothermal conditions (25, 35, and 45 °C). For this purpose, counts were considered before and after heat shock treatment (80 °C for 10 min) and their difference represented the population of vegetative cells as shown in Figures 5.B.3 and 5.B.4, for HHP treatment at 500 and 600 MPa, respectively. The population of the vegetative cells for unpressurized samples throughout storage at all temperatures (25, 35, and 45 °C) was found below the detection limit of the enumeration method (< 1.0 log CFU/mL). This observation confirms that the inoculated population was spores and not vegetative cells of the microorganism. These results are in agreement with Vercamenn et al. [3] who inoculated *A. acidoterrestris* spores in tomato sause (pH= 4.2) and reported 1.0 log cycle vegetative cells population of *A. acidoterrestris* immediately after treatments at 500 and 600 MPa combined with 40 and 60 °C for 10 min. In the case of HHP treatment at 500 MPa, results indicated that the counts of the vegetative cells increased gradually throughout storage at 25 °C, with 2.0, 2.5 and 3.5 log cycles increase for heat-assisted HHP treatment at 25, 45 and 60 °C, respectively. In the case of storage at 35 °C, the counts of the vegetative cells presented lower increase, whereas at 45 °C the population of vegetative cells was limited close to the detection limit of the plating method throughout storage, (with the exception of 60 °C where 1.0 log cycle increase was observed after 192 h of storage). The population of the vegetative cells for HHP treatment at 600 MPa followed by storage at 25 °C presented the same trend as for 500 MPa combined with 25 and 45 °C, with 2.0 and 3.0 log cycles increase, respectively. When treated at 600MPa/60 °C the counts were below the detection limit of the enumeration method for the first 120 h followed by an increase of *ca*. 3.0 log cycles throughout storage. When stored at 35 °C, the vegetative cell population presented also similar trend with the 500 MPa treatment for all applied temperatures, and after 72, 120 and 94 h of storage at 25, 35 and 45 °C respectively, counts were near or below the detection limit. Peach samples stored at 45 °C also presented counts below the detection limit of the enumeration method with heat-assisted HHP treatment at 60 °C throughout storage, whereas when the applied temperature during pressurization was 25 °C and 45 °C, counts fluctuated up to 1 log for the first 43 and 144 h, respectively and then decreased to the detection limit of the enumeration method. Therefore, the present results indicate that the population of the HHP treated vegetative cells in all cases was below or near the detection limit of the method, instantly after pressurization.

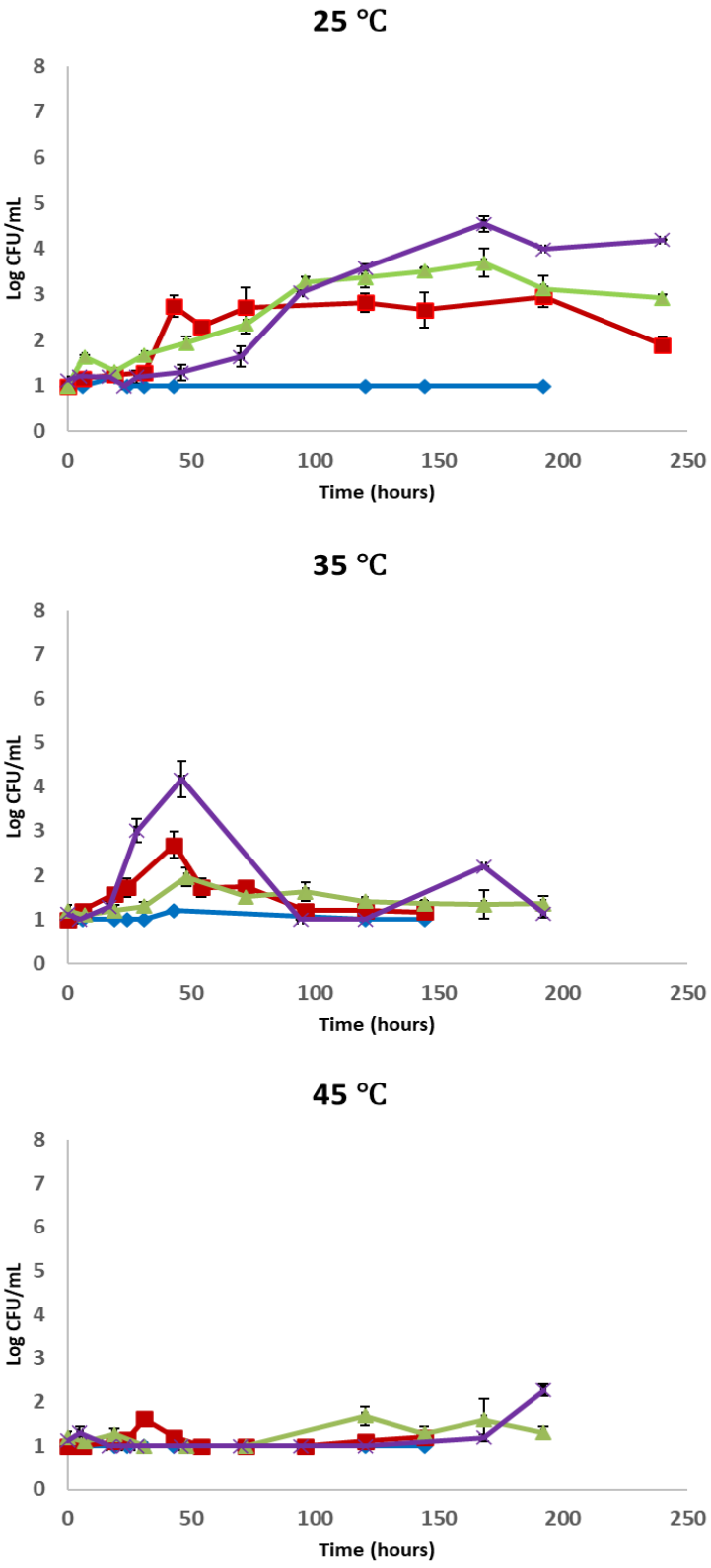

Figure 5.B.3. Survival curves of unpressurized (♦) *A. acidoterrestris* vegetative cells and pressurized at 500 MPa for 10 min combined with temperature at 25 °C (■), 45 °C (▲) and 60 °C (x) during storage of peach juice at 25 °C, 35 °C and 45 °C. Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

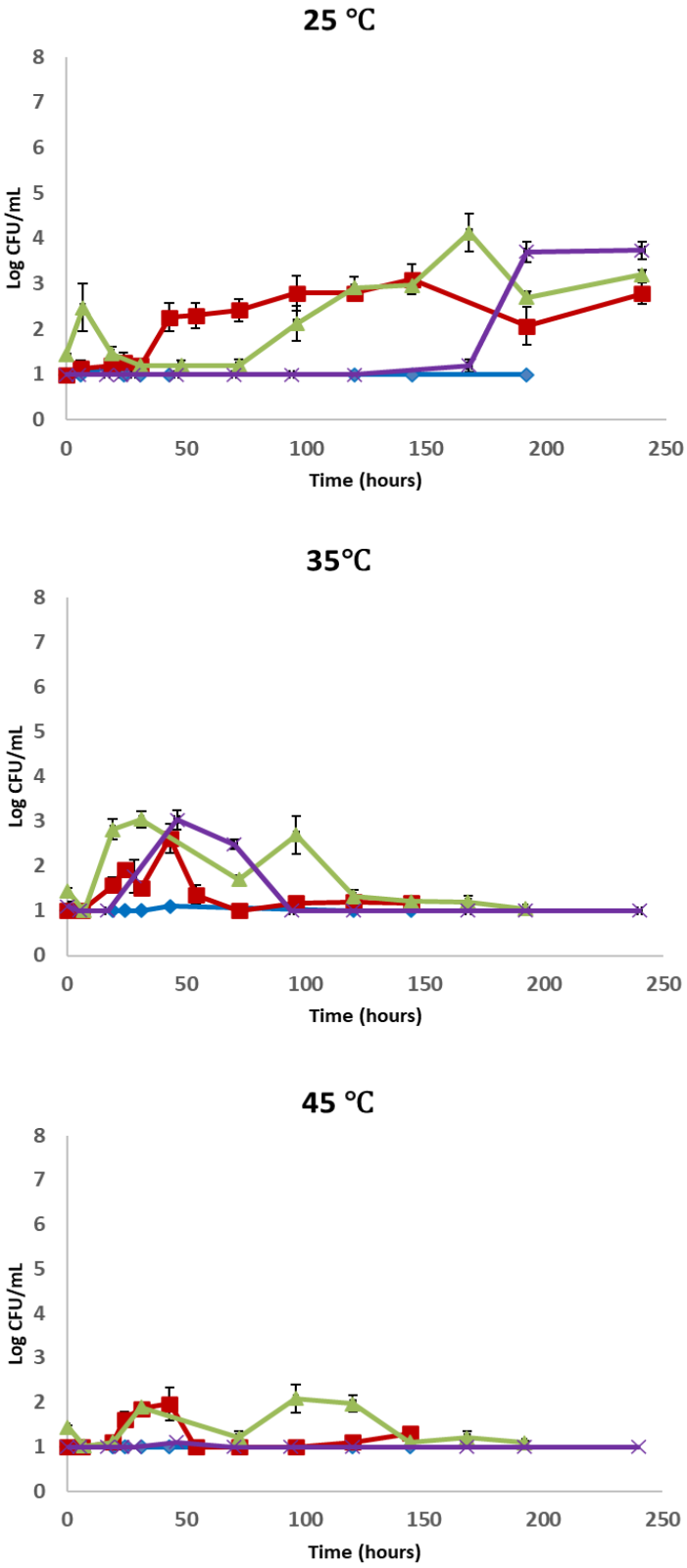

Figure 5.B.4. Survival curves of unpressurized (♦) *A. acidoterrestris* vegetative cells and presssurized at 600 MPa for 10 min combined with temperature at 25 °C (■), 45 °C (▲) and 60 °C (x) during storage of peach juice at 25 °C, 35 °C and 45 °C. Data points are mean values ± standard deviation of duplicate samples from two independent experiments (n = 4)

Taking into account the whole duration of storage, results pointed out that counts of vegetative cells were higher as storage temperature decreased for both HHP treatments (500 and 600 MPa) at all combined temperatures (25, 45 and 60 °C). This observation leads to the conclusion that the vegetative cells of *A. acidoterrestris* can be limited at higher storage temperatures (35 and 45 °C) after HHP treatment.

In conclusion, the inactivation of *A. acidoterrestris* spores in peach juice could be induced by HHP combined with mild heat treatment. Although storage at different isothermal conditions did not present a consistent pattern, results indicated that HHP treatment at 600 MPa combined with 60 °C for 10 min could eliminate the spore population for storage at 25 °C for the studied time period, while treatment at 500 MPa/45 °C could be more effective throughout storage at 45 °C. Furthermore, the vegetative cell population of *A. acidoterrestris* in peach juice samples treated at 600 MPa/60 °C could also be inhibited to the detection limit of the method at all storage temperatures (25, 35 and 45 °C). Therefore, HHP treatments managed to restrict the spore population for longer period time in comparison with unpressurized samples and this leads to the conclusion that HHP can ensure fruit juices with prolonged shelf life at higher temperatures. Consequently, heat assisted HHP treatment offers perspectives to reduce spoilage problems in the fruit juice industry.

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CHAPTER 6

Conclusions and Future Perspectives

Fruit juices are considered to be the most popular representatives of the beverage industry. Their characterization as natural and healthy make them very attractive to consumers who look for convenient fruit juices that can fit in today's busy life style. Furthermore, the high acidity of fruit juices (pH<4.6) does not favor the growth of pathogens and spoilage microorganisms, rendering them more attractive to consumers. However, the fruit juice industry worldwide has reported a major concern for the presence of *Alicyclobacillus* spores. Therefore, the present thesis studied the presence of spoilage microorganisms in orange and peach juices of the Greek market with main focus on the detection of *Alicyclobacillus* in the surveyed samples. After verifying the presence of the bacterium in commercial fruit juices, the isolated strains of *Alicyclobacillus* were discriminated with RFLP-PCR for rapid identification and *Alicyclobacillus acidoterrestris* strains were differentiated from other species. All isolated strains had the ability to produce guaiacol and consequently have the potential to spoil the juices. Further on, HHP was employed as a non thermal pasteurization method in order to control *Alicyclobacillus acidoterrestris*. The spores of the microorganism were subjected to heat-assisted HHP at different levels of pressure and temperature, in order to investigate the best combination of these parameters that could control their presence. Moreover, spores were inoculated in orange and peach juice samples in order to examine the dynamic of the spore and vegetative cell population before and after HHP treatment during storage at different isothermal conditions.

As demonstrated in Chapter 2 commercial refrigerated and pasteurized orange and peach juices of the Greek market presented low contamination from spoilage microorganisms, and at the same time confirmed the presence of *Alicyclobacillus* in Greek juices. Our results revealed that orange juice presented higher contamination from *Alicyclobacillus* spp. compared to peach juice. In addition, no correlation could be established between the contamination incidence and the origin of the fruit juices (super markets, mini markets or street markets).

The isolated colonies from the commercial orange juices were discriminated into 8 clusters with 16S rDNA PCR-RFLP and the use of three restriction endonucleases (*Hha*I, *RSa* I and *HiNF*I). Seven of the groups had different pattern from the reference strain *Alicyclobacillus acidoterrestris* DSMZ 2498. This observation combined with the fact that different patterns also originated from the same juice leads to the conclusion that the colonies may belong to various strains or even to different species.

All isolates had the potential to produce guaiacol as determined by the PECA method. In order to identify if the isolated colonies belonged to *A. acidoterrestris* a single enzyme RFLP PCR method was utilized. This molecular assay succeeded in discriminating *A. acidoterrestris* isolates from *A. acidocaldarius* and *A. hesperidum* within 8 hours with the use of specific conditions. The early detection and identification of *Alicyclobacillus* is of great importance for fruit juice manufacturers, because the spoilage is difficult to be noticed before consumption.

Therefore, it is of great importance to develop control methods for the inactivation of the spores of *Alicyclobacillus* and mainly *A. acidoterrestris* that may be present in fruit juices. For this reason, we studied the effect of HHP on *A. acidoterrestris* spores, which is considered to be the most effective non thermal processing for fruit juices and moreover for spore inactivation. After studying the effectiveness of HHP on spores of two *A. acidoterrestris* strains inoculated in orange juice using different pressures, combined with different temperatures for pressurization time up to 30 min we confirmed the effectiveness for the selected conditions and strains of the processing. In particular results indicated that the higher the pressure and temperature levels applied, the higher the inactivation of bacterial spores. Complete spore inactivation was achieved at the higher pressurization and temperature levels (600 MPa / 70 °C) employed in the study, for both strains. Furthermore, the inactivation kinetics of *A. acidoterrestris* spores was successfully described by means of the Weibull model, although strain variability affected the performance of the model.

The majority of published works focuses on the effectiveness of HHP treatment to inactivate the spores of *A. acidoterrestris* immediately after the application of high pressure. However, it would be of great interest to investigate the dynamics of the spores and vegetative cells of *A. acidoterrestris* throughout storage of HHP treated fruit juices at different isothermal conditions (4, 12 and 25 °C). The used HHP treatment was able to induce spore inactivation of two *A. acidoterrestris* strains inoculated in orange juice and ensure the inhibition of the surviving spores' germination throughout storage at refrigerated temperatures (4 and 12 °C). However, the spore population did not present a consistent pattern during storage for two months at the different isothermal conditions and the changes in the spore population were strain dependent. The remaining vegetative cells could be eliminated throughout storage at low temperatures, although strain variability should be taken into consideration.

In order to study the dynamics of treated spores and vegetative cells of *A. acidoterrestris* at higher storage temperatures (25, 35 and 45 °C) that favor the growth of the bacterium, spores of strain ATCC 49025 were inoculated in peach juice. Although storage at different isothermal conditions did not present a consistent pattern, it was clear that HHP treatment at 600 MPa/60 °C/10 min could eliminate the spore population during storage at 25 °C. The outcome of this study was that the same HHP treatment can induce germination, since the population of the vegetative cells was inhibited to the detection limit of the method at all storage temperatures. Consequently, HHP treatments managed to restrict the spore population for longer period time in comparison with unpressurized samples and this leads to the conclusion that HHP can ensure safer fruit juices with prolonged shelf life at higher temperatures. Therefore, heat assisted HHP treatment offers perspectives to control spoilage problems in the fruit juice industry.

Based on the results of this thesis, the contamination of commercial fruit juices from *Alicyclobacillus* was not rare. The early detection and identification of the bacterium is very important for the fruit juice industry, since spoilage incidents result in economic losses. The need of developing rapid and accurate detection and identification methods is of paramount importance and RFLP PCR used in this study was proved to fulfill these criteria. In order to ensure the high safety and extended shelf life of fruit juices non thermal technologies and their combination with heat have been developed. HHP was proved capable of inactivating the spores of thermoacidophilic bacteria like *A. acidoterrestris* in fruit juices and presents promising perspectives for the fruit juice industry. Besides spores' inactivation, HHP was able to eliminate the vegetative cells throughout storage and therefore enhance spoilage control. Strain variability should be taken into consideration, because it was an important factor affecting the high-pressure tolerance, the dynamics of the spore and vegetative cell populations throughout storage and the performance of the Weibull model. However, future work should be undertaken to include more strains and processing conditions of HHP, including oscillatory high-pressure treatment, so as to ensure the safety and quality of fruit juices.

Appendix

Publication from this thesis

Publication in International Scientific Journals of Science Citation Index

1. Sourri, P.; Doulgeraki, A.I.; Tassou, C.C.; Nychas, G.-J.E. A single enzyme PCR-RFLP assay targeting V1-V3 region of 16S rRNA gene for direct identification of *Alicyclobacillus acidoterrestris* from other *Alicyclobacillus* species. *J. Appl. Genet.* **2019**, *60*, 225–229. https://doi.org/10.1016/j.ijfoodmicro.2008.02.030
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- Sourri, P.J., Argyri, A.A., Tassou, C.C. (2015) Combined effect of high pressure processing and thermal treatment on the inactivation of *Alicyclobacillus* spores in orange juice. 2nd International Conference on Food and Biosystems Engineering (FABE2015), 28- 31 May, Mykonos Island, Greece. P81, pp 185
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Poster Presentations in international conferences

- Sourri, P.J., Argyri, A.A., Nychas, G.E., Tassou, C.C. (2014) Combined Effect Of High Pressure Processing And Thermal Treatment On The Inactivation Of *Alicyclobacillus* Spores In Orange Juice. EFFoST 2014, 7th International Conference on the Food Factory for the Future, 25 - 28 November 2014, Uppsala, Sweden.
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More Publication in International Scientific Journals of Science Citation Index

- Doulgeraki, A.I., Bikouli, V., Argyri, A.A, Chorianopoulos, N., Mitre, E., Charvourou, G., Sourri, P., Tassou, C.C., Oikonomou, A. Exploring the Bacterial Communities of the Kaiafas Thermal Spring Anigrides Nymphes in Greece Prior to Rehabilitation Actions. *Int. J. Environ. Res. Public Health*. **2020** Dec *7*,17(23):9133. doi: 10.3390/ijerph17239133.
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