AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE & HUMAN NUTRITION
LABORATORY OF QUALITY CONTROL & HYGIENE

PhD Thesis

Genotypic, physiological and technological attributes of lactic acid bacteria and yeasts isolated from spontaneously fermented Greek wheat sourdoughs

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«Γονοτυπικά, φυσιολογικά και τεχνολογικά χαρακτηριστικά οξυγαλακτικών βακτηρίων και ζυμών, απομονωθέντων από ελληνικά προζύμια σίτου αυθόρμητης ζύμωσης»

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Abstract

The aim of this thesis was to unravel the microecosystem composition of 13 spontaneously fermented wheat sourdoughs, originating from regions of Greece not previously assessed, via the combination of culture dependent and independent approaches. Then, further investigation of the technological and safety attributes of the associated microbiota was performed, for potential application of the sourdough isolates as starters or adjunct cultures in food fermentations.

In the first study, the microbial ecology of the 13 Greek wheat sourdoughs was identified by combining both culture dependent and independent approaches. Regarding the culture dependent method, conventional plating, clustering by Polymerase Chain Reaction-Random Amplified Polymorphic DNA (PCR-RAPD), identification by PCR species-specific for *Lactiplantibacillus plantarum* and 16S and 26S rRNA gene sequencing for lactic acid bacteria (LAB) and yeasts, respectively, were applied. On the other hand, culture independent approach included DNA and RNA-based PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis of sourdough samples. Results obtained, revealed that LAB populations ranged between 6.28-9.20 log CFU/ g, with *Lp. plantarum* and *Levilactobacillus brevis* dominating or co-dominating in the majority of cases. *Companilactobacillus paralimentarius*, *Fructilactobacillus sanfranciscensis*, *Latilactobacillus sakei*, *Lt. curvatus*, *Lv. zymae*, *Lactococcus lactis*, *Leuconostoc citreum* and *Leu. mesenteroides* were detected, as well. In the case of yeasts, their population ranged between 4.60-6.32 log CFU/ g and consisted mostly of *Saccharomyces cerevisiae*, while the presence of *Pichia membranifaciens*, *Pi. fermentans*, *Wickerhamomyces anomalus* and *Kazachstania humilis* was also recorded. Despite the fact that RNA and DNA-based PCR-DGGE analysis yielded the same microbial profiles, overall PCR-DGGE analysis only partially verified the sourdough microecosystem composition, as depicted by culture dependent approach.

In a further study, a wide set of technological and safety attributes of the sourdough derived LAB and yeasts, were assessed. The under study properties included proteolysis, lipolysis, amylolysis, amino acid decarboxylase, phytase activity, antimicrobial capacity and exopolysaccharide (EPS) production of both LAB and yeasts. Strains exhibited proteolytic, lipolytic and antimicrobial activities, which were initially screened by the well diffusion assay (WDA). Seven LAB and 8 yeast strains hydrolyzed gluten, while 11 LAB were considered lipolytic. In the former case, further Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of gluten fractions was applied, which resulted in partial and strain dependent gluten degradation. In the latter case, a subsequent lipolysis kinetics over 21 days revealed increased fluctuation of enzyme activities, among the different strains under study. Regarding the antimicrobial activity assessment, 13 LAB and 12 yeast strains exhibited inhibitory activity against different molds, which was attributed to non-proteinaceous metabolites. In addition, 21 *Lp. plantarum* strains revealed antibacterial activity against a mixture of *Listeria monocytogenes* strains, belonging to serotype 4b, while the protein nature of the inhibitory compounds was verified, as well. Among them, three *W. anomalus* strains (LQC 10346, 10353, 10360) and 6 *Lp. plantarum* strains (LQC 2320, 2422, 2441, 2485, 2516, 2520), which exhibited more than one technological or safety property, worth further investigation.

The three aforementioned sourdough derived *W. anomalus* strains, namely LQC 10346, 10353 and 10360, which were previously found to produce antimold metabolites of non-protein nature, were further subjected to antimold activity assessment both *in vitro* and *in situ*. For that purpose, the yeast strains were grown at 30 ^{o}C for 24 h in two substrates; the former was Brain Heart Infusion (BHI) broth adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and 6 NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%), while the latter was liquid dough supplemented with the aforementioned 6 NaCl concentrations. WDA revealed that the maximum inhibitory activity was 5120 AU/ mL, obtained by all three strains after growth in both substrates. Then, the responsible volatile compounds were identified by solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS), among which ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate were detected in all samples assessed. In the case of antimold activity assessment of pure

volatile organic compounds (VOCs), the lower minimum inhibitory concentrations (MICs) were recorded for 2,4-di-tert-butyl-phenol and benzaldehyde (0.04 and 0.06 μL/ mL of headspace, respectively). Finally, the breads produced either with monocultures of *W. anomalus* strains or with their co-culture with baker's yeast gained additional mold free shelf life, ranging from 9 to 30 days, compared to the control.

In a next study, 6 sourdough derived bacterial strains, namely LQC 2320, 2422, 2441, 2485, 2516 and 2520, the identity of which was initially assigned to *Lp. plantarum* by 16S rRNA gene sequencing, were further subjected to whole genome sequencing, due to their technological and safety properties. Thus, the raw sequence reads were quality checked, then de novo assembly was performed (assembly of reads into contigs), further organization of contigs to scaffolds occurred and finally scaffolds were annotated. Taxonomic classification of the aforementioned strains revealed their assignment to *Lp. plantarum* subsp. *argentoratensis*. Good quality of both assembled scaffolds and genomes after annotation was exhibited. The total size of genomes ranged within 3.13-3.49 Mb, while the number of protein-coding and the non-coding genes ranged within 3091-3492 and 160-231, respectively.

Given that there has been no previous report on the bioinformatic genome analysis of sourdough derived *Lp. plantarum* subsp. *argentoratensis* strains, the aim of the present study was to bioinformatically analyze and compare the genomes of the 6 bacterial strains, belonging to the subspecies *argentoratensis* (namely LQC 2320, 2422, 2441, 2485, 2516 and 2520). Analysis related to their biotechnological potential revealed that the 6 LAB strains possessed the genes associated with carbohydrate metabolism, membrane transport, metabolism of specific organic acids (namely conversion of citrate to oxaloacetate), acetoin biosynthesis, riboflavin and folate production. Regarding the genomic aspects concerning the food safety, the bioinformatic analysis performed revealed no pathogenic factors, but the presence of antibiotic resistance genes was detected. In addition, the 6 *Lp. plantarum* subsp. *argentoratensis* strains were considered negative producers for the majority of biogenic amines, however the detection of *cadA* encoding cadaverine production, worth's further phenotypical assessment. As far as the stability of genomes was concerned, the presence of plasmids, insertion sequences, prophage

regions and CRISPR*cas* systems was recorded. Finally, all 6 bacterial strains were plantaricin producers.

In previous studies, the 6 *Lp. plantarum* strains, namely LQC 2320, 2422, 2441, 2485, 2516 and 2520, were reported to exert antibacterial activity, due to the production of proteinaceous metabolites, while the presence of a set of genes encoding plantaricins in their genomes was recorded, as well. Thus, the aim of this study was to assess the effect of sourdough related parameters on the plantaricin activity and the transcriptomic response of the plantaricin genes in the 6 *Lp. plantarum* strains. Sourdough related parameters included 3 substrates: de Mann Rogosa and Sharpe (MRS) broth, MRS broth containing the type and amount of carbohydrates found in wheat flour, wheat flour and water extract fortified with carbohydrates to the initial flour concentration; 3 temperatures (20, 30, 37 ^{o}C); 2 initial pH values (5.0, 6.0); 2 NaCl concentrations (0.0, 1.8%); 12 time points (ranging from 0 to 33 h); 6 *Lp. plantarum* strains. The plantaricin activity was exerted in a strain dependent manner. More accurately, the 6 strains were discriminated in two groups in terms of phenotypic features and organization of the *pln* locus. Analysis of variance revealed that growth substrate, temperature, initial pH value and strains had significant effect on plantaricin activity, while NaCl had only marginal effect. Regarding the transcriptomic response of plantaricin genes, both temperature and substrate had a more significant effect on the relative gene transcription, compared to the respective of initial pH value. Weak correlation was recorded between phenotypic assessment and relative gene transcription.

Scientific area: Sourdough micro-ecosystem

Key words: Greek sourdoughs, spontaneous fermentation, culture-dependent approach, culture independent approach, technological properties, safety attributes, whole genome sequencing

Γονοτυπικά, φυσιολογικά και τεχνολογικά χαρακτηριστικά οξυγαλακτικών βακτηρίων και ζυμών, απομονωθέντων από ελληνικά προζύμια σίτου αυθόρμητης ζύμωσης

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Περίληψη

Στόχος της παρούσας διπλωματικής μελέτης ήταν να διερευνηθεί η σύσταση του μικρο-οικοσυστήματος 13 προζυμιών σίτου αυθόρμητης ζύμωσης, τα οποία έχουν συλλεχθεί από περιοχές της Ελλάδας, που δεν έχουν μελετηθεί έως τώρα. Για το λόγο αυτόν, επιλέχθηκαν τόσο κλασικές μέθοδοι όσο και ανεξάρτητες της καλλιέργειας προσεγγίσεις. Στη συνέχεια, διεξήχθη περαιτέρω διερεύνηση των μεταβολικών δράσεων των απομονωθέντων μικροοργανισμών, που σχετίζονται με την τεχνολογία και την ασφάλεια παρασκευής των άρτων, με στόχο την πιθανή εφαρμογή τους ως εναρκτήριες ή συμπληρωματικές καλλιέργειες σε ζυμώσεις τροφίμων.

Στην πρώτη μελέτη, πραγματοποιήθηκε ταυτοποίηση της μικροβιακής οικολογίας των 13 ελληνικών προζυμιών σίτου, συνδυάζοντας κλασικές τεχνικές καθώς και προσεγγίσεις που δεν στηρίζονται στην καλλιέργεια. Όσον αφορά στην κλασική μέθοδο, εφαρμόστηκε η συμβατική καλλιέργεια σε τρυβλία, ομαδοποίηση με πολυμορφισμούς τυχαίως πολλαπλασιασμένου DNA που βασίζεται σε αλυσιδωτή αντίδραση πολυμεράσης (PCR-RAPD), ταυτοποίηση μέσω εξειδικευμένης PCR για το είδος *Lactiplantibacillus plantarum*, καθώς και αλληλούχιση του 16S και 26S rRNA γονιδίου για οξυγαλακτικά βακτήρια και ζύμες, αντίστοιχα. Από την άλλη πλευρά, η μη κλασική προσέγγιση περιλάμβανε ανάλυση με ηλεκτροφόρηση βαθμιδωτής αποδιάταξης βασισμένη σε PCR (PCR-DGGE), με άμεση εκχύλιση τόσο του DNA όσο και του RNA των δειγμάτων προζυμιού. Η ανάλυση των αποτελεσμάτων έδειξε ότι ο πληθυσμός των οξυγαλακτικών βακτηρίων κυμάνθηκε μεταξύ 6.28-9.20 log CFU/ g, με τους *Lp. plantarum* και *Levilactobacillus brevis* να επικρατούν ή να συνεπικρατούν στην πλειονότητα των περιπτώσεων. Επίσης, ταυτοποιήθηκαν και τα είδη *Companilactobacillus paralimentarius, Fructilactobacillus sanfranciscensis, Latilactobacillus sakei, Lt. curvatus, Lv. zymae, Lactococcus lactis, Leuconostoc citreum* και *Leu. mesenteroides*. Στην περίπτωση των ζυμών, ο πληθυσμός τους κυμάνθηκε μεταξύ 4.60-6.32 log CFU/ g και αποτελούνταν κυρίως από *Saccharomyces cerevisiae*, ενώ καταγράφθηκε και η

παρουσία των *Pichia membranifaciens, Pi. fermentans, Wickerhamomyces anomalus* και *Kazachstania humilis*. Παρά το γεγονός ότι η PCR-DGGE ανάλυση με βάση το DNA ή το RNA απέδωσε παρόμοια μικροβιακά προφίλ, η συνολική PCR-DGGE ανάλυση επαλήθευσε μόνο εν μέρει τη σύσταση του μικρο-οικοσυστήματος του προζυμιού, όπως αποτυπώθηκε με την κλασική μέθοδο.

Σε επόμενη μελέτη, τα οξυγαλακτικά βακτήρια και οι ζύμες που απομονώθηκαν από ελληνικά προζύμια, αξιολογήθηκαν ως προς μία σειρά ιδιοτήτων, οι οποίες σχετίζονται με την τεχνολογία και την ασφάλεια παρασκευής των άρτων. Οι υπό μελέτη ιδιότητες περιλάμβαναν την πρωτεόλυση, λιπόλυση, αμυλόλυση, παρουσία αποκαρβοξυλάσης αμινοξέων, δραστηριότητα φυτάσης, αντιμικροβιακή ικανότητα και παραγωγή EPS τόσο από οξυγαλακτικά όσο και από ζύμες. Τα στελέχη εμφάνισαν πρωτεολυτικές, λιπολυτικές και αντιμικροβιακές δράσεις, οι οποίες αρχικά εξετάστηκαν με τη μέθοδο διάχυσης σε στερεό θρεπτικό υπόστρωμα (WDA). Επτά οξυγαλακτικά βακτήρια και 8 ζύμες ήταν ικανά να υδρολύσουν τη γλουτένη, ενώ 11 οξυγαλακτικά βακτήρια χαρακτηρίστηκαν από παρουσία λιπολυτικής δράσης. Στην πρώτη περίπτωση, εφαρμόστηκε περαιτέρω ανάλυση με τη μέθοδο ηλεκτροφόρησης των πρωτεϊνών σε πηκτή πολυακρυλαμιδίου με δωδέκυλο-θειικό νάτριο (SDS-PAGE) των κλασμάτων της γλουτένης, η οποία έδειξε μερική και εξαρτώμενη από το στέλεχος υδρόλυση της γλουτένης. Στην περίπτωση της λιπόλυσης, μια επακόλουθη κινητική λιπόλυσης σε διάστημα 21 ημερών αποκάλυψε αυξημένη διακύμανση της ενζυμικής δραστηριότητας, μεταξύ των υπό μελέτη στελεχών. Όσον αφορά στην αξιολόγηση της αντιμικροβιακής δραστηριότητας, 13 στελέχη οξυγαλακτικών βακτηρίων και 12 στελέχη ζυμών παρουσίασαν παρεμποδιστική δράση ως προς την ανάπτυξη διαφορετικών μυκήτων, η οποία αποδόθηκε σε μη πρωτεϊνικούς μεταβολίτες. Επιπλέον, 21 στελέχη *Lp. plantarum* εμφάνισαν αντιβακτηριακή δράση εναντίον ενός μίγματος 5 στελεχών *Listeria monocytogenes*, που ανήκουν στον ορότυπο 4b, ενώ επαληθεύτηκε και η πρωτεϊνική φύση των παρεμποδιστικών ενώσεων. Μεταξύ των παραπάνω, 3 στελέχη *W. anomalus* (LQC 10346, 10353, 10360) και 6 στελέχη *Lp. plantarum* (LQC 2320, 2422, 2441, 2485, 2516, 2520), τα οποία εμφάνισαν περισσότερες από μία ιδιότητες, αξίζουν περαιτέρω έρευνας.

Τα τρία στελέχη *W. anomalus*, συγκεκριμένα τα LQC 10346, 10353 και 10360, για τα οποία η παραγωγή αντιμυκητιακών ενώσεων μη πρωτεϊνικής φύσης

καταγράφθηκε σε προηγούμενη μελέτη, υποβλήθηκαν σε περαιτέρω αξιολόγηση της παρεμποδιστικής τους δράσης τόσο *in vitro* όσο και *in situ*. Για το σκοπό αυτό, τα στελέχη ζυμών αναπτύχθηκαν στους 30 ^{o}C για 24 ώρες σε δύο υποστρώματα. Το πρώτο ήταν υγρό θρεπτικό υπόστρωμα BHI προσαρμοσμένο σε 6 αρχικές τιμές pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) και 6 συγκεντρώσεις NaCl (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%), ενώ το δεύτερο ήταν ένα υγρό ζυμάρι ενισχυμένο με τις προαναφερθείσες συγκεντρώσεις NaCl. Τα αποτελέσματα της διάχυσης σε στερεό θρεπτικό υπόστρωμα έδειξαν ότι η μέγιστη ανασταλτική δράση ποσοτικοποιήθηκε σε 5120 AU/ mL, η οποία παρουσιάστηκε και από τα τρία στελέχη μετά από ανάπτυξη και στα δύο υποστρώματα. Στη συνέχεια, πραγματοποιήθηκε ταυτοποίηση των πτητικών ενώσεων με SPME-GC-MS, μεταξύ των οποίων ανιχνεύθηκε αιθανόλη, οξικός αιθυλεστέρας, ισοαμυλική αλκοόλη και οξικός ισοαμυλεστέρας, σε όλα τα δείγματα που αξιολογήθηκαν. Στην περίπτωση της αξιολόγησης της παρεμποδιστικής δράστης των καθαρών πτητικών ενώσεων εναντίον της ανάπτυξης του μύκητα, οι χαμηλότερες τιμές ελάχιστης παρεμποδιστικής δράσης καταγράφηκαν για τις 2,4-δι-τριτ-βουτυλ-φαινόλη και βενζαλδεΰδη (0,04 και 0,06 μL/ mL ελεύθερου χώρου, αντίστοιχα). Τέλος, οι άρτοι που παρήχθησαν είτε με μονοκαλλιέργειες στελεχών *W. anomalus* είτε με τη συγκαλλιέργειά τους με μαγιά αρτοποιίας απέκτησαν πρόσθετη διάρκεια ζωής, που κυμάνθηκε από 9 έως 30 ημέρες, σε σύγκριση με το μάρτυρα.

Σε επόμενη μελέτη, 6 στελέχη οξυγαλακτικών βακτηρίων απομονωθέντων από προζύμια, συγκεκριμένα τα LQC 2320, 2422, 2441, 2485, 2516 και 2520, τα οποία αρχικά ταυτοποιήθηκαν ως *Lp. plantarum* μέσω αλληλούχισης του 16S rRNA γονιδίου, επιλέχθηκαν για περαιτέρω αλληλούχιση ολόκληρου του γονιδιώματός τους, λόγω ιδιοτήτων, που σχετίζονται με την τεχνολογία και την ασφάλεια παρασκευής των άρτων. Τα ακατέργαστα τμήματα των αλληλουχιών ελέγχθηκαν ποιοτικά, στη συνέχεια πραγματοποιήθηκε de novo (χωρίς τη χρήση γονιδιώματος αναφοράς) συναρμολόγησή τους σε μια συνεχόμενη γονιδιωματική αλληλουχία (contigs), στην οποία η σειρά των βάσεων είναι γνωστή με υψηλό επίπεδο εμπιστοσύνης. Τέλος, οι συναρμολογηθείσες αλληλουχίες οργανώθηκαν σε ακόμη μεγαλύτερα τμήματα (scaffolds), χρησιμοποιώντας μία γνωστή και ολοκληρωμένη αλληλουχία (completed genome) ως γονιδίωμα αναφοράς (reference-based scaffolding). Μετά την οργάνωση των αλληλουχιών, πραγματοποιήθηκε ο

εντοπισμός των γονιδίων μέσα στις αλληλουχίες DNA (genome annotation). Η ταξινόμηση των προαναφερθέντων στελεχών αποκάλυψε ότι ανήκαν στο υποείδος *Lp. plantarum* subsp. *argentoratensis*. Η ποιοτική ανάλυση των συναρμολογημένων μη ολοκληρωμένων γονιδιωμάτων (draft genomes) και του εντοπισμού των γονιδίων έδειξε ότι ήταν υψηλής ποιότητας. Το συνολικό μέγεθος των γονιδιωμάτων κυμάνθηκε μεταξύ 3,13-3,49 Mb, ενώ ο αριθμός των γονιδίων που κωδικοποιούν πρωτεΐνες και των αντίστοιχων που δεν κωδικοποιούν πρωτεΐνες κυμάνθηκε μεταξύ 3091-3492 και 160-231, αντίστοιχα.

Δεδομένου ότι δεν έχει υπάρξει προηγούμενη αναφορά σε βιοπληροφορική ανάλυση γονιδιώματος του στελέχους *Lp. plantarum* subsp. *argentoratensis* απομονωθέντος από προζύμι, σκοπός της συγκεκριμένης μελέτης ήταν η βιοπληροφορική ανάλυση και σύγκριση των γονιδιωμάτων 6 βακτηριακών στελεχών (LQC 2320, 2422, 2441, 2485, 2516 και 2520), τα οποία ανήκουν στο συγκεκριμένο υποείδος. Η ανάλυση που σχετίζεται με το βιοτεχνολογικό τους δυναμικό αποκάλυψε ότι στα 6 στελέχη οξυγαλακτικών βακτηρίων καταγράφθηκαν τα γονίδια που σχετίζονται με το μεταβολισμό των υδατανθράκων, το σύστημα μεταφοράς διαφόρων ουσιών μέσω της κυτταρικής μεμβράνης, το μεταβολισμό συγκεκριμένων οργανικών οξέων, όπως για παράδειγμα τη μετατροπή του κιτρικού σε οξαλοξικό, τη βιοσύνθεση ακετοΐνης καθώς και την παραγωγή ριβοφλαβίνης και φολικού οξέος. Όσον αφορά στις γονιδιωματικές πτυχές που σχετίζονται με την ασφάλεια των τροφίμων, η βιοπληροφορική ανάλυση που πραγματοποιήθηκε δεν αποκάλυψε παθογόνους παράγοντες, ωστόσο ανιχνεύθηκε η παρουσία γονιδίων με ανθεκτικότητα στα αντιβιοτικά. Επιπλέον, τα 6 στελέχη *Lp. plantarum* subsp. *argentoratensis* αξιολογήθηκαν αρνητικά ως προς τη δυνατότητα παραγωγής της πλειονότητας των βιογενών αμινών. Παρόλα αυτά, η ανίχνευση του γονιδίου *cadA* που κωδικοποιεί την παραγωγή καδαβερίνης, αξίζει περαιτέρω έρευνα σε φαινοτυπικό επίπεδο. Σχετικά με τη σταθερότητα των γονιδιωμάτων, σημειώθηκε η παρουσία διαφόρων κινητών/ μετακινούμενων γενετικών στοιχείων (Mobile Genetic Elements - MGEs), όπως είναι για παράδειγμα τα πλασμίδια, μικρές αλληλουχίες DNA (instertion sequences - IS) που κινούνται εντός ή μεταξύ των γονιδιωμάτων χρησιμοποιώντας τα δικά τους εξιδεικευμένα συστήματα ανασυνδυασμού (recombination systems), οι προφάγοι (γονιδίωμα βακτηριοφάγου ενσωματωμένο στο βακτηριακό γονιδίωμα) και συστημάτων CRISPR*cas*

(αλληλουχίες DNA που παίζουν ρόλο στην άμυνα του μικροοργανισμού). Τέλος, και τα 6 βακτηριακά στελέχη αξιολογήθηκαν θετικά ως προς την παραγωγή πλανταρισινών.

Σε προηγούμενες μελέτες, τα 6 στελέχη *Lp. plantarum* (LQC 2320, 2422, 2441, 2485, 2516 και 2520) χαρακτηρίστηκαν από αντιβακτηριακή δράση, λόγω της παραγωγής μεταβολιτών πρωτεϊνικής φύσης, ενώ καταγράφηκε και η παρουσία ενός συνόλου γονιδίων στο γονιδίωμά τους που κωδικοποιούν πλανταρισίνες. Επομένως, στόχος αυτής της μελέτης ήταν να αξιολογηθεί η επίδραση των παραμέτρων που σχετίζονται με την παρασκευή προζυμιού στην ενεργότητα των πλανταρισινών και στη μεταγραφική απόκριση των γονιδίων που τις κωδικοποιούν, στα 6 στελέχη *Lp. plantarum*. Οι παράμετροι που σχετίζονται με την παρασκευή προζυμιού περιλάμβαναν 3 υποστρώματα: υγρό θρεπτικό υπόστρωμα MRS, υγρό θρεπτικό υπόστρωμα MRS που περιέχει τον τύπο και την ποσότητα υδατανθράκων που απαντώνται στο αλεύρι σίτου, εκχύλισμα αλεύρου-νερού ενισχυμένο με υδατάνθρακες στην αρχική συγκέντρωση του αλεύρου˙ 3 θερμοκρασίες (20, 30, 37 ^{o}C)˙ 2 αρχικές τιμές pH (5.0, 6.0)˙ 2 συγκεντρώσεις NaCl (0.0, 1.8%)˙ 12 χρονικά σημεία (από 0 έως 33 ώρες)˙ 6 *Lp. plantarum* στελέχη. Τα αποτελέσματα έδειξαν διαφορετικό τρόπο δράσης των πλανταρισινών ανάλογα με το στέλεχος. Πιο αναλυτικά, τα 6 στελέχη διακρίθηκαν σε δύο ομάδες όσον αφορά στα φαινοτυπικά χαρακτηριστικά και στην οργάνωση του *pln* γενετικού τόπου. Η ανάλυση διακύμανσης αποκάλυψε ότι το μέσο ανάπτυξης, η θερμοκρασία, η αρχική τιμή pH και το στέλεχος είχαν σημαντική επίδραση στη δραστηριότητα των πλανταρισινών, ενώ το NaCl είχε μόνο οριακή επίδραση. Αναφορικά με τη μεταγραφική απόκριση των γονιδίων που κωδικοποιούν τις πλανταρισίνες, τόσο η θερμοκρασία όσο και το υπόστρωμα είχαν πιο σημαντική επίδραση στη σχετική γονιδιακή μεταγραφή, σε σύγκριση με την αντίστοιχη της αρχικής τιμής pH. Ασθενής συσχέτιση καταγράφηκε μεταξύ της φαινοτυπικής αξιολόγησης και της σχετικής γονιδιακής μεταγραφής.

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To George & Brevis

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1. Introduction

1.1 A brief history of bread

Bread making has been traced back to ancient times. The very first breads, probably flat breads, made from a mixture of cereal flour and water were produced in Mesopotamia, since 8000 B.C. Later, ancient Egyptians were introduced to the bread making process. According to scientific reports, Egypt is considered the motherland of sourdough bread (Cappelle et al., 2013, Arora et al., 2021). More accurately, Egyptians (3000 B.C.) accidentally discovered that a mixture of cereal flour and Nile River water, left to spontaneously ferment in clay pots, increased in volume and was transformed by baking into leavened breads. In addition, they were the first to introduce the use of beer foam in the bread making. Figure 1.1 illustrates the activities involved in bread making process in ancient Egypt.

Figure 1. 1. Egyptian wall painting depicting the activities involved in the bread making process (Jacob, 1997).

The contribution of ancient Greeks to the bread making process and the sensory improvement of the end products, was significant as well. More accurately, the constant trading of Greeks with Egyptians led to the bread familiarization of the former. The use of sourdough is estimated to be adopted from Egyptians back to 800 B.C. (Cappelle et al., 2013). A great variety of breads based on different ingredients, namely sesame seeds, fruit pieces, milk or honey, were produced. Greeks introduced the closed built-of-stone ovens, thus improving the technology

of bread making process. In addition, they were the first to create bakery corporations and establish shifts and rules so as to serve the normal night work of bakers. After contact with Greek civilization, the first organized bakeries in Roman Empire were dated from 172 B.C. Bakeries were recruited by freed slaves, who turned into professional and highly respected bakers (Catzeddu et al., 2011). A representative depiction of bread distribution in ancient Pompei is presented in Figure 1.2.

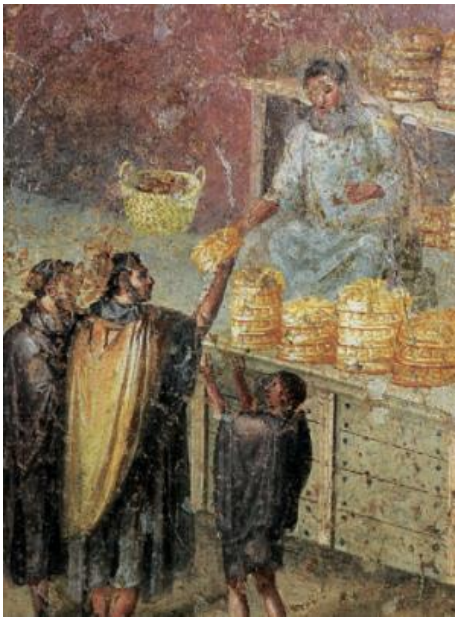

Figure 1. 2. Bread distribution in ancient Pompei (Jacob, 1997).

During the Middle Age, scarce was the progress made in the bread making technology, except for the construction of windmills, while watermills preexisted from Roman times. The commonly known white bread was introduced to the public. At that time, the production of flour without bran led to a time consuming, laborious and concomitantly expensive bread making process, thus constituting bread a privilege of the rich, while the whole grain bread was left to the peasantry. In Britain and Europe quality standards were set and laws were implemented so as to impede the practices of some bread manufactures, who intentionally debased the quality of flour by adding potentially toxic ingredients, such as sand (Hutkins et al., 2006).

Until the 17th century, the technology of bread making met only a slight progress. Leonardo da Vinci was occupied with the improvement of mills operation, while Parmentier approached the field of the bread quality in a scientifically based manner

(Paramithiotis, 2001). The latter founded the "Bakery Academy" in 1780, the first school for bakers, in which he served as a teacher. However, the greatest contribution to the enhancement of bread quality was demonstrated in the 19th century by Muller and Sulzberger, who replaced stone mills by iron roller ones.

Regarding the central Europe, the first finding of sourdough bread is traced back to 3600 B.C., in Switzerland (Cappelle et al., 2013, Gänzle, 2014). Similar were the findings obtained from excavations in Austria and Germany, dating from 1800 and 800 B.C., respectively (Spicher & Stephan, 1982). Sourdough was widely consumed in North America, as well. In brief, sourdough was disseminated in San Francisco during California gold rush (1849) and to western Canada during the Klondike gold rush (1898) (Catzeddu, 2011).

Bread was significant part of the history of World Wars I and II. In more detail, during World War I, the military bread, initially consumed by soldiers, was further introduced to the civilian population. During the World War II, the role of American allies contributed to the prevalence of the white bread (Cappelle et al., 2013). On the other hand, Germans supported the dissemination of rye flour originating bread. After the end of World War II, the technology of bread making met great progress, via the use of motorized equipment and kneaders.

Since then, the development of microbiology and chemical industry contributed to a completely automated bread making process and bread production in industrial scale. Despite the fact that for many centuries sourdough has been replaced by baker's yeast, it currently experiences a revival, due to the increasing consumer demand for clean label products.

1.2 Types of sourdough

Sourdough constitutes the intermediate product of the bread making process. It is the result of spontaneous or starter culture-induced fermentation of a mixture of cereal flour (usually wheat or rye) and water. In the case of wheat, soft wheat (*Triticum aestivum* species) or hard wheat flour (*Triticum durum* species) are used. As far as the sourdough microbiota are concerned, lactic acid bacteria (LAB) and yeasts, are the microorganisms responsible for transforming the mixture into metabolically active (De Vuyst et al., 2009). Depending on the desired technological

attributes of the end products, different technologies are applied and the sourdoughs produced are discriminated into three types, as summarized in Figure 1.3.

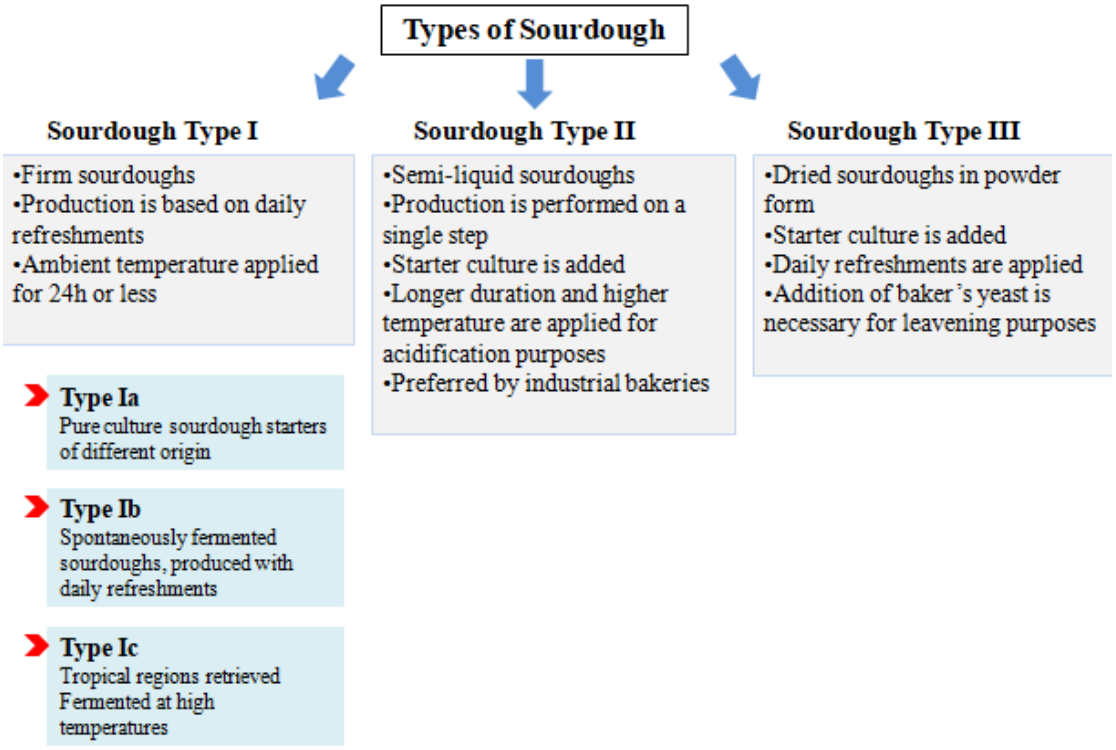

Figure 1. 3. Sourdough classification into three distinct types, based on the technology applied.

1.3 Sourdough Microbiota

From a microbiological point of view, sourdough is a stressful environment, attributed to the low pH value, restricted oxygen availability and its carbohydrate rich content. Sourdough microbiota include mainly LAB and yeasts and occasionally acetic acid bacteria (AAB). In most cases, LAB prevail over yeasts, with the population of the former ranging between 10^7 and 10^9, while the respective of the latter quantified less than 10^7 CFU/g.

Regarding the bacterial community dynamics of the sourdough microecosystem, the stable presence of LAB belonging to the former *Lactobacillus* genus has been extensively reported, while other genera, namely *Leuconostoc*, *Lactococcus*, *Enterococcus*, *Weissella* and *Pediococcus* have been documented as adjunct populations, as well. In more detail, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Fructilactobacillus sanfranciscensis* and

Limosilactobacillus fermentum prevail during sourdough fermentation processes (Minervini et al., 2012). The species diversity of LAB, isolated from wheat based sourdoughs around Europe, is presented in Table 1.1.

Table 1. 1. Overview of LAB species isolated from wheat based European sourdoughs.

Country	LAB species	Reference
Greece	*Fr. sanfranciscensis, Lv. brevis, Lp. plantarum, Lv. zymae, Cp. paralimentarius, W. cibaria, Pd. pentosaceus*	De Vuyst et al. (2002), Paramithiotis et al. (2010)
Albany	*Lp. plantarum, Leu. citreum, Leu. mesenteroides, Lc. lactis, Pd. pentosaceus*	Nionelli et al. (2014)
Bulgaria	*Lv. brevis, Lp. plantarum, Pd. pentosaceus, Pd. parvulus, Pd. acidilactici, E. faecium*	Petkova et al. (2021)
Italy	*Lv. brevis, Lv. parabrevis, Lo. coryniformis, Lp. plantarum, Lc. lactis, Lc. garvieae, E. casseliflavus, E. faecium, Leu. citreum, Leu. fructosus, Pd. pentosaceus, Fr. sanfranciscensis, Cp. paralimentarius, Lv. zymae, Lt. sakei, La. paracasei, Lp. paraplantarum, Lp. pentosus, Fu. rossiae, Leu. mesenteroides, Leu. pseudomesenteroides, Leu. durionis, W. cibaria, Lt. curvatus*	Iacumin et al. (2009), Minervini et al. (2012), Alfonzo et al. (2017), Reale et al. (2019)
Spain	*Lv. brevis, Lp. plantarum, Lb. cellobiosus, Leu. mesenteroides*	Barber et al. (1983), Barber & Báguena (1988, 1989)
France	*Fr. sanfranciscensis, Lt. curvatus, Lv. hammesii, Fr. lindneri, Cp. paralimentarius, Lp. plantarum, Lt. sakei, Lv. spicheri, La. paracasei, Lp. pentosus, Lp. paraplantarum, Lv. brevis, Pd. pentosaceus, Leu. mesenteroides, Leu. citreum, W. cibaria, W. confusa, Lc. lactis, E. hirae*	Robert et al. (2009), Lhomme et al. (2015)
Germany	*Lp. plantarum, Fr. sanfranciscensis, E. faecium, La. casei, Lm. fermentum, Pd. pentosaceus, W. confuse*	Kitahara et al. (2005)
Austria	*Lv. brevis, Lo. coryniformis, Lt. curvatus, Lv. hammesii, Sc. harbinensis, Le. kisonensis, Le. parabuchneri, La. paracasei, Sc. perolens, Lp. plantarum, Lm. pontis, Fu. rossiae, Lt. sakei, Fr. sanfranciscensis, Lv. senmaizukei, Lv. spicheri, Pa. vaccinostercus, Leu. mesenteroides, Leu. citreum, Leu. pseudomesenteroides, Pd. parvulus, Pd. pentosaceus, Str. dentisani, Str. salivarius, W. cibaria, W. viridescens*	Fraberger et al. (2020)
Belgium	*Lv. brevis, Lp. plantarum, Cp. paralimentarius, Fr. sanfranciscensis, Lt. sakei, Le. buchneri, Le. parabuchneri, Cp. crustorum, Lv. hammesii, Lb. helveticus, Cp. nantensis, La. paracasei, Lm. pontis, Fu. rossiae, Lv. spicheri, Lm. fermentum, Leu. mesenteroides, Pd. pentosaceus, W. cibaria, W. confusa*	Van der Meulen et al. (2007), Scheirlinck et al. (2007a, b, 2008)

Cp.: Companilactobacillus, E.: *Enterococcus, Fr.*: *Fructilactobacillus, Fu.*: *Furfurilactobacillus, La.*: *Lacticaseibacillus, Lb.*: *Lactobacillus, Lc.*: *Lactococcus, Le.*: *Lentilactobacillus, Leu.*: *Leuconostoc, Lm.*: *Limosilactobacillus, Lo.*: *Loigolactobacillus, Lp.*: *Lactiplantibacillus Lt.*: *Latilactobacillus, Lv.*: *Levilactobacillus, Pa.*: *Paucilactobacillus, Pd.*: *Pediococcus, Sc.*: *Schleiferilactobacillus, Str.*: *Streptococcus, W.*: *Weissella*

The role of yeasts in bread making process is to ferment flour carbohydrates anaerobically, with further gas production and retention, so as to ensure the

manufacture of a leavened dough. Except for CO_2 production, yeasts significantly affect sensory properties of bread, via inducing the production of precursors for flavour compounds. *Saccharomyces cerevisiae*, *Candida humilis*, *Kazachstania exigua*, *Pichia kudriavzevii*, *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* comprise the 6 most commonly encountered yeast species in sourdough fermentations (De Vuyst et al., 2016). Among them, the frequent isolation of *S. cerevisiae* from spontaneous back-sloppings both in bakery and laboratory environments indicates the autochthonous flour origin of the aforementioned yeast species. Regarding the other yeasts, *W. anomalus* is considered a generalist species, while *C. humilis* and *K. exigua* are regarded sourdough adapted yeasts. The biodiversity of yeasts, originating from different wheat based sourdoughs around Europe, is summarized in Table 1.2.

Table 1. 2. Overview of yeast species isolated from wheat based European sourdoughs.

Country	Yeast species	Reference
Greece	*S. cerevisiae, T. delbrueckii, Pi. membranifaciens, Y. lipolytica, D. hansenii*	Paramithiotis et al. (2000, 2010)
Bulgaria	*S. cerevisiae, K. barnettii, Kl. marxianus, Y. lipolytica, Pi. fermentans*	Petkova et al. (2021)
Italy	*S. cerevisiae, K. unispora, S. bayanus, K. barnettii, K. exigua, C. humilis, C. milleri*	Iacumin et al. (2009), Minervini et al. (2012, 2015)
France	*S. cerevisiae, C. humilis, K. exigua, C. carpophila, H. pseudoburtonii, K. unispora, K. bulderi, R. mucilaginosa, K. servazzii*	Lhomme et al. (2015)
Spain	*S. cerevisiae, Me. Guilliermondii*	Barber et al. (1983) Barber & Báguena (1988, 1989)
Austria	*S. cerevisiae, S. uvarum, T. delbrueckii, K. humilis, K. unispora*	Fraberger et al. (2020)
Belgium	*S. cerevisiae, W. anomalus, K. barnettii, K. unispora, T. delbrueckii, C. glabrata*	Van der Meulen et al. (2007), Vrancken et al. (2010)

C.: *Candida*, *D.*: *Debaryomyces*, *H.*: *Hyphopichia*, *K.*: *Kazachstania*, *Kl.*: *Kluyveromyces*, *Me.*: *Metschnikowia*, *Pi.*: *Pichia*, *R.*: *Rhodotorula*, *S.*: *Saccharomyces*, *T.*: *Torulaspora*, *W.*: *Wickerhamomyces*, *Y.*: *Yarrowia*

1.4 Metabolic traits of LAB and yeasts

1.4.1 Metabolism of carbon sources

The prevalence of specific LAB in sourdough ecosystem is attributed to their ability to ferment certain carbon sources, which is performed either homofermentatively or heterofermentatively. Maltose, glucose, fructose and sucrose are the principal carbohydrates found in flour.

Obligately homofermentative LAB species ferment hexoses, through the Embden-Meyerhof-Parnas (EMP) pathway, yielding lactate as the main end product (Figure 1.4). In the case of obligately heterofermentative lactobacilli, the end products of hexose and pentose fermentation include lactate, acetate and ethanol, generated through the phosphoketolase pathway (Figure 1.5). CO_2 is further generated when hexoses are used as carbon sources. Regarding facultatively heterofermentative LAB species, hexoses and pentoses are fermented through the EMP and phosphoketolase pathway, respectively (Gänzle & Gobbetti, 2013).

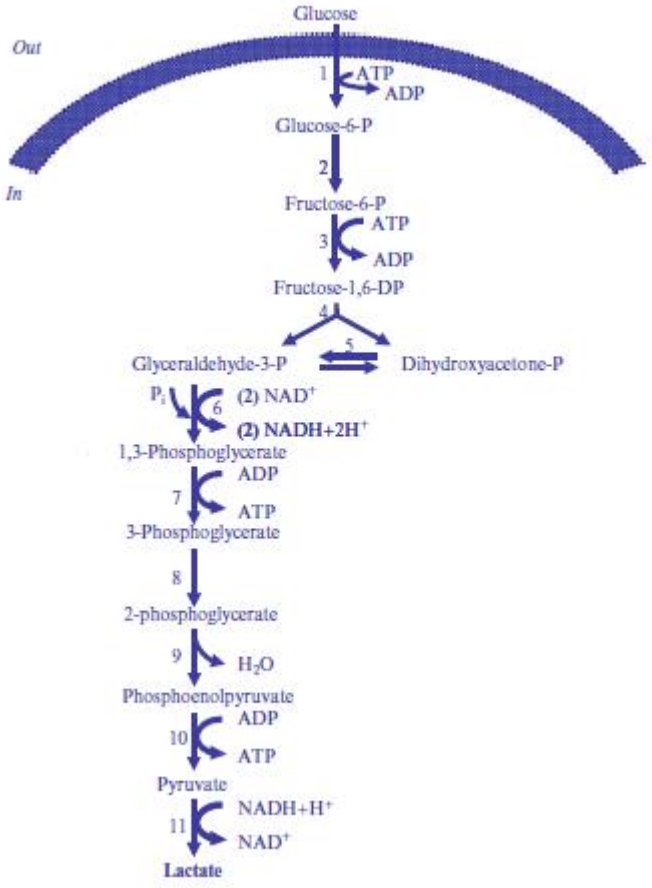

Figure 1. 4. Metabolism of homofermentative LAB in a sourdough microecosystem, via the Embden Meyerhof Parnas (EMP) pathway. 1, Glucokinase; 2, glucose-6-phosphate, isomerase; 3, phosphofructokinase; 4, fructose 1,6-bisphosphate aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde 3-phosphate dehydrogenase; 7, 3-phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, pyruvate kinase; 11, lactate dehydrogenase. Reprinted with permission from Gänzle & Gobbetti (2013).

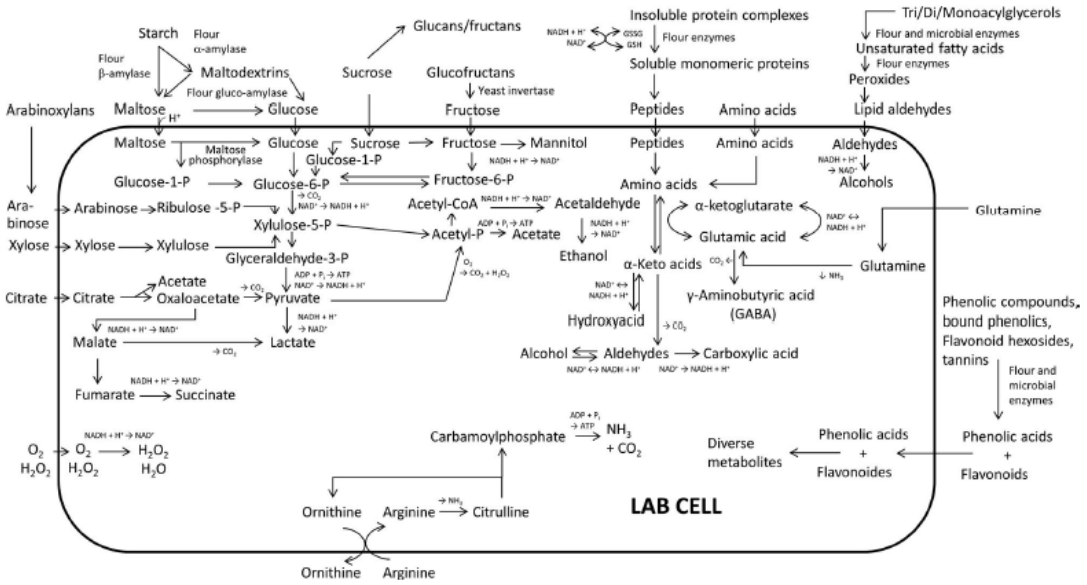

Figure 1. 5. Metabolism of heterofermentative LAB in a sourdough microecosystem. Reprinted with permission from De Vuyst et al. (2021).

Heterofermentative LAB, namely *Lp. plantarum*, *Lv. brevis* and *Fr. sanfranciscensis* prevail in sourdough ecosystems, over the homofermentative respectives, due to specific physiological traits of the former (De Vuyst et al., 2021). More accurately, the presence of maltose phosphorylase in specific lactobacilli, namely *Fr. sanfranciscensis* and *Lv. brevis*, contributes to maltose catabolism and improves their competitiveness during sourdough fermentation process. In more detail, maltose uptake into the cell occurs via a maltose/ H^+ symport system. Then, maltose degradation into glucose and glucose-1-P takes place via maltose phosphorylase, with the latter being further degraded, while glucose is excreted from the cell, so as to avoid internal accumulation. As long as maltose is available, glucose will be excreted from the cell. When maltose depletion occurs, then glucose catabolism initiates. Regarding the utilization of fructose during sourdough fermentation, the *Fr. sanfranciscensis* and *Lv. brevis* use it as an external acceptor of electrons, thereby contributing to the prevalence of heterofermentative lactobacilli in sourdough microecosystem. In more detail, heterofermentative LAB reduce fructose into mannitol and co-factor regeneration is accomplished (NAD^+), by avoiding ethanol synthesis. Regarding the energy yield, extra ATP is produced via the conversion of acetyl phosphate to acetate, thus favoring the competitiveness of LAB species. In the case of sucrose, either LAB catabolize it via intracellular

sucrose phosphorylase (e.g. *Lm. reuteri*) or extracellular glycosyltransferases convert sucrose to homopolysaccharides (De Vuyst et al., 2009).

A strain dependent metabolism of organic acids, namely citrate, has been reported for sourdough LAB, as well (Gänzle & Gobbetti, 2013). The initial transport of extracellular citrate into the cell is mediated by citrate permease. Then, its conversion into acetate and oxaloacetate takes place, by the action of citrate lyase. The fate of oxaloacetate follows two pathways; either conversion to succinate, which is the most typical pathway among lactobacilli, or decarboxylation into pyruvate. In the case of pyruvate, a further conversion to lactate mediated by lactate dehydrogenase occurs. In other cases, an α-acetolactate synthase mediated conversion of pyruvate into α-acetolactate takes place, with a concomitant release of CO_2. Then, α-acetolactate is further reduced to acetoin (enzymatically) or diacetyl (non enzymatically). Several *Fr. sanfranciscensis* strains have been previously shown to utilize citrate as electron acceptor, when maltose was co-present in the medium, with the latter being utilized as carbon source. As long as citrate was present, the metabolic products included lactate and acetate (Gobbetti & Corsetti, 1996, Paramithiotis, 2001). By the time citrate had been depleted, acetate production was replaced by the ethanol formation. In addition, the concomitant catabolism of malate with maltose resulted in increased lactate production by *Fr. sanfranciscensis*.

Regarding yeast carbohydrate metabolism, the initial stage includes the sugar transport into the cell. In the case of *S. cerevisiae*, hexose uptake is mediated via facilitated diffusion, while *Kluyveromyces* species are characterized by a hexose-H+ symport (Dickinson & Kruckeberg, 2006). Concerning fructose transport, the presence of a fructose specific symporter, namely Fsy1, has been recorded in *S. pastorianus* and *S. bayanus*. Regarding the uptake of disaccharides, e.g. maltose, a symport mechanism has been reported in *S. cerevisiae*, followed by maltose hydrolysis to glucose by a-glucosidase. In the case of sucrose, an initial degradation to glucose and fructose by invertase takes place in the periplasm, before their transport inside the cell.

Sourdough yeasts are considered facultative anaerobes, performing both fermentation and respiration (Guerzoni et al., 2013). When oxygen is present, yeasts carry out respiration. In more detail, pyruvate enters the mitochondrial matrix, where it is subjected to oxidative decarboxylation, thus its conversion into acetyl

CoA takes place, via the pyruvate dehydrogenase complex. Citric acid cycle removes electrons from acetyl CoA and further uses them in order to form NADH and $FADH_2$.

Under anaerobic conditions, the fate of pyruvate follows the reactions associated with alcoholic fermentation (Guerzoni et al., 2013). In brief, flour carbohydrates are fermented into pyruvate via the EMP pathway (Figure 1.6). Then, pyruvate is decarboxylated into acetaldehyde, mediated by pyruvate decarboxylase. A further reduction of acetaldehyde to ethanol occurs, catalyzed by alcohol dehydrogenase. In the aforementioned reaction NAD^+ is regenerated. An alternative route leading to the regeneration of NAD^+, however at the cost ethanol, includes the glycerol production. Glycerol formation has practical significance for the regeneration of inorganic phosphorus used in glycolysis.

Generally, carbohydrate metabolism in yeasts is subjected to regulatory phenomena, as follows (De Vuyst et al, 2016):

- **Pasteur effect** associates oxygen with carbohydrate consumption kinetics, thus suggesting that carbohydrates are consumed faster under anaerobic conditions, than the aerobic respectives in *S. cerevisiae*.
- **Crabtree effect** associates glucose concentration with carbohydrate consumption; at elevated glucose levels, fermentative catabolism will take place, even at aerobic conditions.
- **Kluyver effect** is defined as the inability to consume oligosaccharides anaerobically, but aerobically, except for glucose in *Pichia* and *Candida* spp.
- **Custers effect** is defined as the shift to the redox potential of the cell, in the absence of oxygen, leading to faster ethanol and gas formation from glucose in aerobic than in anaerobic conditions in *Brettanomyces* and *Dekkera* spp.

An alternative pathway for glucose catabolism is the pentose phosphate route, which regenerates NADPH implicated in the biosynthesis of fatty acids, amino acids and sugars. Apart from NADPH production, the aforementioned pathway contributes to the production of pentoses, namely riboses, which further improve the biosynthesis of genetic material and co-enzymes, namely NAD^+, $NADP^+$, FAD^+ and FMN.

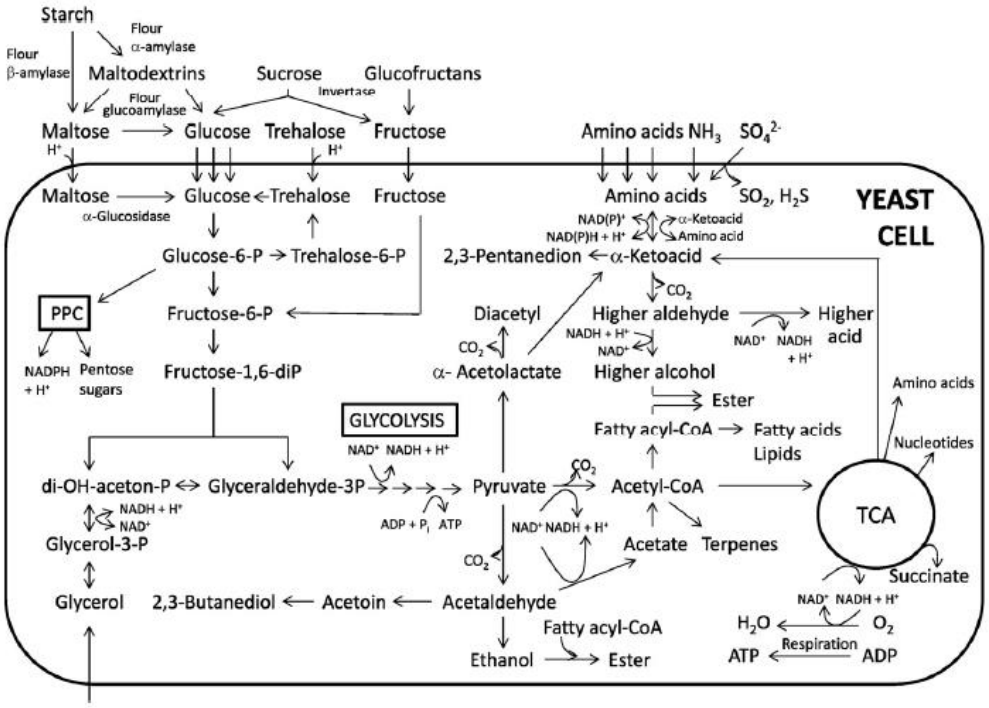

Figure 1. 6. Yeast metabolism in sourdough ecosystem. Reprinted with permission from De Vuyst et al. (2016).

1.4.2. Metabolism of nitrogen sources

Nitrogen is a limiting factor for the growth of LAB in a series of ecosystems. Glutamic acid, isoleucine and valine are necessary for growth of *Lv. brevis* subsp. *lindneri* and *Lp. plantarum* strains. Arginine, methionine and leucine enhance growth, while alanine, glycine, asparagine acid, lysine, histidine, cysteine, tyrosine, serine, threonine and proline do not seem to affect growth (Gobbetti et al., 1994).

In the case of yeasts, nitrogen compounds do not constitute limiting factors. Yeasts are able to utilize each of the aforementioned amino acids, previously reported, as nitrogen sources, except for lysine, cysteine and histidine. Among them, asparagine is the preferred nitrogen source. However, when amino acids and ammonium ions coexist, yeasts prefer the latter (Gobbetti et al., 1998). Given that yeasts carry a series of genes coding for amino acid biosynthesis, they can efficiently grow in the presence of ammonium ions, even as the only nitrogen source (Guerzoni et al., 2013).

Regarding the competition for nitrogen sources among LAB and yeasts, their co-culture in liquid broth, fortified with adequate carbon and vitamin content, enhanced LAB growth, due to the lack of antagonism for nitrogen compounds. In addition,

yeasts secrete amino acids, namely leucine and valine, which enhance LAB growth (Hui & Evranuz, 2012).

1.4.3 Acidification capacity

Rapid acidification capacity is a significant technological property that should be considered, when selecting LAB for food fermentations, such as sourdough (Sáez et al., 2018). The direct impact of acids produced by sourdough LAB on structure forming constitutes, namely gluten and strarch, is the improvement of swelling and solubility of gluten proteins (Zhang et al., 2020). This disentanglement of the gluten protein network during fermentation is attributed to the formation of an intramolecular electrostatic repulsion, which induces gluten proteins to unfold and transit from a tight structure to a more amorphous (Clarke & Arendt, 2005). As a result, the softness of the gluten is achieved, with concomitant swelling and entrapping of water. Except for the primary impact of acidifying conditions on gluten network, the decreased pH values also activate cereal proteases and phytases, while inactivates amylases, as well (Galle et al., 2013). Sourdough and wheat flour derived *Lp. plantarum*, *W. cibaria*, *Lt. sakei* and *Leu. mesenteroides* have displayed good acidification capacity, assessed *in vitro* in flour extract broth (Alfonzo et al, 2013, Sáez et al., 2018). On the other hand, the rate of acidification in a dough will definitely affect the dominance of specific strains, namely *Fr. sanfranciscensis*, the optimum pH of which is close to pH 5.0 (De Vuyst et al., 2014).

1.4.4 Starch hydrolysis

Starch hydrolysis has increased technological significance in bread making process, since its degradation operates as a source of fermentable carbohydrates. The presence of amylase activity in wheat based sourdoughs is involved in starch hydrolysis with concomitant liberation of fermentable sugars, namely maltose, glucose and maltodextrines (Gänzle, 2014). Amylases are discriminated into a-amylases, endo-acting enzymes, which catalyze the hydrolysis of a-1,4-glucosidic bonds of amylose and amylopectin, with oligosaccharides and a-limit dextrins, as the end products and b-amylases, exo-acting hydrolases, which release maltose (Struyf et al., 2016). Glucoamylases or amyloglucosidases, constitute another class

of amylases, acting on a-1,4 glucosidic bonds of a-glucans from the non-reducing ends and on a-1,6 linkages as well, with glucose as the end product. The diagram of starch metabolism is presented in Figure 1.7.

Given that maltose is one of the end products of amylase activity, combined with the fact that it constitutes the most abundant carbon source in wheat based sourdoughs, it is implied that the ability of LAB to catabolize maltose, increases their competitiveness. The presence of maltose phosphorylase (MalP) in strains of *Fr. sanfranciscensis*, coupled with its capacity to use other carbon sources, namely fructose, as an external electron acceptor, improves the dominant character of *Fr. sanfranciscensis* during sourdough fermentations. In some strains, the presence of a glucosidase, namely DexB, which hydrolyses a(1→6)-linked gluco-oligosaccharides has been detected (Gänzle, 2014).

However, it is important to take into account that excessive levels of amylase have been reported as undesirable in rye sourdough fermentations. More accurately in rye flour, increased levels of a-amylase have been reported, which enhance the formation of a sticky crumb and a decreased loaf volume bread, thus inhibition of the amylase activity, via dough acidification, is imperative. This is the reason why among others, sourdough fermentation by LAB has been used as acidification agent in rye flours (Cappelle et al., 2013). Nevertheless, amylase activity has not been considered a common enzymatic property for the majority of LAB species, derived from wheat based fermentations. Consistent with the aforementioned observation, Paramithiotis et al. (2010) reported no amylolytic properties of both LAB and yeast strains, previously isolated from Greek wheat sourdoughs.

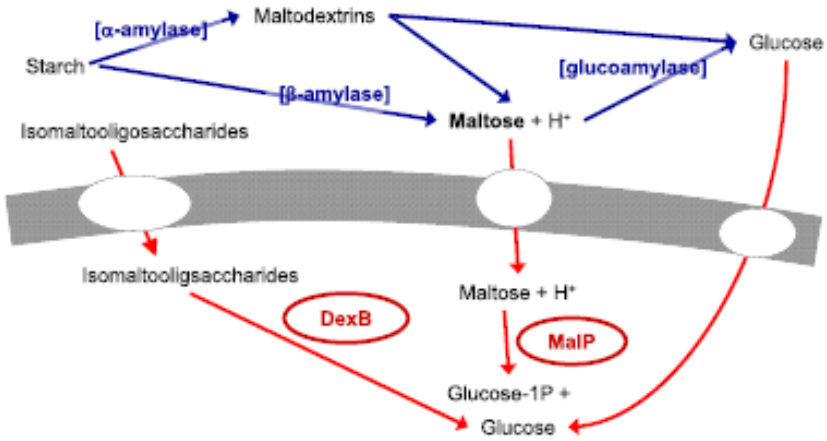

Figure 1. 7. Starch hydrolysis in wheat and rye sourdoughs. Reprinted with permission from Gänzle (2014).

1.4.5 Proteolysis and amino acid metabolism

Gluten hydrolysis during sourdough fermentation is a crucial process strongly affecting the bread quality. Amino acids and peptides liberated during proteolysis, affect leavened baked goods as taste and flavor precursors. On the other hand, extensive hydrolysis of gluten proteins may cause adverse effects on the rheological attributes of wheat doughs.

Given that the majority of sourdough retrieved LAB do not own a cell envelope related proteinase, endogenous cereal proteases are responsible for the initial protein degradation to oligopeptides. Acidic conditions generated, as a result of flour fermentation by LAB, shift the ambient pH value to the optimum one for flour proteinase activation (De Vuyst et al., 2021). In addition, increased levels of thiols improve the solubility of gluten proteins. More accurately, the glutathione reductase activity of certain heterofermentative lactobacilli, namely *Fr. sanfranciscensis*, leads to the partial depolymerization of glutenin macropolymer (GMP), via the reduction of extracellular oxidized glutathione (GSSG) to GSH. In some cases the hydrolysis of increased disulfide bonded allergens has been reported (Gänzle & Zheng, 2019). Further gluten hydrolysis is performed via intracellular peptidases of LAB, in a strain specific way, with increased liberation of free amino acids. Finally, a co-presence of sourdough LAB and fungal enzymes contributes to complete protein degradation. The schematic representation of proteolysis, taking place during sourdough fermentation, is illustrated in Figure 1.8.

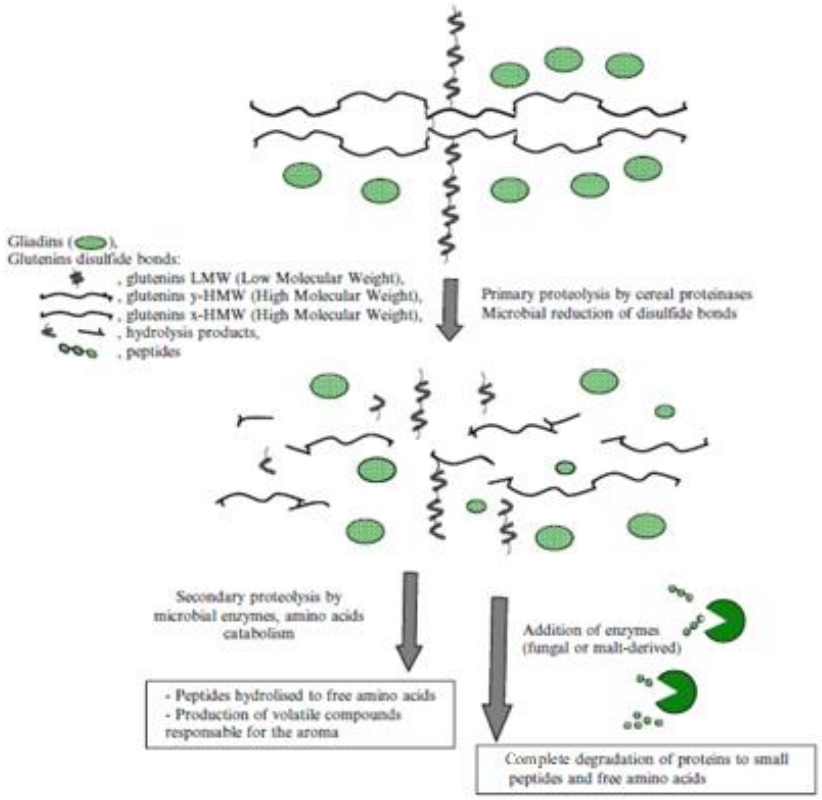

Figure 1. 8. Schematic representation of proteolysis occurring during sourdough fermentation. Reprinted with permission from Gänzle & Gobbetti (2013).

Regarding the peptidase system of LAB, the respective of *Fr. sanfranciscensis* has been characterized to a great extent (Gänzle et al., 2008). In brief, the presence of endopeptidases (e.g. PepO and PepF), aminopeptidases (e.g. PepN and PepC), dipeptidases (e.g. PepD) tripeptidases (e.g. PepT) and proline targeting peptidases (e.g. PepX) have been reported. Gobbetti et al. (1996) reported the characterization of three proteolytic enzymes, namely a cell envelope proteinase, a cytoplasmic dipeptidase and an aminopeptidase, isolated from sourdough derived *Fr. sanfranciscensis* CB1. In addition, Gallo et al. (2005) characterized an X-prolyl dipeptidyl aminopeptidase (PepX) derived from *Fr. sanfranciscensis* CB1.

In addition to proteolysis, amino acid conversions by LAB contribute to pH homeostasis and improvement of the sensory attributes of the end product, through the formation of flavor precursors. Amino acids are catabolized via decarboxylation, transamination, deamidation reactions and the respectives mediated by lyases (Gänzle & Gobbetti, 2013). The arginine deiminase (ADI) pathway found in *Lv. brevis*, *Lt.* sakei, *Lm. fermentum* and *Fu. rossiae* is a step of utmost importance

(Figure 6). In brief, ADI pathway improves the competitiveness of certain LAB via the production of extra ATP, enhances their tolerance towards acidic conditions (proton consumption and ammonia release) and produces ornithine, a precursor of 2-acetyl-1-pyrroline, responsible for the characteristic flavor of bread crust (De Vuyst et al., 2009).

Except for arginine, the metabolism of glutamine, the most common amino acid found in gluten, is of comparable significance, as well. More accurately, certain lactobacilli, e.g. *Lm. reuteri*, have been previously reported to possess glutaminase activity, able to deamidate glutamine to glutamate, with the latter affecting the umami taste of bread (Li et al., 2020). Thus, glutamine deamidation contributes to acid resistance of lactobacilli and sensory improvement of the end products, as well. As far as the fate of glutamate is concerned, either decarboxylation to γ-aminobutyrate (GABA) or oxidate deamination to α-ketoglutarate by a glutamate dehydrogenase is performed, with α-ketoglutarate serving as amino acceptor for the transamination reaction of other amino acids (Gänzle et al., 2007). In the latter case, NADH is regenerated. Several authors have associated LAB strains with glutamate dehydrogenase and glutamate decarboxylase activity in a strain specific manner (Gänzle et al., 2007, Gänzle & Gobbetti, 2013).

In contrast to the enhanced proteolysis attributed to the sourdough fermentation by LAB, dough fermentation by yeasts resulted in decreased levels of free amino acids (Thiele et al., 2002). As previously mentioned, several LAB are able to catabolize arginine to ornithine, thus enhancing the roasty note of wheat bread crust odor, however that was not the case in yeasted doughs, in which no ornithine was detected.

In brief, yeasts convert free amino acids to fusel alcohols, namely 3-methyl-1-butanol and 2-methyl-1-butanol, via the Ehrlich pathway (Guerzoni et al., 2013). Initially, a transamination step takes place, followed by a decarboxylation reaction, which leads to the formation of branched chain aldehydes from the respective oxoacids. Finally, the reduction of branched chain aldehydes to alcohols, commonly known as fusel respectives, occurs. Sourdough derived yeasts, among which *S. cerevisiae*, have been previously reported to synthesize 3-methylbutanol and 2-phenylethanol via leucine and phenylalanine, respectively.

1.4.5.1 Hydrolysis of gluten proteins

Gluten constitutes a complex mixture of storage proteins in wheat, namely gliadins and glutenins, while albumins and globulins are considered metabolic proteins, the technological significance of which is decreased. Based on the electrophoretic mobility of storage proteins, gliadins are subdivided into α/ β, γ and ω-gliadins, while glutenins are discriminated into high (HMW-GS) and low molecular weight subunits (LMW-GS). HMW and LMW-GS are crosslinked with disulfide bonds forming the glutenin macropolymer (GMP). Given that LMW-GS share similarities with α/ β and γ-gliadins, in terms of MW and amino acid content, both gliadins and LMW-GS provide the viscous properties on wheat based dough, whereas HMW-GS are responsible for the strength and elasticity. According to Ogilvie et al. (2021), α/ β and γ-gliadins are characterized by the presence of 2-3% cysteine and 15-20% proline residues, while in the case of ω-gliadin, the C-terminal region has no cysteine residues but higher proline content (20-30%) has been reported. As far as glutenins are concerned, the LMW-GS are characterized by 2-3% cysteine and 30-45% proline, while HMW-GS possess lower cysteine residues (0.5-1.5%) and 15-20% proline residues.

Regarding the degradation rate of gluten proteins, high proline and glutamine content have been detected in both gliadin and glutenin fractions, which enhances their resistance to human gastrointestinal enzymes, namely trypsin, chymotrypsin and pepsin. Thus, proline and glutamine rich polypeptides are liberated, which serve as triggering factors in celiac disease and IgE induced allergies (Fraberger et al., 2020). In brief, the α-gliadin family has been repeatedly shown to contain immunogenic peptides, namely, fragment 31-43, 57-89 and 62-75 and the 33mer corresponding to fragment 57-89 from α2-gliadin (Gobbetti et al., 2007, Scherf et al., 2018). Until now, a strict lifelong gluten free diet has been proposed as the only available treatment for the gluten intolerance. The application of gluten degrading lactobacilli in sourdough fermentations has been reported to positively affect the detoxification of wheat or rye flour. De Angelis et al. (2006) reported the ability of probiotic mixture VSL#3 (among which lactobacilli were included) to completely hydrolyze immunogenic fragments from α2-gliadin, upon prolonged fermentation. An earlier study by Di Cagno et al. (2002) reported the capacity of selected lactobacilli to hydrolyze wheat gliadins, e.g. 33mer and producing a sourdough

bread, the gluten levels of which could be tolerated by celiac disease patients. On the other hand, a recent study by Ogilvie et al. (2021) revealed that sourdough fermentation was not able to reduce the total content of immunogenic peptides, but modified their *in vitro* ability to be digested.

1.4.6 Phytic acid degradation

Among nutritional properties of sourdough LAB and yeast isolates, the detection of phytase activity, has been in the epicenter of intensive study over the past few years (Çakır et al., 2020). Phytic acid or phytate constitutes an anti-nutritional factor, due to its high chelating capacity to form insoluble complexes with essential minerals, thus hindering their bioavailability (Gobbetti et al., 2014). During sourdough fermentation, optimal pH conditions are created and endogenous grain phytases are stimulated. The latter dephosphorylate phytic acid into free inorganic phosphate and myo-inositol phosphate esters, which are characterized by less chelating capacity and thus enhance the nutritional characteristics of the end product. Except for the presence of endogenous phytases found in flour, the phytase capacity has been recorded in bacterial and yeast strains, as well (Ispirli et al., 2018, Karaman et al., 2018). However, it is estimated that the endogenous flour phytase activity is significantly higher than the microbial one (Katina & Poutanen, 2013).

1.4.7 Lipolysis

Lipolysis performed at certain levels during sourdough fermentation is a preferable technological property of starters, since the metabolic products contribute to the bread flavor improvement. However, lipid degradation in sourdoughs has been studied only to a limited extent.

Despite the low concentration of wheat flour lipids (2 %), they seem to play a significant role in bread performance, via improvement of the mixing capacity and volume of the dough (Koehler & Wieser, 2013). Polar phospho- and glycolipids, especially the free ones and not those bound to gluten proteins, contribute to the bread quality. Regarding the interactions between lipids and proteins, free lipids bind to proteins during flour rehydration, thus stabilizing gluten network and improving carbon dioxide retention capacity.

During sourdough bread making process, lipid oxidation results to the liberation of key aroma compounds. In brief, initial hydrolysis starts with lipases, which convert triacyglycerols into free fatty acids (Pico et al., 2015). Then, FFA are either autoxidized during flour storage, or oxidized via cereal lipoxygenase activity, during dough mixing, into lipid peroxides (Gänzle et al., 2007). A chemical conversion of the latter into aldehydes, namely (E)-2-nonenal and (E,E)-2,4- decadienal, occurs, which contribute to the crumb odor. Heterofermentative LAB, such as *Fr. sanfranciscensis*, have been reported to reduce the aforementioned aldehydes to the alcohol respectives, combined with the oxidation of NADH to NAD^+, with further ATP production. In the case of homofermentative LAB, an increase of lipid oxidation due to the presence of hydrogen peroxide has been reported (Vermeulen et al., 2007). In the case of sourdough derived yeasts, lipolytic activity has not been considered a widespread attribute, with some minor exceptions, namely *Yarrowia lipolytica* (Paramithiotis et al., 2000).

1.4.8 EPS production

Exopolysaccharide (EPS) producing LAB strains can successfully drive sourdough fermentation process, favoring both the technological and functional properties of the final product (Dertli et al., 2016, Valerio et al., 2020). EPS are not used as an energy source (due to the absence of EPS hydrolyzing enzymes), but their ecological application is associated with biofilm formation, cell integrity protection, stress resistance, while in some cases prebiotic activities have been recorded. Regarding their technological footprint in the bread industry, EPS application is relied on their capacity to mimic the hydrocolloid properties, thus creating a strong network with the rest dough components. A further improvement of bread volume and restriction of crumb firmness, with concomitant shelf life extension has been reported (Galli et al., 2020).

EPS constitute high molecular weight polymers, which are structurally classified as homopolysaccharides (HoPS) and heteropolysaccharides (HePS) (Milanović et al., 2020). HoPS comprise of only one type of monosaccharide, namely glucose or fructose, thus referred as glucans and fructans, synthesized from sucrose by the activity of glucan- and fructansucrases, respectively. Dextran and levan are two of the most studied HoPSs, composed of glucose and fructose, respectively. On the

other hand, HePS are composed of repeating units of at least two types of monosaccharides, namely galactose and glucose, produced from sugar nucleotides by the action of intracellular glycosyltransferases (Galle & Arendt, 2014, Wolter et al., 2014). Another differentiation between the two types of EPS is found on the location, where the biosynthesis takes place. Regarding HePS, their synthesis is performed in the cytoplasm, while in the case of HoPS an extracellular production has been recorded (Sahin et al., 2019).

Regarding the EPS production by LAB, generally low amounts have been documented. Factors, namely strain, pH, temperature, sucrose content, fermentation time and substrate, exert significant effect on the EPS yields produced *in situ* (Paramithiotis & Drosinos, 2017). On that basis, wheat and rye grains are not considered an ideal source of EPS producing LAB strains, while other grains e.g. chickpea, spelt and buckwheat flour have been reported to serve as good substrates for EPS production. As far as the LAB strains were concerned, *W. cibaria*, *W. confusa* and *Leu. mesenteroides* are regarded good EPS producers. Galli et al., (2020) reported the *in situ* production of dextran by *W. confusa* Gk15, isolated from spontaneously fermented chickpea sourdough. In addition, Wolter et al. (2014) reported that dextran production by *W. cibaria* MG1 was strongly substrate dependent, highlighting that highest yields were recorded in buckwheat and quinoa sourdough. Opposing previous studies supporting that sourdough LAB are associated with HoPS production, while only a marginal part of isolates has been related to HePS production (Gänzle & Gobbetti, 2013). Milanovic et al. (2020) reported that the majority of LAB strains assessed for EPS production were recognized as HePS.

In the case of yeasts, several genera among which *Saccharomyces*, *Candida*, *Pichia*, *Rhodotorula*, *Phomopsis*, *Exophiala*, *Lipomyces* and *Tremella*, have been reported as EPS producers (Sahin et al., 2019, Franco et al., 2020). However, the EPS yield obtained from yeast strains has been significantly lower compared to the respective of LAB, thereby an industrial application is yet to come.

1.4.9 Decarboxylase activity

The ability of candidate microbial cultures to form biogenic amines (BAs), is a food safety factor that should be taken into consideration, prior to their application to food fermentations (Spano et al., 2010, Li et al., 2020). BAs are nitrogenous, low molecular weight organic compounds with an aliphatic, aromatic or heterocyclic structure, formed by the decarboxylation of their corresponding amino acids (Kannan et al., 2020). BAs accumulation in food products stems from uncontrolled enzymatic activity and constitutes a defense mechanism so as to withstand the acidic conditions. Some of the most commonly encountered BAs in foods are histamine, tyramine, putrescine and cadaverine (del Rio et al., 2018). Consumption of fermented foods containing high concentration of BAs would possibly lead to food intoxication, followed by a series of symptoms e.g. cardiac palpitations, while in worst case scenario, a fatal outcome could be encountered. Despite that there is no certain legislation determining the BA content in fermented foods, it should be as low as possible.

In fermented foods, namely dairy products (cheese), wine, meat and fish, the presence of BAs is attributed to the decarboxylase activity of certain LAB strains (Fessard & Remize, 2017). However, BA production is of less concern in sourdough microecosystems, since only few studies have reported the presence of BA producing sourdough derived LAB strains. More accurately, del Rio et al. (2018) reported the capacity of a sourdough derived *Fu. rossiae* strain to produce putrescine from arginine via the ornithine decarboxylase pathway, in order to counteract the acidic conditions of sourdough. *odc* and *potE* were identified in *Fu. rossiae* and acidic conditions induced their expression. However, putrescine content was not quantified. In another study, BA formation after solid state flaxseed fermentation by different LAB was observed, but at too low limits to cause an intoxication incidence (Bartkiene et al. 2014).

In the case of yeasts, despite the fact that both *Saccharomyces* and non *Saccharomyces* yeasts have been associated with the ability to decarboxylate amino acids as a strain specific property (Delgado-Ospina et al., 2021, Fernández-Pacheco et al., 2021), no previous study has linked the BA production or decarboxylase activity with sourdough derived yeasts.

1.4.10 Antimold activity

Despite the fact that bread constitutes one of the most widely consumed foods, mold spoilage, with concomitant increased waste problems, poses a serious concern for both bakery industry and scientific community. A further concern is the mycotoxin production from mold infection, with consequent contamination of grains and flours. The most common molds, related to bread spoilage, originate from the genera *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* (Axel et al., 2017). Given that the majority of mycotoxins are thermally stable, thus surviving the bread making process, a serious threat to human health is posed (Karlovsky et al., 2016). Chemical preservatives, namely sorbate and propionate, are commonly applied in order to suppress mold growth and to confer shelf life prolongation. However, an increasing consumer demand for less chemical additives, thus more "clean label" bakery products, has shift the scientific interest into alternative preservation techniques. In this regard, the application of sourdough technology and plant extracts has been studied.

The use of sourdough technology for biopreservation purposes is based on the release of antimold compounds, derived from the metabolic activities of LAB and yeast strains. In the case of LAB, except for the production of lactic and acetic acid, the synthesis of several low molecular weight compounds, namely phenyl derivatives, cyclic dipeptides, hydroxy fatty acids and fungicidal peptides, has been reported, as well (Gänzle & Gobbetti, 2013). However, it should be emphasized that the amounts of the aforementioned compounds synthesized *in situ* have been recorded at significantly lower levels compared to their minimum inhibitory activity (MIC) (Paramithiotis & Drosinos, 2017). Thus, the inhibitory mode of action is attributed to a synergistic effect of the antimold compounds.

Consistent with the previous statement, Müller et al. (2021) reported lower levels of acetic, lactic, phenyllactic and hydroxyphenyllactic acids produced by four multifunctional sourdough derived *Leu. citreum* strains, compared to their MICs calculated *in vitro*. Therefore, the recorded antimold activity of *Leu. citreum* strains possibly originates from the interaction of the antimold compounds.

In a previous study by Dal Bello et al. (2007), the identification of lactic acid, 3-phenyllactic acid and the two cyclic dipeptides, namely cyclo (L-Leu-L-Pro) and cyclo (L-Phe-LPro), in the cell free supernatant of *Lp. plantarum* FST 1.7 was associated with the antimold activity of the bacterial strain. Sourdough bread fermented by the aforementioned strain displayed comparable antimold effect to the respective retrieved from *Fr. sanfranciscensis*.

As far as the antifungal hydroxy fatty acids were concerned, *Lv. hammesii* was reported to convert linoleic acid to the respective monohydroxy octadecenoic acid, with the latter being characterized by antimold activity (Black et al., 2013). In addition, the aforementioned conversion was recorded during sourdough fermentation, upon medium fortification with linoleic acid. Application of 20% sourdough fermented by *Lv. hammesii* in bread making, gained 2 or 3 days of mold free shelf life.

Regarding the plant based extracts, Luz et al. (2019) reported the production of secondary metabolites in wheat based water soluble extracts, fermented by *Lp. plantarum* and *Lb. delbrueckii* subsp. *bulgaricus*, respectively, able to inhibit mold growth. In the case of water soluble extract fermented by *Lp. plantarum*, the identification of gallic, chlorogenic, caffeic, and syringic acid was recorded, while in the respective of *Lb. delbrueckii* subsp. *bulgaricus*, the presence of phenyllactic and sinapic acid was reported. Breads made with *Lp. plantarum* and *Lb. delbrueckii* subsp. *bulgaricus* sourdoughs gained 1 or 2 days of mold free shelf life, compared to the control. The application of peptides with antimold capacity has been assessed, as well. In an earlier study by Rizzello et al. (2009), the antimold activity of a water soluble extract of amaranth seeds was attributed to the identification of four novel antifungal peptides (agglutinin sequences). However, mold free shelf life prolongation did not exceed seven days. The presence of a pea flour hydrolysate followed by antimold activity was reported in another study by Rizzello et al. (2015), as well. Bread produced via a combination of sourdough fermentation by *Lp. plantarum* 1A7 and the freeze dried pea flour hydrolysate presented the longest shelf life, due to the presence of pea defensins 1, 2, a non specific lipid transfer protein and other peptides liberated during hydrolysis.

The application of yeasts as biocontrol agents in diverse food microecosystems has received considerable attention over the past few years. Several biocontrol

mechanisms, e.g. competition for nutrients, enzyme secretion, killer toxin production, VOCs production and myco-parasitism, have been proposed up to now (Freimoser et al., 2019). However scarce is the literature assessing the suitability of antimold yeast strains in sourdough ecosystems. In more detail, in an earlier study, Coda et al. (2011) reported that a sourdough fermented by *Lp. plantarum* and *W. anomalus* and further incorporated into bread making, resulted in a mold free shelf life of bread by 14 days, upon artificial inoculation. The increased inhibitory activity was attributed to the synergistic effect of ethyl acetate and peptides, produced by *W. anomalus* and *Lp. plantarum*, respectively. In a further study, Coda et al. (2013) reported the fungistatic potential of *Meyerozyma guilliermondii* LCF1353, via the production of the extracellular cell wall degrading enzyme β-1,3-glucanase and ethyl acetate. The application of the aforementioned yeast strain to bread making, combined with *W. anomalus* LCF1695 and *Lp. plantarum* 1A7, resulted in a delay of fungal growth by at least 14 days.

1.4.11 Antibacterial activity of LAB

The ability of LAB to produce antibacterial compounds, namely bacteriocins, has received substantial attention over the past few years as a clean label technology (Bangar et al., 2022). Bacteriocins constitute ribosomally synthesized small proteins or peptides, cationic and hydrophobic, characterized by an antibacterial mode of action against closely related species. Their application to food systems relies on the use of bacteriocinogenic LAB strains or the addition of the bacteriocin itself. Despite that their application in sourdough bread making process, does not seem to fit in terms of shelf life prolongation of baked products, it does contribute to the sourdough stability, through the competitiveness enhancement of the bacteriocin producer (De Vuyst & Leroy, 2007). Thus, the risk of contamination by pathogenic bacteria during processing is significantly minimized and the desired microbiota dominate the food matrix.

1.4.11.1 Bacteriocin classification

Over the past few years different classifications of bacteriocins have been proposed. According to Soltani et al. (2021) two classes were proposed based on the presence

or not of post-translationally modified motifs. However, the classification system generally approved discriminates bacteriocins into four classes as follows.

Class I or Lantibiotics: Bacteriocins comprising of 19-50 amino acids, with intramolecular thioester ring structures, which derive from post-translational modifications (Diep et al., 2009, Kumariya et al., 2019). Nisin is a typical member of class I bacteriocins.

Class II: Small heat stable bacteriocins with non-modified peptides, characterized by the presence of the double glycine processing site in the precursor, which are subdivided into four groups (Todorov et al., 2009).

- Class IIa: Pediocin like bacteriocins, with strong antilisterial capacity
- Class IIb: Two peptide bacteriocins, the activity of which is strongly dependent on the complementary activity of the two peptides
- Class IIc: Peptides requiring the presence of reduced cysteine residues for activity
- Class IId: Unmodified bacteriocins, which do not meet the criteria of the aforementioned classes

Class III: Large heat labile bacteriocins (> 30 kDa in size). Helveticin J is a representative member of this group.

Class IV: Large complex proteins requiring a carbohydrate or lipid moiety for activity. Some recent studies reclassified them as bacteriolysins, thus discriminating bacteriocins into the rest three classes (Mokoena, 2017).

1.4.11.2 Genetic organization of bacteriocins

Bacteriocins can be chromosomally or plasmid encoded, such as plantaricins ST31 and 423, respectively (Todorov et al., 2009).

Regarding class I bacteriocins, the nisin gene cluster, namely *nisABTCIPRKFEG*, includes 11 genes (Dimov et al., 2005). Among them, *nisA* encodes for a precursor peptide, *nisT* encodes for an ABC transporter protein, while *nisR* and *nisK* encode for a response regulator and a histidine protein kinase, respectively. As far as the nisin biosynthesis is concerned, the initial step involves the translation of *nisA* to pre-nisin A peptide. Then, pre-nisin A is further transformed into precursor nisin A,

with the aid of *nisB* and *nisC*. Finally, precursor nisin A is transported out of the cell, which is facilitated by the action of *nisT* and *nisP*, with concomitant leader peptide cleavage so as to generate nisin A.

Genes encoding for class II bacteriocins normally include a structural, an immunity, an ABC transporter gene and one latter encoding for an accessory protein, facilitating the export of bacteriocin from the cell (Todorov et al., 2009). The majority of the aforementioned bacteriocins are produced as inactive precursors with a N-terminal leader sequence, combined with a double glycine site.

1.4.11.3 Mechanism of action of bacteriocins

The ability of bacteriocins to interact with cell membrane and create pores has been well documented (Todorov et al., 2009). The initial interaction is mediated via electrostatic forces among the positively charged amino acid groups of bacteriocins and the negatively charged phospholipid groups in cell membrane. However, in many cases the presence of docking molecules and receptors to interact is necessary. The mechanism of action of bacteriocins is illustrated in Figure 1.9.

Class I bacteriocins or lantibiotics, e.g. nisin, have been reported to bind cell wall precursor lipid II, a docking molecule, thus preventing cell wall biosynthesis and further proceed to pore formation in a wedge like model (O' Bryan et al., 2018, Kumariya et al., 2019). However, membrane permeabilization has been suggested as the core inhibitory mode of action of nisin. An ionic imbalance is generated, proton motive force is partially or totally dissipated and cell death follows.

Class II bacteriocins increase cell membrane permeability via barrel stave or carpet like arrangement (Karpinski and Szkaradkiewicz, 2016). Barrel stave is differentiated from wedge like model in the ability of peptides to expose themselves into the hydrophobic core of membrane (Snyder & Worobo, 2013). In the case of carpet like arrangement, peptides align in parallel to the cell membrane via electrostatic interactions, with no prerequisite aggregate accumulation. Regarding class IIa bacteriocins, the binding to certain receptors in cell membrane, namely mannose permease of the phosphotransferase system is required. As far as class IIb bacteriocins are concerned, pore formation via the barrel stave like arrangement has been reported. In the case of class IIc bacteriocins, several mechanisms of action

have been proposed, among which membrane permeabilization and pheromone activity (O' Bryan et al., 2018).

Mechanism of action of class III bacteriocins includes lysis induction of bacterial cells (Hernández-González et al., 2021). The aforementioned mechanism is exerted via the interaction between bacteriocin and cell membrane components, namely teichoic, with further liberation of autolytic enzymes. Inhibition of biosynthesis of DNA and proteins has been proposed as an additional mode of action class III bacteriocins.

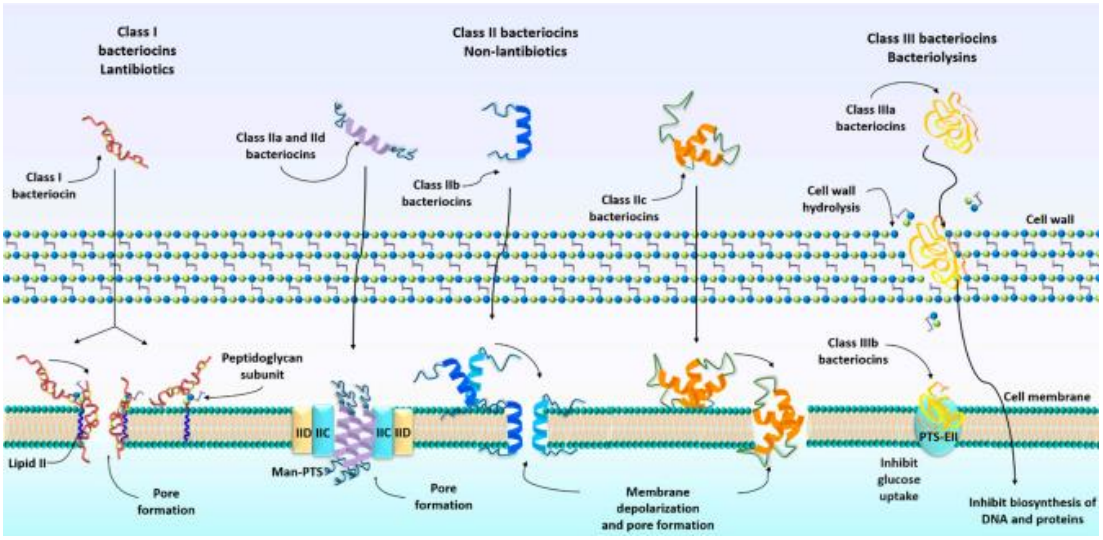

Figure 1. 9. Different modes of action of class I, II and III bacteriocins. Cationic bacteriocins bind either directly or indirectly, via docking molecules and receptors, to the cell membrane and form pores. In other cases, suppression of DNA and protein biosynthesis has been recorded. Reprinted with permission from Hernández-González et al. (2021).

1.4.11.4 Effect of growth-related conditions on bacteriocin production

After a bacteriocin producing LAB strain is identified, the optimal conditions required for bacteriocin production are assessed *in vitro* and then the *in situ* application is further evaluated. Regarding the *in vitro* assessment, different parameters, among which microbial strain, temperature, pH and fermentation medium components, can interfere on growth and bacteriocin production by LAB in diverse food ecosystems.

In the case of temperature and initial pH value, the optimal ones for bacteriocin production do not always coincide with the respectives for growth of the producer strain. That was the case in the study by Mataragas et al. (2003), according to which

the optimum pH and temperature values, namely 6.0-6.5 and 30 ^{o}C, for growth of *Ln mesenteroides* L124 and *Lt. curvatus* L442 were different from those recorded for optimal bacteriocin production, namely pH 5.5 and 25 ^{o}C. Similar were the findings reported by Drosinos et al. (2005), according to which increased bacteriocin production was recorded, upon growth of *Leu. mesenteroides* E131 at lower pH value, namely pH 5.5, compared to that at pH 6.0, which is considered the optimum for bacterial growth. In the case of temperature, opposite results were obtained. In more detail, better bacteriocin production by *Leu. mesenteroides* E131 was recorded after growth at temperatures close to the optimum, namely 25 ^{o}C. From the aforementioned findings it is concluded that suboptimal growth conditions contribute to the maximum bacteriocin production, probably via a better energy utilization. As far as the effect of medium components on bacteriocin production is concerned.

The type and concentration of carbon sources have been found to significantly affect bacteriocin production. Glucose represents a preferred energy source, the presence of which in a growth medium has been directly linked to increased bacteriocin production (Abbasiliasi et al., 2017). However, the diversity of carbon sources have been reported to enhance bacteriocin production, as well. In a previous study, Leroy et al. (2006) reported that the complexity of carbon sources, namely three or four sugars, improved bacteriocin production by *Lb. amylovorus* DC471, compared to single sources. Another study by Todorov et al. (2006) has demonstrated a direct effect of carbon sources on bacteriocin production, exerted in a concentrate dependent manner. Except for the fortification of growth medium with carbon sources, NaCl addition has been shown to induce a stressful environment, via which ultimately the enhancement of bacteriocin production is performed (Abbasiliasi et al., 2017). However, the adverse effects of NaCl addition on bacteriocin production have been recorded as well (Leroy et al., 1999, Himelbloom et al., 2001).

1.4.11.5 Sourdough derived bacteriocinogenic LAB strains

Only few studies have reported the bacteriocin production by sourdough derived LAB strains. In brief, Todorov et al. (1999) reported the production of chromosomally encoded plantaricin ST31 by sourdough originating *Lp. plantarum* ST31, characterized by maximum yields after growth in MRS broth adjusted to pH6

at 30 ^{o}C. In addition, Settanni et al. (2005) reported the two-peptide bacteriocin production by *Lc. lactis* spp. *lactis* M30 during sourdough fermentation. The inhibitory activity of *Lc. lactis* spp. *lactis* M30 towards the indicator strain, namely *Lp. plantarum* 20, after a propagation period of 20 days was indicated, while the starter culture of *Fr. sanfranciscensis* CB1 was unaffected. In another study, the production of plantaricin A by sourdough derived *Lp. plantarum* 400 was reported and the induction rate of *plnA* biosynthesis, based on the microbial co-culture, was assessed (Di Cagno et al., 2010). Results obtained revealed that when *Fr. sanfranciscensis* DPPMA174 was used as microbial partner increased biosynthesis of *plnA* and thus a decrease of species viability was recorded.

1.5 Diverse approaches for microbial characterization

The development of molecular tools has undoubtedly changed the way of studying the microecosystem composition of fermented foods, providing the scientific community with reliable and effective methods for microbial detection, identification and typing. Conventional microbiology has been characterized by strong limits to depict microecosystem diversity, thus culture independent techniques have emerged to offer better insight into microbial ecology of food matrices (Figure 1.10). In the case of detection and identification, they can benefit from both culture dependent and independent approaches. As far as typing is concerned, it is performed on isolated strains and is strictly correlated with culture dependent methods.

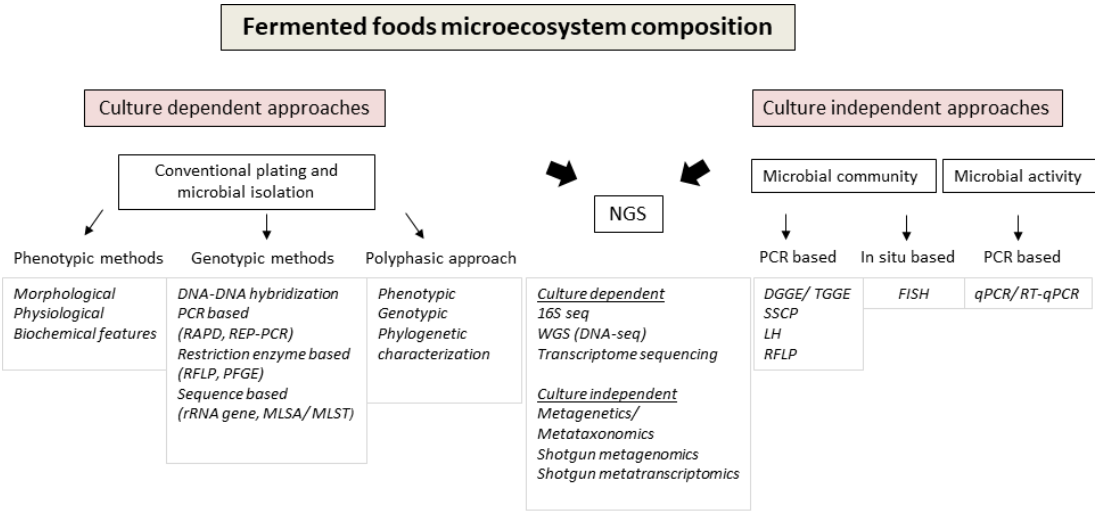

Figure 1. 10. Culture dependent and culture independent approaches applied for microbial community profiling and microbial activity investigation in fermented foods.

1.5.1 Culture dependent methods

Traditionally, the microecosystem composition of fermented foods has been unraveled with culture dependent approaches, namely phenotypic and genotypic (Dolci et al., 2015). Phenotypic approach, which is not directed toward DNA or RNA, is based on morphological features, physiological and biochemical characteristics for microbial classification. However, the laborious and time-consuming classical phenotypic approach led to the evolution of genotypic methods so as to overcome the aforementioned pitfalls. At the same time, a polyphasic approach based on the incorporation of genotypic, phenotypic and phylogenetic characterization, thus exploiting data from different levels of cellular organization, in order to improve the accuracy of microbial taxonomy, was developed (Vandamme et al., 1996). Regarding the genotypic approach, methods based on DNA-DNA hybridization, PCR, enzymatic restriction and on sequence are included and are briefly described as follows.

1.5.1.1 DNA-DNA hybridization

DNA-DNA hybridization (DDH) approach, based on the complementary action of double stranded DNA, is considered the golden standard for prokaryotes classification (Bonatsou et al., 2019, Raina et al., 2019). Denatured DNA molecules retrieved from two bacterial species are brought back to form a heteroduplex molecule, with G-C content and melting temperature recognized as crucial

parameters. A threshold less than 70% has been defined for labeling two isolates as distinct species. However, the time consuming and labor-intensive nature of the pairwise cross-hybridizations combined with the absence of a central strain database hinder the applicability of method for taxonomic purposes.

1.5.1.2 PCR based

Among PCR based approaches, randomly amplified polymorphic DNA (RAPD)-PCR has been widely used as typing method in food micro-ecosystems. The aforementioned technique requires no previous knowledge of the DNA. The method is based on the use of short primers, characterized by random sequence (approximately 10 base pairs in length), which hybridize to distinct location of chromosomal DNA sequences, leading to the amplification of many distinct DNA products (Sharma et al., 2020). In theory, in the same DNA, primers will hybridize at the same points, thus same samples will yield the same band profile. Despite the fact that higher standardization is required for reproducibility, RAPD-PCR has been extensively applied for microbial identification and typing in a wide variety of fermented foods (Giraffa & Carminati, 2008, Dolci et al., 2015).

1.5.1.3 Restriction enzymes based

The application of restriction enzymes in microbial genomes has been previously shown to detect polymorphisms, thus leading to strain differentiation. Restriction Fragment Length Polymorphism (RFLP) is a commonly used technique, the principle behind which is based is the digestion of genomic DNA into further fragments with restriction enzymes. Then, a size based separation via an agarose gel electrophoresis is performed, while a further hybridism with probes and autoradiography detection takes place (Yang et al., 2013).

Another widespread molecular method based on the use of restriction enzymes is Pulsed-Field Gel Electrophoresis (PFGE). PFGE characterized by high discriminatory capacity and reproducibility allows large DNA molecules separation (Bonatsou et al., 2019). After restriction endonucleases are applied on the whole chromosome structure and target DNA is digested, a pulse voltage gradient is generated, followed by periodic shift in the orientation of electric field. However, expensive equipment, time consuming protocols and experience personnel needed

for the analysis of fingerprinting patterns hinder the diffusion of the method (Sharma et al., 2020).

1.5.1.4 Sequence based

Sequencing analysis of the 16S and 26S ribosomal RNA (rRNA) gene are considered the gold standards for bacterial and yeast identification, respectively (Ndoye et al., 2011). Regarding bacterial 16S rRNA gene, it is a highly conserved large molecule (approximately 1500 bp), present in almost all bacteria, thus serving as a suitable target gene for DNA sequencing. The presence of both conserved and hypervariable regions (V1-V9), contributes to the PCR amplification of target sequences via universal primer use and enables microbial discrimination, respectively (Chakravorty et al., 2007, Raina et al., 2019). Based on the desired sequence length, different sub-regions are targeted. More accurately, the V1-V3 region of the rRNA gene, which has been preferred by many researchers due to the acceptable levels of discrimination among the different species of LAB, yields long sequencing reads. On the other hand, the increasing demand for widely used Illumina sequencing platforms has shifted the scientific interest to the V3-V4 region of 16S rRNA gene due to the production of short sequencing reads (Poirier et al., 2018). However, this sub-region is not able to discriminate at species level. Despite the extensive use of 16S rRNA gene sequencing for identification purposes, several drawbacks have been reported, among which the insufficient resolution, concerning the inability to discriminate among closely related species and the presence of multiple copies within the 16S rRNA gene in an individual prokaryotic organism. In the case of yeast identification, except for the D1/ D2 region of 26S rRNA gene, the variable ITS1 and ITS2 regions serve as gene targets, as well (Bonatsou et al., 2019).

Multilocus sequence analysis/ typing (MLSA/ MLST) is another sequence based approach in prokaryote taxonomy used for the surveillance of foodborne pathogens. The principle behind this technique is based on the genetic variation found in a set of housekeeping or protein coding genes (Rong & Huan, 2014). Partial gene sequences are used for phylogenetic trees generation and further phylogeny deduction (Glaeser & Kämpfer, 2015). This approach offers sequence data, which are unambiguous and the allelic profiles can be efficiently compared between laboratories. Compared to 16S rRNA gene sequencing, the aforementioned

approach exhibits higher resolution. However, the absence of generally accepted recommendations for individual MLSA schemes, implies variations in the way MLSA is applied, thus conflicting phylogenies are generated.

1.5.2 Culture independent methods

The emergence of culture independent approaches arose so as to counteract the limitations encountered during classical approach, with the latter being characterized laborious, while the identified isolates may not truly reflect the actual microbial diversity of the given sample. Thus, the application of culture independent methods, based on direct analysis of nucleic acids, without any prior culturing step, has been gaining field over the last years. Among them, PCR-based, *in situ* based, Next Generation Sequencing (NGS) techniques have been quickly recognized as valuable tools for in microbial characterization.

1.5.2.1 PCR based

PCR based approaches, namely Denaturing/ Temperature Gradient Gel Electrophoresis PCR (DGGE/ TGGE-PCR), Single Strand Conformation Polymorphism PCR (SSCP-PCR), Length Heterogeneity PCR (LH- PCR), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Quantitative PCR (qPCR) and Reverse Transcription Quantitative PCR (RT-qPCR) have received considerable attention for profiling microbial populations in food ecosystems (Dolci et al., 2015).

PCR-DGGE

PCR-DGGE has been initially applied for microbial characterization purposes on environmental samples and then the method was further introduced in the food microecosystem (Ercolini et al., 2004). PCR-DGGE is based on the polyacrylamide gel electrophoretic separation of DNA fragments via a denaturing gradient created by urea and formamide. More accurately, amplicons characterized by similar size, but different sequences, are exposed to increasing denaturing conditions, thus a separation is performed, as they partially melt at distinct areas. In other words, different sequences yield distinct melting profiles as the denaturant levels increase and an electrophoretic separation takes place. To avoid complete separation of double stranded DNA fragments into single strands, the addition of a GC clamp with

30-50 base pairs to the primer sets is performed. The final outcome is a series of bands which can be excised and sequenced for microbial identification. However PCR-DGGE can carry many biases, which impede its application for quantification purposes of a-diversity (Neilson et al., 2013). Some indicative artifacts include low detection limit, namely 10^3-10^4 CFU/ mL, comigration of DNA fragments due to close phylogenetic relatedness and preferential amplification of microbial communities. In addition, multiband profile corresponding to single microbial strains and heteroduplex bands, commonly known as phantom respectives, have been frequently encountered during PCR-DGGE, leading to an unreliable estimation of microbial community. For the aforementioned biases, PCR-DGGE has been recommended as a survey tool assisting the comparative analysis of microecosystem composition, coupled with conventional plating and molecular identification, but not for quantification purposes (Scheirlinck et al., 2008, Neilson et al., 2013).

Real Time/ qPCR

Although Real Time PCR (quantitative PCR, qPCR) has not been considered a community profiling method, it can reliably detect individual microbial populations in food matrixes (Dolci et al., 2015). Given that qPCR is not able to effectively discriminate and quantify an increasing number of targets in a single sample, its combination with culture dependent approaches, namely conventional plating and classical PCR, led to successful depiction of microecosystem composition.

Except for qPCR, which uses DNA as a template molecule, RT-qPCR adopted for RNA quantification purposes has met considerable scientific attention. In RT-qPCR, RNA molecules are reverse transcribed into their complementary DNA (cDNA), which are further submitted to standard PCR. The increasing interest for RT-qPCR has been probably attributed to the fact that RNA is a better indicator of microbial vitality, while the ability of DNA to persist after cell death, may intervene in the analysis and lead to an overestimation of the viable microorganisms.

qPCR constitutes an alternative to the conventional PCR, during which specific target sequences are detected and amplified with one exceptional difference; qPCR allows the monitoring of the targeted molecule amplification at that specific time, through fluorescence monitoring (Kralik & Ricci, 2017). In more detail, the

momentary amount of nucleic acids amplified is monitored via the fluorescence intensity of some fluorescent reporter molecules that bind or integrate into the amplified sequence. The intensity of the fluorescence signal increases gradually and proportionally to the amount of product synthesized at each reaction cycle, while the fluorescence measured in each PCR cycle results in the development of an amplification curve. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescence signal to reach a threshold, above which a significant increase in signal is observed leading to a separation from the background level (Kubista et al., 2006). The Ct value is inversely proportional to the initial quantity of the under study product.

Based on whether the relative amount of nucleic acid or the exact transcript copy number is required, relative and absolute quantification is performed, respectively (Livak & Schmittgen, 2001). In the case of absolute quantification, the construction of an absolute standard curve for each PCR product is required so as to compare the signal emitted during the reaction. Regarding the relative quantification, the fluorescent signal of the target gene is compared to the respective of the gene corresponding to a control sample.

For the real time visualization of PCR products, fluorescent reporter molecules are applied, namely non specific DNA binding dyes and specific dye labeled probes (Kralik & Ricchi, 2017). In the former case, SYBR Green I is one of the most commonly applied dyes, which increases its fluorescence signal by over 1000-fold when bound to double stranded DNA (dsDNA). This strategy is both cost effective and easy to use, however its inherent non specificity can yield a misleading estimation of the actual target concentration (Stratagene, 2006). In the case that fluorescently labeled oligonucleotide probes are used, an increased level of specificity has been detected. More accurately, the 5' end of the probe contains a fluorescent reporter dye, while the 3' end a quencher dye is found. During PCR reaction, Taq polymerase cleaves the hybridized probe, thus the separation of reporter dye from the quencher takes place and fluorescence emission occurs (Marmiroli & Maestri, 2007).

1.5.2.2 *In situ* methods

Fluorescence *in situ* hybridization (FISH) constitutes a non PCR method based on rRNA targeted oligonucleotide probes so as to observe the temporal and spatial

microbial distribution in their native environment, namely food matrix (Ndoye et al., 2011). In a previous study Cocolin et al. (2007) combined the culture independent approaches PCR-DGGE and FISH for profiling bacterial populations in food products, fresh and processed and the results obtained were in agreement. However, some limitations encountered include variability associated with the physiological cell state and the inability to identify sequences of low copy number (Giraffa & Carminati, 2008).

1.5.3 NGS methods

Next generation sequencing (NGS) technologies have evolved into indispensable tools for microbial community investigation in various food matrices (De Melo Pereira et al., 2022). NGS methods can be applied in food microbiology as both culture dependent and culture independent approaches. Their culture dependent use aims to the investigation of genome and transcriptome of a single microorganism (Mataragas, 2019). For that purpose, its prior isolation from the food matrix is necessary. Then, 16S rRNA gene sequencing, Whole genome sequencing (DNA-seq) and Transcriptome sequencing (RNA-seq) can be applied, responding to different questions. On the other hand, NGS can be applied for the investigation of taxonomic and functional profiles of microbial communities, as well (culture independent approach). In that case, prior bacterial culturing is not a prerequisite, since nucleic acid is directly extracted from food sample. Then, Metagenetics/ Metataxonomics (16S rRNA gene sequencing) and Shotgun metagenomics/ metatranscriptomics (DNA/ RNA seq) can be employed so as to depict either the taxonomic diversity or both taxonomy and biological function (Mataragas, 2019).

1.5.3.1 A brief history of NGS methods

However, a brief history of sequencing technologies dates back to 1977, when Sanger and Maxam-Gilbert methods were developed, constituting the very first generation sequencing technologies (Sanger et al., 1977, Maxam & Gilbert, 1977). The former, which was adopted as the primary sequencing method, was based on the application of chain terminators (dideoxynucleotides) and the latter, commonly known as chemical degradation approach, relied on the nucleotide cleavage by chemicals. Nevertheless, the time, cost and low throughput of the aforementioned

sequencing methods necessitated the emergence of the second generation sequencing approaches, namely NGS or massively parallel sequencing methods. NGS technologies, characterized by high throughput, reduced cost and no need for electrophoresis, are able to produce millions or billions of parallel sequencing reactions at the same time (Mayo et al., 2014). The NGS approaches currently applied include pyrosequencing, sequencing by synthesis, sequencing by ligation and ion semiconductor sequencing. However, the PCR amplification step required, combined with the relatively short read lengths yielded, with the latter occasionally interfering with genome assembly, led to the demand for even more revolutionary sequencing technologies (Kchouk et al., 2017). Thus, third generation sequencing (TGS) technologies were developed, characterized by the absence of PCR amplification step and longer read length. Single molecule real time (SMRT) method represents the most commonly applied TGS technology (Liu et al., 2012).

1.5.3.2 Similarities and differences among the NGS approaches

Despite the fact that different NGS approaches have been developed over the past few years revolutionizing the microbial ecology investigation, their workflow shares some similarities and is being differentiated in other fields, as well. In more detail, library preparation is a common and prerequisite step, including nucleic acid modification with oligonucleotide adapter ligation, so as to reach an insert size compatible with the sequencing system to be used. Then, after hybridization occurs, rDNA fragments are amplified and cluster generation takes place on a solid surface, via immobilization either on tiny beads and further emulsion PCR or on glass flow cell and bridge PCR amplification. Another common feature of the NGS approaches is the fact that multiple amplifications occur at the same time in different places on a DNA chip and they are monitored individually.

Regarding the differences among the distinct NGS approaches, they are mostly based on the technical part of the sequencing reaction.

Pyrosequencing

Pyrosequencing technique relies on the principle that when a nucleotide is incorporated into the extending DNA strand, pyrophosphate is released and converted into adenosine triphosphate (ATP), which is further turned into light, as a result of luciferin oxidation catalyzed by luciferase (Jay et al., 2011, Mayo et al.,

2014). Thus, the sequence of DNA fragment is deduced, each time a nucleotide is added and subsequently light is emitted. The unincorporated nucleotides are enzymatically degraded.

Sequencing by synthesis

Sequencing by synthesis is based on a reversible terminator chemistry technology (De Melo Pereira et al., 2022). In more detail, each nucleotide is labeled with a fluorescent specific molecule and is reversibly terminated, as well, so as to ensure the incorporation of a single base. Thus, after a single base is incorporated, the non incorporated respectives are washed away and the fluorescence is detected and recorded. Then, the fluorescently labeled terminator group is removed and the next one can be added.

Sequencing by ligation

Sequencing by ligation is differentiated by the two former technologies in the usage of 16 8-mer oligonucleotide probes and DNA ligase, instead of DNA polymerase. Among these 8 bases, the first and second are probe specific, while the rest are considered degenerated bases (De Melo Pereira et al., 2022). Upon annealing, a DNA ligase facilitates the probe ligation to the primer sequence. After wash out of the unincorporated probes, fluorescent signal is detected and recorded (Cai et al., 2013). As soon as the probe ligation is completed, the last three bases are removed and the incorporation of a new probe starts. The aforementioned three-base nucleotide gap necessitates the process repetition by 7 cycles with the use of sequencing primers, characterized by one base setback.

Ion semiconductor sequencing

Ion semiconductor sequencing constitutes an improvement of the pyrosequencing technology. Their differentiation relies on the detection of H^+ ions released during nucleotide addition into the elongating strand (Jay et al., 2011).

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3 Objectives of thesis

Several studies have unraveled the microecosystem composition of sourdoughs up till now. This increased scientific and industrial attention stems from the significant effects of sourdough incorporation on the bread quality, combined with its health promoting properties. The technological, safety, nutritional and health promoting attributes of sourdoughs are directly related to the associated microbial consortium, consisting primarily of LAB and yeasts.

Thus, the very first objective of **Chapter 2** was to expand our knowledge on the microecosystem composition of spontaneously fermented wheat based sourdoughs originating from regions of Greece, which have not been previously assessed. For that purpose, 12 of the 13 sourdoughs, included in the present study, were obtained from Aetolia-Acarnania, Thessaloniki, Arcadia and Salamis island. A combined approach of culture dependent (conventional plating, clustering by PCR-RAPD, identification by PCR species-specific for *Lp. plantarum*, 16S and 26S rRNA gene sequencing for LAB and yeasts, respectively) and independent methods (DNA and RNA based PCR-DGGE) was applied for microbial profiling purposes. Regarding the culture independent methods, except for targeting DNA for PCR-DGGE analysis in order to obtain a picture of the sample history, total RNA extraction and further conversion to cDNA were performed, as well. Given that RNA is rapidly degraded upon cell death, targeting of the specific nucleic acid provides with a better indication of the microbial viability, thereby obtaining the microbial profiling of the under study food matrix at a specific time point. However, RT-PCR-DGGE has been applied to a lesser extent in fermented foods microecosystem analysis, compared to the DNA based PCR-DGGE.

In **Chapter 3**, the technological and safety properties of sourdough derived LAB and yeasts were assessed, since they improve our knowledge on the interactions between the biotic and abiotic parameters within the sourdough ecosystem and may indicate their fitness as starters in food fermentations. Although, the technological properties of sourdough originating microbial strains have been adequately assessed, they are mainly restricted to a narrow set of the under study features, thereby providing with a non completed view of the metabolic capabilities of isolates. Thus, in the present study, a series of attributes, namely proteolytic,

lipolytic, amylolytic, amino acid decarboxylase, phytase activity, antimicrobial capacity and EPS production of both LAB and yeasts, was evaluated. Of particular interest were the proteolysis, lipolysis and antimicrobial capacity, which, in the case of positive results, were further assessed through phenotypic (effect of proteolytic enzymes, pH value, thermal treatment) and molecular approaches (SDS-PAGE, specific PCR).

In **Chapter 4**, the antimold capacity of three sourdough derived *Wickerhamomyces anomalus* strains was further assessed both *in vitro* (modified BHI broth) and *in situ* (liquid dough) by the well diffusion assay. Then, the responsible volatile compounds (VOCs) were identified with SPME-GC-MS and the inhibitory activity of each VOC against mold growth was assessed. Finally, yeasted breads were produced, either with monocultures of *W. anomalus* strains or with cocultures with baker's yeast and the mold free shelf life of breads was evaluated. Despite the fact that the biocontrol activity of *W. anomalus* has been reported, its application on bread making has been only marginally studied. To our knowledge, no previous study has assessed the effect of dough related parameters (substrate, initial pH value and NaCl concentrations) on the antimold activity of *W. anomalus* strains and the extension of mold free shelf life of bread. In the case of substrate, the use of an alternative liquid medium, namely liquid dough, constitutes a very promising and flexible technology, which is simple to use and close to the typical nature of the end products. In addition, liquid dough offers homogenization of environmental compounds, namely nutrients. Thus, the application of non conventional yeasts as biocontrol agents, combined with the use of alternative media, such as liquid dough, could lead to the production of products with increased safety, flavor and texture attributes.

In **Chapter 5**, six sourdough derived *Lp. plantarum* strains, which previously exhibited increased technological and safety potential, were further subjected to Whole Genome Sequence analysis. The dominance of *Lp. plantarum* strains in a wide range of fermented foods has been constantly reported and attributed to their metabolic versatility and stress adaptation. Thus, the Whole Genome Sequencing analysis aimed to depict the genetic potential and improve our understanding towards the genomic and functional attributes of the *Lp. plantarum* strains and their further application as starters in food fermentations. In addition, data obtained

provide insights into the restricted number of available genomes of *Lp. plantarum* subsp. *argentoratensis* strains through the provision of high-quality whole-genome sequences.

In **Chapter 6**, a comparative genomic analysis of the 6 aforementioned sourdough derived *Lp. plantarum* subsp. *argentoratensis* strains was performed. The scope was to obtain a bioinformatic characterization of bacterial strains and to assess the genomic features associated with their biotechnological capacity and safety for potential application as starters. There is no previous report on the bioinformatic analysis of *Lp. plantarum* subsp. *argentoratensis* strains, isolated from sourdoughs, thereby this study constitutes the first comparative genomic and safety assessment of *Lp. plantarum* subsp. *argentoratensis* strains.

In previous Chapters, well diffusion assay and bioinformatic analysis revealed the antibacterial capacity of the 6 *Lp. plantarum* strains against a mixture of 5 *L. monocytogenes* strains belonging to serotype 4b and the presence of plantaricin associated genes, respectively. Thus, in **Chapter 7**, the effect of sourdough related parameters, namely incubation temperature and time, substrate, initial pH value and NaCl content on the growth and plantaricin production by the 6 *Lp. plantarum* strains was assessed. Then, further analysis of the transcriptomic response of the plantaricin genes was performed. Regarding the phenotypic assessment of plantaricin activity, no previous study has examined the fitness of *Lp. plantarum* strains, in terms of cell growth and plantaricin production, under sourdough related conditions. In addition, for the first time the application of a wheat flour and water extract, as a sourdough simulation growth medium, was considered and applied on the plantaricin activity assessment. As far as the transcriptomic response of plantaricin genes was concerned, there is no scientific literature assessing the relative gene transcription in *Lp. plantarum* strains under sourdough simulating conditions. Thus, for the first time, the relative contribution of several plantaricins to the overall antilisterial capacity of *Lp. plantarum* strains was assessed.

Chapter 2

Microbial ecology of Greek wheat sourdoughs identified by a culture dependent and a culture independent approach

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1. Abstract

The aim of the present study was to assess the microecosystem of 13 homemade spontaneously fermented wheat sourdoughs from different regions of Greece, through the combineduse of culture-dependent (classical approach; clustering by RAPD-PCR and identification by PCR species-specific for *Lp. plantarum*, and sequencing of the 16S-rRNA and 26S-rRNA gene, for LAB and yeasts, respectively) and independent approaches (DNA- and RNA-based PCR- DGGE). The pH and Total Titratable Acidity (TTA)values ranged from 3.64–5.05 and from 0.50–1.59% lactic acid, respectively. Yeast and lactic acid bacteria populations ranged within 4.60–6.32 and 6.28–9.20 log CFU/g, respectively. The yeast: LAB ratio varied from 1:23–1:10,000. A total of 207 bacterial and 195 yeast isolates were obtained anda culture-dependent assessment of their taxonomic affiliation revealed dominance of *Lp. plantarum* inthree sourdoughs, *Lv. brevis* in four sourdoughs and co-dominance of these species in two sourdoughs. In addition, *Cp. paralimentarius* dominated in two sourdoughs and *Fr. sanfranciscensis* and *Lt. sakei* in one sourdough each. *Lc. lactis*, *Lt. curvatus*, *Leu. citreum*, *Leu. mesenteroides* and *Lv. zymae* were also recovered from some samples. Regarding the yeast microbiota, it was dominated by *Saccharomyces cerevisiae* in 11 sourdoughs and *Pi. membranifaciens* and *Pi. fermentans* in one sourdough each. *W. anomalus* and *K. humilis* were also recovered from one sample. RNA-based PCR-DGGE provided with nearly identical results with DNA-based one; in only one sample the latter provided an additional band. In general; the limitations of this approach; namely co-migration of amplicons from different species to the same electrophoretic position and multiband profile of specific isolates; greatly reduced resolution capacity that resulted to only partial verification of the microbial ecology detected by culture-dependent approach in the majority of sourdough samples. Our knowledge regarding the microecosystem of spontaneously fermented Greek wheat based sourdoughs was expended, through the study of sourdoughs originating from regions of Greece not previously assessed.

Keywords: Greek wheat sourdough; lactic acid bacteria; yeasts; identification; RAPD-PCR; PCR-DGGE;

2. Introduction

Sourdough is considered as one of the most ancient natural starters, used for the production of leavened baked goods (Gobbetti et al., 2016, De Angelis et al., 2019). Traditionally its preparation includes a mixture of cereal flour, usually wheat or rye and water, with concomitant fermentation by lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2014). Depending on the desired technological characteristics of the final product, different fermentation conditions are applied. Generally, three distinct types of sourdoughs have been defined so far, according to the technology and inoculum applied (De Vuyst et al., 2014, Paramithtiotis & Drosinos, 2017, Van Kerrebroeck et al., 2017). Type I sourdoughs are firm sourdoughs and their production is based on daily refreshments or back-sloppings, performed at ambient temperature, for 24 h or less, to keep the microorganisms metabolically active. Type I sourdoughs are further separated into Type 1a, which comprise of pure culture sourdough starters of different origin; Type 1b, which include spontaneously fermented sourdoughs, produced with daily refreshments; Type 1c, which originate from tropical regions and are fermented at high temperatures. Type II sourdoughs are semi-liquid sourdoughs, performed on a single fermentation step, with the addition of a starter culture. Longer duration and higher temperature, compared to Type I sourdoughs, are applied for acidification purposes. Their production is preferred by industrial bakeries. Finally, Type III sourdoughs are dried sourdoughs, initiated by defined starter cultures and followed by daily refreshments. The addition of baker's yeast is necessary for leavening purposes.

Over the past few years, the microecosystem of spontaneously fermented sourdoughs of different origins has been the epicenter of intensive study (Bartkiene et al., 2020, Boreczek et al., 2020, Comasio et al., 2020). LAB and yeasts represent the sourdough microbiota and their metabolic activity has been reported to exert beneficial effects on the shelf life,texture and taste of breads (Bockwoldt et al., 2020). Several authors have previously reported that the LAB to yeast ratio ranges between 10:1–100:1 (De Vuyst et al., 2017, Fraberger et al., 2020). The type of flour used, percentage of sourdough inoculum, pH, fermentation time, fermentation temperature and number of daily refreshments represent some of the factors determining the microbial diversity of sourdough

ecosystems (Gobbetti et al., 2016, Reale et al., 2019).

Sourdough fermentation is a dynamic process, during which fast acidifying LAB dominate theearly stages of fermentation, then typical sourdough LAB prevail and final stages of fermentation are dominated by highly adapted sourdough LAB (Huys et al., 2013). Type I sourdoughs, in which lower incubation temperatures are applied, obligate heterofermentative lactobacilli (formerly belonging to the *Lactobacillus* genus) such as *Fr. sanfranciscensis*, *Lv. brevis*, *Lm.fermentum* and facultative heterofermentative *Lp. plantarum, Cp. paralimentarius* have been previously reported to dominate sourdough processes (Paramithiotis & Drosinos, 2017, Minervini et al., 2012). Other LAB species belonging to *Leuconostoc*, *Lactococcus*, *Enterococcus, Weissella* and *Pediococcus* genera have been identified as additional populations. Regarding yeast diversity, the 6 most frequently detected species in sourdoughs of different origin are *S. cerevisiae, C. humilis* (reassigned as *K. humilis*), *T. delbrueckii*, *W. anomalus*, *K. exigua* and *Pi. kudriavzevii* (De Vuyst et al., 2016). The stable association between maltose positive *Fr. sanfranciscensis* and maltose negative *K. humilis* due to the lack of antagonism for maltose has been stated by many authors (Van Kerrebroeck et al., 2017, Comasio et al., 2020).

The microecosystem composition of spontaneously fermented Greek wheat sourdoughs has been previously described by de Vuyst et al. (De Vuyst et al., 2002) and Paramithiotis et al. (Paramithiotis et al., 2000, 2010). The dominance of *Fr. sanfranciscensis* in sourdoughs from Attica, Viotia and Thessaly, *Lv. brevis* in sourdoughs from Evia and *Lp. plantarum* in sourdoughs from Peloponnesus has been reported (De Vuyst et al., 2002, Paramithiotis et al., 2010). Regarding yeast diversity of Greek sourdough samples, dominance of *S. cerevisiae* in sourdoughs from Attica, Evia and Viotia and *T. delbrueckii* in sourdoughs from Thessaly and Peloponnesus has been documented (Paramithiotis et al., 2000, 2010). Other LAB such as *Cp. paralimentarius*, *Lv. zymae*, *W. cibaria* and *Pd. pentosaceus* and yeast species such as *Pi. membranifaciens* and *Y. lipolytica* have been identified as complementary populations.

Considering the limitations encountered during conventional plating, which has been recognized as a labor intensive method, frequently followed by incomplete isolation and identification of microorganisms that may depend upon selective

enrichment and subculturing, a great variety of natural food microecosystems has been unraveled with the combined application of both culture-dependent and -independent methods (Blaiotta et al., 2018, Boreczek et al., 2020, Comasio et al., 2020, Anguita-Maeso et al., 2020). In fact, conventional plating and molecular characterization with PCR-RAPD, combined with PCR-DGGE population profiling, have been successfully applied on sourdough ecosystems (Vogelmann et al., 2009, Minervini et al., 2010, Aponte et al., 2013, Ruiz Rodríguez et al., 2016).

The aim of the present study was to elucidate the microecosystem of 13 homemade spontaneously fermented wheat sourdoughs, 12 of which were collected from regions of Greece not previously assessed, namely Aetolia-Acarnania, Thessaloniki, Arkadia and Salamis island. In addition, the combined use of culture-dependent (classical approach, clustering by RAPD-PCR and identification by PCR species-specific for *Lp. plantarum*, and sequencing of the 16S-rRNA and 26S-rRNA gene, for LAB and yeasts, respectively) and independent approaches (DNA- and RNA- based PCR-DGGE) allowed a comparative assessment of their accuracy and complementarity.

3 Materials and Methods

3.1 Sampling

A total of 13 homemade spontaneously fermented wheat sourdough samples were analyzed (Table 2.1). Sourdoughs were prepared according to local traditions; the initial sourdough was prepared by mixing flour, water and the ingredient mentioned in Table 2.1, propagation took place by back-slopping at weekly intervals. Samples were aseptically collected, stored at 4 °C, transported to the laboratory and analyzed the same day.

Table 2. 1. Sourdough samples analyzed in the present study.

Sample No.	Origin	Ingredients [a]
1	Aetolia-Acarnania	Basil
2	Aetolia-Acarnania	Basil
3	Aetolia-Acarnania	Basil
4	Aetolia-Acarnania	Basil
5	Arkadia	Basil
6	Aetolia-Acarnania	Basil
7	Aetolia-Acarnania	Basil
8	Thessaloniki	Milk
9	Thessaloniki	Basil
10	Thessaloniki	No details available
11	Thessaloniki	Yoghurt
12	Viotia	Basil
13	Salamis island	Basil

[a] wheat flour is common ingredient for all samples.

3.2 Physicochemical characterization

The pH value was recorded by immersing the electrode (WTW, Weilheim, Germany) into the sourdough. Sourdough samples (10 g) were homogenized with 90 mL of distilled water using Stomacher apparatus (Seward,London, UK). The acidity (TTA) was titrated using 0.1 N NaOH and expressed in % lactic acid.

3.3 Microbiological analyses

Sourdough samples (10 g) were aseptically homogenized with 90 mL sterile ¼ Ringer solution using Stomacher apparatus. Lactic acid bacteria and yeasts were enumerated by plating serial dilutions on de Mann Rogosa and Sharpe (MRS) agar (LAB M, Lancashire, UK) and Rose Bengal Chloramphenicol (RBC) agar (LAB M), respectively. MRS plates were incubated at 30 ^{o}C for 48 h under microaerophilic conditions and RBC plates at 25 ^{o}C for 5 d under aerobic conditions. From each sample a number of colonies, selected according to the representative sampling scheme of Harrigan and McCance (1976), were purified by successive subculturing on MRS and Brain Heart Infusion (BHI) agar, for LAB and yeasts, respectively. LAB and yeast isolates were stored at -20 ^{o}C in Nutrient broth (LAB M), supplemented with 50% glycerol.

3.4 Culture-dependent assessment of the sourdough microecosystem

3.4.1 Classical identification

The phenotypic identification scheme described by Kurtzman at al. (2011) was employed in the case of yeast isolates. The tests performed included examination of morphological characteristics, ability to ferment carbohydrates (D-galactose, D-glucose, lactose, maltose, and sucrose), assimilate carbon (L-arabinose, cellobiose, citric acid, ethanol, D-galactose, D-glucose, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-ribose, sucrose, a-trehalose, and D-xylose) and nitrogen sources (cadaverine, creatine, ethylamine, imidazole, L-lysine, nitrate, and nitrite), as well as ability to grow at 35, 37 and 40 °C, in the presence of 50 and 60% glucose, 1% acetic acid and 0.,01% cycloheximide. Finally, the ability of the yeast isolates to produce acetic acid, form starch and hydrolyze urea was also examined.

In the case of LAB, phenotypic identification was carried out according to the second edition of the Bergey's Manual of Systematic Bacteriology. It included examination of morphological characteristics, Gram stain, the ability to produce CO_2 from glucose, grow at 15 and 45 ^{o}C, as well as the ability to ferment a range of carbohydrates (cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannitol, melibiose, raffinose, D-ribose, sorbitol, sucrose, a-trehalose, and D-xylose).

3.4.2 Molecular identification

DNA was extracted from the microorganisms according to Doulgeraki et al. (2010). Clustering of both LAB and yeast isolates was performed by PCR-RAPD using M13 as primer, according to Hadjilouka et al. (2014). DNA fragments were separated by electrophoresis in 1.5% agarose gel in 1.0X Tris Acetate EDTA (TAE) at 100 V for 1.5 h and visualized by ethidium bromide staining. Gels were scanned with GelDoc system (BioRad, Hercules, CA, USA). Bionumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used for conversion, normalization and further analysis, applying the Pearson coeffi*c*ient and UPGMA cluster analysis. For species identification, one to threerepresentative microbial strains from each cluster were subjected to sequencing of the V1–V3 region of 16S-rRNA gene and the D1/D2 region of 26S-rRNA gene, for LAB and yeast isolates, respectively, according to Doulgeraki et al. (2010). Species-specific

PCR was also applied according to Berthier and Ehrlich (2006) to separate *Lp. plantarum* from the *Lp. plantarum* group of species.

3.5 Culture-independent assessment of the sourdough microecosystem (PCR-DGGE)

DNA and RNA were extracted from the sourdough samples according to Doulgeraki et al. (2010) in the first case and using the NucleoSpin® RNAkit (Macherey-Nagel, Dueren, Germany) in the second. In the latter case, cDNA was synthesized using the PrimeScript™RT reagent kit (Takara, Kusatsu, Japan). As far as DNA and cDNA fragments are concerned, they were subjected to two PCR reactions. The approximately 250 nucleotides of the 5' end of the 26S rRNA gene and the V6–V8 region of the 16S rRNA gene were amplified by PCR, in a final volume of 50 μL, using NL1 with a GC clamp and LS2 as primers in the first case and U968 with a GC clamp and L1401 in the latter one, in agreement with Paramithiotis et al. (2014, 2016). PCR products were separated using the DCode Universal Mutation Detection System (Bio-Rad) with 8 % (w/v) polyacrylamide gel containing urea-formamide (Applichem, Darmstadt, Germany) as denaturing agents in a concentration gradient from 20 to 60 % in TAE buffer (40 mM Tris– acetate, 2 mM Na_2EDTA H_2O, pH 8.5). Electrophoresis took place at 50 V for 10 min and then 200 V for 4 h. Then, gels were visualized by ethidium bromide staining and photographed using a GelDoc system (Bio-Rad). Species identification was performed by co-migration with reference patterns.

3.6 Statistical analysis

The differences between the sourdough samples based on the measured physicochemical and microbiological parameters were evaluated using the correlation-based Principal Component Analysis (PCA) function embedded in the PAST v4.0 software (Hammer et al., 2001).

4 Results

4.1 Physicochemical and microbiological characterization

In Table 2.2, the physicochemical and microbiological characteristics of thirteen Greek wheat sourdoughs are presented. pH values ranged from 3.64 to 5.05, with sourdough samples 5, 6 and 13 having the more acidic pH values, while samples 10 and 12, presented pH values of approximately 5. TTA values ranged from 0.50 to 1.59 % lactic acid, with the former belonging to sourdough sample 12 and the latter to sample 1. Yeast and LAB populations ranged within 4.60-6.32 and 6.28-9.20 log CFU/g, respectively. Samples 10 and 12 showed a deviation (Figure 2.1) during the fermentation process presenting high pH (around 5.0) and low TTA (low lactic acid production). The causes were the low presence (concentration) of LAB in sample 10 (Table 2.2) and/or the low prevalence (6.25%) of highly acid-producing strains (e.g. *Lp. plantarum*) in sample 12 (Figure 2.4).

Table 2. 2. Physicochemical and microbiological data of 13 Greek wheat sourdoughs.

Sample No	pH	TTA [a]	Yeasts [b]	LAB [b]
1	3.76 (0.01)	1.59 (0.01)	4.60	7.00
2	3.91 (0.13)	0.79 (0.13)	6.20	7.57
3	3.91 (0.07)	0.70 (0.07)	6.32	9.20
4	3.72 (0.01)	0.85 (0.01)	5.23	8.20
5	3.64 (0.07)	0.99 (0.07)	5.36	8.26
6	3.65 (0.01)	0.98 (0.01)	5.30	9.18
7	3.85 (0.01)	1.23 (0.01)	5.28	8.18
8	3.76 (0.04)	1.21 (0.04)	5.08	8.08
9	3.75 (0.01)	1.03 (0.01)	5.94	8.23
10	5.05 (0.01)	0.65 (0.01)	4.78	6.28
11	3.80 (0.06)	1.10 (0.06)	6.08	8.32
12	4.96 (0.03)	0.50 (0.03)	6.30	8.20
13	3.64 (0.04)	0.70 (0.04)	6.30	8.36

All determinations were performed in triplicate. Standard deviation is given in parenthesis.
a TTA: Total Titratable Acidity, % lactic acid; **b** Microbial populations in log CFU/g.

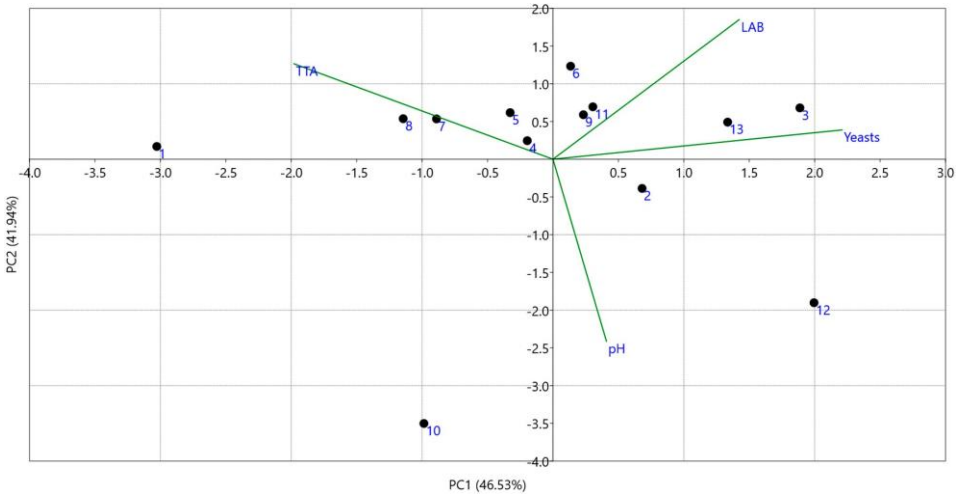

Figure 2. 1. Correlation-based Principal Component Analysis (PCA, biplot) of the sourdough samples with the measured physicochemical and microbiological parameters of pH, total titratable acidity (TTA, in % lactic acid), lactic acid bacteria (LAB, in log CFU/g) and yeasts (in log CFU/g) concentrations.

4.2 Culture-dependent assessment of microbiota

A total of 207 bacterial and 195 yeast isolates were obtained from 13 Greek wheat sourdoughs and subjected to evaluation of their biochemical properties, according to the respective classical identification schemes, as well as PCR-RAPD.

In Tables S2.1–S2.4 the biochemical tests used for the identification of yeast and bacterial strains, respectively, are presented. Based on these data, the yeast isolates were separated into 5 groups. The majority of the isolates (151) were clustered in group 4 and assigned to the *S. cerevisiae* species. The remaining isolates formed 4 groups and were identified as *K. humilis* (group 1), *Pi. fermentans* (group 2), *Pi. membranifaciens* (group 3) and *W. anomalus* (group 4). Most of the bacterial isolates were grouped into two groups, namely 1 and 2. These isolates were assigned to *Lp. plantarum* and *Lv. brevis* species, respectively. The remaining isolates were classified as *Cp. paralimentarius* (group 3), *Lv. zymae* (group 4), *Lt. curvatus* (group 5), *Lt. sakei* (group 6), *Leu. citreum* (group 7), *Leu. mesenteroides* (group 8), *Lc. lactis* (group 9) and *Fr. sanfranciscensis* (group 10).

Application of PCR-RAPD to the bacterial and yeast isolates resulted in their separation into 27 and 20 clusters, respectively (Figures 2.2 and 2.3). Representative bacterial and yeast isolates were subjected to partial 16S and 26S rRNA gene sequencing, respectively, and the resulting taxonomic affiliation is

presented in Tables 2.3 and 2.4. In addition, the identity of the bacterial isolates that were assigned to *Lp. plantarum* by 16S-rRNA gene sequencing, was verified by species-specific PCR.

Table 2. 3. Taxonomic affiliation of bacterial strains based on sequencing of the V1–V3 region of the 16S rRNA gene.

Strain Number	Closest Relative	Accession Number	Identity (%)
LQC 2322	*Cp. paralimentarius*	KX247775.1	100
LQC 2323	*Cp. paralimentarius*	MF540546.1	100
LQC 2338	*Lv. brevis*	MN166306.1	100
LQC 2339	*Lv. brevis*	LC199964.1	100
LQC 2362	*Lv. brevis*	MN720522.1	100
LQC 2381	*Cp. paralimentarius*	MH544805.1	100
LQC 2389	*Cp. paralimentarius*	MH544805.1	100
LQC 2391	*Cp. paralimentarius*	MH544805.1	100
LQC 2394	*Lv. zymae*	KT757254.1	100
LQC 2395	*Cp. paralimentarius*	MF942368.1	100
LQC 2398	*Lv. brevis*	CP031174.1	100
LQC 2404	*Cp. paralimentarius*	KY435699.1	100
LQC 2408	*Fr. sanfranciscensis*	MH704126.1	100
LQC 2410	*Cp. paralimentarius*	KC755102.1	100
LQC 2412	*Lv. brevis*	MN431348.1	100
LQC 2428	*Fr. sanfranciscensis*	LC483557.1	100
LQC 2430	*Lv. brevis*	MN049503.1	100
LQC 2440	*Lv. brevis*	MG646821.1	100
LQC 2456	*Lt. sakei*	MF428782.1	100
LQC 2458	*Lv. brevis*	MN720508.1	99
LQC 2473	*Lt. sakei*	MG462120.1	100
LQC 2475	*Lt. curvatus*	MN720519.1	100
LQC 2494	*Lv. brevis*	KX649032.1	100
LQC 2508	*Leu. citreum*	MG754627.1	100
LQC 2510	*Lc. lactis*	MN368062.1	100
LQC 2511	*Lv. brevis*	MH681603.1	100
LQC 2512	*Leu. mesenteroides*	MG825699.1	100
LQC 2517	*Cp. paralimentarius*	MH544773.1	100
LQC 2537	*Cp. paralimentarius*	MH704124.1	100

Table 2. 4. Taxonomic affiliation of yeast strains based on sequencing of the D1/D2 region of the26S rRNA gene.

Strain Number	Closest Relative	Accession Number	Identity (%)
LQC 10300	*S. cerevisiae*	JQ771733.1	100
LQC 10306	*S. cerevisiae*	JQ771733.1	100
LQC 10308	*S. cerevisiae*	CP025108.1	100
LQC 10313	*S. cerevisiae*	MK397410.1	99
LQC 10341	*S. cerevisiae*	MN462945.1	100
LQC 10345	*K. humilis*	MK262977.1	100
LQC 10347	*Pi. fermentans*	KJ413162.1	98
LQC 10350	*Pi. fermentans*	KM589485.1	99
LQC 10351	*S. cerevisiae*	JQ771733.1	100
LQC 10353	*W. anomalus*	MH479120.1	99
LQC 10355	*Pi. fermentans*	KY296092.1	99
LQC 10361	*W. anomalus*	LC178747.1	99
LQC 10366	*S. cerevisiae*	MK358167.1	100
LQC 10369	*S. cerevisiae*	MG017585.1	100
LQC 10373	*S. cerevisiae*	MG017587.1	100
LQC 10388	*S. cerevisiae*	MG017572.1	100
LQC 10389	*S. cerevisiae*	MK358167.1	99
LQC 10391	*S. cerevisiae*	MK027355.1	99
LQC 10399	*S. cerevisiae*	HM191654.1	100
LQC 10403	*S. cerevisiae*	MF521985.1	100
LQC 10406	*S. cerevisiae*	MG017572.1	100
LQC 10408	*S. cerevisiae*	MF979228.1	100
LQC 10412	*S. cerevisiae*	MH844381.1	100
LQC 10455	*S. cerevisiae*	MG386438.1	99
LQC 10459	*S. cerevisiae*	MG386438.1	99
LQC 10460	*S. cerevisiae*	MG017586.1	99
LQC 10466	*S. cerevisiae*	GU080045.1	99
LQC 10419	*S. cerevisiae*	MG386438.1	100
LQC 10420	*S. cerevisiae*	KF141642.1	100
LQC 10423	*Pi. membranifaciens*	KF141642.1	100
LQC 10432	*S. cerevisiae*	MF979228.1	100
LQC 10441	*Pi. membranifaciens*	KF141642.1	100
LQC 10447	*Pi. membranifaciens*	MK358179.1	99
LQC 10469	*S. cerevisiae*	MF521980.1	100
LQC 10472	*S. cerevisiae*	MN462933.1	100
LQC 10475	*S. cerevisiae*	MF979228.1	100
LQC 10476	*S. cerevisiae*	MK358167.1	100
LQC 10482	*S. cerevisiae*	MG017585.1	99

The majority of bacterial isolates were identified as *Lp. plantarum* (34.94 %) and *Lv. brevis* (34.08 %). In addition, *Cp. paralimentarius* (13.93 %), *Fr. sanfranciscensis* (6.15 %), *Lt. sakei* (5.33 %), *Lt. curvatus* (2.66 %), *Lv. zymae* (0.43 %), *Lc. lactis* (1.51 %), *Leu. citreum* (0.48 %) and *Leu. mesenteroides* (0.48 %) were also detected. As far as yeasts were concerned, *S. cerevisiae* represented the primary microbiota (84.1 %) in the examined sourdoughs, while the presence of *Pi. membranifaciens* (10.3 %), *Pi. fermentans* (2.8 %), *W. anomalus* (2.1 %) and *K. humilis* (0.7 %) was also documented.

The bacterial and yeast microecosystem composition of the sourdough samples examined is presented in Figures 2.4 and 2.5, respectively. Regarding the bacterial biota of the examined sourdoughs, *Lp. plantarum* and *Lv. brevis* were recorded as the dominant species, forming the primary microbiota in sourdoughs 1, 3 and 11 and 4, 8, 12 and 13, respectively. In addition, in sourdoughs 2 and 10,a co-dominance of the two LAB species was observed as they formed the 100% and the 73.7% of the bacterial biota, respectively. On the other hand, *Cp. paralimentarius* dominated sourdoughs 5 and 6 (61.11 and 60 % of the bacterial biota, respectively), while *Fr. sanfranciscensis* was the dominant member of the LAB biota only in sourdough 7 and *Lt. sakei* in sourdough 9. From a microbial diversity point of view, sourdough 12, exhibited a rather diverse LAB micro-community consisting of *Lp. plantarum*, *Lv. brevis*, *Lt. curvatus*, *Lc. lactis*, *Leu. mesenteroides* and *Leu. citreum*.

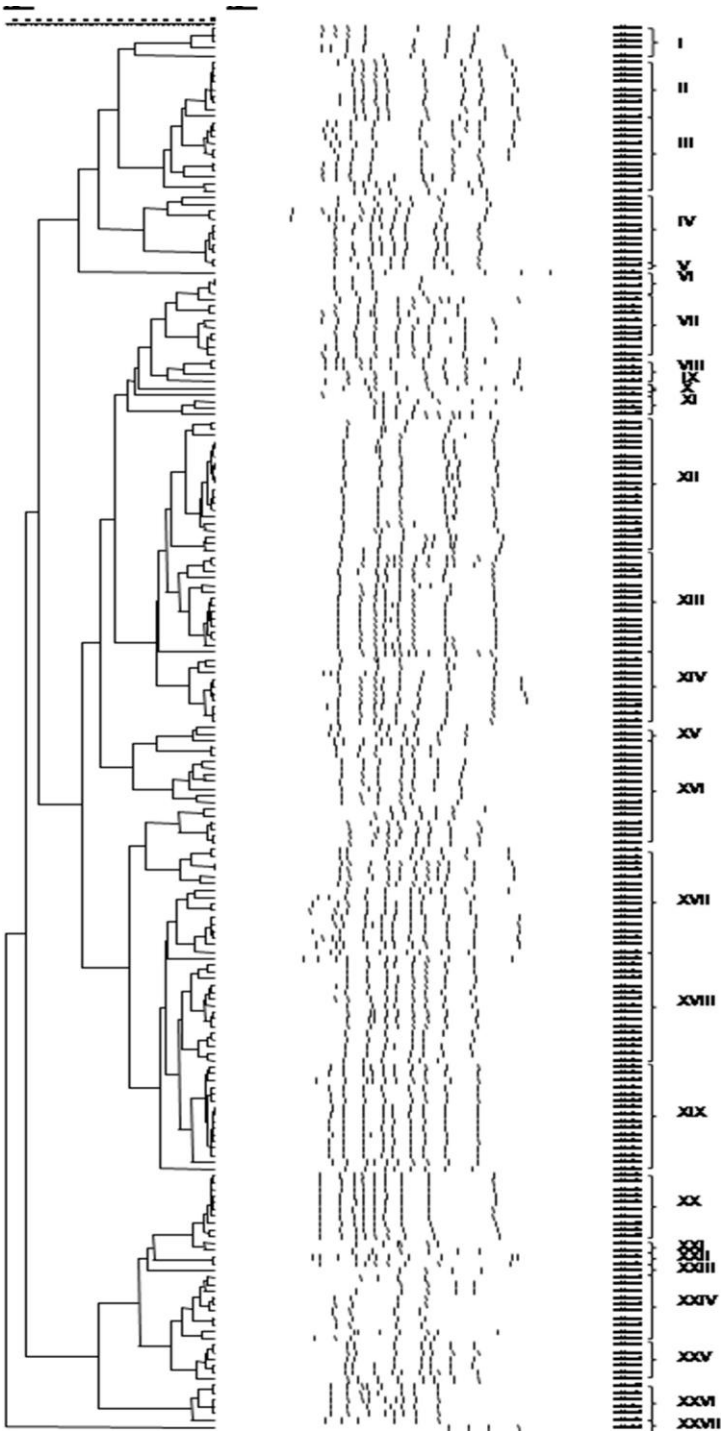

Figure 2. 2. Cluster analysis of PCR-RAPD patterns of bacterial isolates, obtained from thirteen Greek wheat sourdoughs. Distance is indicated by the mean correlation coefficient [r (%)] and clustering was performed by UPGMA analysis. The representative strains selected for 16S rRNA gene sequencing are underlined. Latin numerals designate bacterial species (I, II, III, VIII, XXIII and XXVII: *Cp. paralimentarius*, IV, VI, VII, XII, XIII and XIV: *Lp. plantarum*, V: *Leu. citreum*, IX: *Lv. zymae*, X: *Leu. mesenteroides*, XI: *Lc. lactis*, XV, XVI, XVII, XVIII, XIX and XXV: *Lv. brevis*, XX and XXII: *Lt. sakei*, XXI and XXIV: *Fr. sanfranciscensis*, XXVI: *Lt. curvatus*).

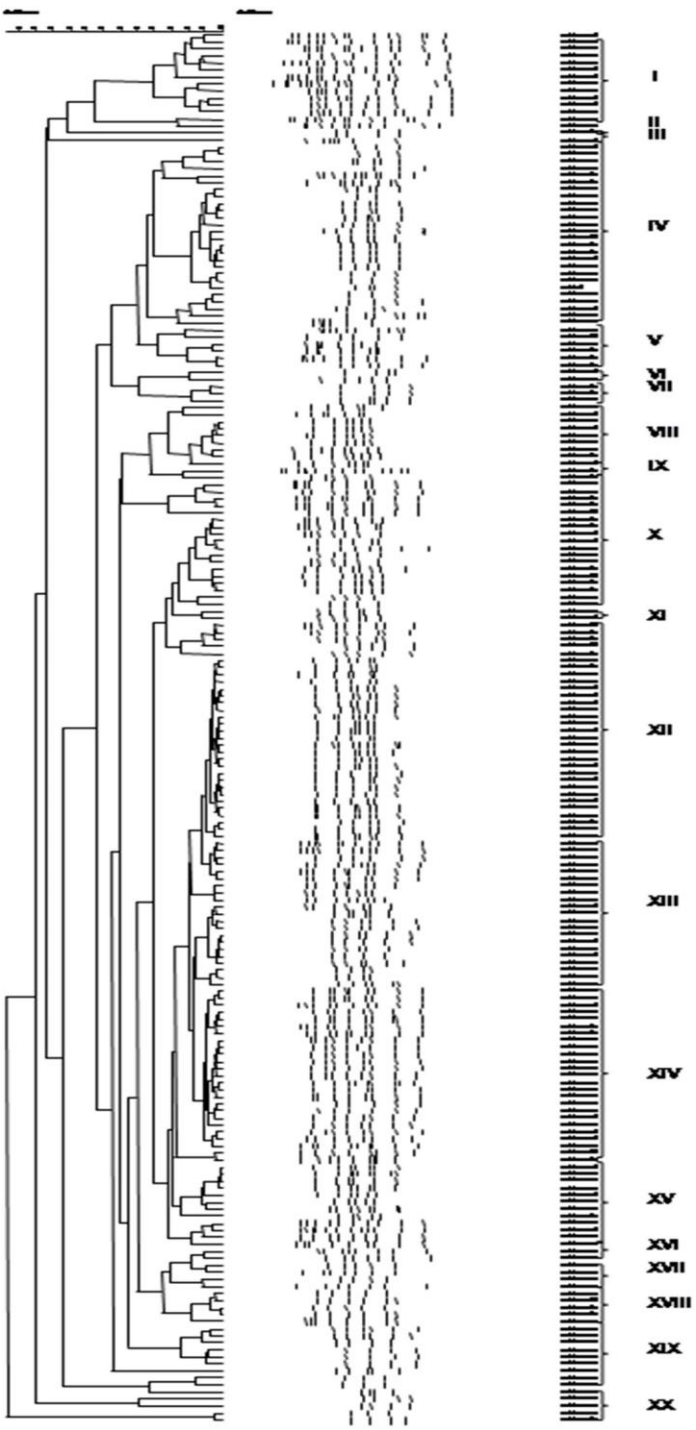

Figure 2. 3. Cluster analysis of PCR-RAPD patterns of yeast isolates, obtained from thirteen Greek wheat sourdoughs. Distance is indicated by the mean correlation coefficient [r (%)] and clustering was performed by UPGMA analysis. The representative strains selected for 26S rRNA gene sequencing are underlined. Latin numerals designate yeast species (I, VI and XVI: *Pi. membranifaciens*, II, IV, V, VIII, X, XII, XIII, XIV, XV, XIX and XX: *S. cerevisiae*, III, IX and XVIII: *Pi. fermentans*, VII and XVII: *W. anomalus*, XI: *K. humilis*).

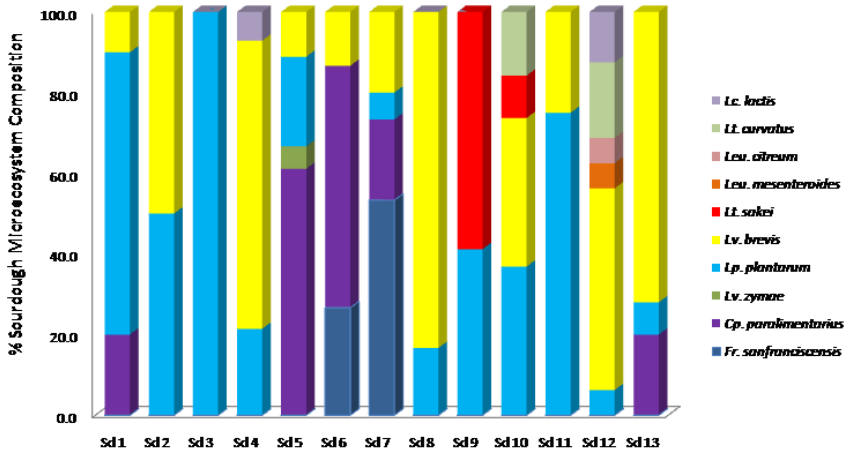

Figure 2. 4. Bacterial microecosystem composition of 13 Greek wheat spontaneous fermented sourdough samples. Sd: sourdough.

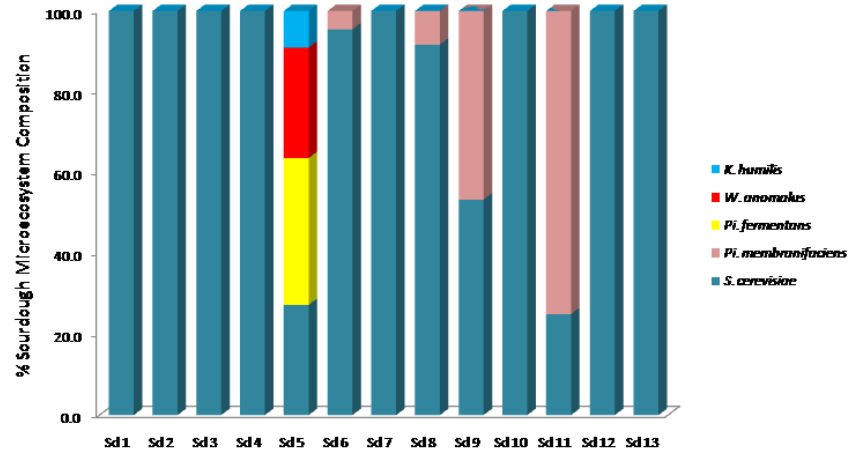

Figure 2. 5. Yeast microecosystem composition of 13 Greek wheat spontaneous fermented sourdough samples. Sd: sourdough.

As far as the yeast microecosystem composition was concerned, *S. cerevisiae* dominated 11 of the 13 wheat sourdoughs (1, 2, 3, 4, 6, 7, 8, 9, 10, 12 and 13), while representing the only species isolated from sourdoughs 1, 2, 3, 4, 7, 10, 12 and 13. *Pi. membranifaciens* was the dominant species of the yeast biota in sourdough 11 and was recorded as secondary microbiota in sourdoughs 6, 8 and 9. Regarding the yeast diversity, sourdough 5 contained four species, with *Pi. fermentans* forming the primary microbiota (36.4%), while *S. cerevisiae*, *W. anomalus* and *K. humilis* were present as additional yeast population.

4.3 Culture-independent assessment of microbiota (PCR-DGGE)

Microbial diversity of 13 Greek wheat sourdoughs was further investigated with PCR-DGGE. In brief, total DNA and RNA were extracted directly from the sourdough samples, cDNA was synthesized by the latter and both were subjected to PCR-DGGE analysis to profile microbial composition. The main limitation encountered was the co-migration of amplicons from different species to the same electrophoretic positions within DNA and cDNA DGGE gels, thus leading to their incomplete discrimination. Four pairs of species, namely *Lp. plantarum* and *Leu. mesenteroides*, *Lv. brevis* and *Lv. zymae*, *Fr. sanfranciscensis* and *Lc. lactis* and finally *Lt. curvatus* and *Lt. sakei*, presented with such a limitation. In addition, the presence of a multiband profile of specific isolates, such as *Fr. sanfranciscensis* and *Leu. mesenteroides*, represented another artifact generated during PCR-DGGE. PCR-DGGE profiles of the examined sourdoughs at both DNA and cDNA level, are shown in Figure 2.6A, 6B. Many similarities were detected between bacterial DNA and cDNA DGGE gels. The only difference was detected in sourdough 9, in the profile of which that originated from DNA, contained an additional band corresponding to *Lt. curvatus* or *Lt. sakei*, which was not present at the cDNA DGGE profile. The culture-independent approach revealed a different bacterial ecology of the examined sourdoughs, compared to the culture-dependent one. In more detail, in both DNA and cDNA DGGE gels, a stable band, corresponding to *Fr. sanfranciscensis* or *Lc. lactis*, was evident at sourdough samples 2, 3, 5, 6, 7, 8, 9, 10, 11, 12 and 13; however, the presence of these bacterial species was verified by culture-dependent approach only for sourdoughs 6 and 7. In addition, the presence of *Cp. paralimentarius* at both DNA and cDNA level was revealed in sourdough 4, opposing conventional plating and molecular identification, which were not able to detect it. In addition, PCR-DGGE only partially verified the microbial ecology detected by culture-dependent approach in the majority of sourdough samples, since bacterial species *Lp. plantarum*, *Lt. curvatus*, *Leu. mesenteroides*, *Leu. citreum* and *Lv. brevis*, *Lt. curvatus* and *Lt. sakei* previously identified in sourdoughs 12 and 10, respectively, were not visible as bands in the gels. Similar was the case for *Cp. paralimentarius*, *Lv. brevis* and *Lp. plantarum* species in sourdough sample 7, which although identified by culture dependent approach, PCR-DGGE failed to detect them.

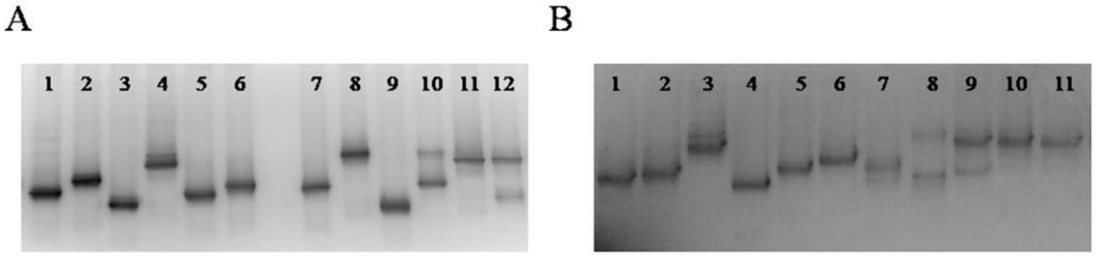

Figure 2. 6. Bacterial DGGE profiles of nucleic acids extracted directly from sourdough samples. A: DNA. Column 1, *Lv. brevis*; Column 2, *Lp. plantarum*; Column 3, *Cp. paralimentarius*; Column 4, *Fr. sanfranciscensis*; Column 5, *Lv. zymae*; Column 6, *Lt. sakei*; Column 7, *Lt. curvatus*; Column 8, *Lc. lactis*; Column 9, *Leu. citreum*; Column 10, *Leu. mesenteroides*; Column 11, Sourdough 3; Column 12, Sourdough 2; B: RNA. Column 1, *Lt. sakei*; Column 2, *Lt. curvatus*; Column 3, *Fr. sanfranciscensis*; Column 4, *Cp. paralimentarius*; Column 5, *Lv. brevis*; Column 6, *Lp. plantarum* 2; Column 7, *Lv. zymae*; Column 8, Sourdough 13; Column 9, Sourdough 5; Column 10, Sourdough 7; Column 11, Sourdough 8.

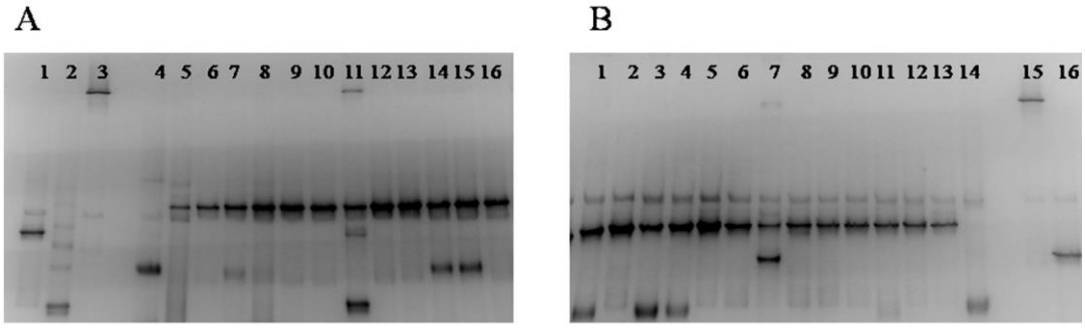

Figure 2. 7. Yeast DGGE profiles of nucleic acids extracted directly from sourdough samples. (A): DNA. Column 1, *K. humilis*; Column 2, *Pi. fermentans*; Column 3, *W. anomalus*; Column 4, *Pi. membranifaciens*; Column 5, sourdough 1; Column 6, sourdough 2; Column 7, Sourdough 11; Column 8, sourdough 3; Column 9, sourdough 12; Column 10, sourdough 4; Column 11, Sourdough 5; Column 12, Sourdough 6; Column 13, Sourdough 7; Column 14, Sourdough 8; Column 15, Sourdough 9; Column 16, Sourdough 10. (B): RNA. Column 1, Sourdough 11; Column 2, Sourdough 1; Column 3, Sourdough 9; Column 4, Sourdough 8; Column 5, Sourdough 2; Column 6, Sourdough 3; Column 7, Sourdough 5; Column 8, Sourdough 4; Column 9, Sourdough 6; Column 10, Sourdough 7; Column 11, Sourdough 10; Column 12, Sourdough 12; Column 13, Sourdough 13; Column 14, *Pi. membranifaciens*; Column 15, *W. anomalus*; Column 16, *K. humilis*

The yeast microecosystem of the sourdough samples analyzed was less complicated than the bacterial one. Yeast DGGE profiles, resulting from direct extraction of DNA and RNA from the examined sourdoughs, are shown in Figure 2.7A, B. No differences in the DGGE profiles of both DNA and RNA extracted from sourdough samples were detected. The limitation of a multiband profile was encountered again for all yeast species. A stable band, belonging to *S. cerevisiae* was present in DGGE gels, in accordance with the results obtained by culture-dependent method. Although yeast DGGE profiles were in complete agreement with the results of conventional plating

and molecular identification, in sourdough 6, the yeast species *Pi. membranifaciens*, previously isolated via culturing method, was not detected by PCR-DGGE.

5 Discussion

Sourdough microecosystem assessment has been the epicenter of thorough study, over the last decades, due to the quality of the sourdough bread and its health promoting attributes (Comasio et al., 2020). Both microbiological stability of the final product and the release of functional compounds during fermentation are strictly determined by the associated microbiota, mainly LAB and yeasts. Spontaneously fermented Greek wheat sourdoughs are classified as type Ib, in which heterofermentative LAB, single or combined with homofermentative ones, are frequently harbored (Paramithiotis et al., 2010, Gänzle et al., 2019). Type I sourdoughs are based on a three-stage preparation procedure, which includes three daily refreshments, to keep microorganisms in a metabolically active state (Huys et al., 2013).

In the present study, the majority of spontaneously fermented Greek wheat sourdoughs exhibited pH values ranging between 3.64 and 3.91 and TTA measurements between 0.70 and 1.59, consistent with previously reported data from Austrian, Italian and Greek sourdoughs (Fraberger et al., 2020, Minervini et al., 2012, Paramithiotis et al., 2010). However, sourdough samples 10 and 12 presented higher pH values, 5.05 and 4.96, respectively, which was also documented in French wheat sourdoughs (Robert et al., 2009), but in this case, the failure of the fermentation process was the most probable reason. pH and acidity values of sourdough samples could be correlated with the metabolic activity of LAB. Regarding LAB and yeast enumeration, the viable cell counts ranged from 6.28–9.20 and from 4.60–6.32 log CFU/g, respectively, consistent with a previous study by Fraberger et al. (2020). In addition, the yeast: LAB ratio of the 13 sourdoughs ranged between 1:23–1:10,000, in agreement with previous findings from European sourdoughs (Fraberger et al., 2020, Lhomme et al., 2015).

The culture-dependent approach, including PCR-RAPD analysis, with M13 primer, has been extensively applied for complete differentiation at species level of microorganisms isolated from sourdough (Minervini et al., 2018, Palla et al., 2020, Üçok et al., 2020) and other food matrices, such as cheese (Caro et al., 2020, Zago et al., 2021), meat (Raimondi et al., 2018, Settanni et al., 2020) and wine (Diaz-

Ozaetaa et al., 2019).The identification of sourdough yeasts and LAB was based on 26S and 16S rRNA gene sequencing, respectively; however, this standard approach does not allow differentiation between closely relatedspecies. This is the reason why species-specific PCR was applied, to specifically detect *Lp. plantarum* species (Berthier et al., 2006). The results obtained by the genotypic clustering through PCR-RAPD were in total agreement with the ones achieved through classical identification procedures, exhibiting the robustness and reliability of the former approach.

As far as the sourdough microecosystem composition was concerned, the number of bacterial species harbored in the 13 wheat sourdoughs ranged from 1 to 6, in agreement with previous data (Dertli et al., 2016, Fraberger et al., 2020). The fluctuated bacterial composition of the analyzed samples could be attributed to numerous intrinsic (e.g., type of flour, thus endogenous enzymes and microorganisms) and extrinsic factors (e.g., propagation process, redox potential, pH, fermentation time and temperature), which are selective factors for the growth rate of specific LAB species (Huys et al., 2013, Reale et al., 2019).

The majority of the examined sourdoughs was characterized by the stable presence of *Lp. plantarum* and *Lv. brevis*. *Cp. paralimentarius* was also frequently detected, as it was present in 5 sourdough samples. The occurrence of these LAB species in Greek wheat sourdoughs, has been previously reported (De Vuyst et al., 2002, Paramithiotis et al., 2010). Their frequent isolation from Italian, Austrian or Belgian sourdoughs has been welldocumented as well (Reale et al., 2019, Comasio et al., 2020, Fraberger et al., 2020). The prevalence of *Lp. plantarum* and *Lv. brevis* in sourdough ecosystem has been attributed to their stress adaptation responses to the household environmental conditions and to their metabolic versatility (Minervini et al., 2018). In particular, the robustness of *Lp. plantarum* is highly associated with its large genome size and its nomadic lifestyle, which both promote its presence in diverse environmental niches (Gänzle et al., 2019).

The obligate heterofermentative *Fr. sanfranciscensis*, which has been widely identified in wheat and rye sourdoughs throughout Europe (Gobbetti et al., 2016, Reale et al., 2019) , was found only in two sourdoughs, namely 6 and 7. In the first, it was detected as part of the secondary microbiota, while in the second, it dominated the bacterial microecosystem. In the same sourdoughs dominance of *S. cerevisiae* was also reported, thereby supporting the firm association between both maltose positive *Fr.*

sanfranciscensis and *S. cerevisiae* in type I sourdoughs (De Vuyst et al., 2014). *Fr. sanfranciscensis* is considered one of the most well adapted lactobacilli in the sourdough habitat and is further characterized by the capacity to use fructose as an external electron acceptor, with concomitant acetate production (Comasio et al., 2020, Huys et al., 2013). The dominance of *Fr. sanfranciscensis* and *Lp. plantarum* subsp. *plantarum* has already been reported in Greek sourdoughs from Thessaly and Peloponnesus, respectively (Paramithiotis et al., 2010). However, Bartkiene et al. (2020) documented that spontaneously fermented sourdoughs usually harbor nomadic microbiota, such as *Lp. plantarum*, while the frequency of *Fr. sanfranciscensis* is considered limited.

Other species such as *Lt. sakei*, *Lt. curvatus*, *Lv. zymae* and LAB cocci, *Lc. lactis*, *Leu. mesenteroides* and *Leu. citreum* were also sporadically present in the examined sourdoughs. Except for *Lv. zymae*, which has already been identified in spontaneously fermented Greek wheat and Italian wheat and rye-based sourdoughs (De Vuyst et al., 2002, Reale et al., 2019) , the rest of the LAB species have not been isolated from Greek wheat sourdoughs previously. *Lt. sakei* has been characterized by psychrotrophic attributes, which could justify its presence in sourdoughs based on daily refreshments, at ambient temperatures (Minervini et al., 2018). Previous studies have reported *Lt. sakei* as additional bacterial biota in Italian wheat (Minervini et al., 2012, Reale et al., 2019) and Finnish fava bean sourdoughs (Coda et al., 2017), while its identification as primary bacterial species in amaranth and buckwheat sourdoughs has been documented as well (Sterr et al., 2009, Moroni et al., 2011). As far as *Lt. curvatus* was concerned, was concerned, its isolation as subdominant species from Italian, Turkish and Austrian wheat based (Minervini et al., 2012, Dertli et al., 2016, Fraberger et al., 2020) and mixed wheat- and rye-based sourdoughs (Comasio et al., 2020), has been reported.

Finally, consistent with the present study, literature data have reported the occurrence of LAB species belonging to *Leuconostoc* and *Lactococcus* genera, as secondary microbiota (Paramithiotis & Drosinos, 2017, Huys et al., 2013). *Leuconostoc* and *Lactococcus* spp. are usually present at the early fermentation stages, since at the late stages of fermentation a decrease in their population has been observed, due to further acidic conditions. Well adapted species of *Leuconostoc* such as *Leu. citreum*, *Leu. mesenteroides* have been previously isolated as additional species from spontaneously fermented wheat sourdoughs (Reale et al., 2019, Fraberger et al., 2020). Concerning

Lc. lactis, its presence in fava bean- and quinoa-based spontaneously fermented sourdoughs, has been reported, usually at the first stages of propagation (Coda et al., 2017). However, Maidana et al. (2020) reported its identification by both culture-dependent and -independent methods between the sixth and tenth refreshment steps of chia sourdough fermentation.

Regarding yeast diversity, the 6 most frequently identified yeast species in type I sourdoughs are *S. cerevisiae, K. humilis, T. delbrueckii, W. anomalus, K. exigua* and *Pi. kudriavzevii* (De Vuyst et al., 2016). In the present study, 12 of the 13 examined sourdoughs harbored one or two yeast species, with *S. cerevisiae* and *Pi. membranifaciens* forming the primary and secondary yeast biota, respectively, consistent with previously reported data concerning Greek sourdoughs (Paramithiotis et al., 2000). However, sourdough 5 exhibited higher species diversity, comprised of *S. cerevisiae, Pi. fermentans, W. anomalus* and *K. humilis*, in a decreasing order of abundance. To our knowledge, it is the first time that identification of *Pi. fermentans, W. anomalus* and *K. humilis* is reported from spontaneously fermented Greek wheat sourdoughs. In the present study, *S. cerevisiae* was retrieved from all 13 sourdough samples, in accordance with previous studies (Fraberger et al., 2020, Boyaci-Gunduz & Erten, 2020). Its prevalence in sourdoughs of different origin has been partially attributed to the extensive use of baker's yeast, however its stable presence during spontaneous laboratory wheat and rye fermentations expresses the autochthonous flour origin of the specific species (Huys et al., 2013). In addition, *S. cerevisiae* ability to ferment the main flour carbohydrates (maltose, glucose, fructose and sucrose), thus justifying its metabolic versatility, has been previously reported (De Vuyst et al., 2016). Finally, opposing literature data supporting the dominant role of *S. cerevisiae* in sourdough ecosystem, a previous study concerning yeast biota of Greek wheat sourdoughs reported the presence of *S. cerevisiae* in one of ten examined sourdoughs, only as secondary yeast population (Paramithiotis et al., 2010).

Despite the fact that *Pi. membranifaciens* has been considered a less frequently isolated yeast species from sourdoughs, its presence in Greek sourdoughs has already been reported (Paramithiotis et al., 2000). Consistent with previous studies, *Pi. membranifaciens* was present in sourdough samples 6, 8 and 9 as secondary yeast biota, with *S. cerevisiae* forming the primary biota. The sub-dominant presence of *Pi. membranifaciens* could partly be attributed to its narrow metabolic profile (glucose

positive). However, in the present study, *Pi. membranifaciens* dominated sourdough 11, while previous data reported its co-dominance with *S. cerevisiae* (Boyaci-Gunduz & Erten, 2020). The presence of *Pi. membranifaciens* in Chinese traditional sourdoughs has been reported as well (Liu et al., 2018).

Pi. fermentans was retrieved as primary yeast biota in 1 of the 13 sourdough samples. It is the first study to report its Greek wheat sourdough origin, as in the case of *W. anomalus* and *K. humilis*. Although *Pi. fermentans* is not characterized by a frequent detection in sourdough samples, recent studies have already reported its identification as dominant or co-dominant yeast biota in Italian spelt, Turkish and Belgian rye fermented sourdoughs (Korcari et al., 2019, Comasio et al., 2020, Boyaci-Gunduz & Erten, 2020), respectively. The lack of metabolic versatility of *Pi. fermentans*, which is explained, in the present study, by its inability to ferment other flour carbohydrates than glucose, was consistent with previous data from Korcari et al. (2019). In the present study, *Pi. fermentans* represented the dominant yeast species isolated from sourdough 5, whereas maltose positive *S. cerevisiae* and *W. anomalus* and maltose negative *K. humilis* were also detected, suggesting a potent competitive interaction.

W. anomalus and *S. cerevisiae* as well, have been reported as generalist yeasts, with high adaptability to stressful conditions in terms of temperature, pH and osmolarity (Cappelli et al., 2014, De Vuyst et al., 2016). As far as *W. anomalus* is concerned, it has been characterized as highly competitive within a variety of ecological niches, which is partly attributed to its ability to ferment many carbon and nitrogen sources (Daniel et al., 2011). On that basis, the identification of *W. anomalus* in sourdough ecosystems of different origin has been repeatedly reported (Vrancken et al., 2010, Huys et al., 2013). In the present study, *W. anomalus* was isolated only from sourdough sample 5, present as secondary yeast biota with *S. cerevisiae*. Korcari et al. (2019) also reported the dominance of *W. anomalus* in spelt fermented sourdough, however its decline in wheat sourdoughs, after 21 back-slopping stages, was documented as well (Oshiro et al., 2019).

K. humilis, a maltose negative yeast species, has been considered as well adapted to the sourdough environment. Its stable association with maltose positive *Fr. sanfranciscensis* has been repeatedly reported in sourdoughs type I, due to the lack of antagonism for the main carbon source, maltose. Unlike *W. anomalus*, *K. humilis* is not considered an opportunistic pathogen since this maltose-negative yeast species cannot

grow at 37 ^{o}C. In this study *K. humilis* was present at 0.7 % of the total yeast isolates, in contrast to previous data reporting the presence of *K. humilis* as primary or secondary yeast biota in wheat and rye sourdoughs (Comasio et al., 2020, Fraberger et al., 2020). The inability of *K. humilis* to adapt to different carbon sources, combined with the detrimental effects of un-dissociated acetic acid or even lactic acid on its growth rate, could account for its low identification rates in the examined sourdoughs (Huys et al., 2013).

Regarding the culture-independent approach, PCR-DGGE has been extensively used for the assessment of microbial dynamics during milk (Sayevand et al., 2018, Maoloni et al., 2020), cheese (Ramezani et al., 2017, Unno et al., 2020) meat (Blaiotta et al., 2018, Cardinali et al., 2018), fish (Osimani et al., 2019) and tequila-based fermentations (Aldrete-Tapia et al., 2020). In the case of sourdough, PCR-DGGE, based on DNA extraction, has been previously employed by Palla et al. (2017), Reale et al. (2019) and Comasio et al. (2020), to elucidatethe sourdough microecosystem composition. In the present study, not only DNA, but also RNA were selected as the target nucleic acids, since DNA may persist in the environment after cell death and may interfere with the analysis, thus leading to the assessment of the history of a sample, rather than the characterization of the microecosystem composition at a given time. Despite the fact that RNA has been considered a better indicator of the microbial viability, compared to DNA, reverse transcription (RT)-PCR-DGGE has drawn less scientific attention, especially in sourdough microecosystem analysis.In fact, Dolci et al. (2013) reported that microecosystem composition in Fontina PDO cheese was better characterized by means of RT-PCR-DGGE, and thus, RNA represents a more informative target than DNA (Dolci et al., 2013, Garofalo et al., 2017). However, in the present study no differences in the bacterial and yeast DGGE profiles of both DNA and cDNA were observed, except for the DNA DGGE profile of sourdough 9, in which a band corresponding to *Lt. curvatus* or *Lt. sakei* was detected; however, this was not visible in the cDNA DGGE gel. Consistent with our present data, Iacumin et al. (2009) reported similar sourdough bacterial and yeast profiles both at DNA and RNA level, respectively, with the exception of a band belonging to *Lc. lactis*, which was only detected in DNA DGGE gel.

In the present study, biodiversity data resulting from PCR-DGGE analysis only partially verified the microbial community fingerprint, obtained from the culture-

dependent approach. As far as bacterial diversity was concerned, several species in sourdough samples, identified through conventional plating and molecular identification, were not detected as bands by PCR-DGGE, while the reverse situation was reported as well. More accurately, bacterial species such as *Fr. sanfranciscensis* or *Lc. lactis*, present as stable DNA and RNA bands in DGGE gels, were not recovered in the corresponding sourdough samples through the culture-dependent approach. In the case of yeast diversity, results obtained from PCR-DGGE analysis, showed almost the same species composition with culture-dependent approach. However, *Pi. membranifaciens*, previously identified in sourdough 6 by traditional method, was not detected in the DGGE gels. These observations outline the significance of applying both culture-dependent and -independent approaches for a more accurate species detection and identification of different sourdough samples.

In general, PCR-DGGE has been associated with a series of artifacts that hinder its use for quantitative assessment and suggest its application as comparative microecosystem analysis technique (Neilson et al., 2013). Co-migration of amplicons with divergent sequences, presence of multiband profile, formation of heteroduplex bands, low limit of detection, preferential amplification of specific DNA templates and limited lengths of DNA fragments amplified are some of the most frequently reported artifacts, generated during PCR-DGGE analysis (Ercolini et al., 2004, Neilson et al., 2013). In the present study, co-migration of *Lp. plantarum* and *Leu. mesenteroides*, *Lv. brevis* and *Lv. zymae, Fr. sanfranciscensis* and *Lc. lactis* and finally *Lt. curvatus* and *Lt. sakei*, analyzed with a gel of 20-60 % denaturing gradient was reported, which could lead to an underestimation of sourdough bacterial diversity. The application of narrower denaturing compounds gradient concentrations has been reported to successfully differentiate microbial populations (Cocolin et al., 2001, Gafan et al., 2005). The co-migration of *Lt. curvatus* and *Lt. sakei* has been previously reported in a 35–70% denaturing gradient gel, which was partly attributed to the close phylogenetic relatedness between *Lt. curvatus* and *Lt. sakei* (Scheirlinck et al., 2008). Another limitation encountered in the present study was the multiband profile of all yeast species *S. cerevisiae, W. anomalus, Pi. fermentans, Pi. membranifaciens* and *K. humilis*. According to Neilson et al. (2013) the multiple DGGE bands displayed for a single species could represent either PCR artifacts, resulting from the amplification of a single sequence or 16S rRNA gene heterogeneous multiple copies. Many authors have already

reported the presence of multiband profile for a single microbial species, obtained through PCR-DGGE analysis (Garofalo et al., 2017, Comasio et al., 2020). A final artifact observed in the present DGGE gels, was the formation of heteroduplex bands in all yeast DGGE profiles of both DNA and RNA extracted from sourdough samples. Heteroduplex molecules are produced in the later PCR cycles, when the concentrations of the amplified products are higher than that of the primers (Kanagawa et al., 2003). Scheirlinck et al. (2008) have also reported the heteroduplex formation through PCR-DGGE analysis ofthe Belgian sourdough ecosystem.

6 Conclusions

The microecosystem of 13 spontaneously fermented Greek wheat sourdoughs, 12 of which originate from regions not previously assessed, was successfully described, and thus, our knowledgeon the respective micro-community was expanded. The observed differences in the physicochemicalparameters of sourdoughs, namely, pH and acidity values, could be attributed to the differences in the microbial population and the prevailing microbial species. Regarding the combined use of culture-dependent and independent techniques that was employed, the biodiversity data resulting from PCR-DGGE analysis could only partially verify the sourdough micro-community as revealed by the culture-dependent approach and could not provide with complementary information.

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Chapter 3

Technological and safety attributes of lactic acid bacteria and yeasts isolated from spontaneously fermented Greek wheat sourdoughs

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1 Abstract

The aim of the present study was to assess the technological and safety potential of 207 LAB and 195 yeast strains isolated from spontaneously fermented Greek wheat sourdoughs. More accurately, the amylolytic, proteolytic, lipolytic, phytase and amino acid decarboxylase activities, along with the production of exopolysaccharides and antimicrobial compounds by the LAB and yeast isolates, were assessed. A well diffusion assay revealed seven proteolytic LAB and eight yeast strains; hydrolysis of tributyrin was evident only in 11 LAB strains. A further Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) indicated partial hydrolysis of gluten. Lipolysis kinetics over 21 days was applied, exhibiting that lipolytic activity ranged from 6.25 to 65.50 AU/mL. Thirteen LAB inhibited *Pe. olsonii* and *Aspergillus niger* growth and 12 yeast strains inhibited *Pe. chrysogenum* growth. Twenty-one *Lp. plantarum* strains exhibited inhibitory activity against *Listeria monocytogenes*, as well as several sourdough-associated isolates. The structural gene encoding plantaricin 423 was detected in 19 *Lp. plantarum* strains, while the structural genes encoding plantaricins NC8, PlnE/F, PlnJ/K, and S were detected in two *Lp. plantarum* strains. None of the microbial strains tested exhibited exopolysaccharide (EPS) production, amino acid decarboxylase, amylolytic or phytase activity. The technological and safety potential of the *Lp. plantarum* and *W. anomalus* strains was highlighted, since some of them exhibited proteolytic, lipolytic, antibacterial and antimold activities.

Keywords: proteolysis; lipolysis; antimicrobial compounds; plantaricins; *Lactiplantibacillus plantarum*; *Wickerhamomyces anomalus*

2 Introduction

Within the past few years, the ever-increasing consumer demand for "clean label" products has shifted the technological interest of baking industries towards the development of more ecologically friendly methods of preserving foods, such as sourdough fermentation (Fraberger et al., 2020). The incorporation of sourdough into bread making imparts positive effects on all aspects of bread quality, namely technological, sensorial, safety and nutritional attributes. The production of microbial metabolites, which further affect the quality of the end product, is highly dependent on the contribution of lactic acid bacteria (LAB) and yeast strains, which form the sourdough microecosystem (Paramithiotis et al., 2010). Thus, suitable starter cultures with defined metabolic properties should be carefully selected to assure the reproducibility of the process at industrial level and, at the same time, develop a bakery product with the desired sensorial traits.

As far as the technological properties are concerned, amylase activity in wheat-based sourdoughs is involved in starch hydrolysis, with concomitant liberation of fermentable sugars, namely maltose, glucose and maltodextrines (Gänzle, 2014). Despite the fact that α-amylase is regularly absent in flours, meaning its supplementation is necessary in order to increase the presence of fermentable sugars excessive levels of amylase have been reported as undesirable (Struyf et al., 2016). However, for the majority of the LAB species derived from wheat-based fermentations, amylase activity has not been considered a common enzymatic property. Apart from amylase activity, amino acids and peptides liberated during proteolysis affect leavened baked goods as both flavor and bioactive compounds (Pétel et al., 2017). Initial protein degradation to oligopeptides is carried out by cereal proteases, namely aspartic proteases, which are activated under acidic conditions (Gänzle, 2014). Further proteolysis is dependent on strain-specific intracellular peptidases of LAB, with the subsequent release of free amino acids (Gänzle, 2014, Gobbetti et al., 2014). The combination of sourdough LAB with mold enzymes has been reported to lead to complete protein hydrolysis, with increased levels of free amino acids (Gänzle & Gobbetti, 2013). Regarding lipolysis in sourdoughs, only scarce literature is available (Paramithiotis et al., 2010, Gänzle & Gobbetti, 2013). Although wheat and rye lipids constitute a small part of the corresponding flours, their degradation into flavor precursors, such as aldehydes and alcohols, strongly affects the rheological properties of the final product. As far as

homofermentative lactobacilli are concerned, an increase in products from lipid oxidation has been reported (Gänzle, 2014). Another enzymatic activity with a nutritional impact, namely phytase activity, results in the degradation of phytic acid present in the cereal grain structure (Çakır et al., 2020, Fekri et al., 2020). Phytic acid is considered an antinutritional factor due to its ability to create insoluble complexes with essential minerals, namely Ca^{2+}, Zn^{2+}, Fe^{2+}, and Mg^{2+}, thus preventing mineral bioavailability (De Angelis et al., 2019). Apart from flour endogenous phytases, several bacterial and yeast isolates have been reported to possess phytase activity, thus contributing to decreased levels of phytate content as well (Çakır et al., 2020, Fekri et al., 2020). The capacity of sourdough LAB to produce exopolysaccharides (EPS) is another trait with technological significance; this is due to their application as a replacement for commercial hydrocolloids in the bread making process, with subsequent improvements in water absorption of the dough, bread rheology, texture, and shelf life (Zhang et al., 2019). Apart from technological contributions, EPS from sourdough LAB have been associated with biofilm formation, pathogen exclusion, and prebiotic activity, too. Generally, sourdough LAB have been reported to produce homopolysaccharides (HoPS), while only few a isolates have been associated with heteropolysaccharide (HePS) production (Ispirli et al., 2020).

The screening of sourdough microorganisms for antimold and antibacterial activity has been an area of increasing focus, aiming to select appropriate starters that can further improve the shelf life and safety of end products, with respect to consumer demands for less chemical preservatives (Axel et al., 2017, Sáez et al., 2018). The capacity of LAB and non-conventional yeast strains to control mold spoilage caused by species of the genera *Aspergillus*, *Penicillium* and *Fusarium* has been thoroughly studied, and is mainly attributed to the synergistic effect of different organic acids, peptides, hydroxyl fatty acids, and phenolic compounds (De Vuyst et al., 2016, Bartkiene et al., 2020). As far as antibacterial capacity is concerned, bacteriocin production has gained much attention the past few years as an alternative biopreservation technique (Kareem et al., 2020). Although LAB strains producing antibacterial compounds are not intended for extending the shelf life of sourdough fermented cereal foods, metabolic products synthesized during the fermentation process have been reported to enhance the stability of sourdough, thus leading to the production of microbiologically safer products (Gänzle & Gobbetti, 2013). An

additional safety attribute to consider is the inability of starters to form biogenic amines (BAs) during sourdough fermentations (Spano et al., 2010, Li et al., 2020). In food fermentations, LAB are the main BAs producers through their decarboxylase activity, thus, an increase in BAs accumulation has been reported (Ladero et al., 2010). In the case of bakery products, although relatively low levels of BAs have been reported (Brandt, 2019), the capacity of sourdough microorganisms to form them has not been adequately assessed.

Several authors have studied the properties of yeast and LAB sourdough isolates. However, in the majority of these studies, only a limited set of properties are included, thus offering a restricted interpretation of the capacity of the isolates and the effect they may have on the quality of the final product. The aim of the present study was to evaluate the technological and safety potential of 207 LAB and 195 yeast isolates. More accurately, the amylolytic, proteolytic, lipolytic, phytase and amino acid decarboxylase activities, along with the production of exopolysaccharides and antimicrobial compounds by the LAB and yeast isolates, were assessed.

3 Materials and Methods

3.1 Microbial strains and culture conditions

A total of 207 lactic acid bacteria and 195 yeast isolates were obtained from thirteen Greek spontaneously fermented wheat sourdoughs (Syrokou et al., 2020). The lactic acid bacteria isolates were identified as follows: *L p . plantarum* (70 isolates); *Lv. brevis* (71 isolates); *Cp. paralimentarius* (30 isolates); *Lv. zymae* (1 isolate); *Lt. curvatus* (6 isolates); *Lt. sakei* 12 isolates); *Leu. citreum* (1 isolate); *Leu. mesenteroides* (1 isolate); *Lc. lactis* (3 isolates); and *Fr. sanfranciscensis* (12 isolates). The yeast isolates were identified as follows: *S. cerevisiae* (161 isolates); *K. humilis* (2 isolates); *Pi. fermentans* (8 isolates); *Pi. membranifaciens* (18 isolates); and *W. anomalus* (6 isolates). All isolates were stored at 20 ◦C in a Nutrient broth supplemented with 50% glycerol (Applichem, Darmstadt, Germany). Before experimental use, lactic acid bacteria and yeast isolates were grown twice in MRS broth, and in BHI broth, and their purity was examined through streaking in MRS agar and BHI agar, respectively. All substrates were from LAB M (Lancashire, UK).

3.2 Technological properties

3.2.1 Production of exopolysaccharides (EPS)

Screening for EPS production was performed according to Smitinont et al. (1999). More accurately, overnight bacteria cultures were used to inoculate MRS agar containing 2% glucose, fructose, maltose or sucrose. Incubation took place at 30 ^{o}C for 3 d. Production of the slimy phenotype was indicative of EPS production.

3.2.2 Amylase activity

Overnight yeast and bacterial cultures were spot-inoculated on the surface of modified BHI and MRS agar, in which glucose was replaced by 2% soluble starch and incubated at 30 ^{o}C for 5 d. Then, the substrate was flooded with Gram's iodine solution (Sigma-Aldrich). Presence of clarification halo around each colony was indicative of amylase activity.

3.2.3 Proteolytic activity

Proteolytic activity was assessed through the agar well-diffusion assay. More accurately, in freshly prepared lawns of a medium consisting of 0.5% tryptone, 0.25% yeast extract, 0.1% glucose, 1% gluten and 1.5% agar, wells were aseptically punched. Overnight yeast and bacterial cultures were centrifuged (12.000 x *g*; 15 min; 4 ^{o}C) to obtain cell-free supernatant (CFS). Then, 25 μL of each CFS were added in each well. Incubation took place at 30 ^{o}C for 5 d. Then, the substrate was stained with 0.05% (w/v) Coomassie Brilliant Blue G-250. The presence of clarification halo around each well was indicative of proteolytic activity.

The strains that exhibited proteolytic activity were subjected to further study. More accurately, overnight culture was centrifuged (12.000 x *g*; 15 min; 4 ^{o}C), washed twice with sterile saline and mixed with a dough made of 10 g wheat flour (T70) and 20 mL tap water. Incubation took place at 30 oC for 24 h. Uninoculated doughs were used as control. After incubation, the albumins, globulins, gliadins and glutenins fractions were obtained according to Di Cagno et al. (2002). Decomposition of the gluten fractions was assessed through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), in 12% polyacrylamide gel, according to Paramithiotis et al. (2000).

3.2.4 Lipolytic activity

Lipolytic activity was assessed through the agar well-diffusion assay. More accurately, the medium consisted of 0.5%, peptone, 0.3% meat extract, 0.5% lecithin, 1% tributyrin, 1.5% agar, with the addition of 2.5 mM $CaCl_2$ and 5 mM $MgSO_4$, according to Carrazco-Palafox et al. (2018). Then wells were aseptically punched. Overnight yeast and bacterial cultures were centrifuged (12.000 x *g*; 15 min; 4 ^oC) to obtain CFS. Twenty-five (25) μL of each CFS were added in each well. Incubation took place at 30 ^oC for 10 d. Presence of clarification halo around each well was indicative of lipolytic activity.

The strains that exhibited lipolytic activity were subjected to further study. More accurately, overnight culture was centrifuged (12.000 x *g*; 15 min; 4 ^oC), washed twice with sterile saline and used to inoculate flasks containing the above medium without agar addition. Incubation took place under shaking (200 rpm) at 30 ^oC for 21 d. The pH value and total titratable acidity of the samples were determined at days 3, 6, 9, 12, 15, 18 and 21. Uninoculated flasks served as control. Lipolytic activity was expressed in AU/mL; one arbitrary unit was defined as the amount of enzyme that catalyzed the release of 1 μmol of fatty acids.

3.2.5 Phytase activity

The protocol described by Anastasio et al. (2010) was used to detect phytase activity. In brief, overnight yeasts and bacteria cultures were inoculated into Chalmers broth that was made without neutral red and with the addition of 1% sodium phytate and incubated at 30 ^oC for 48 h. Then, a loopful was spotted on the surface of Chalmers agar that was made without calcium carbonate and with the addition of 1% hexacalcium phytate and incubated at 30 ^oC for 48 h. After incubation, the plates were flooded with 2% (w/v) cobalt chloride for 20 min. Presence of clarification halo around each colony was indicative of phytase activity.

3.3 Safety properties

3.3.1 Production of antimicrobial compounds

The well diffusion assay was employed to assess the production of antimold compounds by lactic acid bacteria and yeasts. LAB isolates were further tested for antibacterial activity. Overnight cultures were centrifuged to obtain a CFS that was consequently neutralized and treated with catalase. Antibacterial activity was assessed against a mixture of five strains of the foodborne pathogens *L. monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella* serovars. Incubation took place at 37 ^{o}C for 24 h. Growth inhibition of the indicator strains around the wells, exceeding 5 mm, was indicative of the presence of antibacterial substances in the cell free supernatant. The strains that exhibited antibacterial activity against the pathogens were further assessed against a mixture of sourdough isolates of *Lp. plantarum* (LQC 2328, 2330, 2343, 2385, 2462, five isolates), *Lv. brevis* (LQC 2368, 2429, 2484, 2509, 2518, five isolates), *Cp. paralimentarius* (LQC 2323, 2381, 2399, 2517, 2537, five isolates), *Lt. sakei* (LQC 2448, 2452, 2456, 2470, 2473, five isolates), *Lt. curvatus* (LQC 2472, 2475, 2476, 2497, 2498, five isolates), *Fr. sanfranciscensis* (LQC 2402, 2408, 2419, 2425, 2428, five isolates), *Lv. zymae* (2394, one isolate), *Lc. lactis* (LQC 2375, 2499, 2510, three isolates), *Leu. citreum* (LQC 2508, one isolate) and *Leu. mesenteroides* (LQC 2512, one isolate). Antimold activity was examined against *Pe. chrysogenum*, *Pe. olsonii* and *Aspergillus niger* (moldy bread isolates) as described above with the exception that incubation took place at 30 ^{o}C for 5 d.

The protein nature of the antimicrobial substances was examined by assessing the effect of proteolytic enzymes on the antimicrobial activity. More accurately, aliquots of 160 μL CFS were mixed with 40 μL 50 mM phosphate buffer pH 7.5 containing 2 mg mL^{-1} proteinase and 40 μL 50 mM phosphate buffer pH 7.0 containing 2 mg mL^{-1} trypsin and incubated at 37 ^{o}C for 1 h. Then, the antimicrobial activity of the CFS were examined as described above.

To determine the effect of pH on the stability of the antimicrobial compounds, the pH value of CFS was adjusted to 2, 4, 6, 8 and 10 with 3M HCl and 3M NaOH and incubated at 37 ^{o}C for 1 h. To evaluate the thermal stability of the antimicrobial compounds, CFS were treated at 60, 80 and 100 ^{o}C for 10 and 30 min, respectively. Untreated CFS was used as control.

Specific PCR was employed for the detection of the plantaricin structural genes. DNA of the *Lp. plantarum* strains under study was extracted according to Paramithiotis et al. (2010). The protocol described by Omar et al. (2006) was used for the detection of the structural genes encoding plantaricins PlnA, PlnE/F, PlnJ/K, PlnN, NC8, S and W. Detection of the structural gene of plantaricin 423 was performed in 20 μL final reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM each primer (423F: TGT GGT AAA CAT TCC TGC TCT G; 423R: CAC TTT CCA TGA CCG AAG TTA GC) and 1 U Taq polymerase (Kappa Biosystems, Boston, MA, USA); the resulting 86 bp amplicon was detected by electrophoresis in a 2% agarose gel.

3.3.2 Biogenic amine production

The protocol described by Bover-Cid and Holzapfel (1999) was used to assess the amino acid decarboxylase activity of the yeast and bacteria isolates. More accurately, overnight yeast and bacteria cultures were spot inoculated on the surface of a medium consisting per liter of 5 g yeast extract, 5 g tryptone, 5 g meat extract, 5 g glucose, 2.5 g sodium chloride, 2 g ammonium citrate, 1 g Tween 80, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 0.04 g $FeSO_4$, 0.01 g thiamine, 2 g K_2PO_4, 0.1 g $CaCO_3$, 0.05 g pyridoxal-5-phosphate, 0.06 g bromocresol purple, 20 g agar and supplemented with 10 g lysine, tyrosine, ornithine or histidine, pH 5.3. Incubation took place at 30 ^oC for 48 h. Occurrence of color change around the colonies, yellow to purple, was indicative of decarboxylation of the respective amino acid.

3.4 Statistical analysis

One-way ANOVA was employed to assess the statistical significance of the differences observed in the lipolysis kinetics between the microbial strains.

4 Results

4.1 Assessment of technological properties

4.1.1 Proteolytic activity

The agar well diffusion assay revealed that, of the 207 LAB and 195 yeast strains initially screened for proteolytic activity, seven and eight strains, respectively, were able to hydrolyze gluten. A clear halo around the wells was present for these 15 strains, indicating the presence of proteolytic capacity. Regarding LAB isolates, four belonged to *Lp. plantarum* (LQC 2320, 2372, 2464, 2520) and three to *Lv. brevis* (LQC 2469, 2474, 2493). In the case of yeasts, four isolates were assigned to *S. cerevisiae* (LQC 10343, 10378, 10398, 10402), three to *W. anomalus* (LQC 10346, 10353, 10360) and one to *Pi. fermentans* (LQC 10349). The proteolytic capacity of these 15 microbial strains was further evaluated with SDS-PAGE analysis. The SDS-PAGE confirmed the gluten degrading potential of three LAB strains, namely LQC 2320, 2469, and 2520. More accurately, sourdough fermentation with the specific monocultures of LAB strains resulted in significant hydrolysis of albumins and gliadins (Figure 3.1). Hydrolysis was partial or absent towards glutenins. Compared to the albumin control, sourdough fermentation with LAB strains LQC 2520 and 2320 resulted in complete and partial hydrolysis, respectively, of the albumin band with molecular weight (Mw) of ca. 40 kDa. In addition, the band intensity of albumins with Mw ranging between 25–35 kDa decreased, while the disappearance of the albumin bands in the range of 20–25 kDa, due to extensive hydrolysis, was also evident. The appearance of additional albumin bands of lower Mw, ranging between 15–20 kDa and belonging to doughs fermented by LAB strains LQC 2520 and 2320, respectively, was also shown in the electrophoretic analysis.

In the case of gliadins, LAB strains LQC 2320, 2520, and 2469 revealed similar profiles of proteolysis. Compared to the control, complete hydrolysis of gliadin bands corresponding to ca. 40 and 20 kDa was detected in SDS-PAGE. After sourdough fermentation with each of the aforementioned LAB strains, the intensity of gliadin bands with Mw between 20–35 was significantly decreased. As far as the third gluten fraction was concerned, glutenins extracted from dough previously inoculated with LAB strain LQC 2520 revealed additional bands between 35–70, and below 25 kDa. The rest of the glutenins extracted were not affected by LAB fermentation.

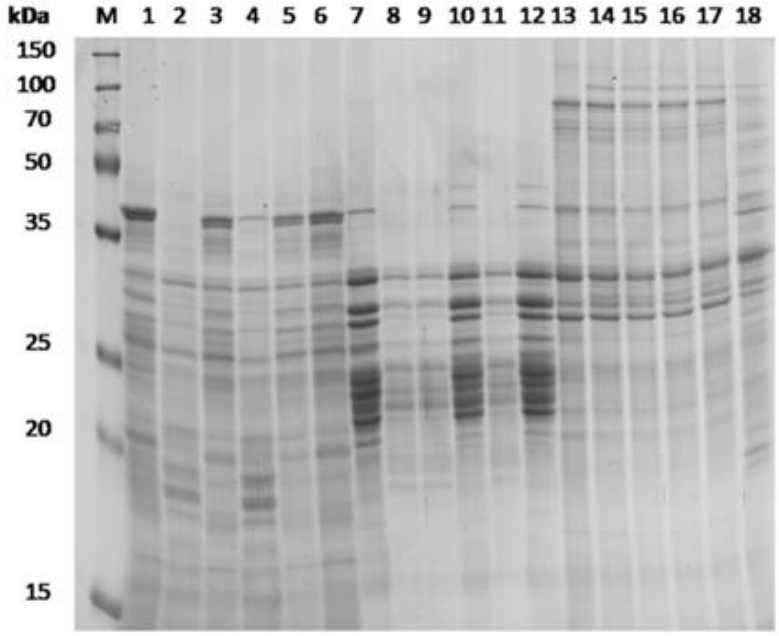

Figure 3. 1. Representative SDS-PAGE analysis of gluten fractions extracted from a mixture of wheat flour and water (1:2), incubated for 24 h at 30 ˚C, after inoculation with different LAB and yeast strains, which previously displayed positive proteolytic activity. M, protein marker; Column 1, albumin fraction extracted from dough without inoculum (control); Columns 2–6, albumin fractions extracted from doughs inoculated with microbial strains LQC 2320, 2474, 2520, 2469, and 10343, respectively; Column 7, gliadin fraction extracted from dough without inoculum (control); Columns 8–12, gliadin fractions extracted from doughs inoculated with microbial strains LQC 2320, 2520, 10343, 2469, and 2474, respectively; Column 13, glutenin fraction extracted from dough without inoculum (control); Columns 14–18, glutenin fractions extracted from doughs inoculated with microbial strains LQC 2320, 2469, 2474, 10343, and 2520, respectively.

4.1.2 Lipolytic activity

Agar well diffusion assay revealed that of the 207 LAB strains tested, 11 were found lipase positive, among which 6 were identified as *Lp. plantarum* (LQC 2320, 2321, 2385, 2397, 2516, 2520), four as *Lv. brevis* (LQC 2374, 2411, 2416, 2432) and one as *Cp. paralimentarius* (LQC 2410). None of the 195 yeast strains tested was able to hydrolyze tributyrin. A further lipolysis kinetics over 21 days was performed, with lipase activity expressed in AU/ mL, as shown in Table 3.1. Data obtained, revealed that *Lp. plantarum* LQC 2321 and 2397 and *Lv. brevis* 2416 presented the highest enzyme activities, 62.75, 65.40 and 55.75, respectively, at the day 18 of incubation, with lipase activities remaining almost constant until day 21 of incubation.

Similarly, *Lv. brevis* LQC 2374, *Cp. paralimentarius* LQC 2410, and *Lp. plantarum* 2516 exhibited the lowest lipase activities until day 21 of incubation (6.25, 17.00 and 14.50 AU/mL, respectively). The other LAB strains exhibited moderate lipase activities, ranging from 22.50 to 47.75, until day 21 of incubation.

Table 3. 1. Lipolysis kinetics of 11 bacterial strains during 21 days of incubation, at 30 ºC.

Sourdough LAB strains	Days						
	3	6	9	12	15	18	21
Lp. plantarum LQC 2320	8.75 ± 1.06 ^{e,E}	23.75 ± 1.06 ^{g,G}	34.00 ± 0.00 ^{g,C}	35.50 ± 0.71 ^{d,D}	36.00 ± 0.00 ^{e,D,E}	37.50 ± 0.71 ^{e,E}	37.25 ± 0.35 ^{e,E}
Lp. plantarum LQC 2321	2.50 ± 0.71 ^{ab,AB}	11.50 ± 0.71 ^{e,E}	42.00 ± 0.00 ^{h,C}	52.50 ± 0.71 ^{g,G}	59.50 ± 0.71 ^{i,E}	62.75 ± 1.06 ^{i,F}	63.00 ± 0.00 ^{i,F}
Lv. brevis LQC 2374	3.00 ± 0.00 ^{a,ABC}	3.25 ± 0.35 ^{ab,A}	3.50 ± 0.00 ^{abc,AB}	4.00 ± 0.00 ^{bc,A}	4.25 ± 0.35 ^{c,B}	6.50 ± 0.71 ^{d,C}	6.25 ± 0.35 ^{d,C}
Lp. plantarum LQC 2385	1.50 ± 0.71 ^{a,A}	5.20 ± 0.42 ^{b,B}	9.55 ± 0.78 ^{bc,C}	14.50 ± 0.71 ^{b,B}	19.50 ± 0.71 ^{d,E}	22.50 ± 0.71 ^{d,F}	22.50 ± 0.71 ^{d,F}
Lp. plantarum LQC 2397	5.50 ± 0.71 ^{d,D}	11.25 ± 1.06 ^{e,E}	49.00 ± 1.41 ^{i,C}	57.50 ± 0.71 ^{h,H}	61.50 ± 0.71 ^{k,E}	65.40 ± 0.85 ^{j,F}	65.50 ± 0.71 ^{j,F}
Cp. paralimentarius LQC 2410	3.00 ± 0.00 ^{abc,ABC}	7.50 ± 0.71 ^{cd,CD}	8.45 ± 0.78 ^{b,B}	14.25 ± 1.06 ^{b,B}	16.00 ± 0.00 ^{c,D}	17.25 ± 0.35 ^{c,D}	17.00 ± 0.00 ^{c,D}
Lv. brevis LQC 2411	9.00 ± 0.71 ^{e,E}	13.25 ± 1.06 ^{f,F}	19.75 ± 0.35 ^{e,C}	34.50 ± 0.71 ^{d,D}	37.50 ± 0.71 ^{f,E}	48.60 ± 0.85 ^{g,F}	47.75 ± 1.06 ^{g,F}
Lv. brevis LQC 2416	5.50 ± 0.71 ^{d,D}	10.50 ± 0.71 ^{e,E}	42.75 ± 0.35 ^{h,C}	49.75 ± 0.35 ^{f,F}	55.25 ± 0.35 ^{h,E}	55.75 ± 0.35 ^{h,E}	55.75 ± 0.35 ^{h,E}
Lv. brevis LQC 2432	1.75 ± 0.06 ^{a,A}	13.50 ± 0.71 ^{f,F}	23.75 ± 1.06 ^{f,C}	41.50 ± 0.71 ^{e,E}	41.75 ± 0.35 ^{g,D}	45.50 ± 0.71 ^{f,E}	46.00 ± 0.71 ^{f,E}
Lp. plantarum LQC 2516	3.50 ± 0.71 ^{bc,BC}	6.25 ± 0.35 ^{bc,BC}	10.50 ± 0.71 ^{c,C}	14.50 ± 0.71 ^{b,B}	14.50 ± 0.71 ^{b,D}	14.75 ± 0.35 ^{b,D}	14.50 ± 0.71 ^{b,D}
Lp. plantarum LQC 2520	4.50 ± 0.71 ^{cd,CD}	8.00 ± 0.71 ^{d,D}	15.50 ± 0.71 ^{d,C}	17.25 ± 0.35 ^{c,C}	18.50 ± 0.71 ^{d,D}	22.50 ± 0.71 ^{d,E}	22.75 ± 0.35 ^{d,E}

The lipase activity is expressed in AU/mL. *Cp.*: *Companilactobacillus*; *Lp.*: *Lactiplantibacillus*; *Lv.*: *Levilactobacillus*. Statistically significant inter-species and intra-species differences are expressed with different letters—a–j and A–F, respectively.

None of the microbial strains tested exhibited EPS production, amylolytic, or phytase activity.

4.2 Assessment of safety properties

4.2.1 Antimicrobial activity

Among 207 bacterial and 195 yeast strains initially evaluated against *Pe. chrysogenum*, *Pe. olsonii* and *A. niger* by agar well diffusion assay, 13 and 12, respectively, were able to inhibit mold growth. In the case of LAB, two *Lp. plantarum* (LQC 2321, 2327), two *Lv. brevis* (LQC 2523, 2529), two *Lt. sakei* (LQC 2448, 2454) and one strain identified as *Fr. sanfranciscensis* (LQC 2419), *Cp. paralimentarius* (LQC 2392), *Lt. curvatus* (LQC 2476), *Lv. zymae* (LQC 2394), *Lc. lactis* (LQC 2499), *Leu. mesenteroides* (LQC 2512) and *Leu. citreum* (LQC 2508), showed extensive inhibition against *Pe. olsonii* and *A. niger*. As far as yeasts were concerned, seven *S. cerevisiae* (LQC 10286, 10298,

10307, 10393, 10404, 10421, 10469), three *W. anomalus* (LQC 10346, 10353, 10360) and two *Pi. fermentans* (LQC 10355, 10356 presented inhibition against *Pe. chrysogenum*. LAB strains exhibited no inhibitory activity against *Pe. chrysogenum*, while *Pe. olsonii* and *A. niger* showed great resistance against all yeast strains.

The antibacterial capacity of LAB strains was also tested against foodborne pathogens *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *Salmonella* serovars. Twenty one *Lp. plantarum* strains (LQC 2320, 2384, 2422, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2506, 2516, 2520) exhibited inhibitory properties against a mixture of *L. monocytogenes* 4b strains. No inhibitory potential of LAB strains evaluated against *S. aureus*, *E. coli* O157:H7 and *Salmonella* serovars was observed. These 21 *Lp. plantarum* strains were further shown to inhibit sourdough isolates of *Lp. plantarum*, *Lv. brevis*, *Cp. paralimentarius*, *Lt. sakei*, *Lt. curvatus*, *Fr. sanfranciscensis* and *Lc. lactis*. No inhibitory activity against *Lv. zymae*, *Leu. citreum* and *Leu. mesenteroides* was observed.

The structural gene encoding plantaricin 423 was detected in nineteen *Lp. plantarum* strains. On the contrary, the structural genes encoding plantaricins NC8, PlnE/F, PlnJ/K, and S were detected in strains LQC 2320 and 2520.

None of the LAB and yeast isolates presented production of biogenic amines.

4.2.2 Effect of proteolytic enzymes, pH and temperature on antimicrobial stability

The CFS obtained from LAB and yeast strains, which previously demonstrated antimicrobial activity, were further assessed for their stability upon enzymatic, pH, and thermal treatment. The antibacterial compounds towards *L. monocytogenes* produced by 21 *Lp. plantarum* strains were of proteinaceous nature, as the inhibitory action was abolished after treatment with at least one of the proteolytic enzymes employed, namely proteinase and trypsin. As far as the effect of thermal and pH treatment was concerned, these antibacterial substances demonstrated a highly thermostable and pH tolerant profile, since activity was retained after any temperature or pH treatment was applied. The antimold compounds produced by both LAB and yeast strains were non proteinaceous, as their activity remained even after enzymatic treatment. Regarding thermal stability, the CFS from all microbial strains tested

completely lost their antimold capacity after thermal treatment. On the contrary, antimold compounds from both LAB and yeast strains exhibited a pH tolerant profile at all different pH values, ranging from 2 to 10.

5 Discussion

In the past few years, increasing demand for large-scale and controllable bread production has driven both scientific and industrial attention towards a more careful selection of sourdough starters. Desired technological and safety properties of candidate starters are those determining the potential use of sourdough microbial strains in the production of baked goods.

Proteolysis during sourdough fermentation has been considered a key process for determining the dough rheology and overall quality of the final product. Thus, the presence of proteolytic activity is a selection criterium of paramount importance among candidate sourdough starters (Fu et al., 2020, Lancetti et al., 2020). Primary gluten hydrolysis is dependent on cereal proteases, while secondary proteolysis is carried out by strain-specific peptidases of sourdough LAB (Galli et al., 2018). Except for LAB acidification, increased levels of thiol groups in the gluten proteins, produced via the glutathione reductase activity of heterofermentative lactobacilli, contribute to the depolymerization of glutenin macropolymer, thus increasing their susceptibility to enzymatic degradation (Gänzle & Gobbetti, 2013). In the present study, the initial test revealed seven proteolytic LAB strains and eight proteolytic yeast strains. However, the SDS-PAGE confirmed the proteolytic capacity for only three LAB strains. Similar results are often reported and may be attributed to insufficient incubation time (Kirilov et al., 2009, Dallas et al., 2016, Syrokou et al., 2019). Lancetti et al. (2020) have already reported the proteolytic capacity of *Lp. plantarum* ES137 and *Pd. acidilactici* ES22, previously isolated from Argentinian grains, by applying SDS-PAGE analysis. More accurately, sourdoughs inoculated with *Lp. plantarum* ES137 and *Pd. acidilactici* ES22 presented a different protein profile in electrophoretic gel, compared to the controls, which is characterized by a decreased band number and intensity. Another study reported that the application of SDS-PAGE for characterization of the protein content of sourdoughs, inoculated with a mixed culture of *Lp. plantarum* and yeast, revealed extensive hydrolysis of

higher molecular weight protein bands, with the concomitant appearance of additional bands of lower molecular weight (Yin et al., 2015). In the case of yeasts, decreased proteolytic activity has been reported by the majority of researchers (Fu et al., 2020) Generally, yeasted doughs are characterized by decreased levels of amino acids compared to doughs fermented with LAB (Thiele et al., 2002).

Lipolysis has been extensively studied in fermented dairy, non-dairy and meat products (Syrokou et al., 2019, García-Cano et al., 2019, Xiao et al., 2020). Despite the fact that a certain level of lipolysis is a desirable attribute of microbial strains, in terms of synthesis of flavor precursors, only a few pieces of scientific literature have reported the microbial screening for lipolytic activity of sourdough microorganisms (Paramithiotis et al., 2010). At first, lipases hydrolyze triacylglycerols into free fatty acids (Pico et al., 2015). Then, unsaturated fatty acids, namely linoleic acid, the major component of cereal lipids, are further degraded into peroxides through either autoxidation during flour storage or cereal lipoxygenase activity during dough mixing (Gänzle et al., 2007). Finally, degradation of peroxides into aldehydes takes place, with the latter being further reduced to alcohols via heterofermentative lactobacilli during sourdough fermentation. The agar well diffusion assay applied in the present study revealed 11 lipase positive LAB strains of the 207 tested; the lipolysis kinetics further applied over 21 days revealed that *Lp. plantarum* LQC 2321 and LQC 2397 and *Lv. brevis* LQC 2416 presented the highest lipolytic activity on day 21 of incubation. None of the yeasts tested exhibited lipolytic activity. To our knowledge, no previous study has reported the presence of lipolytic microbial strains isolated from spontaneously fermented sourdoughs; except for Paramithiotis et al. (2000), who reported the presence of *Y. lipolytica* in Greek sourdough microbiota. *Y. lipolytica* strains are known for their strong lipolytic and proteolytic activity (Fröhlich-Wyder et al., 2019). Regarding LAB, they are considered as weak lipolytic compared to other bacterial species, such as *Pseudomonas* spp., *Acinetobacter* spp. and *Flavonobacterium* spp. Indeed, absence of lipolytic activity among strains of *Lp. plantarum* and *Lv. brevis* was reported by Kamiloğlu et al. (2020) and Ebadi Nehzad et al. (2020). However, another study revealed that, of the 137 bacterial strains initially screened for proteolytic and lipolytic activity, seven exhibited both (García-Cano et al., 2019). Among them, two belonged to *Lp. plantarum*. Zymography was further applied to isolate

the proteins responsible for exhibiting lipolytic activity; however, no lipolytic proteins were detected in the case of *Lp. plantarum*. Regarding *Cp. paralimentarius*, this is the first study to report its lipolytic activity.

Sourdough fermented with antimold LAB and yeast strains has been the epicenter of intensive study over the past few years; it is an alternative biopreservation approach in line with consumer demand for clean label products (Debonne et al, 2020). The most common molds spoiling bread products belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Cladosporium*, and their presence constitutes a major concern for baking industries due to the potential of mycotoxin production, with concomitant long term health risks (Fraberger et al., 2020). Antimold metabolites synthesized by sourdough LAB and yeast strains are responsible for biopreservation effects. In the present study, 13 LAB inhibited *Pe. olsonii* and *A. niger* growth and 12 yeast strains inhibited *Pe. chrysogenum* growth. The antimold activity of the majority of LAB species, tested in the present study, has been previously reported against numerous molds, commonly spoiling bread. More accurately, Fraberger et al. (2020) reported a strain dependent antimold potential of wheat and rye sourdough derived lactobacilli; the majority of the isolates belonging to *Lp. plantarum* presented a wide antimold activity against all mold strains tested. In the case of *Lv. brevis*. isolate S4.5 strongly inhibited *Pe. roqueforti*, while the mold remained resistant against *Lv. brevis* S13.18, S14.3, and S6.13. Isolates belonging to *Fr. sanfranciscensis*, *Lt. sakei* S4.19 and *Lt. curvatus* S4.14, S5.22, S6.15 exhibited very strong inhibition against *F. graminearum*. In addition, *Cp. paralimentarius* S7.5 exhibited very strong inhibitory activity against *A. fumigatus* and *F. graminearum*. An additional study by Manini et al (2016) reported the antimold activity of wheat bran sourdough originating *Lp. plantarum*, *Lv. brevis*, *Lt. curvatus* and *Lt. sakei* against *A. oryzae* and *A. niger*. *Leu. mesenteroides* and *Leu. citreum* strains were able to inhibit both *Aspergillus* species, as well. As far as *Lc. lactis* was concerned, strain CH179 derived from chia flour fermentation, presented inhibitory activity against *A. niger* and *Pe. roqueforti* (Maidana et al., 2020). In the case of *Lv. zymae*, the antimold activity of a sourdough derived strain has not been reported so far, while few studies have documented *Lv. zymae* as inactive against mold growth (Rouxel et al., 2020). Regarding yeasts, literature is focused on their leavening capacity, flavor development and mutualistic interaction with LAB, while only few scientific data have reported their role as antimold

agents (Coda et al., 2011, De Vuyst et al., 2016). More accurately, Jin et al. (2021) reported the absence of antimold activity of sourdough derived *S. cerevisiae* against *A. flavus*. In addition, its combination with antimold *Pd. pentosaceus* and their application as starters in sourdough fermentations, presented the greatest inhibitory effect against *A. flavus*. Regarding *W. anomalus*, a strong inhibitory activity against a range of molds has been reported, when used as starter in dough fermentation. Sourdough fermented with a combination of *W. anomalus* with *Lp. plantarum* exhibited a slightly decreased antimold activity, however sourdough bread delayed mold growth until 28 days of storage. The antimold peptides and ethyl acetate synthesized by *Lp. plantarum* and *W. anomalus*, respectively, were responsible for the extension of mold free shelf life of bread. With respect to *Pi. fermentans*, no previous study has reported the antimold activity of this sourdough originating yeast. However, the antimold potential of a coffee fruit derived strain LPBYB13 was reported against an ochratoxigenic strain of *A. westerdijkiae* on agar tests and a further inhibition of ochratoxin A production in coffee beans was performed (Pereira et al., 2016).

The antibacterial potential of LAB is a significant criterium for the selection of more competitive starters in sourdough fermentations and could determine the microbiological stability and safety of the baked products. Despite the fact that the antibacterial compounds produced by sourdough LAB, namely bacteriocins, bacteriocin-like inhibitory substances (BLIS), and antibiotics, do not extend the mold free shelf life of the end products, they positively affect their microbiological safety by counteracting food contamination during processing (Minervini et al., 2014). *L. monocytogenes*, *S. aureus*, *E. coli* and *Salmonella* serovars are the most common foodborne pathogens. In the present study, of 207 LAB isolates evaluated against food borne pathogens, 21 *Lp. plantarum* strains exhibited inhibitory activity against a mixture of *L. monocytogenes* 4b strains. These 21 *Lp. plantarum* strains further inhibited several sourdough-associated LAB. In agreement with the present data, hull-less barley sourdough-originating *Lp. plantarum* SAB15 exhibited a very strong inhibitory potential against B. subtillis, *B. cereus* and *E. coli*, based on agar diffusion assay (Çakır et al., 2020). In addition, Demirbas et al. (2017) reported the antibacterial activity of Turkish wheat sourdough-derived *Lp. plantarum* ED10 against *B. cereus, S. aureus*, *Y. enterocolitica*, and *E. coli.* The DNA sequence of the structural genes encoding eight plantaricins, namely NC8, PlnA, PlnE/F, PlnJ/K, PlnN, W, S, and

423, has been verified so far. The latter is plasmid-encoded, while the rest are chromosomally-encoded (van Reenen et al., 1998, Diep et al., 2009). Their characteristics and applications have been also reviewed for the majority of them (Kareem et al., 2020, Garcia-Gonzalez et al., 2021). To our knowledge, this is the first study reporting the presence of plantaricins NC8, PlnE/F, PlnJ/K, S, and 423 from sourdough-derived *Lp. plantarum* isolates. Previous studies have reported only the production of plantaricin ST31 (Todorov et al., 1999) and plantaricin A (Di Cagno et al., 2010) from sourdough-derived strains of *Lp. plantarum.*

The presence of phytase and amylase positive sourdough microorganisms is of paramount importance from a technological perspective. During sourdough fermentation, phytases dephosphorylate phytic acid into myo-inositol and phosphoric acid, leading to increased mineral bioavailability and further improvement of the nutritional characteristics of the final product. Regarding amylases, they are responsible for starch hydrolysis, with concomitant formation of fermentable sugars. In the present study, no phytase and amylase positive LAB and yeast strains were detected. In line with the present data, Paramithiotis et al. (2010) detected no amylolytic activities of sourdough LAB and yeast strains tested. On the other hand, several studies have reported the presence of sourdough-derived microbial strains with phytase activity (Carrizo et al., 2016, Palla et al., 2019). Another property with technological im- pact, however, not detected in the present study, was EPS production by LAB strains. On the contrary, previous studies by Milanović et al. (2020) and Manini et al. (2016) reported the presence of EPS producing LAB strains, derived from cereal based substrates and wheat bran sourdoughs, respectively. A last safety attribute assessed in the present study, but what was not detected in either LAB or yeast strains tested was the ability to form BAs. Within permissible limits, BAs exert no adverse health effects on consumers; however, when accumulated in food products, they can possibly present a health risk. Compared to other fermented foods, BA production in sourdough fermentations is of less concern, with BA levels ranging far below those posing health risks (Brandt et al., 2019). In agreement with the previous statement, Bartkiene et al. (2014) reported on BA production in the solid-state fermentation of flaxseed; however, the levels were relatively low to pose health issues.

6 Conclusions

The results obtained in the present study clearly reveal the technological and safety potential of several bacterial and yeast strains. Two *Lp. plantarum* strains, LQC 2320 and 2520, exhibited proteolytic, lipolytic and antibacterial capacity. The presence of structural genes encoding plantaricins NC8, PlnE/F, PlnJ/K, and S was also detected in both LAB strains. In the case of yeasts, three *W. anomalus* strains, LQC 10346, 10353, and 10360, exhibited both proteolytic and antimold potential. These properties deserve further research due to the impact they may have on the quality of sourdough bread.

7 References

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Chapter 4

Effect of dough-related parameters on the antimold activity of Wickerhamomyces anomalus strains and mold free shelf life of bread

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1. Abstract

The aim of the present study was to assess the antimold capacity of three *W. anomalus* strains, both *in vitro* and *in situ*, and to identify the responsible volatile organic compounds. For that purpose, two substrates were applied; the former included BHI broth, adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and supplemented with 6 different NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%), while the latter was a liquid dough, fortified with the 6 aforementioned NaCl concentrations. After a 24 h incubation at 30 °C, the maximum antimold activity was quantified for all strains at 5120 AU/mL, obtained under different combinations of initial pH value and NaCl concentration. A total of twelve volatile compounds were detected; ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate were produced by all strains. On the contrary, butanoic acid-ethyl ester, acetic acid-butyl ester, ethyl caprylate, 3-methyl-butanoic acid, 2,4-di-tert-butyl- phenol, benzaldehyde, nonanal and octanal were occasionally produced. All compounds exhibited antimold activity; the lower MIC was observed for 2,4-di-tert-butyl-phenol and benzaldehyde (0.04 and 0.06 μL/mL of headspace, respectively), while the higher MIC was observed for butanoic acid- ethyl ester and ethyl caprylate (5.14 and 6.24 μL/mL of headspace, respectively). The experimental breads made with *W. anomalus* strains LQC 10353, 10346 and 10360 gained an additional period of 9, 10 and 30 days of mold free shelf life, compared to the control made by commercially available baker's yeast. Co-culture of the *W. anomalus* strains with baker's yeast did not alter the shelf life extension, indicating the suitability of these strains as adjunct cultures.

Keywords: yeasts; adjunct culture; inhibitory capacity; *Pe. chrysogenum*; VOCs

2. Introduction

The application of antagonist yeasts, as biological control agents (BCAs) in the production of clean label products, has been at the epicenter of intensive study over the past few years, as a promising alternative to chemical preservatives. Several possible mechanisms have been reported for the biocontrol activity exerted by yeasts, among which are competition for space and nutrients, biofilm formation and secretion of killer toxins, volatile organic compounds (VOCs) and hydrolytic enzymes (Freimoser et al., 2019). The use of *W. anomalus* strains as antimold agents has received considerable attention due to the antagonistic features of the yeast (Raynaldo et al., 2021).

W. anomalus is an ascomycetous yeast, formerly known as *Hansenula anomala, Pichia anomala* and *Saccharomyces anomalus*, the anamorph name of which is *Candida pelliculosa* (EFSA, 2017). Its frequent isolation from a wide variety of ecological niches, ranging from soil, plants and water to dairy and baked products, indicates the highly competitive nature of this yeast species (Walker, 2011, Cappelli et al., 2021). In addition, the ability of *W. anomalus* strains to ferment many carbon and nitrogen sources, combined with their capacity to survive in harsh conditions, in terms of temperature, initial pH value, water activity and osmotic pressure, suggest a robust character (Daniel et al., 2011). Due to the ability of *W. anomalus* strains to grow in a broad range of temperatures, its implication as an opportunistic pathogen has been claimed and its occasional identification in clinical settings has raised some consideration (Zhang et al., 2021). However, the European Food Safety Authority (EFSA) addressed these safety concerns, by granting the Qualified Presumption of Safety (QPS) status to *W. anomalus* for use in food industries (EFSA, 2021). Regarding the antimold activity assessment of *W. anomalus* strains, several studies have associated their biocontrol activity with VOCs production (Parafati et al., 2015, Khunnamwong et al., 2020), with ethyl acetate and 2-phenylethanol being reported as the major ones (Hua et al., 2014, Oro et al., 2018).

Despite the fact that the application of *W. anomalus* strains for postharvest decay control in fruits and vegetables has extensively been reported (Grzegorczyk et al., 2017, Czarnecka et al., 2019), only scarce is the literature concerning their effect on the shelf life of foodstuff in general, and particularly bread. Bread spoilage represents a serious concern for food industries, leading to significant economic losses and posing a major threat for human health. The most common molds causing bread spoilage

belong to *Penicillium*, *Aspergillus* and *Cladosporium* genera (Gänzle & Gobbetti, 2013). The applicability of *W. anomalus* strains to control their proliferation has only been marginally assessed (Coda et al., 2011).

In our previous study (Syrokou et al., 2021), the inhibitory activity of three *W. anomalus* strains, namely LQC 10346, 10353 and 10360, against the growth of *Pe. chrysogenum*, was reported and attributed to the production of non-proteinaceous compounds. Thus, the aim of the present study was to assess the effect of substrate, initial pH value and NaCl content on the growth and antimold activity of the three *W. anomalus* strains, identify the responsible VOCs and to evaluate the effect on the shelf life of experimental breads.

3. Materials and Methods

3.1 Microbial strains and culture conditions

Three sourdough-derived yeast strains, namely *W. anomalus* LQC 10346, 10353 and 10360 (Syrokou et al., 2020) and one mold strain belonging to the species *Pe. chrysogenum* (moldy bread isolate) were used throughout this study. The yeast strains were able to produce non-proteinaceous compounds with inhibitory activity against the mold strain (Syrokou et al., 2021).

The yeast strains were stored at -20 °C in Nutrient Broth (LAB M, Lancashire, UK), supplemented with 50% glycerol (Applichem, Darmstadt, Germany). The mold strain was stored in BHI agar (LAB M) slants, at 4 °C. Before experimental use, *W. anomalus* strains were grown twice in BHI broth at 30 °C for 48–72 h, while mold was grown on BHI agar, at 25 °C for 5 days.

3.2 Effect of pH and NaCl on growth and antimold activity of *W. anomalus* strains

Overnight cultures of the three *W. anomalus* strains were washed twice with sterile saline, resuspended in the same medium and used to inoculate BHI broth adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and supplemented with NaCl at concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5%). The inoculum level was 6 log CFU/mL. Incubation took place at 30 ◦C for 24 h. Yeast enumeration was performed

by plating serial dilutions on BHI agar. The WDA, as previously described by Syrokou et al. (2021), with minor modifications, was applied to assess the antimold activity. More accurately, wells were aseptically punched, with the aid of a Pasteur pipette, in freshly prepared lawns of BHI agar previously surface inoculated with the *Pe. chrysogenum* strain. Twenty-four-hour yeast cultures were centrifuged (12,000 x g; 15 min; 4 ◦C) to obtain cell-free supernatants (CFS), which were further neutralized and treated with catalase (Sigma-Aldrich, St. Louis, MO, USA). An amount of 25 μL of each CFS was added to each well. Incubation took place at 25 ◦C for 5 days. The antimold activity was quantified by applying the two-fold serial dilution approach on the CFS and expressed in AU/mL. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution, exhibiting a clear inhibition zone, multiplied by 40 to obtain AU/mL. All analyses were performed in triplicate and the average values are presented.

Liquid wheat dough was prepared by mixing sterilized wheat flour (10 g) and tap water (30 mL). The liquid wheat dough at pH 6.0 was supplemented with NaCl at 6 concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5%). Overnight cultures of the three *W. anomalus* strains were washed twice with sterile saline, resuspended in the same medium and used to inoculate liquid wheat dough at 6 log CFU/mL. Incubation took place at 30 ˚C for 24 h. Yeast enumeration and WDA were performed as previously described.

3.3 Identification of yeast volatile compounds by SPME-GC-MS

W. anomalus VOCs obtained after growth of the strains in conditions, which previously revealed the maximum antimold activity, were determined by SPME-GC-MS, according to Xagoraris et al. (2021), with some minor modifications. Regarding the isolation of VOCs, SPME was performed using a divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/ CAR/ PDMS) fiber 50/ 30 μm (Supelco, Bellefonte, PA, USA) with 1 cm length. For fiber activation, heating to 260 °C was applied. At the same time, two mL of *W. anomalus* CFSs were introduced into 15 mL screw top vials with PTFE/silicone septa and water bath followed at 35 °C for 15 min. Then, the needle of the SPME fiber was inserted into the vial and exposed to the sample headspace. The sampling time lasted 15 min. Then, the fiber was removed from the vial and inserted into the gas chromatograph injector for analysis of the isolated VOCs. VOCs were analyzed applying a Trace Ultra gas chromatograph (GC) (Thermo Scientific Inc.,

Waltham, MA, USA), coupled to a mass spectrometer (MS) (DSQII, Thermo Scientific Inc., Waltham, MA, USA). The desorption conditions were as follows: GC inlet temperature 260 °C in the splitless mode for 3 min, with a 0.8 mm injector liner (SGE International Pty Ltd., Ringwood, Australia). The column used was a Restek Rtx-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness) and the flow rate of carrier gas (helium) was 1 mL/min. The column was adjusted to 40 °C for 6 min, then increased to 120 °C at a rate of 5 °C/min, followed by another increase to 160 °C at a rate of 3 °C/min, then heated to 250 °C with a step of 15 °C/min and kept at 250 °C for 1 min. The transfer line and injector temperatures were maintained at 290 and 220 °C, respectively. Electron impact was 70 eV, and mass spectra were recorded at the 35–650 mass range. The peak identification was carried out with the Wiley 275 mass spectra library, its mass spectral data and arithmetic index provided by Adams. VOCs were tentatively identified by comparing their elution order and mass spectra with data from the NIST 98, Wiley 275 mass spectra libraries.

3.4 Antimold activity assessment of pure volatile compounds against *Pe. chrysogenum*

Pure standards of 12 previously identified VOCs, namely ethanol, ethyl acetate, acetic acid-butyl ester, isoamyl alcohol, isoamyl acetate, 3-methyl-butanoic-acid, butanoic acid- ethyl ester, ethyl caprylate, benzaldehyde, octanal, nonanal and 2,4-ditert-butyl-phenol, purchased from Sigma Aldrich, were used for inhibitory activity assessment against *Pe. chrysogenum*, according to Oro et al. (2018), with some modifications. In brief, an agar plug (6-mm diameter), obtained from actively growing culture of *Pe. chrysogenum,* was placed in the center of freshly prepared lawn of BHI agar. Then, different concentrations of the 12 VOCs, ranging from 0.02 to 6.45 μL/mL of headspace, were placed on paper filter (Whatman No. 1, 90 mm diameter), which was placed in the center of the Petri dish lid. Distilled water was used as control. The dishes were immediately closed and sealed with parafilm. Incubation took place at 25 ◦C for 5 days. The concentration of each VOC that inhibited growth of the mold (i.e., growth less than 5 mm around the agar plug) was considered as the MIC of each compound. The experiment was performed in triplicate.

3.5 Bread preparation and shelf life assessment

Liquid wheat dough supplemented with 1.0% NaCl concentration, in which the maximum antimold activity of the yeast strains was recorded, was prepared by mixing 20 g wheat flour and 60 mL tap water. Overnight cultures of the three *W. anomalus* strains were treated as previously described and used to inoculate the liquid wheat dough at 6 log CFU/mL. Each yeast strain was inoculated in monoculture as well as in coculture with commercial baker's yeast. Liquid wheat dough inoculated only with commercial baker's yeast was used as control. Incubation took place at 30 °C for 24 h. Then, 50 g of each incubated liquid dough was added to a mixture of 250 g wheat flour, 150 mL water and 4.5 g NaCl (final NaCl concentration 1%). After mixing, the doughs were placed in separate containers and proofing took place (30 °C for 2.5 h). Baking of doughs took place at 200 °C for 40 min. After baking, the bread loaves were cooled at room temperature for 3 h. Then, slices of ca. 20 g were cut from each loaf and placed in polyethylene bags. The shelf life of bread slices was monitored on a daily basis, at room temperature, until visible signs of mold presence were detected. The shelf life was monitored until 30 d after control bread exhibited visible mold growth. The experiment was performed in triplicate.

3.6 Statistical analysis

The contribution of the initial pH value and NaCl on the antimold activity and population of the three *W. anomalus* strains was evaluated through Multifactor Analysis of Variance. The method of 95% LSD was applied as post-hoc comparison test to discriminate among the means, at $p < 0.05$. All calculations were performed in Statgraphics Centurion XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

4. Results

4.1 Effect of pH and NaCl on growth and antimold activity of *W. anomalus* strains

Antimold activity in the three *W. anomalus* strains, namely LQC 10346, 10353 and 10360, after incubation in BHI broth, adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and 6 NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5%), is

presented in Figure 4.1. After 24 h incubation at 30 °C, the population of yeast strains LQC 10346, 10353 and 10360 ranged between 7.11–7.45, 7.03–7.39 and 7.03–7.46 log CFU/mL, respectively.

The maximum antimold activity, by all three yeast strains, was quantified at 5120 AU/mL. However, the aforementioned activity was achieved at different combinations of initial pH value and NaCl concentrations. More specifically, strain LQC 10346 reached maximum anti-mold activity in BHI broth adjusted to initial pH 6.0, supplemented with 0.0 and 1.0% NaCl. Regarding strain LQC 10353, maximum antimold activity was achieved in BHI broth adjusted to initial pH 4 supplemented with 1.5% NaCl and in BHI broth adjusted to initial pH 4.5 and supplemented with 0.5 and 1.5% NaCl. Finally, the maximum antimold activity of strain LQC 10360 was obtained in BHI broth adjusted to initial pH 5.0, without NaCl addition, and in BHI broth adjusted to initial pH 4.5, supplemented with 2.5% NaCl.

Multifactor Analysis of Variance was applied to assess the effect of initial pH value and NaCl on the antimold activity of each yeast strain. Initial pH value, NaCl concentration and their in-pair interactions were found to significantly affect the antimold activity exhibited by all three strains ($p < 0.05$). The method of 95% LSD was applied as a post-hoc comparison test, so as to elucidate how the antimold activity of the three yeast strains was differentiated among the 6 levels of both pH values and NaCl concentrations (Table S4.1). In the case of strain LQC 10346, the mean antimold activities obtained after growth in BHI broth at pH 3.5, 4.0 and 4.5 were similar in most treatments and that was the case for the activities recorded at pH 5.0 and 5.5. Their differentiation from the mean activity, corresponding to initial pH 6.0, was evident only in a few cases, namely at 0.0 and 1.0% NaCl. Regarding the contribution of the different NaCl concentrations to the mean antimold activity, supplementation of BHI broth adjusted to pH 3.5, 5.0 and 6.0 with 0.0 and 1.0% NaCl revealed overlapping activities, which were differentiated from those at 1.5% NaCl. In the case of strain LQC 10353, the effect of pH values on the antimold activity revealed no statistically significant differences in the majority of cases, with some minor exceptions. More accurately, in BHI broth at 1.5% NaCl, the mean activities corresponding to pH 3.5, 5.0 and 6.0 were similar and were further differentiated from those at pH 4.0 and 4.5. In addition, overlapping mean activities were recorded in the same medium at pH 3.5, 4.5, 5.0 and 5.5, at 2.0% NaCl, which were significantly different from those at pH 4.0 and 6.0, with

the latter two being differentiated in a pH-dependent manner, as well. In terms of the contribution of the different NaCl levels, the mean activities recorded upon growth at pH 5.5 were similar among the different NaCl concentrations. On the other hand, the adjustment of BHI broth to pH 4.0 resulted in a NaCl-dependent antimold capacity. In brief, similar activities were recorded at 0.0, 0.5 and 2.5% NaCl, which were differentiated from those at 1.5% and 2.0%, between which significant differences were displayed as well. Regarding strain LQC 10360, overlapping mean antimold activities were recorded upon growth in BHI broth at pH 3.5 and 4.0, corresponding to 0.0, 0.5, 1.0 and 2.0% NaCl, respectively. Similar was the case for the mean activities at pH 5.0 and 5.5, at 0.5, 1.5, 2.0 and 2.5% NaCl, respectively. Concerning the effect of different NaCl levels on the antimold activity, overlapping means were observed after growth in BHI broth supplemented with 0.0, 0.5 and 1.0% NaCl, at pH 4.0, 4.5 and 5.5, respectively, which were further differentiated from those corresponding to 2.5% NaCl.

Regarding the effect of initial pH value and NaCl on the growth of yeast strains, Multifactor ANOVA revealed statistically significant differences among the mean populations obtained from the different levels of NaCl and initial pH values, either as single factors or as a combination ($p < 0.05$). However, these differences lack biological significance, since yeast populations ranged within less than half a logarithm.

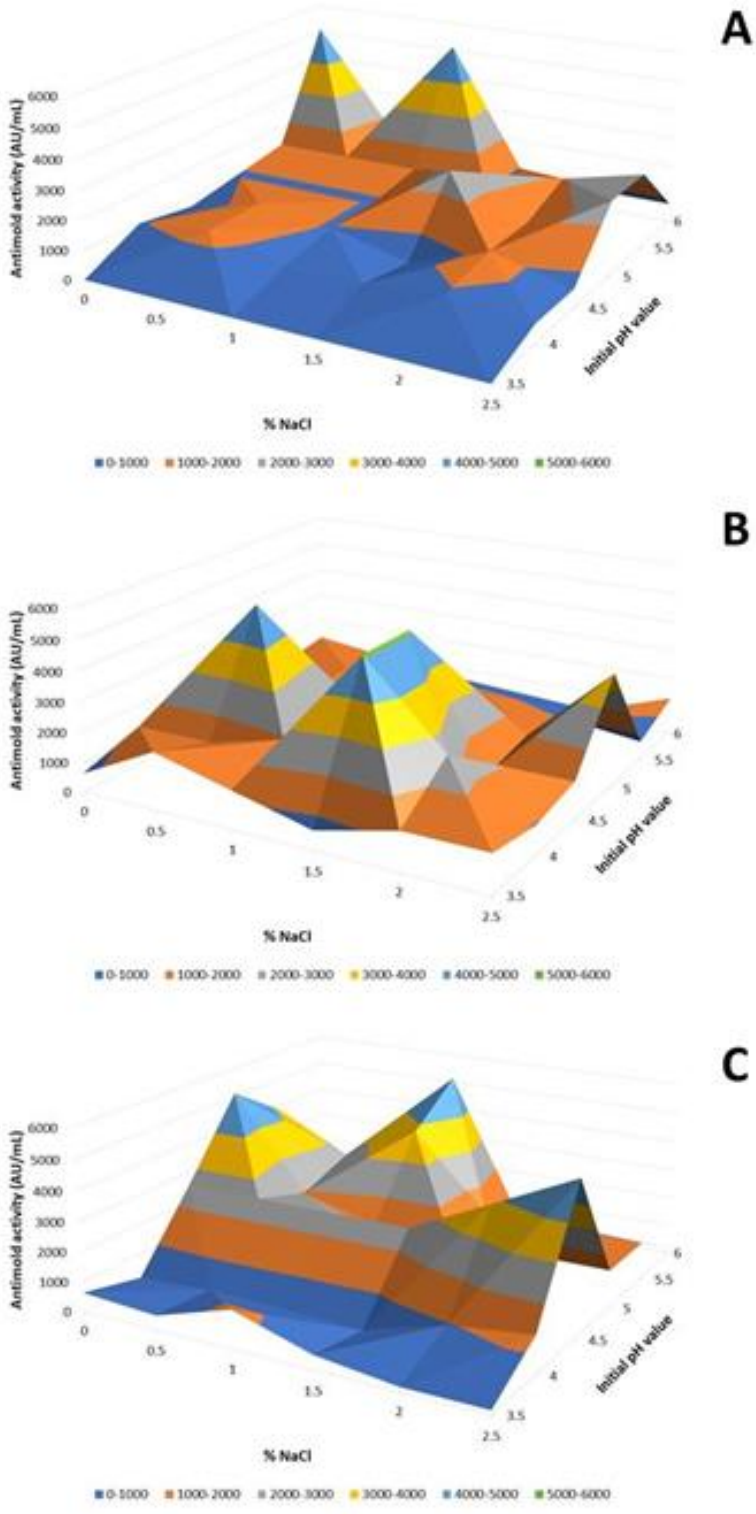

Figure 4. 1. Antimold activity (AU/mL), exhibited by the three *W. anomalus* strains, LQC 10346 (A), 10353 (B) and 10360 (C), after growth in modified BHI broth adjusted to 6 initial pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and 6 NaCl concentrations (0.0, 0.5, 1.0%, 1.5, 2.0, 2.5%), at 30 ˚C for 24 h.

As far as the antimold activity of the three *W. anomalus* strains, namely LQC 10346, 10353 and 10360 was concerned, the 24 h incubation at 30 °C in liquid dough supplemented with 6 NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5%) resulted in populations ranging between 7.28–7.45, 7.21–7.32 and 7.26–7.37 log CFU/mL, respectively. The maximum antimold activity of all strains was quantified at 5120 AU/mL and was recorded in liquid dough supplemented with 1.0% NaCl (Table S4.2). The statistically significant contribution of the NaCl concentration on the antimold activity was also exhibited ($p < 0.05$).

4.2 Identification of yeast volatile compounds by SPME-GC-MS

Cell-free supernatants of the three *W. anomalus* strains, grown under conditions in which the highest inhibitory activity against *Pe. chrysogenum* was observed, were subjected to SPME-GC-MS analysis. The qualitative analysis of VOCs is summarized in Table 4.1. Twelve compounds were detected, among which three alcohols (ethanol, isoamyl alcohol, 2,4-di-tert- butyl-phenol) three aldehydes (benzaldehyde, nonanal, octanal), one acid (3-methyl-butanoic acid), and five esters (isoamyl acetate, ethyl acetate, butanoic acid-ethyl ester, acetic acid-butylester, ethyl caprylate) were identified. Ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate were detected in all samples. Ethyl acetate was the dominant compound in all samples, with ethanol and isoamyl alcohol being in relative abundance as well; the % area of these three compounds ranged between 77.6% and 89.2% of the total peak area of the samples. The restof the volatile compounds were not produced in all conditions tested. Acetic acid-butyl ester and 2,4-di-tetr-butyl-phenol were produced by all yeast strains, butanoic acid-ethyl ester and ethyl caprylate were only produced by strain LQC 10346, 3-methyl-butanoic acid and octanal by strain LQC 10353, while nonanal by both LQC 10346 and 10353. Finally, benzaldehyde wasproduced by strains LQC 10353 and 10360.

The incubation of yeast strains in liquid wheat dough resulted in the production of fewer volatile compounds, compared to those identified after growth in BHI broth. More accurately, the stable presence of ethanol, ethyl acetate, isoamyl alcohol and isoamyl acetate was detected in all samples assessed, in a strain- and substrate-independent manner. Additionally, 2,4-di- tert-butyl-phenol was identified in fewer samples; however, it was retrieved from incubation in both substrates. Regarding the VOCs identified in the *W. anomalus* CFS after growth in BHI broth, the presence of acetic

acid-butyl ester, butanoic acid-ethyl ester, ethyl caprylate, benzaldehyde, nonanal, octanal, and 3-methyl-butanoic acid was detected as well, in a strain, substrate, initial pH value and NaCl-dependent manner.

Generally, the most abundant compound in every sample assessed, generated by % area, was ethyl acetate (ranging from 43.50 to 74.31%). In addition, isoamyl alcohol and ethanol were constantly detected in significant amounts as well (ranging from 9.14 to 17.67% and from 5.23 to 24.81% of total area, respectively). Acetic acid-butyl ester and nonanal exceeded 1.0% of total area in samples detected. Similar was the case for benzaldehyde, the abundance of which was recorded at 1.72% and 2.98% of total area in two assessed samples, but in another one, was found less than 1.0%. Regarding the abundance of isoamyl acetate, only in some cases, it exceeded 1.0% of the total area.

Table 4. 1. VOCs produced by three *W. anomalus* strains, after a 24 h incubation at 30 °C, in modified BHI broth and liquid wheat dough at different pH value and NaCl combinations. Identification was performed by SPME-GC-MS.

Strain/ Substrate	pH value	NaCl (%)	Compound	Kovats Index	Area (%)
LQC 10346/ BHI broth	6.0	0.0	Ethanol	< 800	9.11
			Ethyl acetate	< 800	63.43
			isoamyl alcohol	< 800	15.10
			Butanoic acid-ethyl ester	< 800	0.24
			Acetic acid-butyl ester	814	1.81
			isoamyl acetate	879	0.62
			Nonanal	1105	1.25
			Ethyl caprylate	1197	0.33
	6.0	1.0	Ethanol	< 800	6.28
			Ethyl acetate	< 800	73.74
			isoamyl alcohol	< 800	9.14
			Butanoic acid-ethyl ester	< 800	0.16
			Acetic acid-butyl ester	814	1.48
			isoamyl acetate	879	1.57
			Ethyl caprylate	1197	0.20
LQC 10353/ BHI broth	4.0	1.5	Ethanol	< 800	11.17
			Ethyl acetate	< 800	56.58
			isoamyl alcohol	< 800	15.01
			3-methyl-butanoic acid	854	0.33
			isoamyl acetate	879	0.61
			Benzaldehyde	965	1.72
			2,4-di-tert-butyl-phenol	1505	0.44
	4.5	0.5	Ethanol	< 800	5.23
			Ethyl acetate	< 800	74.31
			isoamyl alcohol	< 800	9.68
			Acetic acid-butyl ester	814	1.75
			isoamyl acetate	879	2.69
	4.5	1.5	Ethanol	< 800	6.87
			Ethyl acetate	< 800	70.48
			isoamyl alcohol	< 800	11.79
			isoamyl acetate	879	1.24
			Benzaldehyde	965	0.66

			Octanal	1002	0.38
			Nonanal	1105	1.05
LQC 10360/ BHI broth	4.5	2.5	Ethanol	< 800	17.76
			Ethyl acetate	< 800	43.50
			isoamyl alcohol	< 800	16.39
			isoamyl acetate	879	0.40
			Benzaldehyde	965	2.98
			2,4-di-tert-butyl-phenol	1505	0.19
	5.0	0.0	Ethanol	< 800	10.82
			Ethyl acetate	< 800	63.40
			isoamyl alcohol	< 800	12.40
			Acetic acid-butyl ester	814	1.69
			isoamyl acetate	879	0.62
			2,4-di-tert-butyl-phenol	1505	0.59
	6.0	1.0	Ethanol	< 800	15.93
			Ethyl acetate	< 800	51.64
			isoamyl alcohol	< 800	17.67
			Acetic acid-butyl ester	814	1.81
			isoamyl acetate	879	0.36
LQC 10346/ Liquid wheat dough	6.0	1.0	Ethanol	< 800	20.01
			Ethyl acetate	< 800	51.60
			isoamyl alcohol	< 800	11.42
			isoamyl acetate	879	0.66
			2,4-di-tert-butyl-phenol	1505	0.35
LQC 10353/ Liquid wheat dough	6.0	1.0	Ethanol	< 800	24.81
			Ethyl acetate	< 800	44.05
			isoamyl alcohol	< 800	14.26
			isoamyl acetate	879	0.61
			2,4-di-tert-butyl-phenol	1505	0.31
LQC 10360/ Liquid wheat dough	6.0	1.0	Ethanol	< 800	20.36
			Ethyl acetate	< 800	57.27
			isoamyl alcohol	< 800	10.83
			isoamyl acetate	879	0.57

4.3 Antimold activity assessment of pure volatile compounds against *Pe. chrysogenum*

In Table 4.2, the MIC of pure VOCs against *Pe. chrysogenum* is presented. All compounds but ethanol exhibited antimold activity. Benzaldehyde and 2,4-di-tert-butyl-phenol were characterized by the lowest MICs, with only 0.06 and 0.04 μL/mL of headspace, respectively. The rest of the aldehydes, namely nonanal and octanal, could also effectively suppress the growth of *Pe. chrysogenum*, with a MIC of 0.34 and 0.42 μL/mL of headspace, respectively. As far as esters were concerned, higher MICs were recorded, with butanoic acid-ethyl ester and ethyl caprylate needing 5.14 and 6.24 μL/mL of headspace, respectively, to inhibit mold growth.

Table 4. 2. Antimold activity of pure VOCs against *Pe. chrysogenum*.

Volatile Organic Compounds	MIC (μL/mL of Headspace)
Alcohols	
Ethanol	-
Isoamyl alcohol	0.97 (0.00) ^{e}
2,4-di-tert-butyl-phenol	0.04 (0.00) ^{a}
Aldehydes	
Octanal	0.42 (0.02) ^{c}
Nonanal	0.34 (0.00) ^{b}
Benzaldehyde	0.06 (0.00) ^{a}
Acids	
3-methyl-butanoic acid	0.67 (0.03) ^{d}
Esters	
Isoamyl acetate	2.15 (0.00) ^{g}
Ethyl acetate	2.01 (0.01) ^{f}
Butanoic acid-ethyl ester	5.14 (0.03) ^{i}
Acetic acid-butyl ester	4.93 (0.03) ^{h}
Ethyl caprylate	6.24 (0.00) ^{j}

Statistically significant differences are indicated with different letters a-j ($p < 0.05$), according to the method of 95% LSD applied as post-hoc comparison test.

4.4 Bread preparation and shelf life assessment

Bread produced with 20% addition of liquid dough, inoculated with the monocultures of the three *W. anomalus* strains or their cocultures with baker's yeast, gained an additional period of mold free shelf life, ranging from 9 to 30 days, compared to the control (bread made only with commercial yeast). More accurately, the greatest prolongation of bread shelf life was recorded with the addition of strain LQC 10360, which led to a shelf life extension of 30 days. On the other hand, bread made with the incorporation of strain LQC 10353 presented an increased shelf life of 9 days. A similar shelf life extension (10 days) was recorded with the addition of strain LQC 10346. Co-culture with baker's yeast did not alter the shelf life extension.

5. Discussion

Mold spoilage in bread represents an ongoing safety and quality concern for various food industries. The use of lactobacilli as biocontrol agents in the sourdough-bread-

making process has been extensively assessed and reported to confer shelf life prolongation and a delay in the staling of baked products, in a strain-specific manner (Axel et al., 2017). However, the application of yeasts as antimold agents for the production of bakery products has been only marginally studied (Coda et al., 2011, Rahman et al., 2022).

The ability of *W. anomalus* strains to thrive in NaCl-rich mediums and at a wide range of initial pH values has already been reported (Sakandar et al., 2018, Ciafardini et al., 2019). The NaCl and pH-tolerant profile of this species was verified in our study as well. The antimold activity was attributed to compounds of a non-protein nature (Syrokou et al., 2021); thus, the production of killer toxins was excluded and the production of VOCs was further considered. Many studies have correlated the inhibitory activity of *W. anomalus* strains with the production of VOCs (Parafati et al., 2015, Grzegorczyk et al., 2017). However, only a few of them reported VOCs identification. In our study, the most abundant com- pounds produced by yeasts were ethyl acetate, ethanol and isoamyl alcohol, while the stable presence of isoamyl acetate was detected as well. In agreement with our observations, a recent study by Khunnamwong et al. (2020) reported that the biocontrol activity of *W. anomalus* strains against five phytopathogenic fungi was mainly attributed to the synthesis of VOCs, with isoamyl alcohol and isoamyl acetate being the most abundant. In addition, Oro et al. (2018) demonstrated that VOCs synthesized by *W. anomalus* exhibited the highest inhibitory activity against *Botrytis cinerea* (87% total inhibition), while *Pe. digitatum* was suppressed only at 1.5%. Consistent with our results, the main VOC produced by *W. anomalus* was ethyl acetate. In another study, by Hua et al. (2014), the biocontrol activity of a *W. anomalus* strain, WRL-076, was attributed to 2-phenylethanol, which further suppressed the spore germination and aflatoxin production of *Aspergillus flavus*. In an earlier study by Masoud et al. (2005), *W. anomalus* strains, grown both on malt yeast glucose peptone and coffee agar, were found to inhibit the growth of *A. ochraceus* and concomitant ochratoxin A production. Regarding VOCs production during coffee processing, the main esters identified with GC-MS were ethyl acetate and isobutyl acetate, while the abundance of isoamyl alcohol was also reported.

It has been proposed that the biocontrol activity of VOCs against mold species decreases, according to the following order: organic acid > aldehyde > alcohol > ether

> ketone > ester > lactone (Maruzzella et al., 1961). However, this is not always the rule, since there are cases op- posing the aforementioned statement (Kurita et al., 1981, Calvo et al., 2020). This was similar to our study, where the MIC of 3-methyl-butanoic acid was higher than the respective of 2,4-di-tert-butyl-phenol and benzaldehyde, which required only 0.04 and 0.06 μL/mL, respectively. The increased antimold effect of 2,4-di-tert-butyl-phenol has probably been attributed to the free functional group (hydroxyl), coupled to the presence of hydrophobic alkyl groups (Kurita et al., 1981). Alkyl groups contribute to the antimold activity of phenol, since their hydrophobicity enhances the affinity for cell membranes, thus, impairing the integrity and leading to disruption. Varsha et al. (2015), reported that at 400 μg/disc, an inhibition zone of 1.5 cm against *Pe. chrysogenum*, was evident, while Padmavathi et al. (2015) demonstrated that a complete suppression of fungal growth was exhibited, with an MIC of 100 μg/mL. In the case of ethanol, its mold- inhibiting effects have already been reported (Morita et al., 2019). According to Dao & Dantigny (2011), the concentration of ethanol applied, combined with the microorganism assessed, exert distinct effects on mold inhibition. Results obtained in our study revealed that 98% ethanol could not prevent the growth of *Pe. chrysogenum.* Similarly, Rogawansamy et al. (2015) reported that 70% ethanol was ineffective to suppress the growth of *Pe. chrysogenum* and *A. fumigatus*, while in another study, by Druvefors & Schnürer (2005), ethanol was found to exert a minor but synergic effect on the antimold activity of *W. anomalus* J121. In general, ethanol is a short-chain alcohol, the inhibitory activity of which is lower against mold species, compared to longer-chain alcohols, namely isoamyl alcohol, due to the increased affinity of the latter for the cell membrane (Ando et al., 2015).

Among the aldehydes assessed, benzaldehyde had the most pronounced antimold effect in our study, while nonanal and octanal needed a higher MIC in order to inhibit the growth of *Pe. chrysogenum*. In accordance with our results, Calvo et al. (2020) demonstrated that only 0.063 mL/L of benzaldehyde was necessary to suppress the growth of *B. cinerea* and *Pe. expansum*. In addition, Zhang et al. (2017) reported that the MIC of nonanal for the complete suppression of *Pe. cyclopium* was 0.35 μL/mL, while octanal needed 0.50 μL/mL for total mycelial growth inhibition of both *Pe. italicum* and *Pe. digitatum* (Tao et al., 2014). However, in another study by Pishawikar & More (2017), the opposite results were demonstrated. More accurately, analogs with aromatic aldehydes exhibited decreased inhibitory activity compared to those

with aliphatic aldehydes against *Candida albicans*. The reason for that was the presence of structural characteristics, which interacted via hydrophobic or Van der Waals forces with the target molecules.

Regarding the antimold activity of the other VOCs, higher MICs were needed to suppress the growth of *Pe. chrysogenum*. In brief, the MICs corresponding to isoamyl alcohol and isoamyl acetate for mold inhibition were 0.97 and 2.15 μL/mL, respectively. These results were in agreement with the previous findings by Ando et al. (2012), which demonstrated that the amount of isoamyl acetate used for the complete inhibition of *Pe. chrysogenum* was two-times higher than the respective isoamyl alcohol. The reason for this could be related to the presence of reactive hydroxyl groups in the case of isoamyl alcohol, thus, being less volatile, in contrast to esters, which are characterized by increased volatility due to the absence of free functional groups. Among esters, ethyl acetate has been reported to suppress the growth of *B. cinerea* at 8.97 mg/cm^3 and gray mold on strawberry fruit at 0.718 mg/cm^3 (Oro et al., 2018), while according to Masoud et al. (2005), at 48 μg/L of headspace, it was able to reduce the growth of *A. ochraceus* only at 15%. The association of butanoic acid-ethyl ester, acetic acid-butyl ester and ethyl caprylate with the volatile metabolome of microbial strains with antimold activity has been documented, as well (Belinato et al., 2018, Guo et al., 2019, Emanuel et al., 2020).

As far as the use of *W. anomalus* strains on sourdough bread making was concerned, only a few studies have assessed their application as starter cultures in sourdough fermentations. More accurately, Coda et al. (2011) shed some light by reporting that dough produced with a co-culture of *Lp. plantarum* 1A7 and *W. anomalus* LCF1695 exhibited good organoleptic features and further contributed to the mold free shelf life extension of bread slices, up to 28 days of storage at room temperature. Similar were the findings obtained in our study, according to which, bread made with the *W. anomalus* strain LQC 10360 gained one additional month of mold free shelf life, compared to the control. Finally, the suitability of all *W. anomalus* strains to be used as an adjunct culture in breadmaking was exhibited, since the co-presence of *W. anomalus* and baker's yeast displayed the same antimold effect compared to monoculture trials.

6 Conclusions

In this work, the antimold capacity of three sourdough-derived *W. anomalus* strains, as well as their suitability as adjunct cultures in bread making, were exhibited. The former was attributed to a set of VOCs produced, some of which were common among all strains, and growth conditions were examined. Regarding their applicability as starter cultures in dough preparation, with further use in bread making, the antimold capacity of the yeast strains was retained, even in the presence of commercial baker's yeast, providing an additional mold-free shelf life of up to 30 days. Thus, the findings of the present study included, among others, the production of yeasted breads with extended shelf life; however, the combination of the antimold *W. anomalus* strains with selected LAB, for further application in sourdough fermentation, is yet to be conducted. From a technological perspective, the collaboration among non-conventional yeasts and acidifying LAB will lead to the production of leavened sourdough products, characterized by microbiological stability and desired flavor attributes. Therefore, moving towards the production of green label products, the assessment of antimold starter cultures, both *in vitro* and *in situ*, is mandatory.

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Chapter 5

High quality draft genome sequence data of six Lactiplantibacillus plantarum subsp. argentoratensis strains isolated from various Greek wheat sourdoughs

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1. Abstract

Lp. plantarum is a species found in a wide range of foods and other commodities. It can be used as starter or adjunct culture in fermented foods. Herein the annotated high-quality draft genome (scaffolds) of 6 *Lp. plantarum* subsp. *argentoratensis* strains (LQC 2320, LQC 2422, LQC 2441, LQC 2485, LQC 2516 and LQC 2520) isolated from various Greek wheat sourdoughs is presented. Raw sequence reads were quality checked, assembled into larger contiguous sequences and scaffolds were annotated. The total size of the genomes ranged from 3.13 Mb to 3.49 Mb and the GC content from 45.02% to 45.13%. The total number of coding and non-coding genes were between 3268 and 3723 (3091 to 3492 protein-coding genes, 62 to 107 repeat-region, 54 to 59 tRNAs and 2 to 5 rRNAs, 20 to 30 crispr-repeats, 17 to 26 crispr-spacers and 2 to 4 crispr-arrays). The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession numbers JAEQMR000000000, JAEQMQ000000000, JAEQMP000000000, JAEQMO000000000, JAEQMN000000000 and JAEQMM000000000. The version described in this paper is version JAEQMR010000000, JAEQMQ010000000, JAEQMP010000000, JAEQMO010000000, JAEQMN010000000 and JAEQMM010000000. Raw sequence reads have been submitted in the Sequence Read Archive (SRA) under the Bio-Project accession number PRJNA689714 (BioSample accession numbers SAMN17215143, SAMN17215144, SAMN17215145, SAMN17215146, SAMN17215147 and SAMN17215148 and SRA accession numbers SRR13357463, SRR13357464, SRR13357465, SRR13357466, SRR13357467, SRR13357468).

2. Introduction

Lp. plantarum species is a microorganism found in a wide range of food commodities. Therefore, analysis of the genome of the *Lp. plantarum* subsp. *argentoratensis* strains will provide insights regarding their genomic and functional features and their potential use as a starter and/or adjunct culture. Data obtained could be of interest for third parties dealing with sourdough fermentations and/or other fermented foods, as well as with lactic acid bacteria as starters. In addition, the data retrieved from the sequencing analysis of genomes of the aforementioned *Lp. plantarum* subsp. *argentoratensis* strains will be available to scientific community for applying other bioinformatics approaches such as comparative genomics to investigate the genome evolution of this species and other technological characteristics. Thus, the present study contributes to the limited number of available genomes of the *Lp. plantarum* subsp. *argentoratensis* strain by providing high-quality whole-genome sequences.

3. Materials and Methods

Lp. plantarum subsp. *argentoratensis* strains were cultured in MRS broth (LAB M, Lancashire, UK) and incubated overnight at 30°C. DNA was extracted from the microorganisms according to Syrokou et al. (2020). The genomic DNA was sequenced by Novogene Genomics Service (Novogene Co., Ltd, UK). At each step of the procedure (sample test, library preparation, and sequencing) quality control was performed. Agarose gel electrophoresis and Qubit 2.0 were employed to test DNA degradation and potential contamination, and to quantify the DNA concentration, respectively (sample quality control step). For the library construction and quality control, the genomic DNA was randomly fragmented by sonication, then DNA fragments were end polished, A-tailed, and ligated with the full-length adapters of Illumina sequencing and followed by further PCR amplification with P5 and indexed P7 oligos. The PCR products as the final construction of the libraries were purified with AMPure XP system (Beckman Coulter, IN, USA). Then libraries were checked for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and quantified by real-time PCR. The qualified libraries were sequenced using paired-end (2 × 150 bp) libraries in the Illumina Novaseq 6000 sequencer (Illumina, CA, USA). Before assembling, adapter-free raw reads were quality checked with the FastQC

v0.11.5 (Andrews, 2010) tool of the KBase web service (Arkin et al., 2018). Different de novo assemblers such as SPAdes v3.13.0 (Bankevich et al., 2012), MEGAHIT v1.2.9 (Li et al., 2015), IDBA-UD v1.1.3 (Peng et al., 2012) and MaSuRCA v3.2.9 (Zimin et al., 2013), as implemented in the KBase web service, as well as Unicycler (Wick et al., 2017), as implemented in the PATRIC v3.6.8 assembly web service (Davis et al., 2020), were compared and the best assembler according to the Quality Assessment Tool (QUAST) v4.4 (Gurevich et al., 2013) (KBase) was selected to assemble reads into contigs. Pilon tool (Walker et al., 2014) accessible in PATRIC v3.6.8 assembly web service was used for polishing bacterial assembly. Taxonomic assignment of the assemblies was done through the Genome Taxonomy Database tool kit v1.1.0 (GTDB-Tk) (Chaumeil et al., 2019) of the KBase and KmerFinder v3.2 (Hasman et al., 2014) of the CGE Server (http://www.genomicepidemiology.org/). Contigs were organized into scaffolds using the Multi-Draft based Scaffolder (MeDuSa) v1.6 web server (Bosi et al., 2015). The scaffolds were ordered and orientated based on the complete genomes of *Lp. plantarum* subsp. *argentoratensis* DSM 16365 (GCA_003641165.1, ASM364116v1) and *Lp. plantarum* WCFS1 (GCA_000203855.3, ASM20385v3) used as reference genomes. A re-implementation of the algorithm of CheckM tool (Parks et al., 2015), offered by PATRIC v3.6.8, and BUSCO v3 (Simão et al., 2015) analysis with lactobacillales_odb9 dataset, facilitated through the GenomeQC web service (Manchanda et al., 2020), were employed to assess the genome quality at contig and scaffold level. In addition, potential bacterial mis-assemblies, after scaffolding, were evaluated with the Skew Index Test (SkewIT) web app (Lu et al., 2020). Genome annotation of the scaffolds was performed using the Rapid Annotation using Subsystem Technology tool kit (RASTtk) (Brettin et al., 2015) as implemented in the PATRIC v3.6.8 annotation web service. Quality of the genome annotation was assessed through the quality metrics provided by PATRIC annotation web service as well as through GenomeQC web service (BUSCO v3 with lactobacillales_odb9 dataset). Annotation based on the NCBI Prokaryotic Genome Annotation Pipeline, performed during the genome submission in the GenBank, is also available at the NCBI website (https://www.ncbi.nlm.nih.gov/).

4. Results

Herein the high-quality draft genome of 6 *Lp. plantarum* subsp. *argentoratensis* strains, isolated from Greek wheat sourdoughs (Syrokou et al., 2020), is presented. FastQC tool showed that the adapter-free raw reads were of high quality and therefore de novo assembly was performed without sequence trimming. Different assemblers were employed and QUAST revealed that in overall Unicycler provided the best assembly (Fig. 5.1). Quality metrics, genomic and functional characteristics of the genomes after scaffolding are shown in Tables 5.1 and 5.2 and Figure 5.2 CheckM, BUSCO and GC skew analysis confirmed the high quality of the genomes at scaffold level. Genome completeness (100%) and contamination (0.0% to 4.8%) levels were above and below the corresponding limits, respectively (>90% and <10%) (Table 5.1). Based on the BUSCO analysis, the percentage of BUSCO genes are displayed in Table 5.1 and the assembled scaffolds were free of contamination (i.e., the assembled sequences were screened against the NCBI UniVec database to quickly identify sequences of vector origin or those of adaptors or linkers). The SkewI metric ranged between 0.933 and 0.993 (Fig. 5.3. Table 5.1), which is far above the threshold value of 0.857 for the genus of *Lactobacillus* (Fig. 5.3). Quality of genome annotation was also good as represented by the genome annotation consistency indices and BUSCO evaluation (Table 5.2). The number of protein coding genes annotated was 3091 to 3492 while the non-coding genes were between 160 and 231 (Table 5.2, Fig. 5.2). Subsystem analysis (set of proteins that perform a specific biological process or form a structural complex) depicted that almost 40% of the annotated protein-coding genes associated with metabolism followed by protein processing (ca. 15%) (Fig. 5.4). Finally, specialty genes related to transporters and antibiotic resistance were also identified (Table 5.2, Fig. 5.2).

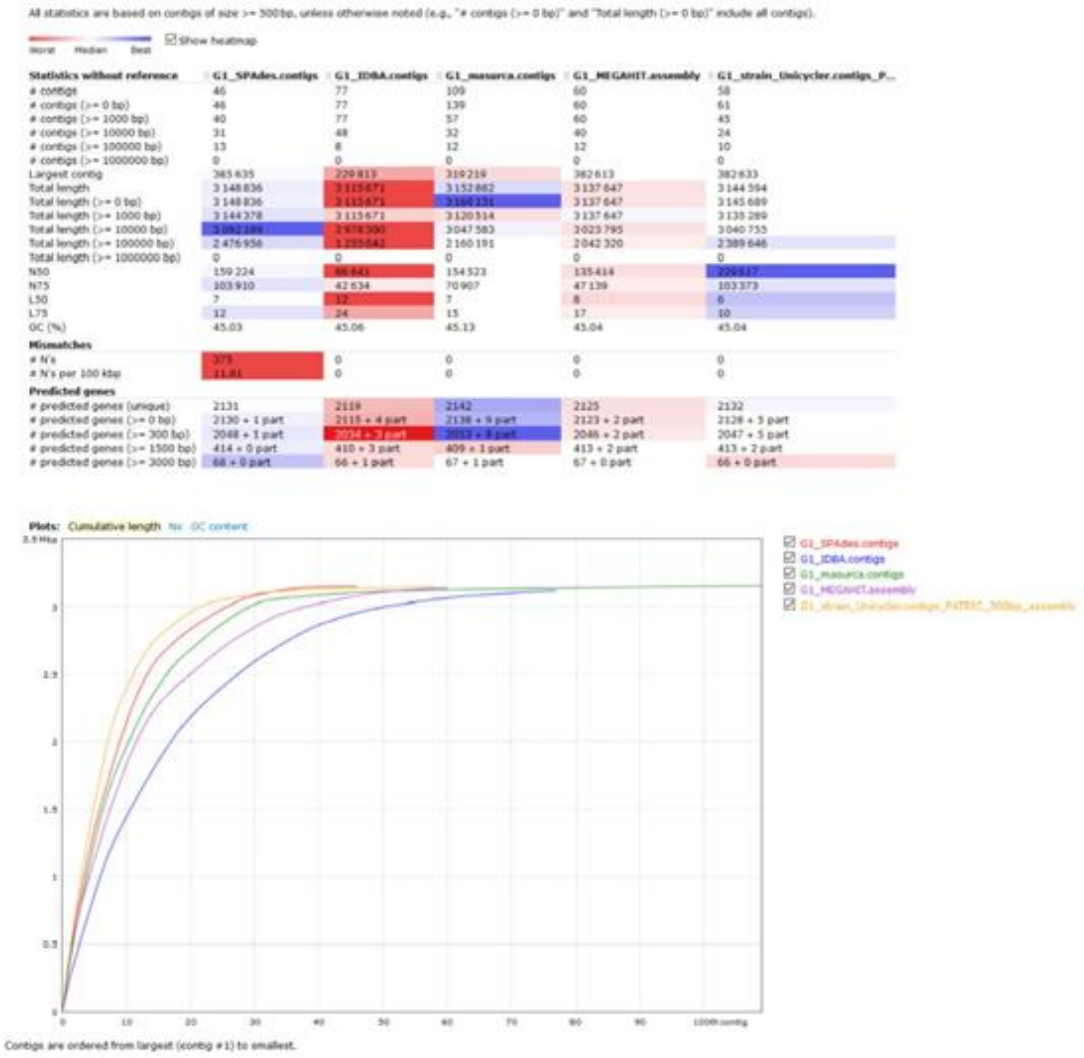

Figure 5. 1. UAST report comparing different assemblers for the *Lp. plantarum* subsp. *argentoratensis* LQC 2441 strain (as signed as G1_strain).

Table 5. 1. Characteristics and quality metrics of the six *Lp. plantarum* subsp. *argentoratensis* genomes after genome assembly into scaffolds[a].

Strain	No of scaffolds	Genome length (bp)	N50 (bp)	GC content (%)	SkewI metric	CheckM		BUSCO			
						Completeness (%)	Contamination (%)	Complete and single copy (%)	Complete and duplicate copy (%)	Fragmented (%)	Missing (%)
LQC 2441	19	3,147,789	2,993,011	45.04	0.982	100	0.2	99.8	0.0	0.0	0.2
LQC 2485	46	3,494,755	3,148,808	45.02	0.934	100	4.8	99.3	0.5	0.0	0.2
LQC 2422	20	3,128,861	2,990,528	45.09	0.983	100	0.2	99.8	0.0	0.0	0.2
LQC 2320	8	3,181,752	3,129,011	45.13	0.991	100	0.0	100	0.0	0.0	0.0
LQC 2516	19	3,148,153	3,000,101	45.04	0.993	100	0.2	99.8	0.0	0.0	0.2
LQC 2520	10	3,175,498	3,140,405	45.12	0.987	100	0.0	100	0.0	0.0	0.0

[a] The percentage of Ns for each genome was 0.07% (LQC 2441), 0.12% (LQC 2485), 0.07% (LQC 2422), 0.05% (LQC 2320), 0.07% (LQC 2516) and 0.04% (LQV 2520).

Table 5. 2. Quality and functional properties of the six *Lp. plantarum* subsp. *argentoratensis* genomes after genome annotation[a].

Strain	Protein-coding genes (CDS)	Non-coding						Consistency		BUSCO			
		repeat-region	tRNA	rRNA	crispr-repeat	crispr-spacer	crispr-array	Coarse (%)	Fine (%)	Complete and single copy (%)	Complete and duplicate copy (%)	Fragmented (%)	Missing (%)
LQC 2441	3098	78	54	2	23	20	3	98.3	96.5	98.4	1.4	0.0	0.2
LQC 2485	3492	107	59	5	30	26	4	98.3	94.6	93.7	6.1	0.0	0.2
LQC 2422	3091	71	54	2	25	22	3	98.3	96.5	98.4	1.4	0.0	0.2
LQC 2320	3131	62	54	2	21	19	2	98.2	96.8	98.6	1.4	0.0	0.0
LQC 2516	3109	79	54	2	20	17	3	98.4	96.8	98.4	1.4	0.0	0.2
LQC 2520	3132	63	54	2	21	19	2	98.2	96.8	98.6	1.4	0.0	0.0

[a] Total number of genes for each genome was 3278 (LQC 2441), 3723 (LQC 2485), 3268 (LQC 2422), 3291 (LQC 2320), 3284 (LQC 2516) and 3293 (LQC 2520) of which the number of specialty genes was 38 (11 transporters and 27 antibiotic resistance for LQC 2441), 41 (11 transporters and 30 antibiotic resistance for LQC 2485), 38 (11 transporters and 27 antibiotic resistance for LQC 2422), 39 (12 transporters, 1 drug target and 26 antibiotic resistance for LQC 2320), 38 (11 transporters and 27 antibiotic resistance for LQC 2516) and 39 (12 transporters, 1 drug target and 26 antibiotic resistance for LQC 2520).

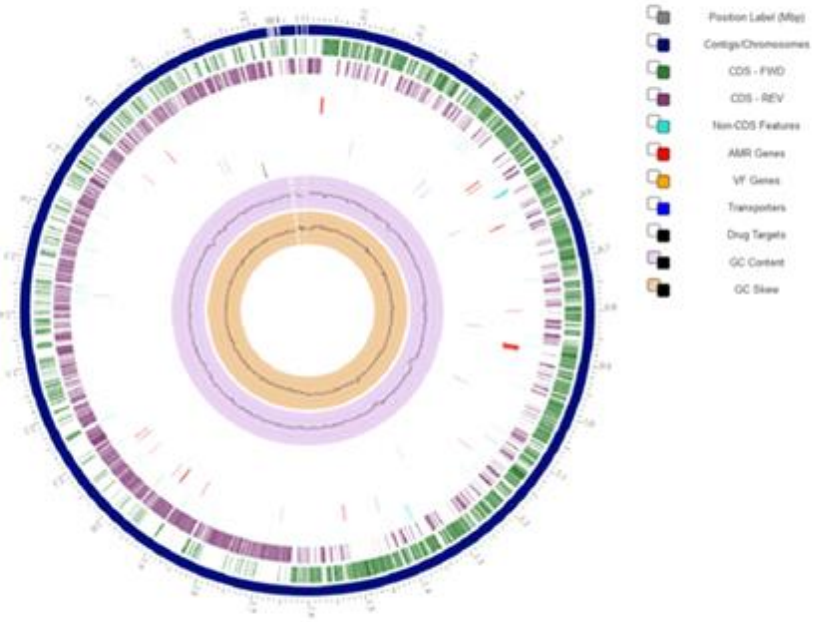

Figure 5. 2. Circular view of the genome of *Lp. plantarum* subsp. *argentoratensis* LQC 2320 strain.

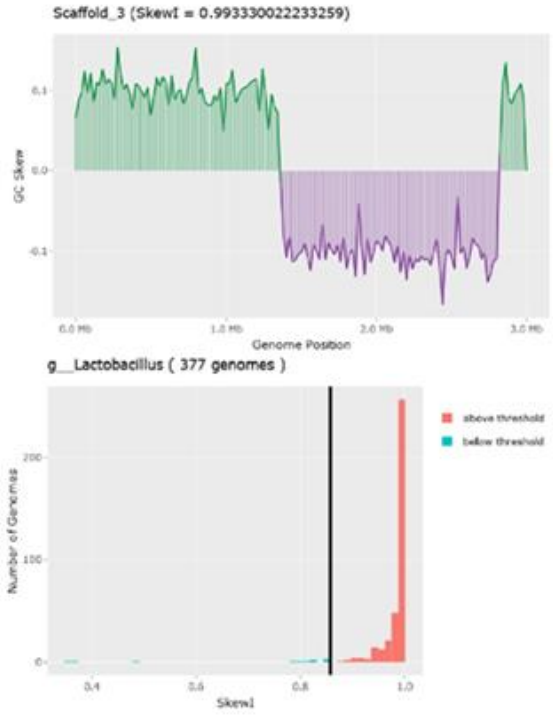

Figure 5. 3. GC skew analysis of the genome of *Lp. plantarum* subsp. *argentoratensis* LQC 2516 strain (above) and skewI threshold value for the genus of *Lactobacillus* (below).

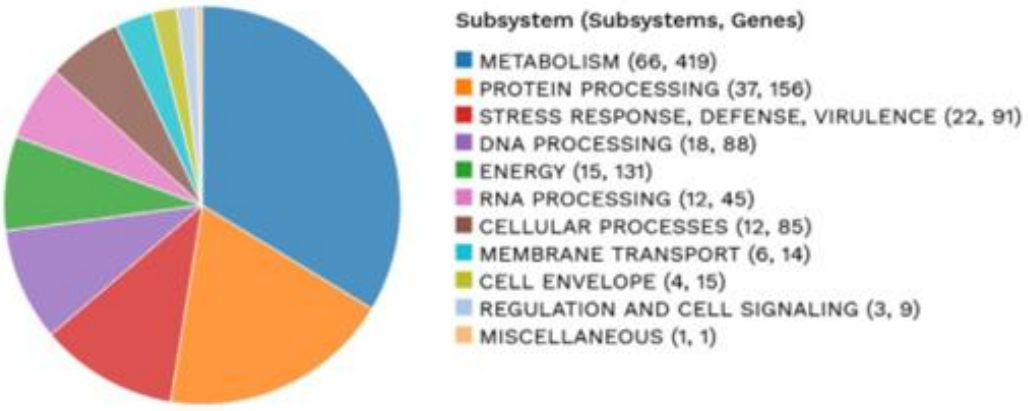

Figure 5. 4. Subsystem analysis of the genome of *Lp. plantarum* subsp. *argentoratensis* LQC 2520 strain.

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Chapter 6

A comparative genomic and safety assessment of six Lactiplantibacillus plantarum subsp. argentoratensis strains, isolated from spontaneously fermented Greek wheat sourdoughs for potential biotechnological application

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1. Abstract

The comparative genome analysis of 6 *Lp. plantarum* subsp. *argentoratensis* strains previously isolated from spontaneously fermented Greek wheat sourdoughs is presented. Genomic attributes related to food safety have been studied according to the European Food Safety Authority (EFSA) suggestions for the use of lactic acid bacteria (LAB) in the production of foods. Bioinformatic analysis revealed a complete set of genes for maltose, sucrose, glucose, and fructose fer- mentation; conversion of fructose to mannitol; folate and riboflavin biosynthesis; acetoin production; conversion of citrate to oxaloacetate; and the ability to produce antimicrobial compounds (plantaricins). Pathogenic factors were absent but some antibiotic resistance genes were detected. CRISPR and *cas* genes were present as well as various mobile genetic elements (MGEs) such as plasmids, prophages, and insertion sequences. The production of biogenic amines by these strains was not possible due to the absence of key genes in their genome except lysine decarboxylase associated with cadaverine; however, potential degradation of these substances was identified due to the presence of a blue copper oxidase precursor and a multicopper oxidase protein family. Finally, comparative genomics and pan-genome analysis showed genetic differences between the strains (e.g., variable *pln* locus), and it facilitated the identification of various phenotypic and probiotic-related properties.

Keywords: bioinformatics; fermentation; foods; lactic acid bacteria; starter culture; whole genome sequencing

2. Introduction

Lactic acid bacteria (LAB) are microorganisms that possess important technological characteristics and, therefore, are used in food fermentations as starters or even as adjunct cultures providing the desired quality and organoleptic properties and assuring the safety of the fermented products. Traditionally made fermented foods, i.e., without the addition of a commercial starter culture, constitute a significant source of isolating wild LAB strains comprising the indigenous flora of the products performing the spontaneous fermentation. Although LAB have been acknowledged as safe for human and animal consumption (Generally Recognized as Safe-GRAS by the Food and Drug Administration-FDA and Qualified Presumption of Safety-QPS by the European Food Safety Authority—EFSA) (EFSA, 2007, Leuschner et al., 2010), infection is still possible—especially for immunocompromised individuals (Kayser et al., 2003, Cannon et al., 2005, Salminen et al., 2006). Thus, the safety assessment of microbial strains aimed to be used as potential food additives is always timely, and it is highly recommended for investigation.

Since they constitute the majority of the respective micro-community, LAB and yeasts play a critical role during sourdough fermentation. There are different types of sourdoughs, based on the applied fermentation and technological processes (Siepmann et al., 2018). Type I sourdough, from which the 6 *Lp. plantarum* subsp. *argentoratensis* strains under study were isolated, is characterized by the dominance of *Fr. sanfranciscensis*, *Lv. brevis*, *Lm. fermentum*, *Lp. plantarum* and *Cp. paralimentarius* (Syrokou et al., 2020). In the latter work, Greek wheat sourdoughs collected from various geographical regions were analyzed by using culture-dependent and culture-independent molecular techniques. These 6 strains, initially identified as *Lp. plantarum* by 16S rRNA gene sequencing, were further selected for whole-genome sequencing based on their phenotypic and technological properties (Syrokou et al., 2021). Taxonomic classification of the strains based on the sequenced genome revealed that the isolated colonies belong to the subspecies *argentoratensis*. Sequenced genomes of *Lp. plantarum* subsp. *argentoratensis* are not readily available, especially strains isolated from sourdoughs.

Nowadays, as the sequencing technology has advanced and the cost of genome sequencing has decreased, the use of bacterial whole-genome sequencing as a diagnostic tool is more feasible than a few years ago. When a genome is available

completed or as a draft of high quality, its bioinformatic analysis offers an unprecedented way of investigating the biotechnological potential and safety of the isolated sequenced strains (Sun et al., 2015, EFSA, 2018) Recently, Carpi et al. (2022) performed a detailed pan-genome analysis of *Lp. plantarum* using 127 complete genomes. Another three related works have been conducted using the draft genomes of *Lp. plantarum* strains (Choi et al., 2018, Inglin et al., 2018, Evanovich et al., 2019). However, the main limitation of those studies is that acquired antibiotic resistance (AR) genes were annotated using only the Comprehensive Antibiotic Resistance Database (CARD) and/or ResFinder databases, resulting in many cases without any hits or in only one hit. In those works, the authors concluded that AR genes were not detected in the *Lp. plantarum* genomes. Chokesajjawatee et al. (2020) mentioned that the limitation of the AR genes search using the CARD and ResFinder databases for non-pathogenic bacteria should be marked because both databases are mainly focused on the AR genes of pathogenic bacteria and those of the non-pathogenic are usually not included, resulting in their inability to identify the AR genes in *Lp. plantarum* genomes at the default. On the contrary, the search of the AR genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database proved to be efficient for this purpose.

Consequently, in this study the genomes of 6 *Lp. plantarum* subsp. *argentoratensis* strains, isolated from spontaneously fermented Greek wheat sourdoughs, were bioinformatically analyzed and compared in relation to their biotechnological features and their safety for potential application to foods. In addition, there is no report on the bioinformatic analysis of the *Lp. plantarum* subsp. *argentoratensis* genomes, isolated from spontaneously fermented Greek sourdoughs, and from sourdoughs in general, considering the very limiting number of the available genomes of this subspecies in the NCBI database. The bioinformatic analysis of the genome of the *Lp. plantarum* subsp. *argentoratensis* strains performed in the current work will provide insights regarding their genomic and functional features and their potential use as a food additive.

3. Materials and Methods

3.1 Microbial strains

The bacterial strains LQC 2320, LQC 2422, LQC 2441, LQC 2485, LQC 2516, and LQC 2520 of *Lp. plantarum* subsp. *argentoratensis* have been isolated from traditional Greek wheat sourdoughs (Syrokou et al., 2020). Information on the whole-genome sequencing of the strains and the subsequent bioinformatics processing of the raw reads (fastq files), up to genome assembly and annotation, can be found in the work of Syrokou et al. (2021) as well as in the NCBI database (GenBank) under the accession numbers JAEQMR01, JAEQMQ01, JAEQMP01, JAEQMO01, JAEQMN01 and JAEQMM01, respectively.

3.2 Strains relatedness

Overall genome-related index (OGRI) analysis (Chun & Rainey, 2014) was performed according to Mataragas (2020). Species relatedness was examined by comparing the average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values between the current strains and the type strains of *Lp. paraplantarum* DSM 10667, *Lp. plantarum* subsp. *plantarum* ATCC 14917, *Lp. plantarum* subsp. *argentoratensis* DSM 16365, and *Lp. pentosus* DSM 20314, downloaded from the GenBank database. The above phylogenetic neigh- bors were chosen following the workflow of genome-based classification at the species level proposed by Chun et al. (2018): first, the full length of the 16S rRNA gene sequence was extracted from the whole-genome sequences of each studied strain using the ContEst16S tool (Lee et al., 2017), and then the extracted sequences were loaded on the 16S-based ID platform of the EzBioCloud server (Yoon et al., 2017) for identifying a bacterial isolate based on the 16S rRNA gene database of type strains. Bacterial identifications that displayed a high 16S sequence similarity value (≥ 98.7%) were further selected for OGRI analysis. The ANI values (threshold for species identification 95–96%) were calculated with the Orthologous Average Nucleotide Identity Tool (OAT) (Lee et al., 2015) while dDDH values (threshold for species and subspecies identification 70% and 79%, respectively) with the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff & Göker, 2019). The TYGS was also used for establishing a bootstrapped genome-based phylogenetic relationship of the strains.

3.3 Phenotypic characterization and pan-genome analysis

Phenotype assignments to bacterial genomes of *Lp. plantarum* subsp. *argentoratensis* strains were achieved through the Traitar web service, accounting for 67 diverse traits (Weimann et al., 2016). Pan-genome analysis was conducted using the BPGA v1.3.0 software (Chaudhari et al., 2016). For the core-genome extraction, the default values of the tool were used; USEARCH software v10.0.240 (Edgar, 2010) with a threshold of sequence identity equal to 0.5 (50%). The functional genes from the core-, accessory- and unique-genome of the *Lp. plantarum* subsp. *argentoratensis* strains were clustered to each COG and KEGG category with the USE- ARCH software, against the respective databases, embedded in the BPGA pipeline, ap- plying the default parameters. Finally, the clusters of functional genes (pan-matrix), pre-sented as a binary matrix (presence or absence), were introduced in the GENE-E software (https://software.broadinstitute.org/GENE-E/) (accessed on 6 September 2021) for visualizing the genes against the genomes. A hierarchical clustering (HC) of genes and genomes was performed using the option one minus the Pearson correlation distances available in the GENE-E. The web services PATRIC v3.6.8 (Davis et al., 2020), KofamKOALA (Aramaki et al., 2020), and eggNOG- mapper (Huerta-Cepas et al., 2017) were used to annotate the predicted proteins obtained during the pan-genome analysis of *Lp. plantarum* subsp. *argentoratensis* strains. The results of eggNOG-mapper also offered CAZymes analysis towards the CAZymes database (Lombard et al., 2021).

3.4 Genomic aspects related to food safety

A safety assessment of the *Lp. plantarum* subsp. *argentoratensis* strains was performed by using whole-genome analysis and following the guidelines and recommendations of Chokesajjawatee et al. (2020) and the EFSA FEEDAP Panel (2018), respectively.

Antibiotic resistance and potential pathogenicity of the microbes were checked with the Resistance Gene Identifier (RGI) tool of the CARD (Jia et al., 2017) and PathogenFinder (Cosentino et al., 2013), respectively, following the analytical approaches of Rodrigo-Torres et al. (2019). Moreover, the egg-NOG mapper results based on the KEGG database was searched for undesirable genes as suggested by Chokesajjawatee et al. (2020). Bacteriocin-encoding genes were inspected using the BAGEL4 webtool (Van Heel et al., 2013). Annotation results from PATRIC v3.6.8,

KofamKOALA and eggNOG-mapper were screened for the ability of the strains to produce biogenic amines. Finally, the stability of the genomes was evaluated by investigating the following genetic features: (a) plasmids and insertion sequences (IS) were analyzed by PlasmidFinder (Carattoli et al., 2014) and MobileElementFinder (Johansson et al., 2021), respectively. MobileElementFinder was also able to annotate acquired antimicrobial resistance genes using ResFinder (Zankari et al., 2017, Bartolaia et al., 2020) and virulence genes using VirulenceFinder (Joensen et al., 2014); (b) regions of Clustered Palindromic Inter- spaced Palindromic Repeats (CRISPR) with *cas* genes were detected with the CRISPRCas- Finder webserver (Couvin et al., 2018) using the default parameters; and (c) the presence of prophages was searched by means of the PHASTER webtool (Arndt et al., 2016). According to Chokesajjawatee et al. (2020), the WHO CIA list (list of critically important antimicrobials of World Health Organization) served as a reference to evaluate the ability of the microbial strains to produce antimicrobial drugs of clinical importance via the KofamKOALA and egg-NOG-mapper results of the KEGG database.

4. Results and Discussion

4.1 Strain relatedness

The complete (100%) sequence of the 16S rRNA gene was extracted from the sequenced genome of all strains using the ContEst16S webtool and its length was 1567 bp. It should be noted, however, that although the genomes were of high quality (number of contigs was far below the limit of 200, the sequence length of each contig was ≥ 500 bp, and the genome coverage was much higher than the threshold of 60–80x and the recommended 100x) they were not closed because it is well known that the genome of lactic acid bacteria contains repeat regions and other mobile elements that make it difficult to finish the genome using only short read sequences (Schmid et al., 2018, Rodrigo-Torres et al., 2019).

The 16S-based ID platform of the EzBioCloud server revealed a high sequence similarity with the following type strains: *Lp. pentosus* DSM 20314^{T} (99.87%), *Lp. paraplantarum* DSM 10667^{T} (99.80%), *Lp. plantarum* subsp. *argentoratensis* DSM 16365^{T} (99.80%) and *Lp. plantarum* subsp. *plantarum* ATCC 14917^{T} (99.80%). The results from the 16S-based ID showed additional strains with sequence similarity above the threshold of

98.70% (*Lp. fabifermentans* DSM 21115, 99.19%; *Lp. daowaiensis* 203-3, 99.17%; *Lp. pingfangensis* 382-1, 99.16%; *Lp. garii*, FI11369, 99.10%; *Lp. nangangensis* 381-7, 99.07%; *Lp. daoliensis* 116-1A, 99.03%; *Lp. herbarum* TCF032-E4, 98.99%, *Lp. xiangfangensis* LMG 26013, 98.92%; and *Lp. plajomi* NB53, 98.79%) but these were not selected further for ANI comparisons because the results obtained from the TYGS webtool indicated a very poor pairwise dDDH values (< 23%) between these strains and the strains of the current study. The calculated ANI values between the genomes of this work and the closely related type strains (16S rRNA gene sequence similarity ≥ 99.80%) are displayed in Fig. 6.1. Based on the threshold ANI value of 95-96% and the calculated ones between the genomes, the 6 microbial strains, isolated from traditional Greek wheat sourdoughs, were identified as *Lp. plantarum* subsp. *argentoratensis*. In addition, the results obtained from the TYGS platform on the pairwise comparisons of user genomes and type-strain genomes, restricted to the above-mentioned four type strains, confirmed that these strains belong to the species of *Lp. plantarum* subsp. *argentoratensis* (dDDH value > 79%) (Table 6.1, Fig. 6.2). According to the Qualified Presumption of Safety (QPS) list of EFSA (EFSA Scientific Committee, 2007) the species *Lp. plantarum* has received the QPS status.

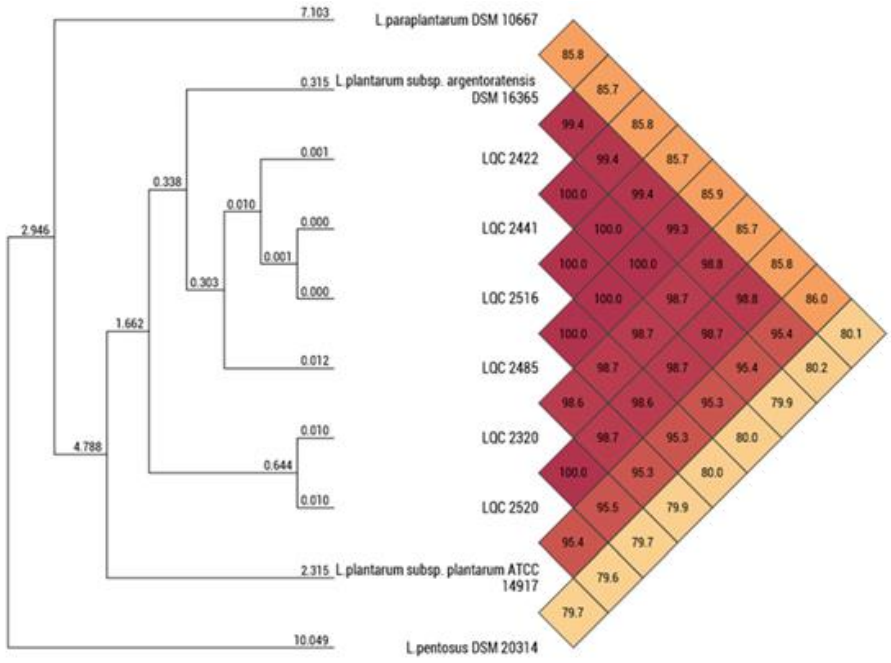

Figure 6. 1. Calculated ANI values showing the relatedness between the sequenced genomes of the strains isolated from traditional Greek wheat sourdoughs (LQC 2422, LQC 2441, LQC 2516, LQC 2485, LQC 2320, and LQC 2520) and the type strains restricted to those displayed a very high 16S rRNA gene similarity (≥ 99.80%).

Table 6. 1. Pairwise comparisons (dDDH values) between the genomes of the strains isolated from traditional Greek wheat sourdoughs (LQC 2422, LQC 2441, LQC 2516, LQC 2485, LQC 2320, and LQC 2520) and the genomes of the type strains restricted to those displayed a very high 16S rRNA gene similarity (≥ 99.80%).

a/a	Strains	1	2	3	4	5	6	7	8	9	10
1	LQC 2422	-									
2	LQC 2441	**99.9**	-								
3	LQC 2516	**100**	**100**	-							
4	LQC 2485	**99.5**	**99.5**	**99.5**	-						
5	LQC 2320	**89.2**	**89.2**	**89.2**	**88.9**	-					
6	LQC 2520	**89.2**	**89.2**	**89.2**	**88.9**	**100**	-				
7	*Lp. paraplantarum* DSM 10667	31.1	31.2	31.2	31.5	31.1	31.2	-			
8	*Lp. plantarum* subsp. *argentoratensis* DSM 16365	**95.1**	**95.1**	**95.2**	**94.0**	**89.9**	**90.5**	31.5	-		
9	*Lp. plantarum* subsp. *plantarum* ATCC 14917	62.5	62.4	62.4	62.3	63.1	63.0	31.1	62.8	-	
10	*Lp. pentosus* DSM 20314	24.4	24.4	24.4	24.5	24.0	24.0	24.4	24.9	23.8	-

Values in bold indicate that the threshold value for subspecies (79%) or species (70%) delineation is exceeded.

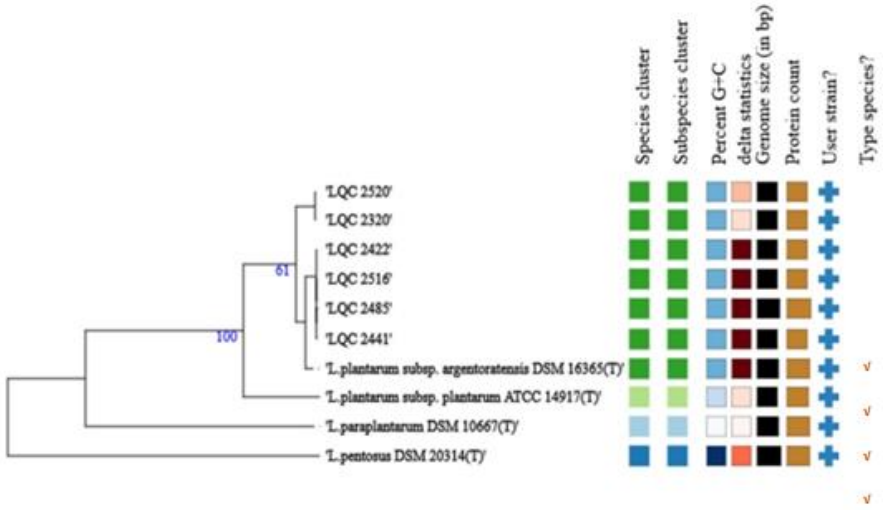

Figure 6. 2. A bootstrapped genome-based phylogenetic relationship of the strains isolated from traditional Greek wheat sourdoughs (LQC 2422, LQC 2441, LQC 2516, LQC 2485, LQC 2320, and LQC 2520) and the type strains restricted to those displayed a very high 16S rRNA gene similarity (≥ 99.80%). The numbers above branches are pseudo-bootstrap support values > 60% from 100 replications. Matrix on the right (columns from left to right), dDDH species (> 70%) and subspecies (> 79%) cluster, strains with the same color belong to the same species or subspecies group; GC content (%), min 43.9 (*Lp. paraplantarum* DSM 10667T) and max 46.3 (*Lp. pentosus* DSM 20314T), strains with the same color have similar GC content (e.g., strains of this study and *Lp. plantarum* subsp. *argentoratensis* DSM 16365T have a GC content of 45.0–45.1); delta (δ) values (< 1) showing the tree-likeness of the data set, if a strain has an exceptionally high value this indicates that this genome should be removed because it negatively affects phylogenetic inference; Genome size in Mb, min 3.13 (LQC 2422) and max 3.67 (*Lp. pentosus* DSM 20314T); Protein content, min 2958 (LQC 2422) and max 3310 (*Lp. pentosus* DSM 20314T); Strains provided by the user (cross symbol); and Type species (tick symbol).

4.2 Phenotypic characterization and pan-genome analysis

4.2.1 Phenotypic properties

Based on the phenotype prediction (Figure 6.3), the 6 strains were: Gram-positive, catalase-negative, non-motile and non-spore forming bacilli; unable to grow at 42 ◦C and produce NH3 from arginine; and negative to indole production, H2S formation, urease activity, and oxidase test. These properties are very common in the genus of *Lactobacillus* (Thakur et al., 2017). Regarding the sugar fermentation profile of the isolates, these showed a positive pattern for maltose, melibiose, sorbitol, mannose, glucose, salicin, lactose, raffinose, esculin, sucrose, trehalose, and mannitol, but they were negative for cellobiose, xylose, myo-inositol, and arabinose fermentation (Figure 6.3). However, genome annotation showed that the strains harbored the respective genes (EC 2.7.1.205 and EC 3.2.1.86) for the utilization of cellobiose. These predictions are in line with the experimental phenotypic tests conducted on the isolates by Syrokou et al. (2020) who found that the *Lp. plantarum* subsp. *argentoratensis* strains can ferment a wide range of carbohydrates, including the cellobiose. Moreover, genome annotation analysis showed that the strains were able to ferment fructose and convert fructose to mannitol, a well-known low-calorie sweetener. Also, the strains LQC 2320 and 2520 were able to catabolize rhamnose and reduce nitrate to nitrite. The latter is mediated by the nitrate reductase which under certain circumstances may lead to nitric oxide (NO) synthesis, a property of great importance during fermented sausage manufacturing. The *Lp. plantarum* AJ2 strain isolated from naturally fermented sausage possessed the ability to reduce the residual levels of nitrite and nitrate (Sawitski et al., 2008).

Alkaline phosphatase production by all strains and gas (CO_2) production from glucose by the LQC 2485 strain was projected by the phypat + PGL predictor only, while the phypat showed a positive result toward acetate utilization (strains LQC 2441, LQC 2422, LQC 2516, and LQC 2485) and growth in 6.5% NaCl (strains LQC 2520 and LQC 2320) (Figure 6.3). However, positive results predicted only by the phypat should be evaluated experimentally. Weimann et al. (2016) noted that the phypat predictor tends to give more false-positive results when draft genomes are used. Alkaline phosphatase production by *Lp. plantarum* has been linked with the increased degradation rate of the organophosphorus pesticides present in wheat dough or skimmed milk (Dordevic et al., 2013, Zhang et al., 2014). Other important phenotypic properties predicted by Traitar

were the strains' ability to utilize the citrate as a carbon source, which may lead to the formation of aroma precursor compounds; the strains' ability to produce the enzyme beta-galactosidase (ONPG); and the positive reaction to the Voges–Proskauer (VP) test. Beta-galactosidases belong to glycoside hydrolase (GH) groups. Genome annotation revealed that all strains had the GH2 group of beta-galactosidase and especially the LacLM type encoded by two genes, *lacL* and *lacM* (EC 3.2.1.23) (the other type is the LacZ, encoded by the *lacZ* gene). While *Lp. plantarum* predominantly possess the LacLM type (Iqbal et al., 2010), the LacZ type is found in other LAB (Kittibunchakul et al., 2019). The enzyme hydrolyzes lactose and, therefore, it is used in the dairy industry for health-related applications like the production of lactose-free products or prebiotic galactooligosaccharides (GOS). The addition of beta-galactosidase-producing lactobacilli as probiotics to dairy products can assist lactose-intolerant individuals. The addition of GOS stimulates the growth of probiotics and regulates the gut microflora (Roberfroid et al., 2010). The positive VP test, which was negatively correlated with the methyl red test (the MR test was predicted as negative) (Figure 6.3), indicates the presence of acetoin, which is the precursor of 2,3 butanediol and other aromatic compounds. Thus, the strains do not use the mixed acid fermentation pathway, i.e., production of several organic acids from glucose utilization, but they use the metabolic pathway that leads to the formation of 2,3 butanediol, in addition to lactate production. Thakur et al. (2017) found that a *Lp. plantarum* strain isolated from pickle samples displayed the opposite phenotype, i.e., MR+ and VP . Usually, a bacterial culture is positive to only one pathway, either VP+ or MR+. Indeed, the results on the genome annotation of *Lp. plantarum* subsp. *argentoratensis* confirmed the presence of a complete set of genes for acetoin production and conversion of citrate to oxaloacetate. The latter coincided with the predictions made by Traitar. Finally, 15 GHs (glycoside hydrolases) and 9 GTs (glycosyltransferases) families contained in the CAZymes database were spotted, indicating a high variability in GH- and GT-encoding.

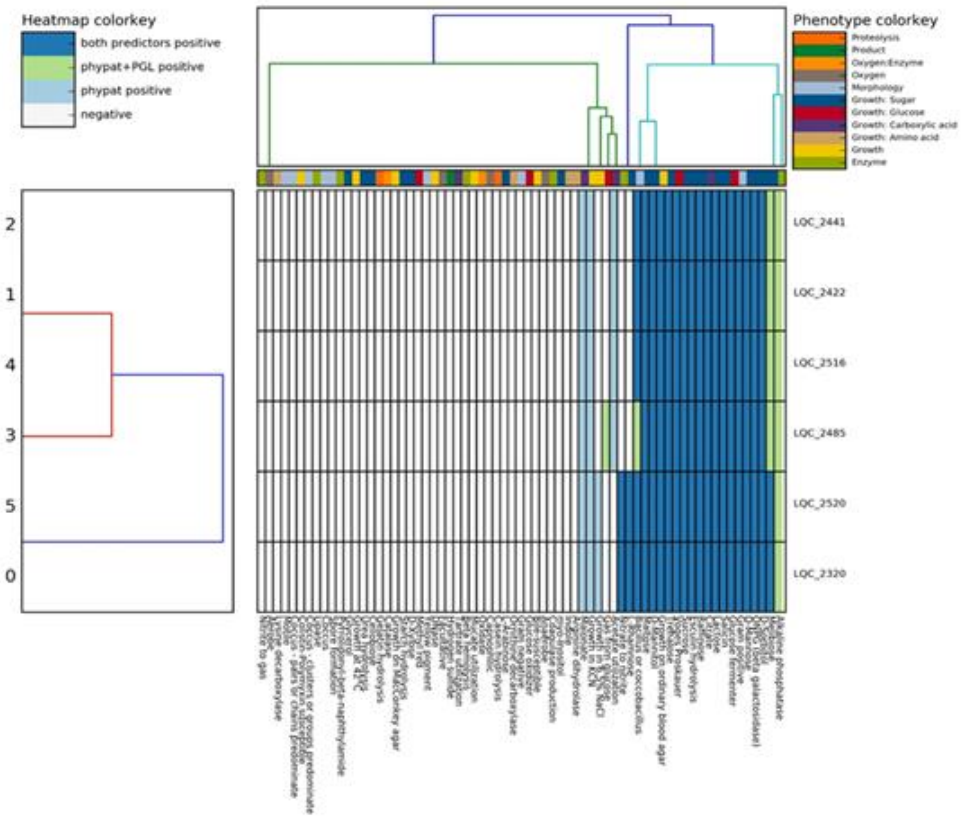

Figure 6. 3. Heatmap of the phenotypic features of the strains isolated from traditional Greek wheat sourdoughs (LQC 2422, LQC 2441, LQC 2516, LQC 2485, LQC 2320, and LQC 2520) as predicted by the Traitar webtool.

4.2.2 Pan-genome analysis and annotation

According to Heaps' law (Tettelin et al., 2008), the curve fitting of the pan-genome resulted in an estimated a value of 0.10 ($a < 1$) which means that when a new genome is added to the pan- genome, new genes are added as well increasing in this way its size (open pan-genome). The increase of pan-genome by 0.10 implies potential evolution-related changes in the *Lp. plantarum* subsp. *argentoratensis* genomes (horizontal gene transfer-HGT, gene gain or gene loss) to cope with the adaptation to various environmental conditions. The pan- genome of the 6 strains comprised of 3239 genes in total, from which 2454 genes were in the core-genome, 550 genes in the accessory-/unique-genome, and 235 genes were unique (Figure 6.4). The strain LQC 2485 harbored the highest number of unique genes (202) compared to the other strains. Hence, gene exchange activities associated with potentially different approaches of adaptation and response to environmental conditions are happening at the highest rate in this specific strain. For instance, the genome annotation performed revealed the presence of a

multiple-sugar metabolism (msm) gene cluster analogous to the described system found in *Streptococcus mutans* (Russell et al., 1992, McLaughlin et al., 1996). This multiple-sugar ABC transporter were comprised of two permease proteins (*msmG* and *msmF*) and a substrate- binding protein (*msmE*). Another feature found in the unique-genome of the LQC 2485 strain is the presence of a pyruvate dehydrogenase (quinone) (EC 1.2.5.1), which stimulates the conversion of pyruvate to acetate.

The hierarchical clustering of the gene matrix (presence/absence) (Figure 6.5), acquired during the pan-genome analysis of the *Lp. plantarum* subsp. *argentoratensis* genomes, was like the clustering obtained based on the WGS (Figure 6.2). However, the LQC 2485 was quite distinct from the other two groups probably due to the high number of unique genes harbored by this strain. The LQC 2320 and LQC 2520 isolates formed a separate group from the rest of the strains (LQC 2422, LQC 2441, and LQC 2516) attributed to the presence/absence of a group of genes in each microbial cluster (Figure 6.5). Thus, HGT, gene gain, or gene loss phenomena potentially have occurred between the *Lp. plantarum* subsp. *argentoratensis* strains and other microbial clades. Indeed, the LQC 2320 and LQC 2520 strains possessed a genes cluster related to the reduction of nitrate to nitrite encountered in *Staphylococcus carnosus* (Fedke et al., 2002) (nitrate/nitrite transporter, NarT; oxygen-sensing two component system sensor histidine kinase, NreBC; and respiratory nitrate reductase alpha, beta, gamma, and delta chain; *narGHIJ*, EC 1.7.99.4), and the utilization of L-rhamnose (transcriptional regulator of rhamnose utilization, AraC family, L-rhamnose permease, RhaY, major facilitator superfamily—MFS; L-rhamnose mutarotase, RhaM, EC 5.1.3.32; L-rhamnose isomerase, RhaA, EC 5.3.1.14; L-rhamnulose kinase, RhaB, EC 2.7.1.5; and L-rhamnulose-1-phosphate aldolase, RhaD, EC 4.1.2.19); both features were predicted bythe Traitar and annotated by the respective web-services. On the other hand, the LQC 2422, LQC 2441, LQC 2485, and LQC 2516 strains harbored the genes cluster associated with the maltodextrin-specific ABC transporter, i.e., the ATP-binding protein MdxE, the membrane-spanning components MdxF and MdxG, and the energizing ATPase MsmX (Schönert et al., 2006), and the utilization of glycerate (phosphotransferase System—PTS, 2-O-alpha-mannosyl-D- glycerate-specific IIA, IIB, and IIC component, EC 2.7.1.195; glycerate kinase, EC 2.7.1.31; and mannosylglycerate hydrolase, EC 3.2.1.170). Annotation showed the presence of 2-dehydro-3-deoxy-D-gluconate-5-dehydrogenase (EC 1.1.1.127) and 2-deoxy-D-

gluconate- 3-dehydrogenase (EC 1.1.1.125) in the genome of the above four strains where the resulting compound enters the pentose phosphate pathway. Finally, all strains harbored a PTS- system, fructose-specific IIA, IIB, and IIC component (EC 2.7.1.202) and a PTS-system, mannose-specific IIA, IIB, IIC, and IID component (EC 2.7.1.191), but only these four strains had a PTS-system, fructose- and mannose- inducible IIA, IIB, IIC, IID, and IIE component. This phosphoenolpyruvate: fructose phosphotransferase system constitutes an alternative way of entering mannose into *Streptococcus salivarius* (Pelletier et al., 1994).

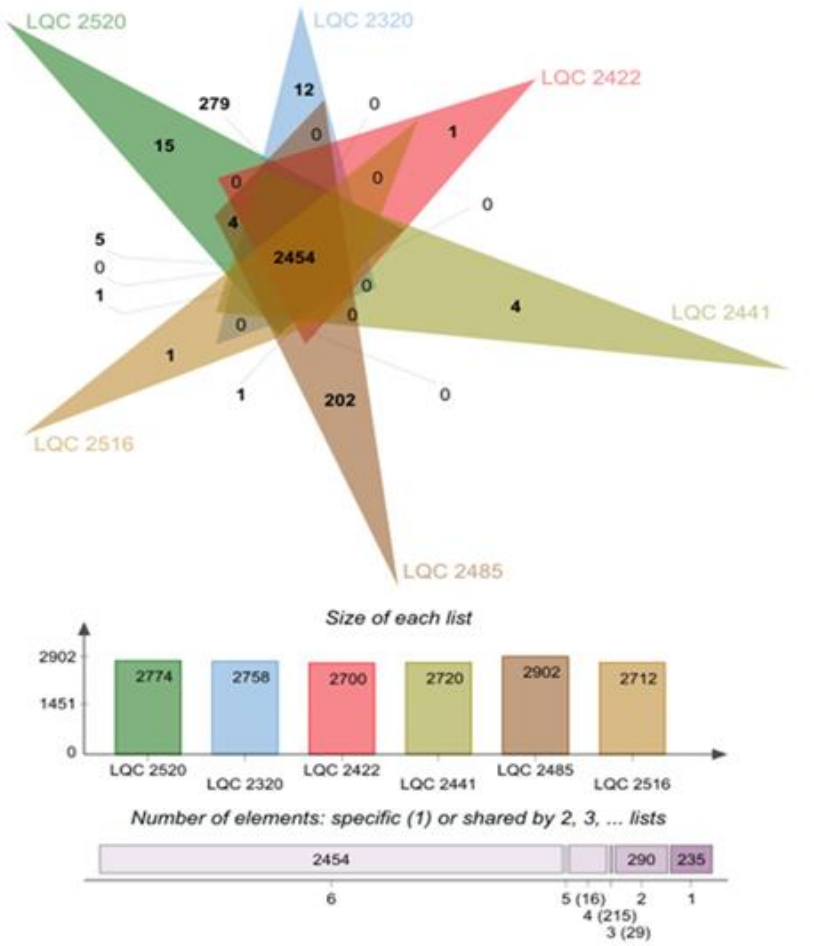

Figure 6. 4. Venn diagram (A) showing the number of genes located in the core-, accessory- and unique genome of each *Lp. plantarum* subsp. *argentoratensis* strain. The plots below the Venn diagram showthe total number of genes in each genome of the strains (B) and the total number of genes shared by the respective number of genomes (C). Venn diagram was constructed using the jvenn webtool (Bardou et al., 2014).

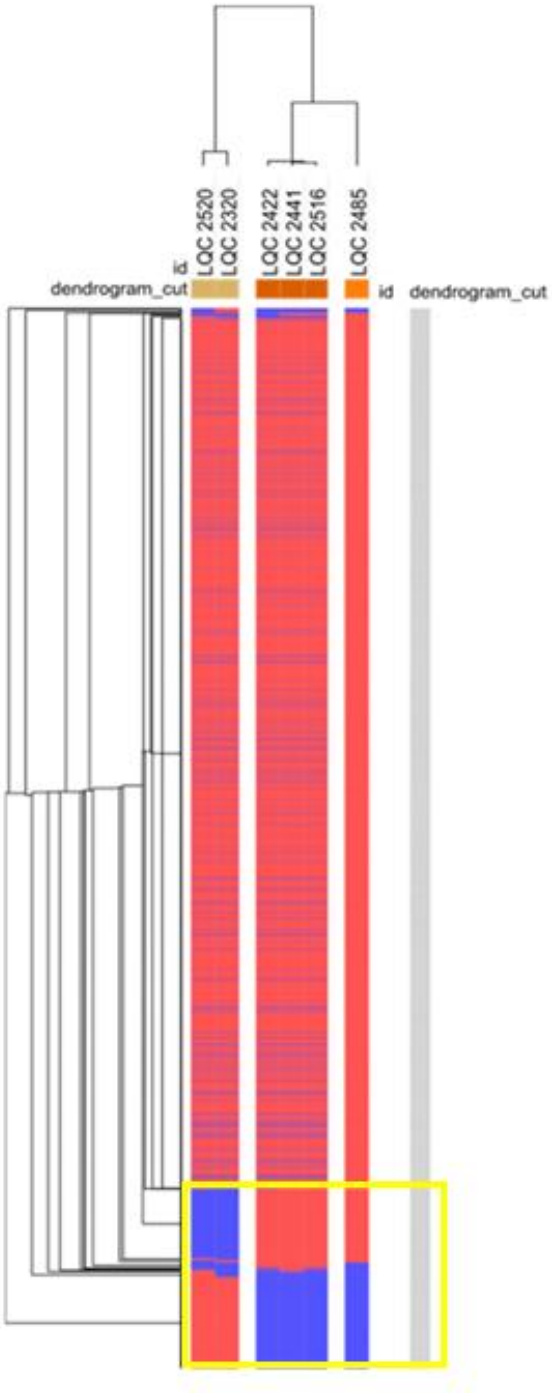

Figure 6. 5. Heat-map of the genes presence (red) and absence (blue) of the six *Lp. plantarum* subsp. *argentoratensis* genomes. Yellow box marks the difference in the genes presence/absence between the two microbial clusters.

Gene assignment to the different COG functional groups depicted that the genes of translation, ribosomal structure and biogenesis (J), amino acid transport and metabolism(E), and nucleotide transport and metabolism (F) were enhanced in the core-genome of the *Lp. plantarum* subsp. *argentoratensis* species (Figure 6.6A), i.e., genes

implicated in microbial housekeeping processes (Chun et al., 2017). Genes related to cell wall/membrane/envelop biogenesis (M), DNA replication, recombination and repair (L), and carbohydrate transport and metabolism (G) were enhanced in the accessory-/unique-genome, i.e., genes implicated in energy metabolism and DNA repair (Figure 6.6A) (Chun et al., 2017). Moreover, the genes involved in carbohydrate metabolism and membrane transport were the most abundant in the *Lp. plantarum* subsp. *argentoratensis* strains confirming the capacity to metabolize different carbohydrates, a feature which could be strain-dependent (higher abundance of genes in the accessory-/unique-genome compared to the core-genome) (Figure 6.6B). For example, as discussed above, the Traitar platform predicted that the LQC 2320 and LQC 2520 strains ferment L-rhamnose, but the others do not have this ability. Another observation worth-mentioning is the relatively low gene abundance in the lipid transport and metabolism category (Figure 6.6), suggesting that the strains' lipolytic activity is not high. Indeed, experimental results obtained by Syrokou et al. (2020) on the lipolytic activity of the strains under study confirm: strains LQC 2422, LQC 2441 and LQC 2485 exhibited no lipolytic activity, strains LQC 2520 and LQC 2320 showed moderate activity (22.75 AU/mL and 37.25 AU/mL, respectively), and strain LQC 2516 showed low activity (14.50 AU/mL).

All strains displayed the presence of a complete set of genes for riboflavin and folate biosynthesis. Similarly, *Lp. plantarum* strains isolated from quinoa sourdough (Carrizo et al., 2016) or a traditional maize-based fermented beverage (chicha) (Rodrigo-Torres et al., 2019) had this ability as well. All *Lp. plantarum* subsp. *argentoratensis* strains seem to produce the foldase protein PrsA encoded by the *prtM* gene, which is responsible for the cleavage of proteins and the subse- quent utilization of the large polypeptides by the microbial cells. The Opp (*oppA*, *oppA2*, *oppBCDF*) and Dtp (*dtpT*) gene clusters identified in their genomes widen the peptide up- take mechanisms. These peptides are subsequently hydrolyzed by various peptidases and proteases such as oligoendopeptidases (*pepE*, *pepF*, *pepF2*, *pepO*, *pepC*, and *pepN*), proline- specific peptidases (*pepX*, *pepI*, *pepQ*, and *pip*), di- and tri- peptidases (*pepV*, *pepT*, and *pipD*), and other enzymes (*map*-methionine aminopeptidase; *mccF*-LD-carboxypeptidase; *est*- serine aminopeptidase; *ydcK-SprT* family; *ddpX*-hydrolysis of the D-alanyl-D-alanine dipeptide; *vanY*-D-alanyl-D-alanine carboxypeptidase; and

ctpA, *dacA*, *amd*, *gluP*, *lepB*, *trpG*, *ytzB*, *ymfH*, *ymfF*, *glbL*, *htpX*, *yvpB*, peptidases belonging to different families).

Various probiotic-related genes were identified in the genomes of the 6 strains suggesting their potential probiotic properties (Li et al., 2016, Goel et al., 2020). These genes were associated with stress response and immunomodulation (*uspA*, *dltA*, *dltB*, *dltC*, *dltD*), salt-stress (nitrate/sulfonate/bicarbonate ABC transporter), acid-tolerance (*gadB*), DNA protection (ImpB/MucB/SamB and Dps family proteins, *clpB*, *clpC*, *clpL*, *msrB*, *luxS*), and adhesion ability (mucin-binding protein—MucBP domain, collagen binding domain protein which encodes an adhesion potentially related to colonization and competition against pathogens, LPxTG-motif cell wall anchor domain protein, *fbpA*, and *groS*). Adhesion could also be related to exopolysac- charides (EPS) or surface proteins. However, no *cps* cluster (*cps1*, *cps2*, *cps3*, *cps4*) was recognized; only the *cps2J* gene (accessory-genome) from cluster 2 and the *cps4J* gene (core- genome) from cluster 4. On the contrary, the surface protein Ef-Tu was identified, which acts as an adhesion factor. Such probiotic-related properties have been found in other *Lp. plantarum* strains such as ZJ316 (Li et al., 2016) and microbes isolated from Indian fermented foods (Goel et al., 2020). Finally, choloylglycine hydrolase family proteins (*pva1*, *pva2*, *pva3*, *cbh*; EC 3.5.1.24) referred to as bile salt hydrolases (BSH) were detected indicating bile adaptation. This observation confirms the prediction made by the Traitar platform on bile sensitivity of the strains which were negative. Although, bile salt resistance has been used as a criterion, among others, for the selection of a strain as probiotic, this activity could be potentially damaging to the human host and, therefore, the use of the BSH feature as a desirable property of the probiotic microorganism is controversial (Begley et al., 2006, Chokesajjawatee et al., 2020).

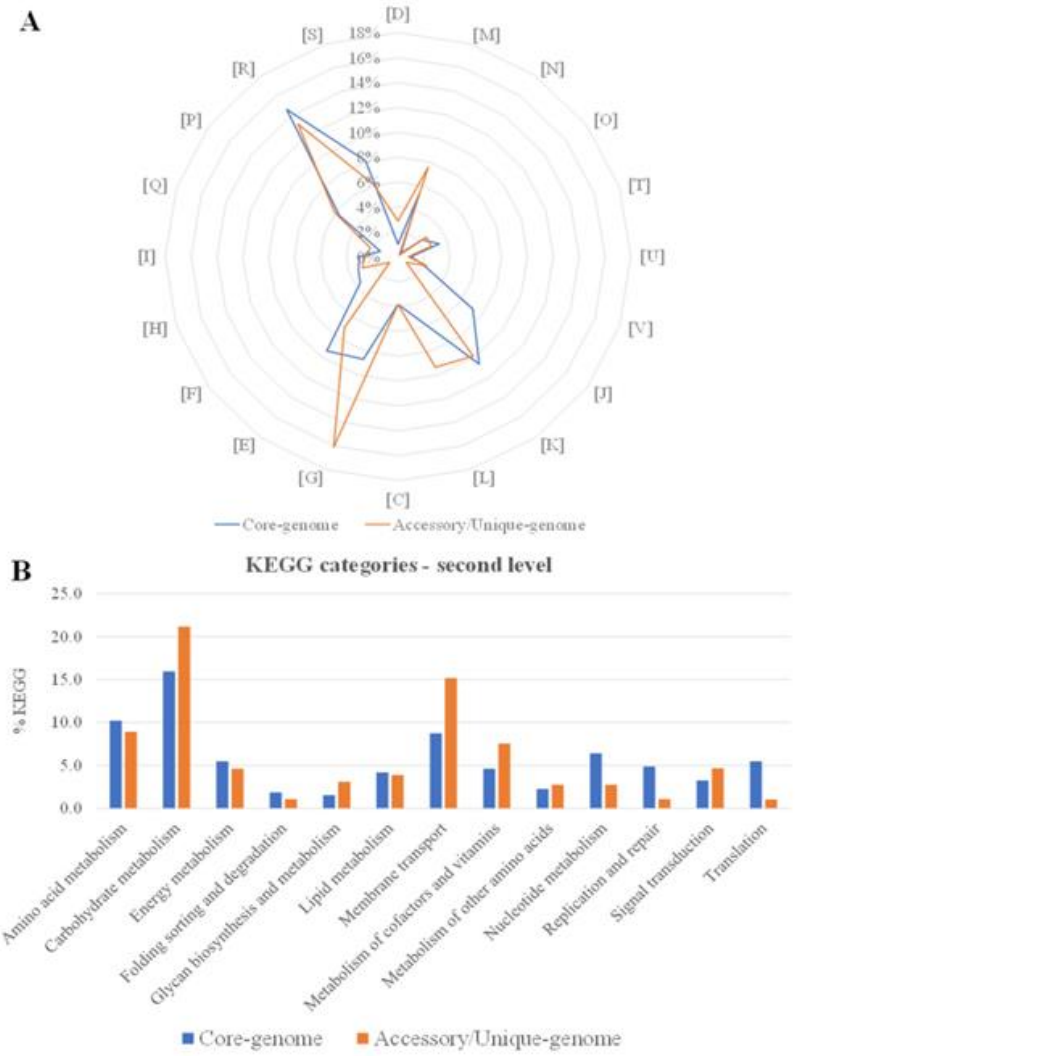

Figure 6. 6. Plots displaying the distribution of the COG (A) and KEGG (B) functional categories in the core- and accessory-/unique-genome of the *Lp. plantarum* subsp. *argentoratensis* strains.

4.2.3 Safety assessment

It is well known that bacteria may produce various biogenic amines (BAs) in foods, leading to health side-effects (Benkerroum, 2016). Therefore, the candidate microbial cultures should be previously assessed for their ability to produce such compounds before their application to fermented foods as starters because there is the risk of biogenic amines accumulation owing to LAB intensive metabolic activity. To identify the presence of the most important BAs-related genes in the genome of the 6 *Lp. plantarum* subsp. *argentoratensis* strains, the KEGG database through the egg-NOG mapper results was searched. Genes associated with tyramine, phenylethylamine, putrescine, spermidine, spermine, histamine, agmatine, and

tryptamine production were not located in any of the queried genomes. The *arcB* gene (ornithine carbamoyl transferase) was present in the genome of the strains but the rest of the gene cluster (i.e., *arcA*, *arcC*, and *arcD*) implicated in the ornithine conversion (decarboxylation) to putrescine was lacking. Thus, the 6 strains can be considered as putrescine-negative producers. On the other hand, the *cadA* gene (lysine decarboxylase), which is associated with the production of cadaverine (CAD), was found in all strains under study. The *cadBC* genes involved in the regulation and transportation system of the CAD were not found. Nevertheless, the homologous *pot* gene cluster (*potABCD*) was available in all genomes, encoding for the putrescine/ornithine antiporter system, which can be used instead of the *cadB* antiporter system of CAD/lysine (Benkerroum, 2016). However, the concentration of CAD should be experimentally determined in order to evaluate the true risk of CAD accumulation. Moreover, the strains harbored some genetic properties related to BA degradation, such as the presence of a blue copper oxidase (CueO) precursor and a multicopper oxidase protein family.

The *Lp. plantarum* subsp. *argentoratensis* strains are able to produce D-lactate because of the presence of D-lactate dehydrogenase and LarA which is implicated in lactate racemization. Hence, the consumption of foods containing LAB with D-lactate producing capabilities may cause problems for people with a high risk of D-lactate acidosis (Chokesajjawatee et al., 2020), and a precaution is needed. The screening against the CARD and PathogenFinder databases resulted in no identification of antibiotic resistance- or virulence- related genes, respectively. The microorganisms were not predicted as human pathogens since the strains' probability of being a human pathogen was low, ranging from 4.6 to 8.4%. Chokesajjawatee et al. (2020) noted that one limitation of the CARD database is its focus on the AMR of pathogenic microorganisms, while the AMR-related elements of non-pathogenic microbes are not considered; and, therefore, the search for any antibiotic resistance property against the KEGG database is recommended. Indeed, the results showed seven AMR-related elements (Table 6.2). However, further experimental investigation on these large families of antibiotics is needed because the possession of the respective genes does not necessarily warrant their resistance as well. For instance, Chokesajjawatee et al. (2020) found that the presence of a macrolide resistance gene *msrA* and two beta-lactamase genes (*penP* spotted on two different locations) in the genome of *Lp.*

plantarum BCC 9546 did not warrant resistance to erythromycin and ampicillin, respectively. According to the authors, this could be attributed to several factors such as the gene expression level and the substrate specificity of the expressed product. Similarly, the identification of hemolysin III protein, the product of the *hly* gene, is another example (Chokesajjawatee et al., 2020). All of these undesirable genes (Table 6.2) located in the genome of the 6 strains are commonly encountered in several *Lp. plantarum* strains including the reference strain WCFS1 and various probiotics (JDM1, ST-III, and 299V) (Chokesajjawatee et al., 2020). Such observations are suggesting the widespread presence of the related genes within the species without jeopardizing, however, their safety level compared to the existing starter/probiotic cultures. The isolated bacteria were bioinformatically examined for the production of antimicrobial drugs, particularly those deemed as clinically important. Based on the performed KEGG database search, no strain displayed the ability to produce any of the concerned antimicrobials.

Table 6. 2. AMR (antimicrobial resistance) genes identified in the *Lp. plantarum* subsp. *argentoratensis* genomes.

a/a	AMR element	KEGG KO	Gene (EC No)	Description
1	Bacitracin	K06153	*uppP*, EC 3.6.1.27	Catalyzes the dephosphorylation of the undecaprenyl diphosphate (UPP). Confers resistance to bacitracin ABC transporter, permease protein, probably the 2 or 3 component bacitracin resistance efflux pump, BcrAB or BcrABC Chloramphenicol acetyltransferase
2	Bacitracin	K01992	-	D-alanyl-D-alanine-carboxypeptidase Catalyzes hydrolysis of the D-alanyl-D-alanine dipeptide Beta-lactamase enzyme family
3	Phenicol	K19271	*catA*, EC 2.3.1.28	Mediates bacterial resistance to the antibiotics streptomycin and spectomycin
4	Vancomycin	K07260	*vanY*, EC 3.4.17.14	
5	Vancomycin	K08641	*ddpX*, EC 3.4.13.22	
6	Beta-Lactam	K17836	*bla1*, *bla2*, EC 3.5.2.6	
7	Aminoglycoside	K00984	*aadA*, EC 2.7.7.47	

Another significant factor for the safety assessment of the candidate starters/probiotics is their genome stability. The presence of various mobile genetic elements (MGEs) such as plasmids, insertion sequences (ISs), and prophages may act as vehicles of the

horizontal transfer of virulence and/or AMR genes. The conducted analysis returned no virulence or AMR genes within the intact prophage regions (all strains contained two complete phage regions except LQC 2485), plasmids (at least one plasmid was identified; rep28 and repUS73 in LQC 2441, LQC 2485, LQC 2422, and LQC 2516; rep28 in LQC 2320 and LQC 2520), and IS (at least one IS was identified except of strain LQC 2485 in which no IS was located; ISLsa1—IS30 family in LQC 2441 and LQC 2422; ISP1—ISL3 family in LQC 2520; ISP2—IS1182 family and ISLsa1—IS30 family in LQC 2516; ISP1—ISL3 family and ISLpl1—IS30 family in LQC 2320). Similar MGEs have been frequently found in other *Lp. plantarum* strains (Liu et al., 2015, Rodrigo-Torres et al., 2019, Chokesajjawatee et al., 2020) that pose no safety risk. The *Lp. plantarum* subsp. *argentoratensis* genomes contained two complete phage regions (intact) and some phage remnants (incomplete). Briefly, LQC 2320 and LQC 2520 had two intact regions of 26.2 Kb (total proteins, 29; phage proteins, 27; hypothetical proteins, 2; tRNA, 0; phage-related keywords found in protein names in the region, terminase, portal, head, capsid, tail, plate, and lysin; putative phage attachment site, no; most common phage, PHAGE_Lactob_jlb1; GC, 43.84%) and 52 Kb (total proteins, 71; phage proteins, 47; hypothetical proteins, 24; tRNA, 6; phage-related keywords found in protein names in the region, lysin, tail, head, portal, terminase, and integrase; putative phage attachment site, yes; most common phage, PHAGE_Lactob_Lj965; GC, 41.63%). The LQC 2485 possessed one intact region of 106.7 Kb (total proteins, 120; phage proteins, 91; hypothetical proteins, 29; tRNA, 6; phage-related keywords found in protein names in the region, integrase, lysin, terminase, portal, head, tail, protease, and capsid; putative phage attachment site, yes; most common phage, PHAGE_Lactob_Sha1; GC, 42.18%). Finally, PHASTER identified two intact regions for the strains LQC 2422, LQC 2441, and LQC 2516 of 50.4 Kb (total proteins, 51; phage proteins, 45; hypothetical proteins, 6; tRNA, 2; phage-related keywords found in protein names in the region, integrase, tail, terminase, head, portal, protease, capsid, and lysin; putative phage attachment site, yes; most common phage, PHAGE_Lactob_Sha1; GC, 42.65%) and 51.3 Kb (total proteins, 65; phage proteins, 46; hypothetical proteins, 19; tRNA, 5; phage-related keywords found in protein names in the region, lysin, tail, head, portal, terminase, and integrase; putative phage attachment site, yes; most common phage, PHAGE_Lactob_Lj965; GC, 41.69%).

The results from the screening of CRISPR sequences are presented in Table 6.3. Each genome had four Class 1 CRISPR systems, except strain LQC 2485 that had five with different evidence levels (1, 3 or 4). In all cases, only two CRISPR regions of evidence level 4 had one *cas* gene cluster per CRISPR array in vicinity, which provided immunity against foreign DNA. The TypeIE CRISPR*cas* system has been associated with the discrimination of self from non-self DNA (Westra et al., 2013). This could be a means to avoid acquiring virulence and/or AMR genes through the horizontal gene transfer phenomenon (Marraffini et al., 2008). The remaining CRISPR regions were of low evidence level or without *cas* genes nearby; and, therefore, these regions were not a real or a functional CRISPR array, respectively (Couvin et al., 2018, Rodrigo-Torres et al., 2019).

Table 6. 3. CRISPR*cas* system identified in the *Lp. plantarum* subsp. *argentoratensis* genomes.

Strain	Total CRISPR regions	CRISPR regions with EL1 or 2[a]	CRISPR regions with EL 3 or 4	*cas* gene clusters nearby CRISPR regions[b]	Type of *cas* gene cluster	Total *cas* genes[c]
LQC 2320	4	2 of EL1	2 of EL4	2	CAS-typeI and CAS-typeIE	11
LQC 2422	4	1 of EL1	1 of EL3 & 2 of EL4	2	CAS-typeI and CAS-typeIE	11
LQC 2441	4	1 of EL1	1 of EL3 & 2 of EL4	2	CAS-typeI and CAS-typeIE	11
LQC 2485	5	1 of EL1	4 of EL4	2	CAS-typeI and CAS-typeIE	11
LQC 2516	4	1 of EL1	1 of EL3 & 2 of EL4	2	CAS-typeI and CAS-typeIE	11
LQC 2520	4	2 of EL1	2 of EL4	2	CAS-typeI and CAS-typeIE	11

[1] When the evidence level (EL) is 1 or 2 then the CRISPR region is not likely to be a real CRISPR array. [2] Only two CRISPR regions of evidence level (EL) 4 had in vicinity *cas* gene clusters (one gene cluster per CRISPR array). [3] CAS-typeI gene cluster, four *cas* genes; CAS-typeIE, seven *cas* genes.

The screening of the 6 genomes with the BAGEL4 platform indicated a variable *pln* locus encoding for plantaricins and spanning in a region of 8.2 kb (LQC 2485, LQC 2441, and LQC 2422), 20.2 kb (LQC 2516), and 28.9 kb (LQC 2320 and LQC 2520) (Figure 6.7). The organization of the *pln* locus for the strains LQC 2485, LQC 2441, LQC 2422, and LQC 2516 resembled that of the *Lp. plantarum* 423 strain consisting of four genes, *plaABCD* (Van Reenen et al., 2003, 2006). Plantaricin 423 is a small pediocin-like plasmid-encoded protein that belongs to the class IIa bacteriocins with a double-glycine leader peptide and strong antilisterial activity. The strains LQC 2320 and LQC 2520 harbored genes encoding for three class IIb bacteriocins, *plnJK*

(two-peptide plantaricin), *plnEF* (two-peptide plantaricin), and *NC8βα* (inducible plantaricin) (Diep et al., 2009). The structure of the *pln* operon in these strains was found to be like that of *Lp. plantarum* strain NC8 (Maldonado et al., 2003, 2004). More accurately, the presence of the operons *plnJKLR*, *plnEFI*, and *plnC8αβc* was verified; while, in the case of the transport operon, the presence of only three genes—*plnG*, *plnH*, and *plnS*—was evident, followed by *plnY*, which was absent in *Lp. plantarum* NC8. As far as the regulatory operon was concerned, the presence of three genes—*plNC8-IF*, *plNC8-HK*, and *plnD*—encoding an induction pheromone, a histidine protein kinase, and a response regulator, respectively, was observed. The presence of *plnM* was also reported. Regarding the *plNC8-IF*, it was indicated as a small open reading frame (ORF) labelled by BAGEL4 as sORF_12 (Figure 6.7C). Its sequence was blasted using the BLAST protein tool with the default settings and the results returned a plantaricin precursor peptide, induction factor (98%, *Lp. plantarum*).

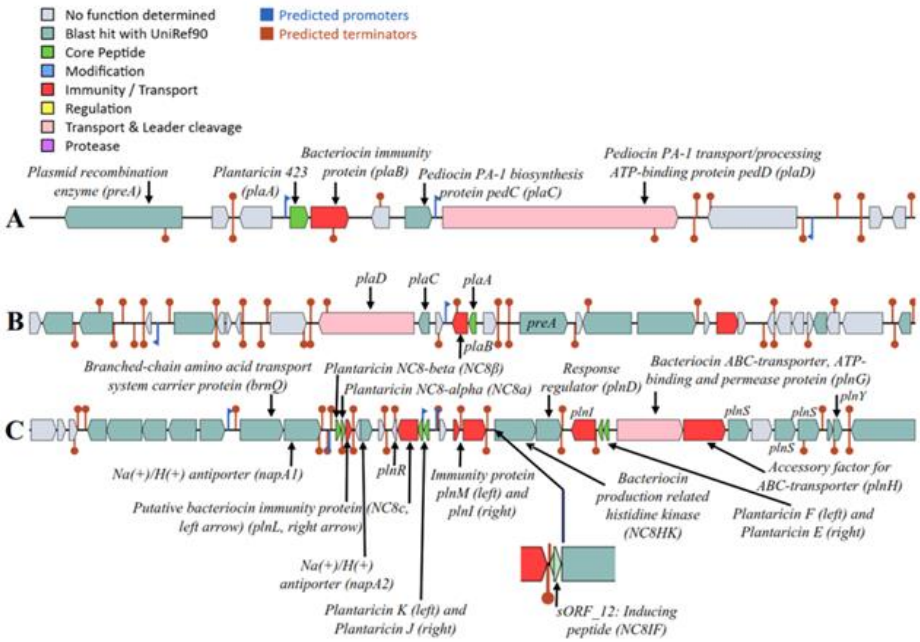

Figure 6. 7. Organization of the pln loci of the six *Lp. plantarum* subsp. *argentoratensis* strains. (A) LQC 2485, LQC 2441 and LQC 2422, (B) LQC 2516, and (C) LQC 2320 and LQC 2520.

5 Conclusions

This work deals with the comparative genomic analysis of 6 *Lp. plantarum* subsp. *argentoratensis* strains previously isolated from Greek wheat sourdoughs. The analysis showed that the lactic acid bacteria possessed properties with biotechnological/probiotic potential (e.g., as a starter culture in fermented foods). Their safety based on the EFSA recommendations was evaluated. Regarding biogenic amines production, the 6 strains encoded a lysine carboxylase gene, *cadA*, associated with cadaverine production but the real risk of such production and accumulation should be confirmed phenotypically. No virulence factors or production of antimicrobial drugs were detected in any of the queried genomes. Genes related to antibiotic resistance were found but the experimental validation of this resistance was not performed. Our aim was the bioinformatic characterization of the *Lp. plantarum* subsp. *argentoratensis* strains and the evaluation of their safety to have a reference study for any future validation experiment involving the current bacteria. Similar resistance antibiotic genes, however, are frequently encountered in strains of the same species including probiotics (WCFS1, JDM1, ST-III, and 299V) (Chokesajjawatee et al., 2020). All microorganisms were able to produce plantaricins belonging to different classes. Finally, plasmids, prophage regions, insertion sequences, and CRISPR*cas* systems were identified in the 6 genomes. Consequently, the in-silico analysis performed and the genomic results obtained guarantee the safety of the microbial strains for food applications.

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Chapter 7

The effect of incubation temperature, substrate and initial pH value on plantaricin activity and the relative transcription of pln genes of six sourdough derived Lactiplantibacillus plantarum strains

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1 Abstract

The aim of the present study was to assess the effect of sourdough related parameters on the growth and plantaricin activity of 6 *Lp. plantarum* strains against a mixture of 5 *L. monocytogenes* strains and to analyze the transcriptomic response of their *pln* genes. Parameters included 3 substrates (MRS broth, mMRS broth, WFE), 3 temperatures (20, 30, 37 °C), 2 initial pH values (5.0, 6.0), 2 NaCl concentrations (0.0, 1.8%) and 12 time points (ranging from 0 to 33 h). The transcriptomic response of the plantaricin genes to the aforementioned parameters was assessed after 21 h of growth. In general, plantaricin activity was strain dependent with that of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516, harboring four *pln* genes, namely, *pln423* (*plαA*), *plαB*, *plαC* and *plαD*, reaching 2560 AU/mL. However, strains LQC 2320 and 2520, in which 18 *pln* genes were detected, namely, *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, exhibited plantaricin activity barely reaching 160 AU/mL. Substrate, temperature, initial pH value and strains significantly affected plantaricin activity, while NaCl had only a marginal effect. Similarly, growth substrate and temperature had a more pronounced effect than initial pH value on gene transcription. A strong correlation between the transcription of the genes belonging to the same locus was observed; however, only a weak correlation, if any, was observed between plantaricin activity and the transcription of the genes assessed.

Keywords: transcriptomic response; *pln* locus; bacteriocins

2 Introduction

Sourdough fermentation is regarded as one of the most ancient and simultaneously ecologically friendly preservation methods. It is a dynamic process, strongly dependent on the metabolites released by highly adapted microorganisms, namely, lactic acid bacteria (LAB), leading to microbiologically stable and safe end products. The ability of sourdough starters to produce bacteriocins has been at the epicenter of intensive study over the past few years, as a clean label technology. Despite the fact that bacteriocin production does not extend the mold free shelf life of sourdough related products, it does positively affect the stability of sourdough, through the prevention of contamination by pathogenic bacteria, and particularly *L. monocytogenes*. The latter is particularly important, since extended fermentation times and the diversity of raw materials used in dough making process increases the risk of cross-contamination in the bakery environment.

By definition, bacteriocins are ribosomally synthesized single proteins or protein complexes capable of inhibiting organisms closely related to the producing strains (Barbosa et al., 2016, Garcia-Gonzalez et al., 2021). Bacteriocins produced by *Lp. plantarum* strains, namely, plantaricins, have gained much industrial and scientific attention. The stable presence of *Lp. plantarum* in spontaneously fermented foods, ranging from pickled vegetables to wine, attributed to its metabolic versatility and stress adaptation responses, has been extensively reported (Da Silva Sabo et al., 2014). In addition, its frequent isolation from type I sourdoughs, with the latter characterized by daily back-sloppings at ambient temperature (< 30 ˚C) and a fermentation time of less than 24 h, has been well documented (Paramithiotis et al., 2010, Corsetti, 2013, Syrokou et al., 2020).

The genetic organization of the *pln* locus of several *Lp. plantarum* strains has been unraveled up till now (Diep et al., 1996, Kleerebezem et al., 2003, Maldonado et al., 2003, Navarro et al., 2008, Rojo-Bezares et al., 2008, Barbosa et al., 2021, Tenea & Ortega, 2021). The *pln* locus is organized in 5 or 6 operons, with variable degrees of conservation (Diep et al., 2009, Tai et al., 2015). The genes encoding the bacteriocin production and associated functions include a structural, an immunity, an ABC transporter and one coding for an accessory protein, while the detection of regulatory genes has been reported as well (Todorov et al., 2009). The stimulation

of gene transcription is performed by a signal transducing network, which includes a secreted induction peptide (IP), a histidine protein kinase (HPK) and a response regulator (RR) (Straume et al., 2009). HPK functions as the receptor for IP, with the former transferring the signal to RR through several phosphorylation reactions. Once phosphorylated, RR stimulates the transcription of genes associated with bacteriocin biosynthesis. A common attribute among several *Lp. plantarum* strains, namely, C11, WCFS1 and J51, is the presence of the regulatory operon *plnABCD*, which encodes a quorum sensing system (Diep et al., 2009). In more detail, the regulatory operon *plnABCD* encodes an IP, namely, PlnA, an HPK, namely, PlnB and two cytoplasmic RRs, namely, PlnC and PlnD. Instead of *plnABCD*, the presence of the regulatory operon *plNC8-IF-HK-D*, encoding an IP, namely, *plNC8-IF*, an HPK, namely, *plNC8-HK* and an RR, namely, *plNC8-plnD*, was found in strains NC8 and J23. In our previous study (Syrokou et al., 2022), the genomic analysis of *Lp. plantarum* strains LQC 2320 and 2520 revealed the presence of the regulatory operon *plNC8-IF-HK-D* and three bacteriocin operons, namely, *plnJKLR*, *plnEFI* and *plNC8αβc*. In the case of the transport operon, only *plnG*, *plnH* and *plnS* were detected, followed by *plnY*. The presence of *plnM* was detected as well. On the contrary, *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 harbored *pln423* (*plαA*), *plαB*, *plαC* and *plαD* in their *pln* locus.

Although the organization of the *pln* locus has been studied to some extent, the literature is scarce on the transcriptomic response of plantaricin associated genes under fermentation conditions. More accurately, Paramithiotis et al. (2019) shed some light by studying the relative transcription of *pln* genes during the lactic acid fermentation of radish (*Raphanus sativus*) roots by an *Lp. plantarum* strain. Regarding sourdough fermentations, no previous study has assessed the relative gene transcription in *Lp. plantarum* at simulated industrial sourdough preparation conditions. Therefore, the aim of this study was to assess the effect of sourdough related parameters applied during type I sourdough fermentations, namely, incubation temperature and time, substrate composition, NaCl concentrations and initial pH values, on the growth and plantaricin activity of the aforementioned 6 *Lp. plantarum* strains and to further analyze the transcriptomic response of their *pln* genes.

3 Materials and Methods

3.1 Bacterial strains and culture conditions

Six Greek wheat sourdough derived *Lp. plantarum* strains, LQC 2320, 2422, 2441, 2485, 2516 and 2520, which previously exhibited antibacterial activity against *L. monocytogenes* (Syrokou et al., 2021), were used in the present study. Five *L. monocytogenes* strains, LQC 15186, 15187, 15188, 15189 and 15190, isolated from minced pork meat and assigned to serotype 4b (Andritsos et al., 2013), were used as indicator strains. Long term storage of bacterial strains took place at -20 ^{o}C in Nutrient Broth (LAB M, Lancashire, UK), supplemented with 50% glycerol. Before experimental use, *Lp. plantarum* and *L. monocytogenes* strains were grown twice in MRS broth (LAB M) and BHI broth (LAB M) at 30 ^{o}C for 24 h and 37 ^{o}C for 24 h, respectively.

3.2 Effect of parameters related to sourdough preparation on growth and plantaricin activity kinetics of *Lp. plantarum* strains

All plantaricin activity assays were performed by applying a WDA against a mixture of the aforementioned 5 *L. monocytogenes* strains. Growth of all *L. monocytogenes* strains at equal or at least comparable populations was verified as follows: 24 hour individual cultures of the 5 *L. monocytogenes* strains were inoculated into a common BHI broth at 7 log CFU/mL each and incubated at 37 ^{o}C for 24 h. Bacterial enumeration was performed by plating serial dilutions on BHI agar (LAB M). All colonies present in the final dilution were selected and further subcultured in BHI broth at 37 ^{o}C for 24 h. Then, DNA was extracted according to Paramithiotis et al. (2010) and amplification by PCR of two genomic regions belonging to the Listeria Pathogenicity Island 1, which has proven effective in differentiating capacity between these strains (Hadjilouka et al., 2018), took place. The genomic regions were *plcB* gene, and the intergenic region *plcB-orfX*, which were amplified according to Hadjilouka et al. (2018) PCR took place using KAPA SYBR Fast qPCR Master Mix (2 x) for ABI Prism (Kapa Biosystems, Wilmington, MA, USA) and StepOnePlus Real-Time PCR System (Applied BiosystemsTM, Waltham, MA, USA). After amplification, the melting temperature of each amplicon of each isolate was determined by melting curve analysis and compared with the respective of the pure cultures that were also included in the analysis. Melting curve analysis was as follows: 95 ^{o}C for 15 s, then 60 ^{o}C for 1 min and raise to 95 ◦C at

0.3 ºC/s. Two biological replicates were assessed; each PCR reaction was performed with ca. 0.1 μg of DNA.

The substrates used in the present study were MRS broth, MRS broth modified to contain glucose 1.47 g/L, fructose 0.52 g/L, maltose 9.87 g/L and sucrose 8.35 g/L (mMRS), i.e., the carbohydrates of wheat flour at relevant concentrations (Paramithiotis, 2001) and wheat flour-water extract (WFE). The latter was prepared according to Gobbetti et al. (1998) and Rizzello et al. (2009) modified as follows: wheat flour was suspended in tap water, at ratio 1:5 and an 18 h incubation took place under shaking (200 rpm) at 30 ºC. Then, the supernatant that was obtained by centrifugation (12,000 x g; 20 min; 4 ◦C) was further fortified with wheat flour carbohydrates at their initial concentration and, finally, the wheat flour-water extract was sterilized at 121 ºC for 15 min. Each of the aforementioned substrates was adjusted according to the rest sourdough related parameters, namely, two pH values: 5.0 and 6.0, adjusted with NaOH 3 M; two NaCl concentrations, 0.0 and 1.8%; three incubation temperatures 20, 30 and 37 ºC and incubation time up to 33 h.

Overnight cultures of the 6 *Lp. plantarum* strains were used to inoculate the aforementioned substrates at final population of ca. 7.5 log CFU/mL. Sampling was performed at regular time intervals (every three hours), the pH value was recorded and bacterial enumeration was performed by plating serial dilutions on MRS agar. For the antilisterial activity assessment, WDA was applied at the same time intervals, using as indicator strains a mixture of the aforementioned 5 *L. monocytogenes* strains, according to Syrokou et al. (2021). In brief, overnight cultures of the indicator strains were inoculated (1% each) into molten BHI agar cooled to 45–47 ºC. Then, the agar was dispensed into Petri dishes and allowed to solidify. Wells were then aseptically punched with the aid of Pasteur pipette. In each well, 25 μL of each CFS, which were obtained by centrifugation (12,000 x g; 10 min; 4 ºC) and subsequent neutralization and treatment with catalase (Sigma-Aldrich, St. Louis, MO, USA), were added. Incubation took place at 37 ºC for 24 h. The plantaricin activity was quantified by applying the two fold serial dilution approach on the CFS and expressed in AU/mL. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution, exhibiting a clear inhibition zone, multiplied by 40 to obtain AU/mL. All analyses were performed in duplicate and the average values are presented.

3.3 Effect of parameters related to sourdough preparation on the transcription of plantaricin genes

The high-quality draft genome data of the 6 *Lp. plantarum* strains were presented in previous studies (Syrokou et al., 2021, 2022). Results obtained revealed the presence of 18 plantaricin genes, namely, *plNC8a, plNC8b, plNC8c, plnL, plnR, plnJ, plnK, plnE, plnF, plnH, plnS, plnY, plNC8-IF, plNC8-HK, plnD, plnI, plnM* and *plnG*, in the *pln* loci of two *Lp. plantarum* strains, namely, LQC 2320 and 2520, while the plantaricin genes *pln423* (*plαA*), *plαB, plαC* and *plαD* were detected in *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516. Primers were designed to detect these genes (Table 7.1) and their specificity was verified by PCR and gel electrophoresis using the conditions mentioned in Table 1. In the case of *plαC*, the PCR efficiency of the primer pairs examined was too low (< 1.70) and, thus, the relative transcription of this gene was not assessed.

The effects of sourdough associated parameters on bacterial growth, pH value and plantaricin activity of the 6 *Lp. plantarum* strains were assessed every three hours, up to 33 h, as previously described. At the same time intervals, samples were collected, centrifuged (12,000 x *g*; 10 min; sample temperature) and the biomass was mixed with 200 μL of RNAlater® solution (Ambion, Waltham, MA, USA). Samples from a specific time point (21 h) were subjected to RT-qPCR analysis as follows: RNA was extracted using the NucleoSpin® RNA Kit (Macherey–Nagel, Duren, Germany) and cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan) according to the instructions of the manufacturer. Real-time qPCR was performed using KAPA SYBR qPCR Kit Master Mix (2 x) for ABI Prism (Kapa Biosystems, Boston, MA, USA). Primers and PCR conditions are presented in Table 7.1. Here, *16S*, *IGS* and *rpob* were evaluated as housekeeping genes; *plNC8a, plNC8b, plNC8c, plnL, plnR, plnJ, plnK, plnE, plnF, plnH, plnS, plnY, plNC8-IF, plNC8-HK, plnD, plnI, plnM, plnG, pln423* (*plαA*), *plαB* and *plαD* were selected as target genes. Two biological replicates were analyzed and two RT reactions were performed for each sample, containing ca. 0.1 μg RNA each; each cDNA was used for the assessment of the transcription of the genes under study.

3.4 Statistical analysis

Processing of the Ct values obtained by qPCR was performed according to Hadjilouka et al. (2019). The calculated fold changes were converted to their $\log_2$ values for further processing. The stability values of the reference genes were calculated with NormFinder v0.953 (Andersen et al., 2004); *IGS* was the most stably transcribed gene and, therefore, it was used for normalization. Regulation of a gene was considered only when the $\log_2$ value of the fold change ($\log_2$FC) was below -1 (downregulation) or above 1 (upregulation), assessed through one-sample *t*-test ($p < 0.05$). The correlation between the transcription of the genes under study was assessed with the Pearson product moment correlation coefficient (r). The contribution of the sourdough related parameters, namely, initial pH value, NaCl content, growth substrate, growth temperature and bacterial strain on plantaricin activity, bacterial population and pH reduction, was evaluated through multifactor analysis of variance. All calculations were performed in Statgraphics Centurion XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

Table 7. 1. Primer sequences and respective amplicon sizes used for the gene transcription assay.

Genes	Primer	Sequence	Amplicon size (bp)	PCR efficiency	References
Reference genes					
16S	16SF	GATGCATAGCCGACCTGAGA	114	2.05	Van der Veen & Abee, (2010)
	16SR	CTCCGTCAGACTTTCGTCCA			Hadjilouka et al. (2014)
IGS	IGSF	GGCCTATAGCTCAGCTGGTTA	135	2.03	Rantsiou et al. (2012)
	IGSR	GCTGAGCTAAGGCCCCGTAAA			
Rpob	rpobF	CCGCGATGCGAAAACAAT	69	2.04	Olesen et al. (2009)
	rpobR	CCWACAGAGATACGGTTATCRAATGC			
Plantaricin genes					
plNC8a	plNC8aF	GGCGGTGATTTAACAACCAAG	70	2.05	this study
	plNC8aR	AATTCCAACGTGCTTTCTTGC			
plNC8b	plNC8bF	CGGATCAGTCCCAACTTCAGTA	80	2.01	this study
	plNC8bR	TTTCAATCGTTTTGCGATGCT			
plNC8c	plNC8cF	AGCGTAAAAGCAGCAGTGAATA	98	2.01	this study
	plNC8cR	AGTACGTGGCAAATGCCTAAAA			
pln423 (plaA)	423F	TGTGGTAAACATTCCTGCTCTG	86	2.06	this study
	423R	CACTTTCCATGACCGAAGTTAGC			
plaB	plaBF	CGGTGAAAAACCCTGAGGCA	151	1.98	this study
	plaBR	TAGCTACCGTTCCAACCTGC			
plaD	plaDF	GCCAAAACAACTGCTGACGG	95	1.96	this study
	plaDR	TCCATATCAGCACGCACAGC			
plNC8-HK	plNC8-HKF	GGTGAAAAACCCTGAGGCAT	148	2.00	this study
	plNC8-HKR	GCTACCGTTCCAACCTGCT			
plNC8-IF	plNC8-IFF	ATAAGCTTGATGTCGGGGTTG	70	2.00	this study
	plNC8-IFR	GATGGCCTCCAAGTGCTTTT			
plnD	plnDF	GTGGTTTTGTTGAGTACATCGAAAT	126	1.98	this study
	plnDR[a]	GCATCGGAAAAATTGCGGATAC			Ben Omar et al. (2006)
plnE	plnEF	TGGTTTTAATCGGGGCGGT	87	2.03	this study
	plnER	ATACCACGAATGCCTGCAAC			
plnF	plnFF	TGCTATTTCAGGTGGCGTTT	94	2.08	this study
	plnFR	GCTAATGACCCAATCGGCAG			
plnG	plnGF[a]	TGCGGTTATCAGTATGTCAAAG	453	1.96	Ben Omar et al. (2006)
	plnGR[a]	CCTCGAAACAATTTCCCCC			
plnH	plnHF	AACTGTTCAACCGACCGGAA	90	2.07	this study
	plnHR	ACTCGCGCACCTTCAACTAA			
plnI	plnIF	CTGGCTGCCATTAGTGTCCA	100	2.09	this study
	plnIR	GAGCTTCCATTGGCCCGTTA			
plnJ	plnJF	TTGAACGGGGTTGTTGGGG	81	2.03	this study
	plnJR	GCCAGCTTCGCCATCATAAA			
plnK	plnKF	GGCCGTCGGAGTCGTAAAAA	90	2.05	this study
	plnKR	ATCCCTTGAACCACCAAGCA			
plnL	plnLF	GGGTGCATCGTATTTGCGTG	113	2.01	this study
	plnLR	TTTGCAGATCGCCATGAAGC			
plnM	plnMF	AGCAGTGGGAAGATGCTTGA	109	2.02	this study
	plnMR	TGCCAACCTGCTTTACCTGT			
plnR	plnRF	GCGCTTATTGTCGTTTTCGC	88	2.01	this study
	plnRR	CAGCAGCCCCATCACTAAGC			
plnS	plnSF	TATGGCACCGGCGTATCTTT	121	2.02	this study
	plnSR	AACTCGTGCTGTATGCCGAT			
plnY	plnYF	GATTGGGGTACCCACGTCAC	91	2.07	this study

plnYR	AAAGAATCGTCCTAGCCGCA	

Thermocycling conditions: initial denaturation at 95 ºC for 20 s and then 40x (95 ºC for 10 s, 60 ºC for 30 s, 72 ºC for 30 s). Melting curve analysis: 95 ºC for 15 s, then 60 ºC for 1 min and raise to 95 ºC at 0.3 ºC /s.

4 Results

4.1 The effect of parameters related to sourdough preparation on growth and plantaricin activity kinetics of *Lp. plantarum* strains

The melting temperatures of the *plcB* gene and the *plcB-orfX* intergenic region of the *L. monocytogenes* strains LQC 15186, 15187, 15188, 15189 and 15190 were calculated at 80.6093, 80.5373, 80.6099, 80.6114 and 80.4620 ºC and at 79.8077, 79.3593, 79.5099, 79.3637 and 79.8074 ºC, respectively. A total of 50 colonies present in the final dilution after the incubation of the mixture of the 5 *L. monocytogenes* strains at 37 ºC for 24 h were isolated and purified by successive subculturing. Their DNA was extracted and the aforementioned genomic regions were amplified and their melting temperature was assessed. Twelve isolates presented identical melting temperatures for both genomic regions with strain LQC 15186, eight isolates with strain LQC 15190 and ten strains for each of the strains LQC 15187, 15188 and 15189. Therefore, it was indicated that the *L. monocytogenes* strains were grown at equal populations and, thus, the WDA was successfully performed.

The effect of sourdough related parameters, namely, 3 growth substrates (MRS broth, mMRS broth, WFE), 3 incubation temperatures (20, 30, 37 ºC), 2 initial pH values (5.0, 6.0), 2 NaCl concentrations (0.0, 1.8%) and 12 time points (spanning from 0 to 33 h), on the plantaricin activity, growth and pH reduction of the 6 *Lp. plantarum* strains are presented in Figures S7.1–S7.6.

Plantaricin activity revealed a strain dependent profile. The strains under study were differentiated into two groups: the first one consisted of strains LQC 2422, 2441, 2485 and 2516 and the second one of strains LQC 2320 and 2520. The strains belonging to the former group were the first to exhibit plantaricin activity (ranging from 80 to 320 AU/mL), after 3 h of incubation, regardless of the treatment applied. In the case of strains LQC 2320 and 2520, detectable levels of plantaricin activity were observed after 6 h of incubation in MRS and mMRS broth and after 9 h in WFE, in all cases quantified as 80 AU/mL. The highest level of plantaricin activity (2560 AU/mL) produced by strains LQC 2422, 2441, 2485 and 2516 was detected after 15 and 18 h of growth in mMRS

broth at pH 6.0, at 30 and 37 ^{o}C, respectively, regardless of the NaCl concentration applied. In both cases, the plantaricin activity remained stable until 21 h. In MRS broth with initial pH 6.0, a slightly decreased maximum plantaricin activity (1920 AU/mL) was observed after the growth of the aforementioned strains both at 30 and 37 ^{o}C for 18 h, with the activity remaining stable until 21 h. Growth in WFE revealed substantially decreased maximum plantaricin activity, ranging from 320 to 640 AU/mL. In all cases, lower levels of plantaricin activity were detected towards the end of the monitored period of incubation. Regarding *Lp. plantarum* strains LQC 2320 and 2520, much lower plantaricin activity levels were recorded, varying between 80 and 160 AU/mL, after any treatment was applied. As far as the effect of sourdough related parameters on growth was concerned, good growth rates of all 6 bacterial strains were demonstrated at any incubation temperature applied, during 33 h of incubation in MRS and mMRS broth. More accurately, the initial bacterial populations were 7.50 log CFU/mL (0 h) and by the end of the monitored period more than 9 log CFU/mL were recorded. During the same period of incubation in WFE, lower population levels were evident (< 9 log CFU/mL). Depending on the initial culture pH, the 6 bacterial strains displayed similar final pH values (< 3.5), after growth both at 30 and 37 ^{o}C, irrespective of the substrate applied, while growth at 20 ^{o}C revealed higher final pH values (> 4.0).

Multifactor analysis of variance was applied to estimate the contribution of the sourdough related parameters on the plantaricin activity every 3 h over a period of 33 h. The independent factors, namely, bacterial strains, temperature, substrate and initial pH value, significantly affected the variability of plantaricin activity at the majority of time points tested ($p < 0.05$). A significant contribution of NaCl on plantaricin activity was only detected at 24 h ($p < 0.05$). The mean plantaricin activity obtained either from the single contribution of each significant factor or from the in pair interactions between significant factors, resulted in the substantial interpretation of the effect of sourdough related parameters on plantaricin activity (Table S7.1). In the case of bacterial strains, in agreement with the biological interpretation of the results, the mean plantaricin activities observed for LQC 2320 and 2520 were similar and were differentiated from the other four bacterial strains, namely, LQC 2422, 2441, 2485 and 2516, at any time point assessed (Table S7.2). Given that the multifactor analysis of variance for plantaricin activity assessment exhibited similar significant effects among the 12 time points tested, some representative figures illustrating the aforementioned effects are further presented,

corresponding to the time point of 21 h. The selection of the aforementioned time point was not random, since the incubation of all 6 *Lp. plantarum* strains for 21 h revealed the maximum plantaricin activities. Regarding the in pair contribution of strains, namely, LQC 2320 and 2520, and incubation temperatures at the time point of 21 h, the mean plantaricin activities were overlapping, implying a temperature independent production. Regarding strains LQC 2422, 2441, 2485 and 2516, the mean plantaricin activities at 30 and 37 ^{o}C were also overlapping but were differentiated from those at 20 ^{o}C (Figure 7.1A1). Regarding the in pair effect of strains and substrates, the mean plantaricin activities observed for strains LQC 2320 and 2520 in MRS and mMRS broth were overlapping but were differentiated from those in WFE. In the case of strains LQC 2422, 2441, 2485 and 2516, a substrate dependent grouping of mean plantaricin activities was demonstrated (Figure 7.1A2).

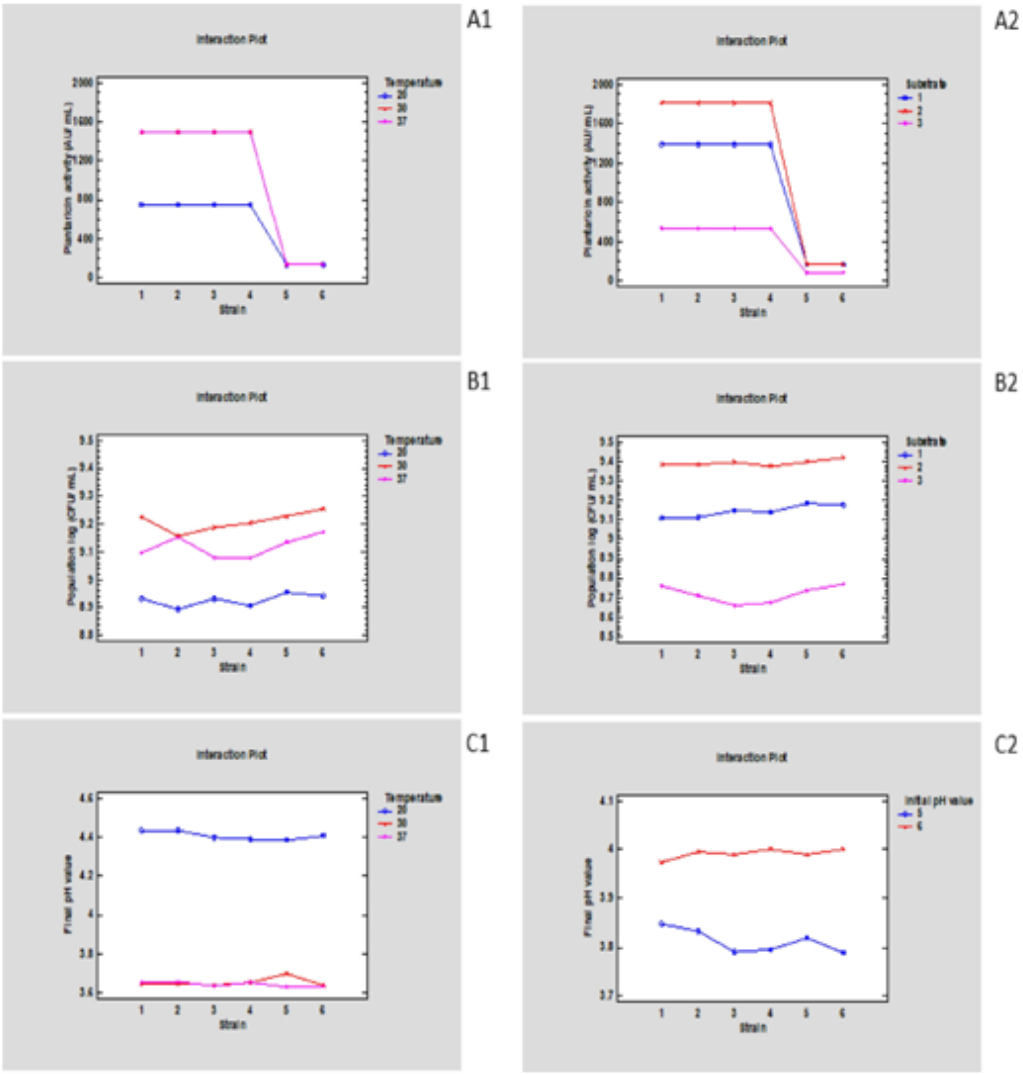

Figure 7. 1. Interaction plots depicting the mean plantaricin activity, population and final pH value, obtained from the combination of two sourdough related parameters. (A1,A2) The effect on mean plantaricin activity (AU/mL), resulting from the in pair interaction of strains with temperatures and substrates, respectively, after growth for 21 h. (B1,B2) The effect on mean population, log (CFU/mL), resulting from the in pair interaction of strains with temperatures and substrates, respectively, after growth for 21 h. (C1,C2) The effect on mean final pH value, resulting from the in pair interaction of strains with temperatures and initial pH value, respectively, after growth for 21 h. Six *Lp. plantarum* strains were included, namely, LQC 2441, 2422, 2516, 2485, 2320 and 2520 corresponding to 1, 2, 3, 4, 5 and 6, three incubation temperatures, namely, 20, 30 and 37 ◦C, three substrates, namely, MRS broth, mMRS broth and WFE corresponding to 1, 2 and 3 and two initial pH values (5.0 and 6.0).

Regarding the effect of sourdough related conditions on the growth of the 6 *Lp. plantarum* strains, in most cases a strain independent profile was exhibited. More accurately, multifactor analysis of variance revealed that substrate, temperature and initial pH value had a significant effect on population for the majority of time points tested ($p < 0.05$). Strain was found to significantly affect population only at 6 and 27 h.

On the other hand, NaCl was not a significant factor. Regarding the in pair contribution of strains and temperatures, the 21 h incubation of the 6 bacterial strains at 30, 37 and 20 ^{o}C revealed, in most cases, a temperature dependent mean population. However, the incubation of strain LQC 2422 at 30 and 37 ^{o}C, resulted in identical mean populations (Figure 7.1B1). As far as the in pair effect of strains and substrates on the mean population was concerned, a substrate dependent grouping of means was exhibited after 21 h of incubation, among which no interaction was evident (Figure 7.1B2). This absence of interaction indicates that the effect of the substrate on the final population is not differentiated among the different strains, which is justified by the presence of parallel lines. In addition, the in pair effect of substrates and temperatures, namely, growth in WFE at 20, 30 and 37 ^{o}C, resulted in mean populations different from those obtained in MRS and mMRS broth at the aforementioned temperatures (Table S7.1). This observation is consistent with the biological interpretation of the effect on population, exerted after incubation of strains in WFE, in which case a decreased growth of bacterial strains (< 9 log CFU/mL) was recorded, irrespective of the initial pH value, incubation temperature and NaCl concentration applied.

As far as the effect of the sourdough related parameters on the pH profile was concerned, multifactor analysis of variance demonstrated that substrate, temperature and initial pH were significant factors in a time independent way, while NaCl was also found to significantly affect the final pH value after incubation for 6, 9, 21, 24, 27, 30 and 33 h ($p < 0.05$). In the case of strain, a statistically significant effect on the final pH value was observed for the majority of the time points assessed, except for 21 h. As in the case of plantaricin activity and population, the representative figures depicting the effect of the in pair contribution of significant factors to the final pH values, refer to the time point of 21 h. The incubation of the 6 *Lp. plantarum* strains, at the three temperatures, revealed that the mean final pH values observed for 30 and 37 ^{o}C were overlapping for all strains but LQC 2320 and were differentiated from those at 20 ^{o}C (Figure 7.1C1). Regarding the in pair effect of initial pH values and strains on the final pH values, an initial pH based grouping of means was performed. The aforementioned grouping of means indicated no interaction between the initial pH values and strains, which further demonstrates that the effect of the initial pH on the final pH value is not differentiated among the different strains (Figure 7.1C2). The mean final pH values obtained from the aforementioned treatments are presented in Table S7.1.

4.2 The effect of parameters related to sourdough preparation on the transcription of plantaricin genes

RT-qPCR analysis was applied to assess the effect of the sourdough related parameters on the transcriptomic response of plantaricin genes in the 6 *Lp. plantarum* strains. Strains LQC 2320 and 2520 harbored 18 plantaricin genes in their locus, namely, *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, while the presence of only four plantaricin genes, namely, *pln423* (*plαA*), *plαB*, *plαC* and *plαD*, was recorded in *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516. For the reasons described in Section 3.3, the relative transcription of *plαC* was not evaluated in the present study. Given the fact that plantaricin activity assessment was performed at regular time intervals, up to 33 h, a specific time point needed to be selected for further RT-qPCR analysis. The incubation time of 21 h was selected since it was the time point at which the maximum plantaricin activities produced by all 6 *Lp. plantarum* strains, after any treatment applied, intersect. In addition, it coincides with the incubation time required for artisanal sourdough preparation. Thus, samples obtained from 21 h incubation were selected for RT-qPCR analysis. Regarding the contribution of NaCl to the variability of the plantaricin activity, it was excluded from RT-qPCR analysis, since it was characterized as not significant. The effects of temperature, substrate, pH value and strain on plantaricin gene transcription are exhibited in Figures S7.7–S7.17 and are summarized in Tables 7.2 and 7.3.

Table 7. 2. Number of samples in which the relative transcription of the genes under study was below, above or within the threshold set under the effect of sourdough related parameters on *Lp. plantarum* strains LQC 2320 and LQC 2520.

		LQC 2320			LQC 2520			
	log_2(FC)	**Effect of temperature[1]**	**Effect of substrate[2]**	**Effect of pH[3]**	**Effect of temperature**	**Effect of substrate**	**Effect of pH**	**Effect of strain[4]**
plNC8α	**< -1**	2 (11.1)	2 (11.1)	2 (22.2)	0 (0.0)	4 (22.2)	0 (0.0)	3 (16.7)
	-1 to 1	12 (66.7)	12 (66.7)	7 (77.8)	9 (50.0)	14 (77.8)	8 (88.9)	12 (66.7)
	> 1	4 (22.2)	4 (22.2)	0 (0.0)	9 (50.0)	0 (0.0)	1 (11.1)	3 (16.7)
plNC8β	**< -1**	2 (11.1)	3 (16.7)	1 (11.1)	1 (5.6)	5 (27.8)	0 (0.0)	1 (5.6)
	-1 to 1	9 (50.0)	10 (55.6)	8 (88.9)	8 (44.4)	13 (72.2)	7 (77.8)	15 (83.3)
	> 1	7 (38.9)	5 (27.8)	0 (0.0)	9 (50.0)	0 (0.0)	2 (22.2)	2 (11.1)
plNC8c	**< -1**	2 (11.1)	2 (11.1)	2 (22.2)	1 (5.6)	5 (27.8)	0 (0.0)	5 (27.8)
	-1 to 1	11 (61.1)	12 (66.7)	7 (77.8)	8 (44.4)	13 (72.2)	8 (88.9)	10 (55.6)
	> 1	5 (27.8)	4 (22.2)	0 (0.0)	9 (50.0)	0 (0.0)	1 (11.1)	3 (16.7)
plnR	**< -1**	4 (22.2)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	-1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	12 (66.7)
	> 1	7 (38.9)	3 (16.7)	0 (0.0)	8 (44.4)	0 (0.0)	2 (22.2)	3 (16.7)
plnL	**< -1**	4 (22.2)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	-1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	12 (66.7)
	> 1	7 (38.9)	3 (16.7)	0 (0.0)	2 (11.1)	0 (0.0)	2 (22.2)	3 (16.7)
plnK	**< -1**	5 (27.8)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	4 (22.2)
	-1 to 1	6 (33.3)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	11 (61.1)
	> 1	7 (38.9)	3 (16.7)	0 (0.0)	8 (44.4)	0 (0.0)	2 (22.2)	3 (16.7)
plnJ	**< -1**	1 (5.6)	7 (38.9)	0 (0.0)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	-1 to 1	10 (55.6)	8 (44.4)	8 (88.9)	7 (38.9)	11 (61.1)	7 (77.8)	12 (66.7)
	> 1	7 (38.9)	3 (16.7)	1 (11.1)	9 (50.0)	0 (0.0)	2 (22.2)	3 (16.7)
plnE	**< -1**	3 (16.7)	3 (16.7)	0 (0.0)	3 (16.7)	4 (22.2)	0 (0.0)	1 (5.6)
	-1 to 1	14 (77.8)	11 (61.1)	9 (100.0)	12 (66.7)	13 (72.2)	7 (77.8)	14 (77.8)
	> 1	1 (5.6)	4 (22.2)	0 (0.0)	3 (16.7)	1 (5.6)	2 (22.2)	3 (16.7)
plnF	**< -1**	3 (16.7)	6 (33.3)	0 (0.0)	2 (11.1)	6 (33.3)	0 (0.0)	1 (5.6)
	-1 to 1	8 (44.4)	6 (33.3)	9 (100.0)	9 (50.0)	12 (66.7)	7 (77.8)	14 (77.8)
	> 1	7 (38.9)	6 (33.3)	0 (0.0)	7 (38.9)	0 (0.0)	2 (22.2)	3 (16.7)
plnH	**< -1**	2 (11.1)	8 (44.4)	2 (22.2)	2 (11.1)	6 (33.3)	0 (0.0)	5 (27.8)
	-1 to 1	9 (50.0)	5 (27.8)	7 (77.8)	7 (38.9)	10 (55.6)	7 (77.8)	10 (55.6)
	> 1	7 (38.9)	5 (27.8)	0 (0.0)	9 (50.0)	2 (11.1)	2 (22.2)	3 (16.7)
plnS	**< -1**	3 (16.7)	6 (33.3)	1 (11.1)	2 (11.1)	6 (33.3)	0 (0.0)	3 (16.7)
	-1 to 1	9 (50.0)	7 (38.9)	8 (88.9)	7 (38.9)	12 (66.7)	7 (77.8)	12 (66.7)
	> 1	6 (33.3)	5 (27.8)	0 (0.0)	9 (50.0)	0 (0.0)	2 (22.2)	3 (16.7)

plnY	**< -1**	1 (5.6)	6 (33.3)	1 (11.1)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	-1 to 1	8 (44.4)	9 (50.0)	8 (88.9)	7 (38.9)	12 (66.7)	7 (77.8)	14 (77.8)
	> 1	9 (50.0)	3 (16.7)	0 (0.0)	10 (55.6)	0 (0.0)	2 (22.2)	3 (16.7)
plNC8-IF	**< -1**	4 (22.2)	6 (33.3)	0 (0.0)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	-1 to 1	13 (72.2)	10 (55.6)	9 (100.0)	12 (66.7)	12 (66.7)	7 (77.8)	13 (72.2)
	> 1	1 (5.6)	2 (11.1)	0 (0.0)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plNC8-HK	**< -1**	4 (22.2)	6 (33.3)	0 (0.0)	2 (11.1)	6 (33.3)	0 (0.0)	1 (5.6)
	-1 to 1	13 (72.2)	10 (55.6)	9 (100.0)	11 (61.1)	12 (66.7)	7 (77.8)	13 (72.2)
	> 1	1 (5.6)	2 (11.1)	0 (0.0)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plnD	**< -1**	4 (22.2)	6 (33.3)	0 (0.0)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	-1 to 1	13 (72.2)	10 (55.6)	8 (88.9)	12 (66.7)	12 (66.7)	7 (77.8)	13 (72.2)
	> 1	1 (5.6)	2 (11.1)	1 (11.1)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plnM	**< -1**	5 (27.8)	7 (38.9)	1 (11.1)	3 (16.7)	6 (33.3)	0 (0.0)	4 (22.2)
	-1 to 1	7 (38.9)	6 (33.3)	7 (77.8)	5 (27.8)	11 (61.1)	6 (66.7)	10 (55.6)
	> 1	6 (33.3)	5 (27.8)	1 (11.1)	10 (55.6)	1 (5.6)	3 (33.3)	4 (22.2)
plnI	**< -1**	3 (16.7)	7 (38.9)	2 (22.2)	2 (11.1)	6 (33.3)	1 (11.1)	3 (16.7)
	-1 to 1	9 (50.0)	6 (33.3)	7 (77.8)	6 (33.3)	12 (66.7)	6 (66.7)	12 (66.7)
	> 1	6 (33.3)	5 (27.8)	0 (0.0)	10 (55.6)	0 (0.0)	2 (22.2)	3 (16.7)
plnG	**< -1**	3 (16.7)	5 (27.8)	1 (11.1)	1 (5.6)	7 (38.9)	1 (11.1)	3 (16.7)
	-1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	6 (33.3)	9 (50.0)	5 (55.6)	12 (66.7)
	> 1	8 (44.4)	5 (27.8)	1 (11.1)	11 (61.1)	2 (11.1)	3 (33.3)	3 (16.7)
Total	**< -1**	55 (17.0)	101 (31.2)	19 (11.7)	30 (9.3)	107 (33.0)	2 (1.2)	46 (14.2)
	-1 to 1	172 (53.1)	154 (47.5)	139 (85.8)	150 (46.3)	211 (65.1)	124 (76.5)	221 (68.2)
	> 1	97 (29.9)	69 (21.3)	4 (2.5)	144 (44.4)	6 (1.9)	36 (22.2)	57 (17.6)

Percentage is given in parenthesis. [1]the effect of incubation temperature was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain at 20, 30 and 37 ºC pairwise using the lower temperature as control. [2]the effect of substrate was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in MRS, mMRS and WFE using MRS as control. In the comparison between WFE and mMRS, the latter was used as control. [3]the effect of pH was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in pH 5.0 and 6.0, using the latter as control. [4]the effect of strain was assessed by comparing the transcription of the genes under study after 21 h growth of *Lp. plantarum* strains LQC 2320 and 2520 using the former as control.

Table 7. 3. Number of samples in which the relative transcription of the genes under study was below, above or within the threshold set under the effect of sourdough related parameters on *Lp. plantarum* strains LQC 2441, LQC 2422, LQC 2485 and LQC 2516.

	log_2(FC)	LQC 2441			LQC 2422			LQC 2485			LQC 2516			
		Effect of temperature[1]	**Effect of substrate[2]**	**Effect of pH[3]**	**Effect of temperature**	**Effect of substrate**	**Effect of pH**	**Effect of temperature**	**Effect of substrate**	**Effect of pH**	**Effect of temperature**	**Effect of substrate**	**Effect of pH**	**Effect of strain[4]**
pln423	< -1	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.1)	0 (0.0)	1 (11.1)	3 (16.7)	0 (0.0)	1 (11.1)	2 (11.1)	1 (5.6)	1 (11.1)	5 (4.6)
	-1 to 1	17 (94.4)	10 (55.6)	7 (77.8)	15 (83.3)	7 (38.9)	7 (77.8)	13 (72.2)	8 (44.4)	7 (77.8)	14 (77.8)	6 (33.3)	7 (77.8)	46 (42.6)
	> 1	1 (5.6)	8 (44.4)	2 (22.2)	1 (5.6)	11 (61.1)	1 (11.1)	2 (11.1)	10 (55.6)	1 (11.1)	2 (11.1)	11 (61.1)	1 (11.1)	57 (52.8)
plaB	< -1	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.1)	0 (0.0)	1 (11.1)	3 (16.7)	0 (0.0)	1 (11.1)	2 (11.1)	1 (5.6)	1 (11.1)	7 (6.5)
	-1 to 1	17 (94.4)	10 (55.6)	7 (77.8)	15 (83.3)	7 (38.9)	7 (77.8)	13 (72.2)	8 (44.4)	7 (77.8)	14 (77.8)	5 (27.8)	7 (77.8)	47 (43.5)
	> 1	1 (5.6)	8 (44.4)	2 (22.2)	1 (5.6)	11 (61.1)	1 (11.1)	2 (11.1)	10 (55.6)	1 (11.1)	2 (11.1)	12 (66.7)	1 (11.1)	54 (50.0)
plaD	< -1	5 (27.8)	8 (44.4)	2 (22.2)	2 (11.1)	6 (33.3)	7 (77.8)	4 (22.2)	7 (38.9)	2 (22.2)	5 (27.8)	3 (16.7)	1 (11.1)	41 (38.0)
	-1 to 1	9 (50.0)	5 (27.8)	3 (33.3)	11 (61.1)	10 (55.6)	2 (22.2)	9 (50.0)	9 (50.0)	4 (44.4)	9 (50.0)	12 (66.7)	5 (55.6)	52 (48.1)
	> 1	4 (22.2)	5 (27.8)	4 (44.4)	5 (27.8)	2 (11.1)	0 (0.0)	5 (27.8)	2 (11.1)	3 (33.3)	4 (22.2)	3 (16.7)	3 (33.3)	15 (13.9)
Total	< -1	5 (9.3)	8 (14.8)	2 (7.4)	6 (11.1)	6 (11.1)	9 (33.3)	10 (18.5)	7 (13.0)	4 (14.8)	9 (16.7)	5 (9.3)	3 (11.1)	53 (16.4)
	-1 to 1	43 (79.6)	25 (46.3)	17 (63.0)	41 (75.9)	24 (44.4)	16 (59.3)	35 (64.8)	25 (46.3)	18 (66.7)	37 (68.5)	23 (42.6)	19 (70.4)	145 (44.8)
	> 1	6 (11.1)	21 (38.9)	8 (29.6)	7 (13.0)	24 (44.4)	2 (7.4)	9 (16.7)	22 (40.7)	5 (18.5)	8 (14.8)	26 (48.1)	5 (18.5)	126 (38.9)

Percentage is given in parenthesis. 1 The effect of incubation temperature was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain at 20, 30 and 37 ◦C pairwise using the lower temperature as control. 2 The effect of substrate was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in MRS, mMRS broth and WFE using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. 3 The effect of pH was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in pH 5.0 and 6.0, using the latter as control. 4 The effect of strain was assessed by comparing the transcription of the genes under study after 21 h growth of the *Lp. plantarum* strains LQC 2441, 2422, 2485 and 2516 pairwise.

Temperature increase revealed a pronounced effect on the relative gene transcription of strain LQC 2520, whereas no such impact was recorded regarding the other strains. More specifically, regarding strain LQC 2520, upregulation was evident in 44.4% of the samples assessed. The most pronounced upregulation was observed for *plnG*, which was upregulated in 61.1% of the cases, followed by *plnY*, *plnI* and *plnM*, which were upregulated in 55.6% of the cases. On the other hand, the genes that were least affected by temperature increase were *plNC8-IF*, *plnD* and *plnE*, the relative transcription of which remained stable in 66.7% of the cases. Regarding strain LQC 2320, the relative transcription of genes *plnY* and *plnG* were most affected by temperature increase, since they were upregulated in the 50% and 44.4% of the samples assessed, respectively. As in the case of strain LQC 2520, the genes that were least affected by temperature increase were *plNC8-IF*, *plNC8-HK*, *plnD* and *plnE*, the relative transcription of which remained stable in 72.2% of the three former cases and 77.8% of the latter one, respectively. Regarding both strains, during growth in MRS and mMRS broth, a temperature increase from 20 to 30 ^{o}C, resulted in the upregulation of the majority of the genes under study, irrespective the initial pH value. This was also the case during growth in mMRS broth with an initial pH value of 5, when the temperature increased from 20 to 37 ^{o}C. As far as strains LQC 2441, 2422, 2485 and 2516 were concerned, the relative transcription of *plαD* seemed to be more affected than that of *pln423* and *plαB*, since the relative transcription of the former was stable in fewer cases than the respective of the two latter.

The transcription of the genes under study in all strains seemed to be affected by the growth substrate, with the exception of strain LQC 2520, the relative transcription of which remained between -1 and 1 $\log_2$(FC) in 65.1% of the cases, which in the present study was not considered as regulation. More specifically, in the case of strain LQC 2520, the relative transcription of the genes *plnR*, *plnL*, *plnK*, *plnJ* and *plnG* indicated downregulation in 38.9% of the cases. On the contrary, the relative transcription of *plNC8a* remained stable in 77.8% of the cases, while the respective of *plNC8b*, *plNC8c* and *plnE* in 72.2% of the samples assessed. As far as strain LQC 2320 was concerned, upregulation was evident when the transcript levels obtained during growth at 37 ^{o}C in mMRS broth were compared with the ones in MRS broth, irrespective of the initial pH value. The most affected genes were *plnH*, the relative transcription of which indicated downregulation in 44.4% of the samples assessed, as

well as *plnR*, *plnL*, *plnK*, *plnJ*, *plnM* and *plnI*, the relative transcription of which indicated downregulation in 38.9% of the cases. On the other hand, the relative transcription of the genes *plNC8a* and *plNC8c* seemed to be the least affected, since in 66.7% of the samples assessed, their relative transcription remained between -1 and 1 $\log_2$(FC). Similar was the case for *plnE*, which remained unaffected in 61.1% of the samples assessed. Regarding both strains LQC 2320 and 2520, downregulation of the majority of the genes under study was observed during growth at 30 ºC in WFE, compared to the respective in MRS and mMRS broth, with an initial pH value of 6. In the case of bacterial strain LQC 2320, a similar transcriptomic response was observed at pH 5.0, as well. As far as strains LQC 2422, 2485 and 2516 were concerned, the relative transcription of *plαD* seemed to be less affected than that of *pln423* and *plαB*, since the relative transcription of the former was stable in more cases than the respective of the two latter. On the contrary, the opposite was evident in bacterial strain LQC 2441, in which the relative transcription of *pln423* and *plαB* was less affected than the respective of *plαD*.

Decrease in the initial pH value from 6 to 5 had a rather restricted effect on the relative gene transcription, since their $\log_2$(FC) value ranged between 1 and -1 in the majority of the cases. As far as strain LQC 2320 was concerned, *plnE*, *plnF*, *plNC8-IF* and *plNC8-HK* were completely unaffected by a decrease in the initial pH value. Regarding strain LQC 2520, the least affected genes were *plNC8a* and *plNC8c*, the relative transcription of which remained stable in 88.9% of the samples assessed. On the other hand, the relative transcription of *plnM* and *plnG* indicated upregulation in 33.3% of the cases. More specifically, upregulation of the majority of genes under study was recorded upon a decrease in the initial pH value both in mMRS broth at 37 °C and WFE at 30 ºC. Regarding strains LQC 2422, 2441, 2485 and 2516, the relative transcription of *plαD* was more affected than that of *pln423* and *plαB*. In brief, regarding strains LQC 2422, 2485 and 2516, a decrease in the initial pH value in MRS broth at 30 ºC, resulted in downregulation of *plαD*, while in strains LQC 2441 and 2516, upregulation of the respective in MRS broth at 20 ºC, was recorded.

The effect of strain was assessed by comparing the gene transcript levels between the strains included in the present study, pairwise. Regarding strains LQC 2520 and 2320, no statistically significant differences were observed in 68.2% of the samples assessed. The gene that was least affected was *plNC8b*, since the $\log_2$(FC) value ranged between

-1 and 1 in the 83.3% of the cases. Similar was the case for *plnE*, *plnF* and *plnY*, the relative transcription of which remained stable in 77.8% of the cases. Notably, strain effect during growth in MRS broth at 37 ºC, revealed significant differences in the transcription of all genes under study, irrespective of the initial pH value. Regarding the comparison between the gene transcript levels of strains LQC 2422, 2441, 2485 and 2516, significant differences were observed in *pln423*, *plαB* and *plαD*. Strain effect resulted in upregulation of both *pln423* and *plαB* in 52.8 and 50.0% of the samples assessed, respectively. As far as the relative transcription of *plαD* was concerned, downregulation was evident in 38.0% of the cases.

In Table S3, the Pearson product moment (r) correlations between plantaricin activity and relative transcription of the 18 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2320 and 2520 are presented. A total of 18 correlations were evaluated; only in 8 cases, namely, between plantaricin activity and the transcriptomic response of *plnJ, plnS plnY, plNC8-IF, plNC8-HK, plnD, plnM* and *plnI* weak positive correlation ($0.3 \leq r < 0.5$) was detected. In the rest of the cases, no statistically significant correlations were observed. On the contrary, positive correlation was observed between the transcription of the genes under study. More accurately, a total of 153 correlations were evaluated and, in all cases, positive correlation was detected. This was characterized as very strong in 102 cases ($0.8 \leq r < 1$), strong in 18 cases ($0.7 \leq r < 0.8$), moderate in 25 cases ($0.5 \leq r < 0.7$) and weak in 8 cases ($0.3 \leq r < 0.5$). In Table S4, the Pearson product moment (r) correlations between plantaricin activity and relative transcription of the three plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 are presented. Weak negative correlation was recorded between plantaricin activity and *pln423* and *plαB*, respectively. In addition, very strong correlation between the transcription of *pln423* and *plαB* was also evident.

5 Discussion

The application of bacteriocin producing LAB in food industries has attracted considerable scientific interest, over the past few years, as a clean label technology (Silva et al., 2018). However, the suitability of bacteriocinogenic LAB for more complex ecosystems, such as sourdough, has been studied only to some extent

(Messens et al., 2002, Neysens et al., 2003, Leroy et al., 2006). Bacteriocin production counteracts food contamination during processing and further contributes to the microbial stability and safety of the end products. *Lp. plantarum* has been consistently reported to prevail spontaneous sourdough fermentations and its bacteriocinogenic potential has been documented as well (Todorov et al., 2011, Parlindungan et al., 2021). However, the suitability of *Lp. plantarum* in terms of cell growth and bacteriocin production, under sourdough related conditions, has not been evaluated so far.

The WDA for plantaricin activity assessment revealed that the 6 *Lp. plantarum* strains were clustered into two groups, most likely based on the plantaricin associated genes present in their *pln* plocus. More accurately, *Lp. plantarum* strains LQC 2320 and2520, harboring 18 *pln* genes, exhibited maximum plantaricin activity, barely reaching 160 AU/mL, while maximum plantaricin activity produced by strains LQC 2422, 2441, 2485 and 2516, in which four *pln* genes were present, was quantified as 2560 AU/mL. Among the 18 genes harbored in strains LQC 2320 and 2520, the presence of *plNC8-IF*, *plNC8-HK* and *plnD*, constituting the regulatory operon *plNC8-IF-HK-D*, which induces the transcription of the other operons and its own operon as well, was evident. On the other hand, the remaining four bacterial strains harbored the genes encoding plantaricin 423, a structural protein (PlαA), a putative immunity one (PlαB) and two putative translocation proteins (PlαC, PlαD) (Van Reenen et al., 2006). Plasmid encoded plantaricin 423 belongs to the class IIa or pediocin like bacteriocins, with strong antilisterial activity.

Temperature constitutes a crucial factor in cell growth and bacteriocin production. Several authors have suggested that maximum plantaricin activity levels are recorded at temperature values below the optimum one for cell growth (Mataragas et al., 2003, Sidooski et al., 2019). However, this is not a rule, since there are cases of maximum bacteriocin activity levels recorded at temperature values close to the optimum for cell growth (Drosinos et al., 2008). In this study, temperatures applied for the preparation of the so-called type I sourdoughs, namely, 20 and 30 ^{o}C, were used as sourdough related parameters. In addition, 37 ^{o}C, corresponding to the temperature at which the highest counts of *Lp. plantarum* strains were obtained (Śliżewska et al., 2020, Parlindungan et al., 2021), was included in the present study as well. Results obtained in the present study revealed that, for strains LQC 2441, 2422, 2485 and 2516, growth

at 30 and 37 ºC, and not at 20 ºC, facilitated bacteriocin production. In the case of bacterial strains LQC 2320 and 2520, growth temperature did not play a crucial role. Consistent with our results, Parlindungan et al. (2021) reported that both 30 and 37 ºC were the optimal temperatures for bacteriocin production by *Lp. plantarum* B21. In addition, Todorov et al. (2005) reported similar bacteriocin ST26MS and ST28MS production by *Lp. plantarum* strains, both at 30 and 37 ºC.

Initial pH values have a pronounced effect on bacteriocin activity. According to the literature data, commonly recorded maximum bacteriocin activity levels correspondto initial pH values ranging from 5.5 to 6.0 (Drosinos et al., 2008). In the present study, two pH values, namely, 5.0 and 6.0, close to the pH of a wheat flour and water mixture, were included.The data obtained revealed lower levels of plantaricin activity by the four aforementioned *Lp. plantarum* strains after growth in a medium adjusted to an initial pH of 5.0, compared to that at pH 6.0. This is consistent with the previous data from Todorov (2006, 2008), according to which higher levels of bacteriocin activity were obtained in MRS broth adjusted to pH 6.0, compared to that at pH 5.0.

Regarding growth medium, there is a consensus that it constitutes one of the most significant factors in bacteriocin production. In the present study, three substrates, namely, MRS broth, mMRS broth and WFE, were included. Decreased population levels and plantaricin activity were recorded after the growth of the 6 bacterial strains in WFE, compared to the respective in MRS and mMRS broth. This can be assigned to the presence of specific nutrients in MRS and mMRS broth, which are missing from WFE. More specifically, yeast extract that serves as a nitrogen source for cell growth with an abundance of different amino acids, elements such as magnesium and phosphorus and even Tween 80 have been reported to positively interfere with bacteriocin production in a strain dependent way (Todorov et al., 2005). In agreement with our results, Paramithiotis et al. (2019) reported a decreased plantaricin activity during radish fermentation, compared to growth in MRS broth. In addition, Todorov et al. (2006) reported decreased activity levels as well, after the growth of bacteriocin producing *Lp. plantarum* strains in BHI broth, M17 broth, 10%, w/v soy flour or 10%, w/v molasses. The aforementioned observation was probably attributed to the absence of specific nutrients from the medium. As far as the effect of sugars on bacteriocin activity was concerned, Leroy et al. (2006) reported that supplementing a sourdough

simulation medium with a combination of three (6.67 g/L of glucose, fructose, maltose, respectively) or four sugars (5 g/L of glucose, 5 g/L of fructose, 10 g/L of maltose, 2.5 g/L of sucrose) resulted in higher bacteriocin activity levels by *Lactobacillus amylovorus* DCE 471, compared to single energy sources. However, a direct comparison with MRS broth would be interesting. Other studies by Todorov et al. (2006, 2008) revealed that carbohydrates, applied as sole energy sources, affect bacteriocin production, in a concentration and strain dependent way. More accurately, in some cases, the fortification of MRS broth with glucose, sucrose or maltose at 20 g/L yielded higher bacteriocin activity by *Lp. plantarum* strains, compared to the control, while in other cases supplementation of the medium with 20 or 50 g/L did not improve bacteriocin activity. However lower concentrations of glucose, such as 5 or 10 g/L, resulted in decreased bacteriocin activity, probably due to rapid consumption of the carbon source. Similar was the case when bacterial strains grew in the presence of 20 g/L of fructose.

Medium components, such as NaCl, constitute stress factors that, at specific concentrations, could be beneficial for bacteriocin production (de Vuyst et al., 1996). NaCl has a significant role in dough preparation, since it affects the sensory characteristics of the dough and strengthens its structure as well. In the present study, two NaCl concentrations were applied, namely, 0.0 and 1.8%, with the latter content retrieved from traditional bread recipes of Greece. In the majority of the cases, the different NaCl concentrations had no statistically significant effect on neither bacteriocin activity nor the growth of the 6 *Lp. plantarum* strains. Regarding the former, the effect seems to be, at least, species dependent. Indeed, the enhancement of lac- ticin 481 production by *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30 (Uguen et al., 1999) and amylovorin L471 by *Lb. amylovorus* DCE 471 (Neysens et al., 2003) were reported with the addition of 0.2 M and 10 g L−1 NaCl, respectively, in the growth medium. Similarly, Leal-Sanchez et al. (2002) reported that the best conditions for plantaricin S production by *Lp. plantarum* LPCO10 included the addition of 2.3–2.5% NaCl. On the contrary, the addition of 1% NaCl negatively affected the production of sakacin K by *Lt. sakei* CTC94 (Leroy et al., 1999) and the addition of 2% NaCl negatively affected the production of carnobacteriocin B2 by *Carnobacterium piscicola* A9b (Himelbloom et al., 2001) and curvacin A by *Lt. curvatus* L471 (Verluyten et al., 2004). Consistent with our results, Drosinos et al. (2005) reported similar maximum bacteriocin titres by

Leu. mesenteroides E131 both at 0.0 and 2.5% NaCl. The exact mechanism by which NaCl affects bacteriocin production is yet to be fully elucidated; however, in some cases, interaction with induction factors has been exhibited (Nilsen et al., 1998, Messens et al., 2002, Nilsson et al., 2002). Similarly, the effect of NaCl on bacterial growth can also be species or strain dependent (Leroy et al., 1999, Uguen et al., 1999, Himelbloom et al., 2001, Leal-Sanchez et al., 2002, Verluyten et al., 2004). Notably, bacterial growth is not always correlated with bacteriocin activity (Todorov et al., 2016, Yang et al., 2018).

Regarding the relative transcription of the 18 *pln* genes under sourdough simulated conditions, temperature and growth substrate had a pronounced effect on the transcript levels of genes, harbored in bacterial strains LQC 2520 and 2320, respectively. In the case of bacterial strains LQC 2422, 2441, 2485 and 2516, substrate and strain were found to significantly affect the relative transcription of the three *pln* genes. In a previous study, Paramithiotis et al. (2019) reported that the *pln* gene transcription was not affected by the experimental conditions, namely, radish fermentation vs. growth in MRS broth. Earlier studies by Hurtado et al. (2011) and Doulgeraki et al. (2013) assessed the effect of different NaCl concentrations on the expression of *pln* genes, a parameter which was excluded from RT-qPCR analysis in our study, since only a marginal proportion of the variance observed could be attributed to NaCl addition.

In our study, *plnE, plNC8-IF, plNC8-HK, plnD* were some of the least affected genes for the majority of treatments applied and were further characterized by a very strong correlation between their transcriptomic responses. The fact that *plNC8-IF*, *plNC8-HK* and *plnD* encode an induction peptide, a histidine protein kinase and a response regulator, respectively, of the regulatory operon, probably justifies their similar transcriptomic profiles. On the other hand, *plnG* harbored in bacterial strains LQC 2320 and 2520 and *plαD* found in strains LQC 2422, 2441, 2485 and 2516, were the most affected genes by culture conditions. Strong and very strong correlations were recorded between the transcriptomic response of genes corresponding to the same operons, namely, bacteriocin operons (*plnEFI*, *plnRLKJ*, *plNC8αβc*), truncated transport operon, (*plnGHS*), followed by *plnY*. However, in the gene cluster *pln423*, *plαB* and *plαD*, only the correlation between the transcriptomic responses of the two former genes was very strong. As far as the correlation between plantaricin activity and

gene transcription was concerned, a weak correlation was evident only in some cases. This observation is consistent with Hurtado et al. (2011) and Paramithiotis et al. (2019), which attributed the inability to correlate bacteriocin activity with gene expression, to post-transcriptional, post-translational modifications and physicochemical parameters interfering in the WDA, as well. Although an obvious correlation between WDA and relative gene transcription was not established, the status of unregulated *plNC8-IF*, *plNC8-HK* and *plnD* (constituting the regulatory operon) upon temperature increase and pH decrease, was in agreement with the temperature and initial pH independent plantaricin activity exhibited by both strains LQC 2320 and 2520. Similar was the case for the downregulation of *pln* genes after growth of bacterial strain LQC 2320 in WFE at 30 and 37 ^{o}C at pH 6.0 was consistent with the phenotypically determined decreased plantaricin activity of the strain, after growth in WFE.

Summarizing the results of the present study, there are two major findings that need to be taken into consideration in future studies. The first refers to the specificity of the bacteriocins produced. It seems that the specificity of Pln423 against *L. monocytogenes* played a decisive role in the increased antilisterial activity of the cell free supernatants of the strains that carry the *pln423* gene. Thus, this gene may serve as a target in genetic screening approaches. In addition, antimicrobial activity in general may result from a mixture of bacteriocins, with possibly variable levels of specificity to the indicator microorganism used. In the present study, the physical clustering of the *pln* genes into a single cluster is probably the reason for their comparable transcriptomic response, which was observed in many cases, to the environmental stimuli examined. However, there were also cases in which differences in the transcriptomic responses were observed. These cases probably arise from differences in the transcriptional organization between the different loci, which needs to be further assessed. The second finding refers to the lack of correlation between gene transcription and plantaricin activity. This may be attributed to post-transcriptional or post-translational modifications, to the compromised stability of mRNA and the resulting proteins (Picard et al., 2009) as a result of the environmental stimuli examined, or to physicochemical parameters interfering with WDA. Such effects need to be fully elucidated, especially in food systems, in order to enable optimization of bacteriocin activity.

6 Conclusions

Plantaricin activity is a strain dependent property; the 6 strains included in the present study were differentiated into two groups on the basis of their phenotype and this was supported by the organization of their *pln* locus. Growth substrate, temperature, initial pH value and bacterial strains were found to have a significant effect on plantaricin activity; on the contrary, NaCl had only a marginal effect. Similarly, growth substrate and temperature seemed to have a more pronounced effect than initial pH value, on the transcription of the genes under study, always in a strain dependent manner. Only weak correlation was observed between plantaricin activity and the transcription of some of the genes assessed. However, a strong correlation between the transcription of the genes belonging to the same locus was observed.

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Chapter 8

Conclusions and future perspectives

The microecosystem composition of 13 spontaneously fermented wheat based sourdoughs originating from regions of Greece, not previously investigated, was unraveled. Thus, our knowledge on the associated microbiota was expanded. The fluctuation observed in the physicochemical parameters of sourdough samples, namely pH and TTA values, was associated with the respective micro-community and the dominant populations. Results obtained from the culture dependent assessment, revealed that *Lp. plantarum*, *Lv. brevis* and *S. cerevisiae* formed the primary bacterial and yeast biota, respectively, in the majority of sourdough samples. As far as the combined application of both culture dependent and culture independent approaches was concerned, RNA and DNA based PCR-DGGE analysis could only partially verify the results obtained with the classical method, thus the yield of complementary information was not successful. To resolve this, the use of different culture independent methods, among which shotgun metagenomic sequencing, would be an interesting alternative, providing with both qualitative and quantitative data on microbial diversity of sourdough samples. Thereby, the combined use of culture dependent methods with a different set of culture independent techniques, including NGS-based ones, would reflect the actual taxonomic structure of the sourdough microbiota in both confirmative and complementary manner.

The technological and safety potential of the sourdough retrieved LAB and yeasts were further demonstrated. Three *W. anomalus* strains, exhibited both proteolytic and antimold potential. In addition, 6 *Lp. plantarum* strains were characterized by antibacterial activity against a mixture of *L. monocytogenes* strains and other sourdough isolates, as well, due to the production of proteinaceous metabolites. Thus, this study constituted the basis for further research on the aforementioned properties, due to the impact they may exert on the quality of sourdough related products. Given that in a further study (Chapter 6; Comparative genomic and safety assessment of *Lp. plantarum* strains) the presence of the complete set of genes for folate and riboflavin biosynthesis was detected, the experimental phenotypic assessment of B-group vitamin synthesis by sourdough isolates would be interesting to be conducted.

Further antimold activity assessment of the three *W. anomalus* strains studied both *in vitro* and *in situ* was assigned to a series of VOCs, some of which were common among the three yeasts. Concerning the suitability of *W. anomalus* strains to be used as starter or adjunct cultures in bread making process, the antimold activity of yeast strains was

retained even in co-culture with baker's yeast, leading to a shelf life prolongation up to 30 days. Further *in situ* studies are required so as to evaluate the impact of the aforementioned strains on the sensory properties of the yeasted breads. In addition, it would be of great interest to assess the co-presence of *W. anomalus* strains with selected LAB for further application in sourdough breads. The combination of biocontrol yeasts with acidifying LAB would generate microbiologically stable products, meeting consumer's demand for less chemical preservatives.

In a subsequent study, whole genome sequencing of 6 sourdough derived *Lp. plantarum* strains was performed, due to their technological and safety attributes and further taxonomic classification revealed their assignment to the subspecies *argentoratensis*. Considering the few available genomes corresponding to *Lp. plantarum* subsp. *argentoratensis* strain, this study provides with data that could be used for further bioinformatic analysis by third parties interested in sourdough fermentation. Then, a comparative genomic and safety assessment of the aforementioned 6 bacterial strains was performed, given that there has been no previous study on the bioinformatic analysis of genomes belonging to sourdough derived *Lp. plantarum* subsp. *argentoratensis* strain, thus being of utmost importance the provision of a reference report for any further validation experiment. Results obtained, demonstrated the technological potential of the under study bacterial strains. Regarding their safety assessment, no virulence factors were detected, while the presence of genes associated with antibiotic resistance was reported. In the case of biogenic amine evaluation, the detection of *cadA* encoding cadaverine production was recorded, however the safety concern could be counterbalanced by the presence of proteins with degrading potential found in their genomes. In addition, phenotypic determination of cadaverine production should be performed, so as to evaluate the aforementioned safety concern. CRISPR and cas genes were detected, while MGEs were present, as well. Finally, the presence of a variable *pln* locus in the genomes of the 6 *Lp. plantarum* subsp. *argentoratensis* strains was demonstrated, highlighting their ability to produce plantaricins.

Finally, the effect of sourdough related parameters on the plantaricin activity and the relative transcription of the 6 aforementioned *Lp. plantarum* strains was assessed. Although, *Lp. plantarum* has been extensively reported to dominate in a wide range of food fermentations, due to its metabolic capacity and adaptability, no previous study

has evaluated the plantaricin activity at conditions associated with sourdough preparation. In addition, the application of a wheat flour and water extract as a sourdough simulation growth medium for plantaricin activity assessment purposes, has not been previously assessed. Regarding the relative transcription of *pln* genes, scarce is the literature concerning their transcriptomic response under actual sourdough fermentation conditions. Thus, in this study, the effect of a set of genes encoding plantaricins to the overall plantaricin activity of *Lp. plantarum* strains was investigated. Results demonstrated that the 6 bacterial strains were differentiated in two distinct groups based on the phenotypic tests and the genes harbored in their *pln* locus. Growth substrate, temperature, initial pH value and bacterial strains significantly affected plantaricin activity. However, it should be kept in mind that decreased activities and populations were exhibited after growth in wheat flour and water extract, which is probably attributed to the absence of specific nutrients from the medium. Regarding the NaCl contribution, analysis of variance revealed that only a marginal proportion of plantaricin activity could be explained by NaCl and thus, the latter was excluded from RT-qPCR analysis. Similar with phenotypic assessment, temperature and growth substrate exerted a more pronounced effect than initial pH value on the relative gene transcription. A strong correlation was evident among genes assigned to the same locus. However, in the case of plantaricin activity and relative gene transcription, only weak correlation was recorded. A further research on the additional molecular mechanisms regarding port-transcriptional and/or post-translational modifications affecting the phenotype would improve our understanding towards overall plantaricin activity.

Appendix

Supplementary material

Table S2.1. Biochemical tests used for the identification of yeast isolates.

Phenotypic groups	No. of isolates	Carbohydrate fermentation					Nitrogen source assimilation						
		gal	glu	lac	mal	suc	cad	ctn	eth	imi	lys	nta	nti
1	2	2[1]	2	0	0	0	0	0	0	nd	0	0	0
2	8	0	8	0	0	0	nd	8	nd	8	8	0	nd
3	18	0	18	0	0	0	18	nd	nd	nd	18	0	nd
4	151	120	151	0	151	151	0	145	0	151	0	0	0
5	6	8	8	0	8	8	nd	8	nd	8	8	8	nd

cad.: cadaverine; ctn.: creatine; eth.: ethylamine; gal.: D-galactose; glu.: D-glucose; imi.: imidazole; lac.: lactose; lys.: L-lysine; mal.: maltose; nta.: nitrate; nti.: nitrite; suc.: sucrose.

nd: not determined

[1]number of positive strains

Table S2.2. Biochemical tests used for the identification of yeast isolates.

Phenotypic groups	No. of isolates	Carbon source assimilation															
		ara	cel	cit	eth	gal	glu	lac	mal	man	mel	raf	rha	rib	suc	tre	xyl
1	2	0[1]	0	0	0	2	2	0	0	0	0	0	0	0	0	2	0
2	8	0	0	8	8	0	8	0	0	0	0	0	0	0	0	0	8
3	18	0	0	0	16	0	18	0	0	0	0	0	0	0	0	0	0
4	151	0	0	0	151	120	151	0	151	0	0	151	0	0	151	151	0
5	6	6	6	6	6	6	6	0	6	6	0	6	0	6	6	6	0

ara.: L-arabinose; cel.: cellobiose; cit.: citrate; eth.: ethanol; gal.: D-galactose; glu.: D-glucose; lac.: lactose; mal.: maltose; man.: D-mannitol; mel.: melibiose; raf.: raffinose; rha.: L-rhamnose; rib.: D-ribose; suc.: sucrose; tre.: a,a trehalose; xyl.: D-xylose

[1]number of positive strains

Table S2.3. Biochemical tests used for the identification of yeast isolates.

Phenotypic groups	No. of isolates	Ability to grow							Acetic acid production	Starch formation	Urea hydrolysis
		35 ºC	37 ºC	40 ºC	50% glu	60% glu	1% acetic acid	0.01% cycloheximide			
1	2	0[1]	0	0	0	0	0	2	0	0	0
2	8	8	8	8	8	8	0	0	0	0	0
3	18	18	0	0	0	0	0	0	0	0	0
4	151	151	150	132	151	0	0	0	0	0	0
5	6	6	6	6	6	6	0	0	0	0	0

glu.: glucose

[1]number of positive strains

Table S2.4. Biochemical tests used for the identification of lactic acid bacteria isolates.

Phenotypic groups	No. of isolates	morphology	CO_2 production	Ability to grow		Acid production from											
				15ºC	45ºC	cel	gal	lac	mal	man	mel	raf	rib	sor	suc	tre	xyl
1	70	bacilli	w	70	0	70[1]	nd	nd	nd	68	70	65	70	70	69	nd	0
2	71	bacilli	71	71	0	0	71	nd	71	nd	71	71	70	nd	71	0	0
3	30	bacilli	0	30	30	28	nd	nd	nd	0	0	0	27	0	30	nd	0
4	1	bacilli	1	1	0	0	1	nd	1	nd	0	0	1	nd	0	0	0
5	6	bacilli	0	6	0	6	nd	nd	nd	0	0	0	5	0	6	nd	0
6	12	bacilli	0	6	0	0	nd	nd	nd	0	10	0	12	0	12	nd	0
7	1	cocci	1	1	0	nd	0	0	1	1	1	0	0	nd	1	1	0
8	1	cocci	1	1	0	nd	1	0	1	1	1	1	1	nd	1	1	0
9	3	cocci	0	3	0	nd	3	3	3	nd	3	0	3	nd	nd	nd	nd
10	12	bacilli	12	12	0	0	12	nd	12	nd	0	0	12	nd	12	0	0

cel.: cellobiose; gal.: D-galactose; lac.: lactose; mal.: maltose; man.: D-mannitol; mel.: melibiose; raf.: raffinose; rib.: D-ribose; sor.: sorbitol; suc.: sucrose; tre.: a,a trehalose; xyl.: D-xylose

all isolates were able to produce acid from D-glucose;nd.: not determined; w.: weak;

[1]number of positive strains

Table S4.1: Antimold activity (AU/ mL) of three *W. anomalus* strains, recorded after a 24h growth at 30 ºC, in modified BHI broth at six different pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) and six NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%).

Strain	**Antimold activity (AU/ mL)**					
LQC 10346	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 3.5	640 (0.00) $^{a, A}$	960 (452.55) $^{a, A, B}$	640 (0.00) $^{a, A}$	1920 (905.10) $^{b, c, B}$	640 (0.00) $^{a, A}$	960 (452.55) $^{a, A, B}$
pH 4.0	960 (452.55) $^{a, b, A, B}$	1280 (0.00) $^{a, B}$	640 (0.00) $^{a, A}$	640 (0.00) $^{a, A}$	960 (452.55) $^{a, b, A, B}$	640 (0.00) $^{a, A}$
pH 4.5	640 (0.00) $^{a, A, B}$	1280 (0.00) $^{a, C}$	960 (452.55) $^{a, b, B, C}$	320 (0.00) $^{a, A}$	1280 (0.00) $^{a, b, C}$	640 (0.00) $^{a, A, B}$
pH 5.0	960 (452.55) $^{a, b, A}$	960 (452.55) $^{a, A}$	960 (452.55) $^{a, b, A}$	2560 (0.00) $^{c, B}$	1280 (0.00) $^{a, b, A}$	2560 (0.00) $^{b, B}$
pH 5.5	1280 (0.00) $^{b, A}$	1280 (0.00) $^{a, A}$	1280 (0.00) $^{b, A}$	960 (452.55) $^{a, b, A}$	1920 (905.10) $^{b, A, B}$	2560 (0.00) $^{b, B}$
pH 6.0	5120 (0.00) $^{c, C}$	1920 (905.10) $^{a, B}$	5120 (0.00) $^{c, C}$	960 (452.55) $^{a, b, A, B}$	640 (0.00) $^{a, A}$	640 (0.00) $^{a, A}$
LQC 10353	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 3.5	640 (0.00) $^{a, A}$	1920 (905.10) $^{a, B}$	1280 (0.00) $^{a, b, A, B}$	640 (0.00) $^{a, A}$	1280 (0.00) $^{b, A, B}$	1280 (0.00) $^{a, A, B}$
pH 4.0	1280 (0.00) $^{a, A}$	1280 (0.00) $^{a, A}$	1920 (905.10) $^{b, A, B}$	5120 (0.00) $^{c, C}$	2560 (0.00) $^{c, B}$	960 (452.55) $^{a, A}$
pH 4.5	960 (452.55) $^{a, A}$	5120 (0.00) $^{b, B}$	1280 (0.00) $^{a, b, A}$	5120 (0.00) $^{c, B}$	960 (452.55) $^{b, A}$	1280 (0.00) $^{a, A}$
pH 5.0	960 (452.55) $^{a, A}$	1280 (0.00) $^{a, A}$	640 (0.00) $^{a, A}$	960 (452.55) $^{a, b, A}$	1280 (0.00) $^{b, A}$	3840 (1810.19) $^{b, B}$
pH 5.5	960 (452.55) $^{a, A}$	1280 (0.00) $^{a, A}$	960 (452.55) $^{a, b, A}$	1280 (0.00) $^{b, A}$	960 (452.55) $^{b, A}$	640 (0.00) $^{a, A}$
pH 6.0	1280 (0.00) $^{a, C}$	960 (452.55) $^{a, B, C}$	640 (0.00) $^{a, A, B}$	480 (226.27) $^{a, A, B}$	320 (0.00) $^{a, A}$	1280 (0.00) $^{a, C}$
LQC 10360	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 3.5	640 (0.00) $^{a, A}$	480 (226.27) $^{a, A}$	1920 (905.10) $^{a, b, c, B}$	480 (226.27) $^{b, A}$	160 (0.00) $^{a, A}$	160 (0.00) $^{a, A}$
pH 4.0	160 (0.00) $^{a, A}$	320 (0.00) $^{a, A}$	240 (113.14) $^{a, A}$	160 (0.00) $^{a, A}$	960 (452.55) $^{a, B}$	1280 (0.00) $^{b, B}$
pH 4.5	2560 (0.00) $^{b, c, A}$	2560 (0.00) $^{b, A}$	2560 (0.00) $^{b, c, A}$	2560 (0.00) $^{d, A}$	3840 (1810.19) $^{b, A, B}$	5120 (0.00) $^{c, B}$
pH 5.0	5120 (0.00) $^{d, B}$	1920 (905.10) $^{b, A}$	1280 (0.00) $^{a, b, A}$	1280 (0.00) $^{c, A}$	1280 (0.00) $^{a, A}$	1280 (0.00) $^{b, A}$
pH 5.5	3840 (1810.19) $^{c, d, B}$	2560 (0.00) $^{b, A, B}$	3840 (1810.19) $^{c, d, B}$	1280 (0.00) $^{c, A}$	1280 (0.00) $^{a, A}$	1280 (0.00) $^{b, A}$
pH 6.0	1280 (0.00) $^{a, b, C}$	320 (0.00) $^{a, B}$	5120 (0.00) $^{d, D}$	240 (113.14) $^{a, b, A, B}$	240 (113.14) $^{a, A, B}$	160 (0.00) $^{a, A}$

Statistically significant differences in the antimold activity of yeast strains, exerted by the effect of pH values and NaCl concentrations, are indicated with different letters a-d and A-D, respectively ($p < 0.05$). The method of 95% LSD was applied as post-hoc comparison test.

Table 4.2: Antimold activity (AU/ mL) of three *W. anomalus* strains, recorded after a 24h growth at 30 ºC, in liquid dough at six NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5%).

Strain	**Antimold activity (AU/ mL)**					
LQC 10346	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 6.0	1280 (0.00) ^{A}	960 (452.55) ^{A}	5120 (0.00) ^{C}	2560 (0.00) ^{B}	2560 (0.00) ^{B}	2560 (0.00) ^{B}
LQC 10353	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 6.0	1920 (905.10) $^{A, B}$	3840 (1810.19) $^{B, C}$	5120 (0.00) ^{C}	1920 (905.10) $^{A, B}$	1280 (0.00) ^{A}	1920 (905.10) $^{A, B}$
LQC 10360	**NaCl 0.0%**	**NaCl 0.5%**	**NaCl 1.0%**	**NaCl 1.5%**	**NaCl 2.0%**	**NaCl 2.5%**
pH 6.0	960 (452.55) ^{A}	1280 (0.00) ^{A}	5120 (0.00) ^{C}	1920 (905.10) $^{A, B}$	1280 (0.00) ^{A}	3840 (1810.19) $^{B, C}$

Statistically significant differences in the antimold activity of yeast strains, exerted by the effect of NaCl concentrations, are indicated with different letters A-C ($p < 0.05$). The method of 95% LSD was applied as post-hoc comparison test.

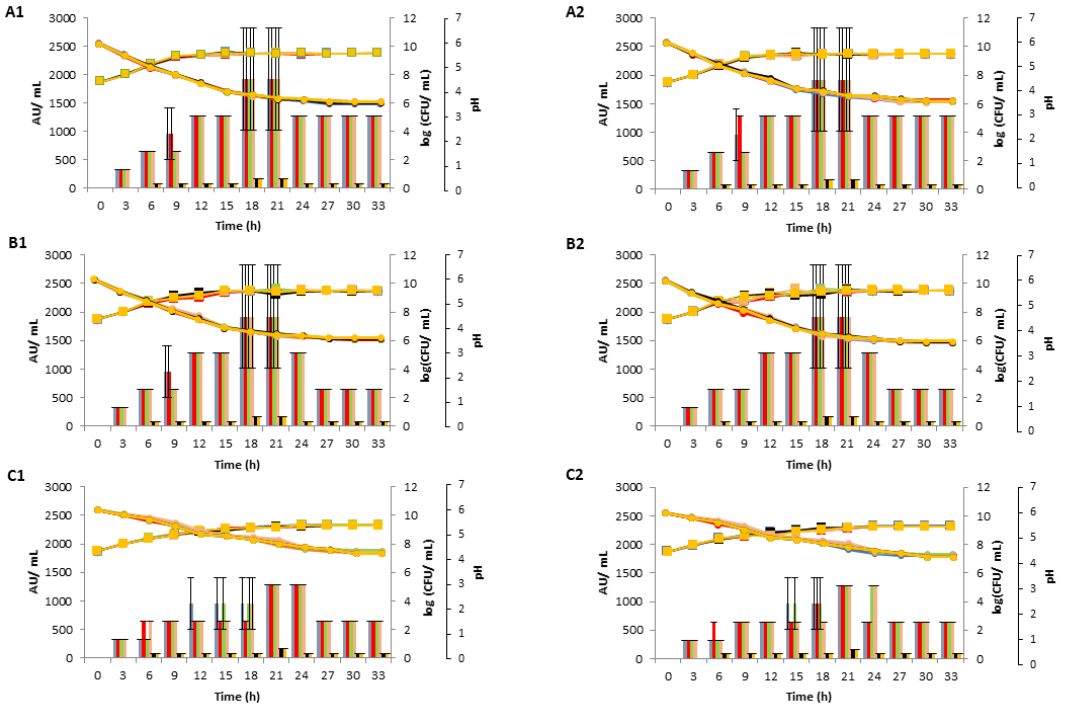

Figure S7.1. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in MRS broth adjusted to initial pH 6.0, at 30 (A), 37 (B) and 20 ºC (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

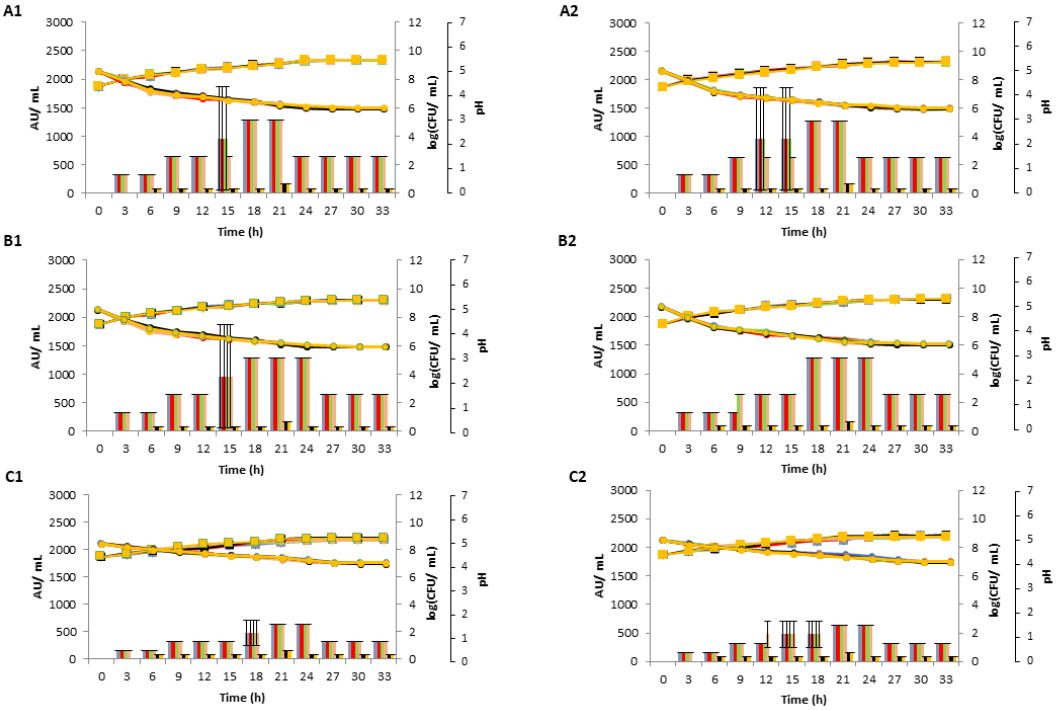

Figure S7.2. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in MRS broth adjusted to initial pH 5.0, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

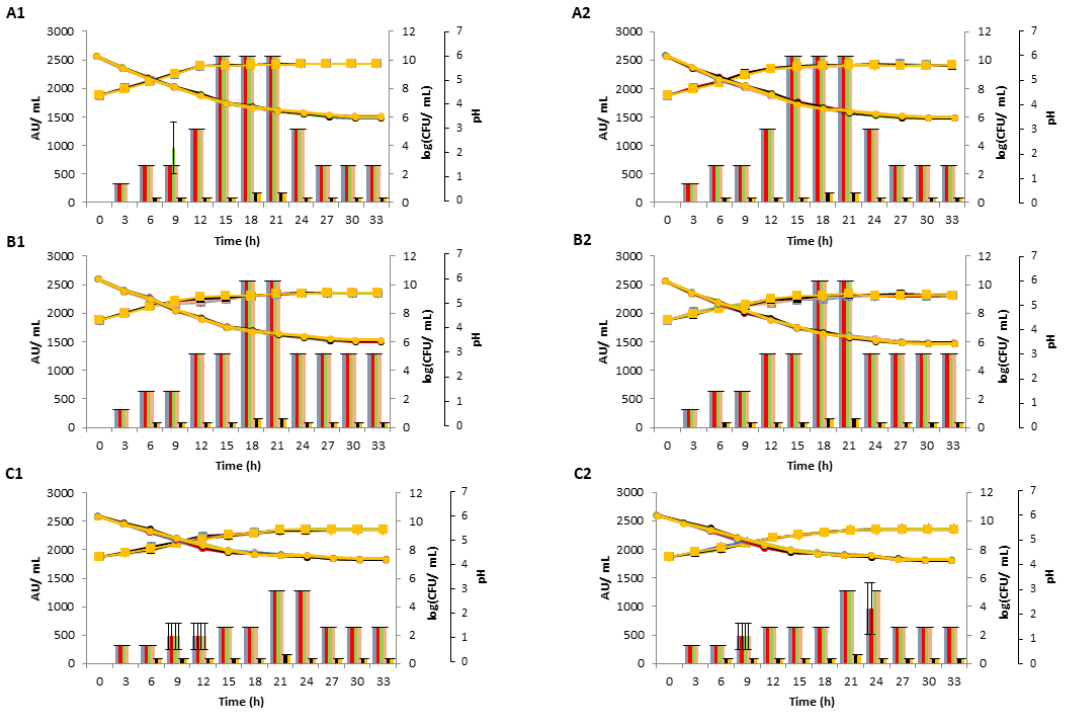

Figure S7.3. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in mMRS broth adjusted to initial pH 6.0, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

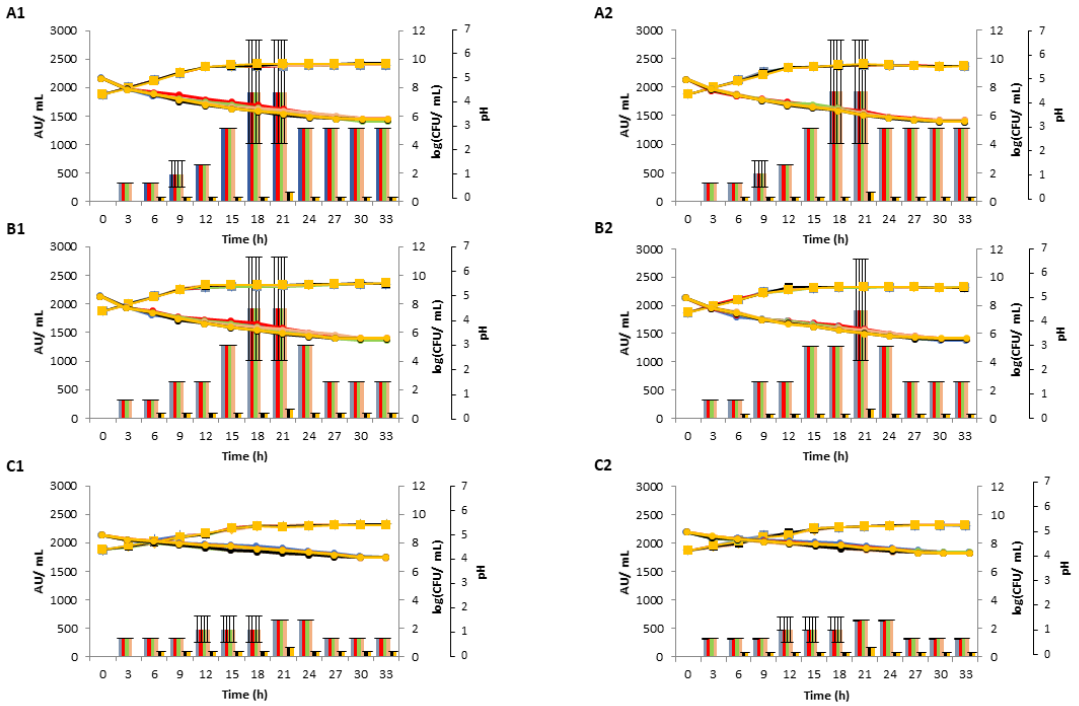

Figure S7.4. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in mMRS broth adjusted to initial pH 5.0, at 30 (A), 37 (B) and 20 ºC (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

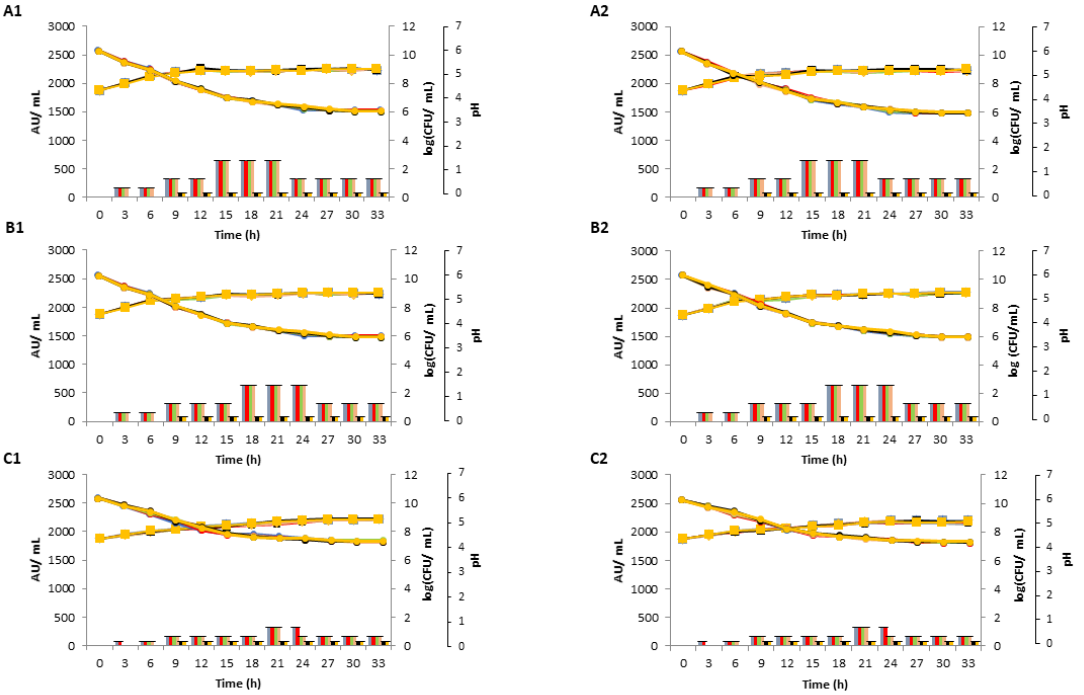

Figure S7.5. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in WFE broth adjusted to initial pH 6.0, at 30 (A), 37 (B) and 20 ºC (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

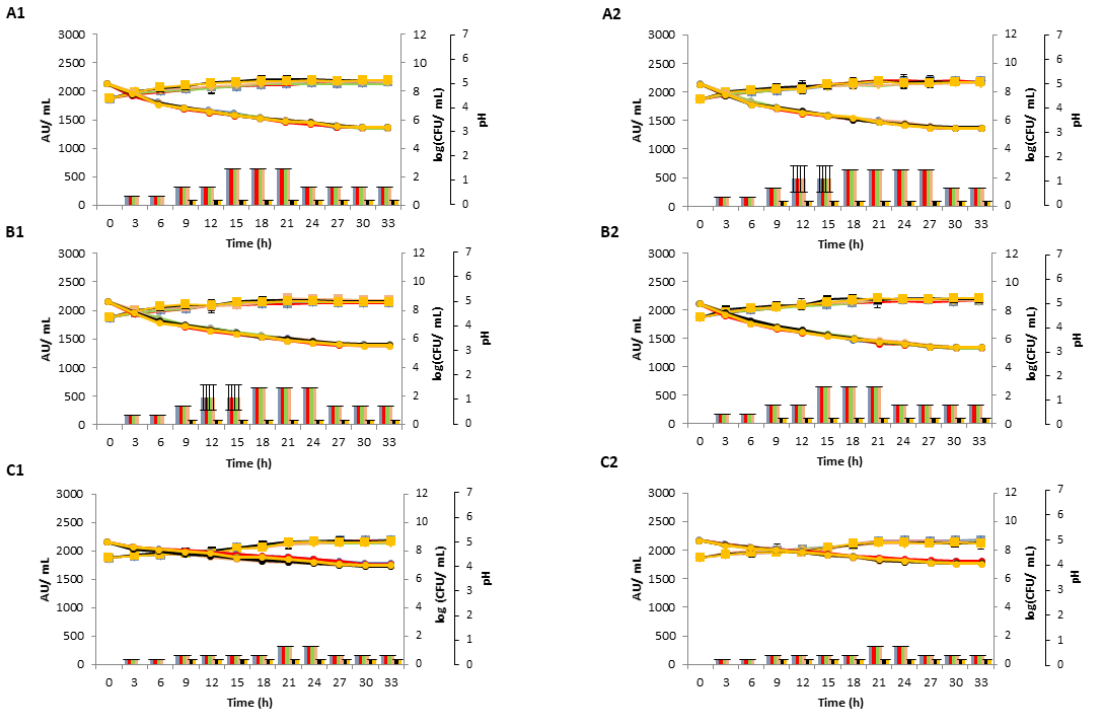

Figure S7.6. Growth expressed in log (CFU/ mL) (■), pH reduction (●) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in WFE broth adjusted to initial pH 5, at 30 (A), 37 (B) and 20 ºC (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8 %) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively.

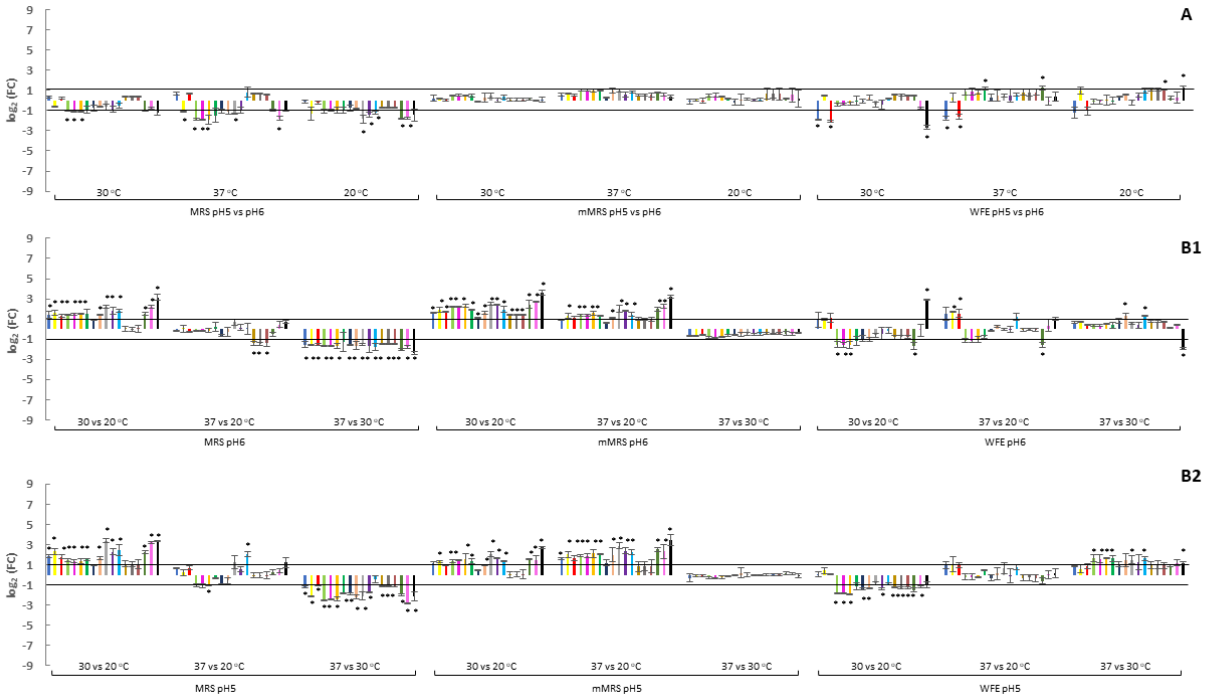

Figure S7.7. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B1) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of the aforementioned genes, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at initial pH 6.0, for 21 h. The lowest temperature was used as control. (B2) Effect of temperature increase on the relative gene transcription, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at initial pH 5.0, for 21 h. The lowest temperature was used as control. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

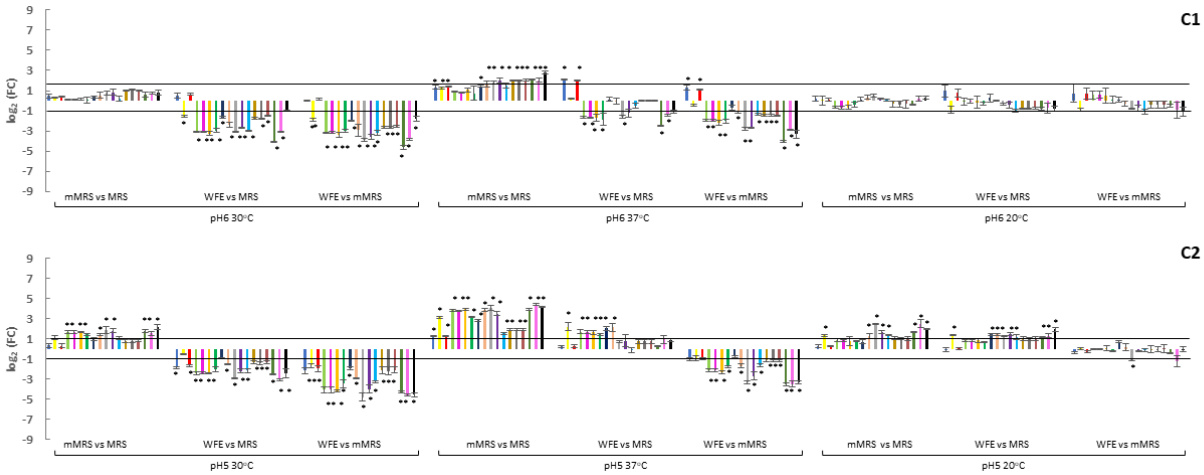

Figure S7.8. (C1) Effect of substrate on the relative transcription of *plNC8a, plNC8b, plNC8c, plnL, plnR, plnK, plnJ, plnE, plnF, plnH, plnS, plnY, plNC8-IF, plNC8-HK, plnD, plnI, plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE at initial pH 6.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. (C2) Effect of substrate on the relative transcription of the plantaricin genes at the aforementioned conditions, at initial pH 5.0. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a, plNC8b, plNC8c, plnL, plnR, plnK, plnJ, plnE, plnF, plnH, plnS, plnY, plNC8-IF, plNC8-HK, plnD, plnI, plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

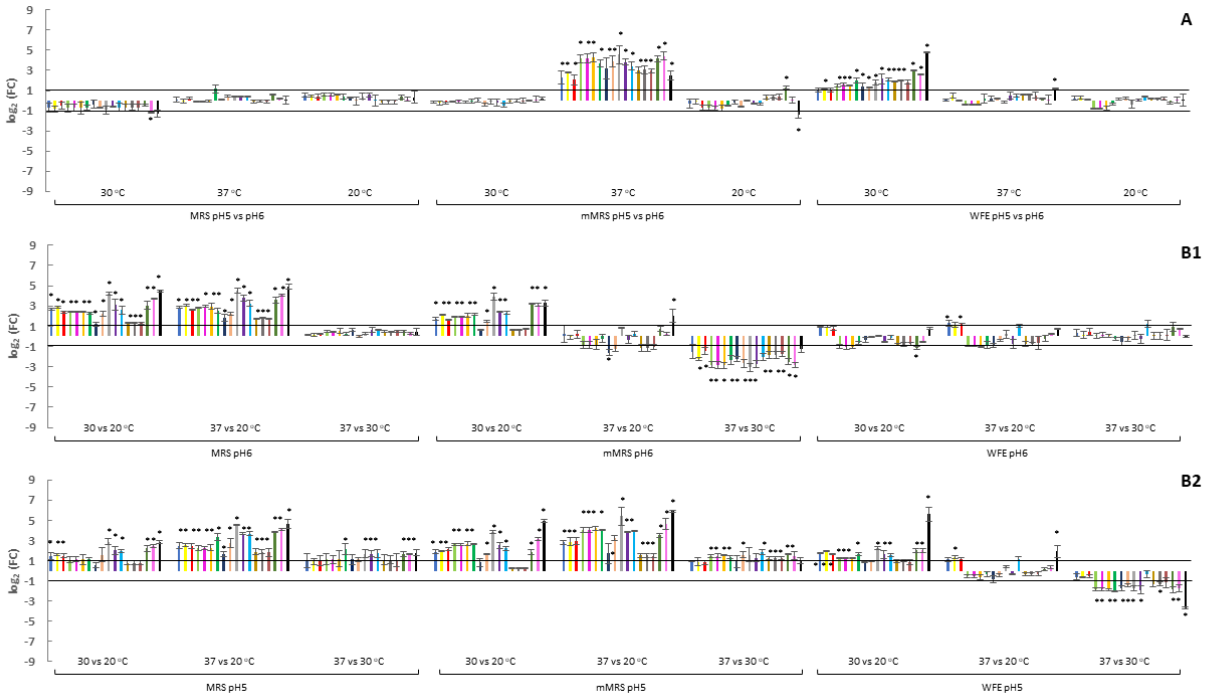

Figure S7.9. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B1) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of the aforementioned genes, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at initial pH 6.0, for 21 h. The lowest temperature was used as control. (B2) Effect of temperature increase on the relative gene transcription, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at initial pH 5.0, for 21 h. The lowest temperature was used as control. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

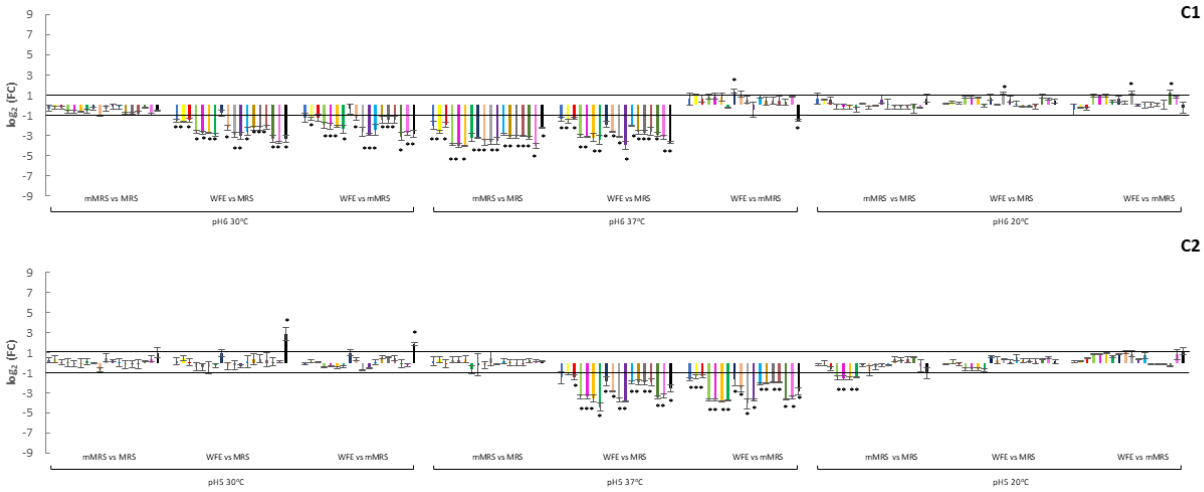

Figure S7.10. (C1) Effect of substrate on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE at initial pH 6.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. (C2) Effect of substrate on the relative transcription of the plantaricin genes at the aforementioned conditions, at initial pH 5.0. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

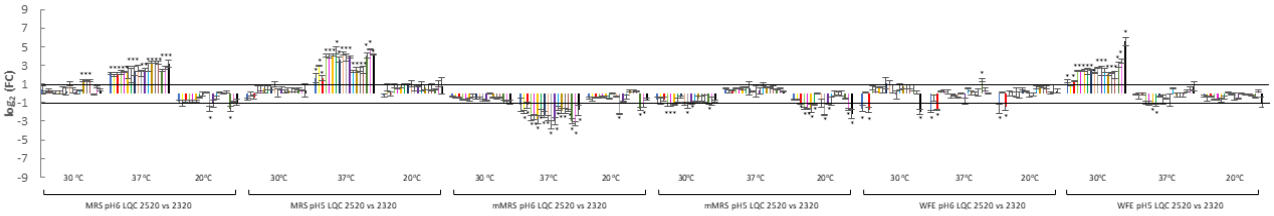

Figure S7.11. Effect of strain on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, was obtained by comparing the transcription of the aforementioned genes after growth of *Lp. plantarum* strains LQC 2320 and 2520 in MRS, mMRS broth and WFE, both at pH 5.0 and pH 6.0, at 30, 37 and 20 ºC, for 21 h, using LQC 2320 as control. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

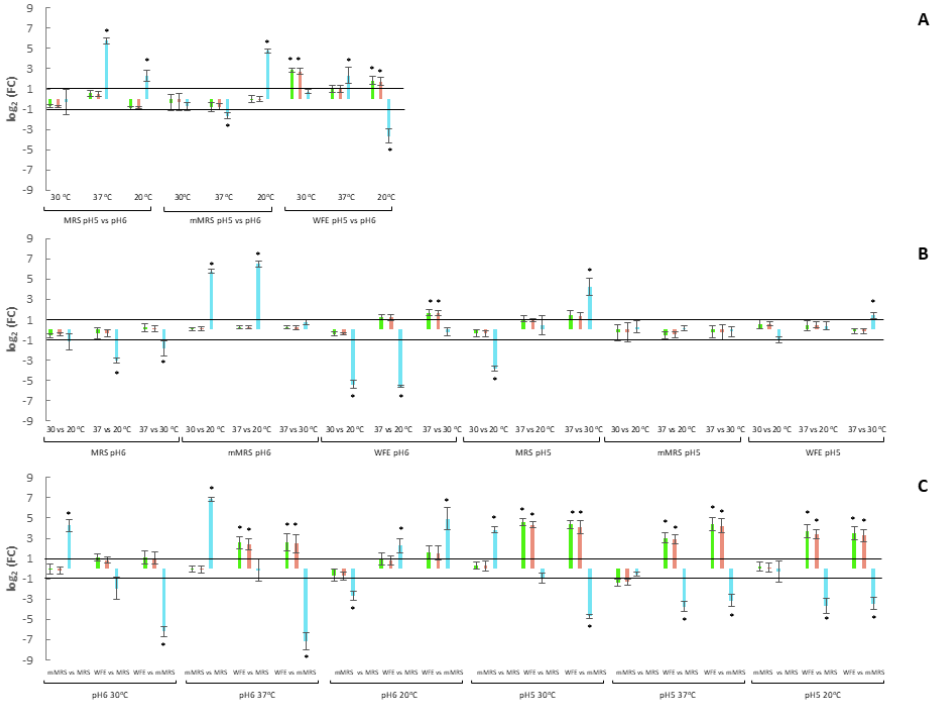

Figure S7.12. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2441 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2441 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2441 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA)*, *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

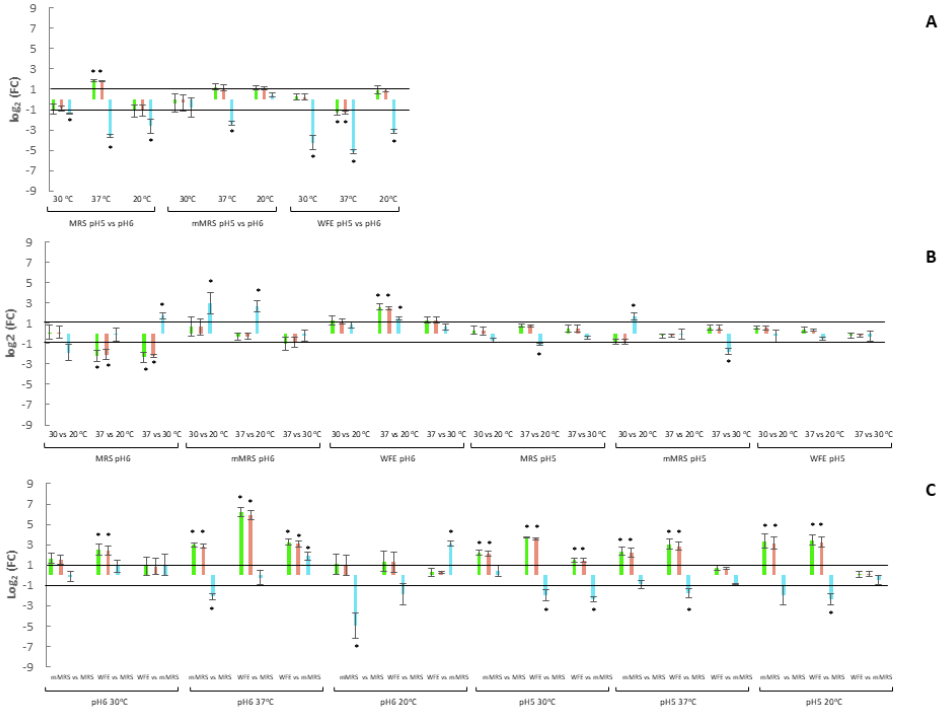

Figure S7.13. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2422 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2422 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2422 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA)*, *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

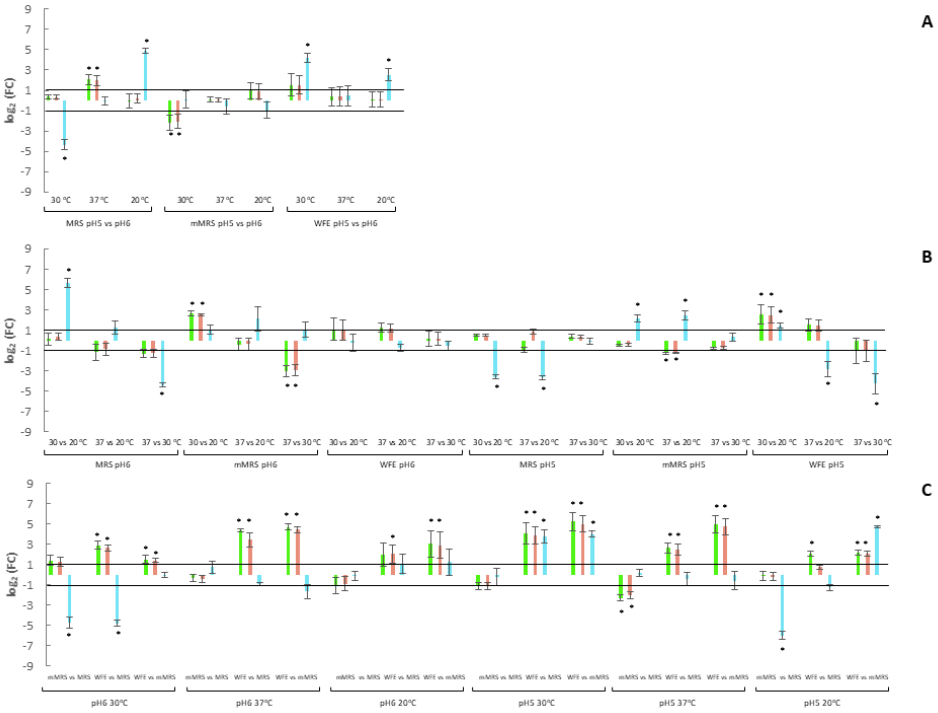

Figure S7.14. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423 (plαA), plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of *pln423 (plαA), plαB* and *plαD*, during growth of strain LQC 2516 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423 (plαA), plαB* and *plαD*, during growth of strain LQC 2516 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA), plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

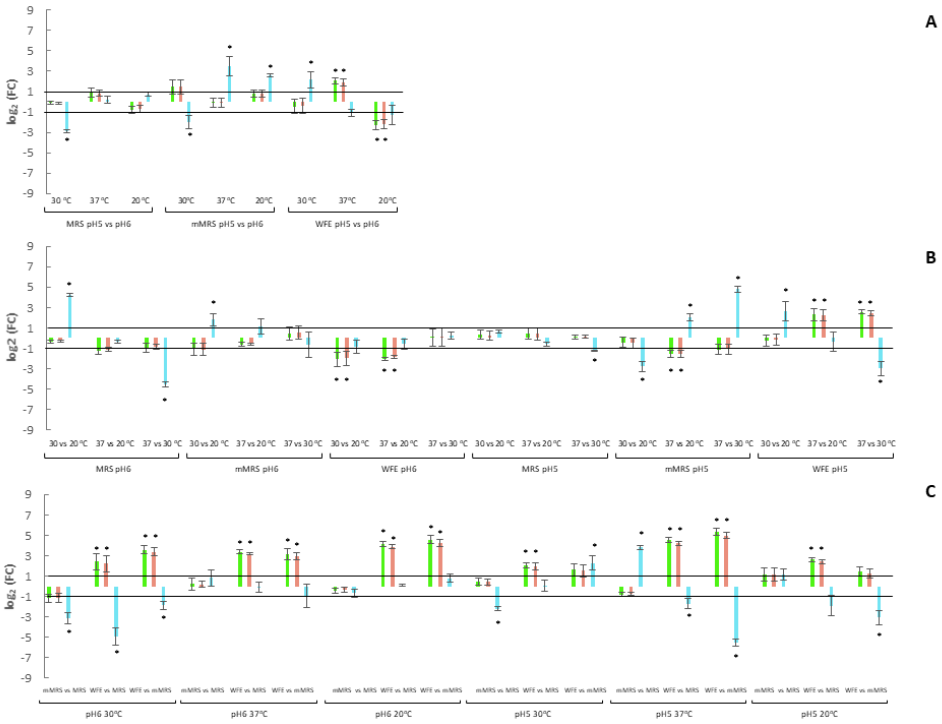

Figure S7.15. (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2485 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Growth at pH 6.0 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 ºC, on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2485 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD*, during growth of strain LQC 2485 in MRS, mMRS broth and WFE, both at initial pH 6.0 and pH 5.0, at 30, 37 and 20 ºC, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA)*, *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

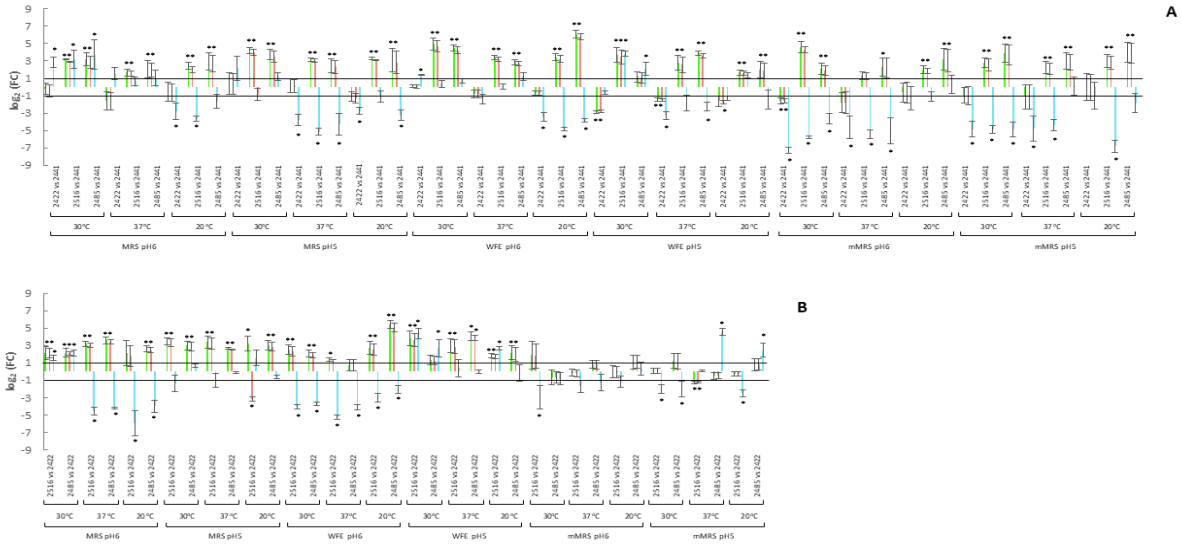

Figure S7.16. (A) Effect of strain on the relative transcription of *pln423 (plαA), plαB* and *plαD* during growth of the *Lp. plantarum* strains LQC 2441, 2422, 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Strain LQC 2441 was used as control. (B) Effect of strain on the relative transcription of *pln423 (plαA), plαB* and *plα*D during growth of the *Lp. plantarum* strains LQC 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Strain LQC 2422 was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA), plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

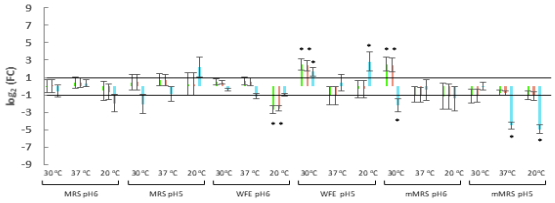

Figure S7.17. Effect of strain on the relative transcription of *pln423 (plαA)*, *plαB* and *plαD* during growth of the *Lp. plantarum* strains LQC 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 ºC, for 21 h. Strain LQC 2485 was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423 (plαA)*, *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$.

Table S7.1. Mean plantaricin activity (AU/ mL), population (log CFU/ mL) and final pH value, with 95.0% confidence intervals. The means were obtained from the in-pair interactions of sourdough related parameters. Independent factors included: 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 corresponding to 1, 2, 3, 4, 5 and 6, three incubation temperatures, namely 20, 30 and 37 ºC, three substrates, namely MRS broth, mMRS broth and WFE corresponding to 1, 2, 3 and two initial pH values, namely 5 and 6.

Mean plantaricin activity				
Level	**Count**	**Mean**	**Lower Limit**	**Upper Limit**
Strain by Temperature				
1,20	24	746.667	630.103	863.23
1,30	24	1493.33	1376.77	1609.9
1,37	24	1493.33	1376.77	1609.9
2,20	24	746.667	630.103	863.23
2,30	24	1493.33	1376.77	1609.9
2,37	24	1493.33	1376.77	1609.9
3,20	24	746.667	630.103	863.23
3,30	24	1493.33	1376.77	1609.9
3,37	24	1493.33	1376.77	1609.9
4,20	24	746.667	630.103	863.23
4,30	24	1493.33	1376.77	1609.9
4,37	24	1493.33	1376.77	1609.9
5,20	24	133.333	16.7698	249.897
5,30	24	133.333	16.7698	249.897
5,37	24	133.333	16.7698	249.897
6,20	24	133.333	16.7698	249.897
6,30	24	133.333	16.7698	249.897
6,37	24	133.333	16.7698	249.897
Strain by Substrate				
1,1	24	1386.67	1270.1	1503.23
1,2	24	1813.33	1696.77	1929.9
1,3	24	533.333	416.77	649.897
2,1	24	1386.67	1270.1	1503.23
2,2	24	1813.33	1696.77	1929.9
2,3	24	533.333	416.77	649.897
3,1	24	1386.67	1270.1	1503.23
3,2	24	1813.33	1696.77	1929.9
3,3	24	533.333	416.77	649.897
4,1	24	1386.67	1270.1	1503.23
4,2	24	1813.33	1696.77	1929.9
4,3	24	533.333	416.77	649.897
5,1	24	160.0	43.4364	276.564
5,2	24	160.0	43.4364	276.564
5,3	24	80.0	-36.5636	196.564
6,1	24	160.0	43.4364	276.564
6,2	24	160.0	43.4364	276.564
6,3	24	80.0	-36.5636	196.564
Mean population				
Level	**Count**	**Mean**	**Lower Limit**	**Upper Limit**
Strain by Temperature				
1,20	24	8.9325	8.87466	8.99034
1,30	24	9.22417	9.16633	9.282
1,37	24	9.09542	9.03758	9.15325
2,20	24	8.89583	8.838	8.95367
2,30	24	9.155	9.09716	9.21284
2,37	24	9.15208	9.09425	9.20992
3,20	24	8.93333	8.8755	8.99117
3,30	24	9.18917	9.13133	9.247
3,37	24	9.08042	9.02258	9.13825
4,20	24	8.90833	8.8505	8.96617
4,30	24	9.20333	9.1455	9.26117

4,37	24	9.07708	9.01925	9.13492
5,20	24	8.95583	8.898	9.01367
5,30	24	9.23	9.17216	9.28784
5,37	24	9.13333	9.0755	9.19117
6,20	24	8.9425	8.88466	9.00034
6,30	24	9.2525	9.19466	9.31034
6,37	24	9.17083	9.113	9.22867
Strain by Substrate				
1,1	24	9.10875	9.05091	9.16659
1,2	24	9.38167	9.32383	9.4395
1,3	24	8.76167	8.70383	8.8195
2,1	24	9.11208	9.05425	9.16992
2,2	24	9.38083	9.323	9.43867
2,3	24	8.71	8.65216	8.76784
3,1	24	9.14625	9.08841	9.20409
3,2	24	9.39583	9.338	9.45367
3,3	24	8.66083	8.603	8.71867
4,1	24	9.13875	9.08091	9.19659
4,2	24	9.375	9.31716	9.43284
4,3	24	8.675	8.61716	8.73284
5,1	24	9.18417	9.12633	9.242
5,2	24	9.39417	9.33633	9.452
5,3	24	8.74083	8.683	8.79867
6,1	24	9.175	9.11716	9.23284
6,2	24	9.41917	9.36133	9.477
6,3	24	8.77167	8.71383	8.8295
Substrate by Temperature				
1,20	48	8.89958	8.85869	8.94048
1,30	48	9.28042	9.23952	9.32131
1,37	48	9.2525	9.2116	9.2934
2,20	48	9.27417	9.23327	9.31506
2,30	48	9.60125	9.56035	9.64215
2,37	48	9.29792	9.25702	9.33881
3,20	48	8.61042	8.56952	8.65131
3,30	48	8.74542	8.70452	8.78631
3,37	48	8.80417	8.76327	8.84506
Mean final pH value				
Level	**Count**	**Mean**	**Lower Limit**	**Upper Limit**
Strain by Temperature				
1,20	24	4.43167	4.40092	4.46241
1,30	24	3.645	3.61425	3.67575
1,37	24	3.65667	3.62592	3.68741
2,20	24	4.435	4.40425	4.46575
2,30	24	3.65083	3.62009	3.68158
2,37	24	3.65833	3.62759	3.68908
3,20	24	4.39583	4.36509	4.42658
3,30	24	3.63917	3.60842	3.66991
3,37	24	3.63667	3.60592	3.66741
4,20	24	4.38667	4.35592	4.41741
4,30	24	3.65583	3.62509	3.68658
4,37	24	3.6525	3.62175	3.68325
5,20	24	4.38167	4.35092	4.41241
5,30	24	3.69917	3.66842	3.72991
5,37	24	3.6325	3.60175	3.66325
6,20	24	4.40417	4.37342	4.43491
6,30	24	3.64083	3.61009	3.67158
6,37	24	3.63667	3.60592	3.66741
Initial pH by Strain				
5,1	36	3.84778	3.82267	3.87288
5,2	36	3.83389	3.80879	3.85899
5,3	36	3.79056	3.76545	3.81566
5,4	36	3.79611	3.77101	3.82121
5,5	36	3.81889	3.79379	3.84399

5,6	36	3.78778	3.76267	3.81288
6,1	36	3.97444	3.94934	3.99955
6,2	36	3.99556	3.97045	4.02066
6,3	36	3.99056	3.96545	4.01566
6,4	36	4.00056	3.97545	4.02566
6,5	36	3.99	3.9649	4.0151
6,6	36	4.0	3.9749	4.0251

Table S7.2. Mean plantaricin activity for each strain, with 95.0 % confidence intervals. The treatment was applied at regular time intervals of 3 h, over a period of 33 h. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to 1, 2, 3, 4, 5 and 6, respectively.

Incubation time	Level-Strain	Count	Mean	Lower Limit	Upper Limit
3 h	1	72	248.889	244.24	253.537
	2	72	248.889	244.24	253.537
	3	72	248.889	244.24	253.537
	4	72	248.889	244.24	253.537
	5	72	0	-4.64843	4.64843
	6	72	0	-4.64843	4.64843
6 h	1	72	320.0	307.392	332.608
	2	72	337.778	325.17	350.385
	3	72	320.0	307.392	332.608
	4	72	328.889	316.281	341.497
	5	72	53.3333	40.7257	65.941
	6	72	53.3333	40.7257	65.941
9 h	1	72	480.0	457.236	502.764
	2	72	480.0	457.236	502.764
	3	72	471.111	448.347	493.875
	4	72	462.222	439.459	484.986
	5	72	80.0	57.2363	102.764
	6	72	80.0	57.2363	102.764
12 h	1	72	653.333	623.95	682.717
	2	72	640.0	610.617	669.383
	3	72	644.444	615.061	673.828
	4	72	640.0	610.617	669.383
	5	72	80.0	50.6168	109.383
	6	72	80.0	50.6168	109.383
15 h	1	72	866.667	819.473	913.861
	2	72	848.889	801.695	896.083
	3	72	862.222	815.028	909.416
	4	72	848.889	801.695	896.083
	5	72	80.0	32.806	127.194
	6	72	80.0	32.806	127.194
18 h	1	72	1137.78	1067.88	1207.68
	2	72	1137.78	1067.88	1207.68
	3	72	1137.78	1067.88	1207.68
	4	72	1137.78	1067.88	1207.68
	5	72	97.7778	27.8772	167.678
	6	72	97.7778	27.8772	167.678
21 h	1	72	1244.44	1177.15	1311.74
	2	72	1244.44	1177.15	1311.74
	3	72	1244.44	1177.15	1311.74
	4	72	1244.44	1177.15	1311.74
	5	72	133.333	66.0353	200.631
	6	72	133.333	66.0353	200.631
24 h	1	72	862.222	834.187	890.258
	2	72	862.222	834.187	890.258
	3	72	862.222	834.187	890.258
	4	72	880.0	851.965	908.035
	5	72	80.0	51.9645	108.035
	6	72	80.0	51.9645	108.035
27 h	1	72	586.667	550.065	623.268
	2	72	586.667	550.065	623.268
	3	72	586.667	550.065	623.268
	4	72	586.667	550.065	623.268
	5	72	80.0	43.3982	116.602
	6	72	80.0	43.3982	116.602
30 h	1	72	586.667	550.065	623.268
	2	72	586.667	550.065	623.268
	3	72	586.667	550.065	623.268
	4	72	586.667	550.065	623.268

	5	72	80.0	43.3982	116.602
	6	72	80.0	43.3982	116.602
33 h	1	72	586.667	550.065	623.268
	2	72	586.667	550.065	623.268
	3	72	586.667	550.065	623.268
	4	72	586.667	550.065	623.268
	5	72	80.0	43.3982	116.602
	6	72	80.0	43.3982	116.602

Table S7.3. Pearson product moment (r) correlations between the relative transcription of the 18 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2320 and 2520 and plantaricin activity.

Variables		ΔAU	*plNC8a*	*plNC8β*	*plNC8c*	*plnL*	*plnR*	*plnK*	*plnJ*	*plnE*	*plnF*	*plnH*	*plnS*	*plnY*	*plNC8IF*	*plNC8HK*	*plnD*	*plnM*	*plnI*	*plnG*
ΔAU	**r**	**1**																		
	p	**0**																		
plNC8a	**r**	0.063	**1**																	
	p	0.725	**0**																	
plNC8β	**r**	0.161	**0.840**	**1**																
	p	0.363	**< 0.0001**	**0**																
plNC8c	**r**	0.045	**0.996**	**0.838**	**1**															
	p	0.802	**< 0.0001**	**< 0.0001**	**0**															
plnL	**r**	0.316	**0.617**	**0.806**	**0.614**	**1**														
	p	0.069	**0.000**	**< 0.0001**	**0.000**	**0**														
plnR	**r**	0.316	**0.621**	**0.805**	**0.618**	**1.000**	**1**													
	p	0.089	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**													
plnK	**r**	0.327	**0.613**	**0.803**	**0.610**	**0.999**	**0.999**	**1**												
	p	0.059	**0.000**	**< 0.0001**	**0.000**	**< 0.0001**	**< 0.0001**	**0**												
plnJ	**r**	**0.375**	**0.668**	**0.835**	**0.666**	**0.971**	**0.971**	**0.972**	**1**											
	p	**0.029**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**											
plnE	**r**	0.017	**0.437**	**0.643**	**0.433**	**0.823**	**0.818**	**0.822**	**0.760**	**1**										
	p	0.923	**0.010**	**< 0.0001**	**0.011**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**										
plnF	**r**	0.247	**0.633**	**0.834**	**0.631**	**0.944**	**0.942**	**0.946**	**0.929**	**0.905**	**1**									
	p	0.158	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**									
plnH	**r**	0.310	**0.806**	**0.904**	**0.800**	**0.887**	**0.886**	**0.889**	**0.916**	**0.671**	**0.871**	**1**								
	p	0.074	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**								
plnS	**r**	**0.395**	**0.718**	**0.872**	**0.712**	**0.943**	**0.942**	**0.946**	**0.966**	**0.772**	**0.931**	**0.955**	**1**							
	p	**0.021**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**							

plnY	**r**	**0.366**	**0.806**	**0.905**	**0.802**	**0.835**	**0.835**	**0.839**	**0.890**	**0.606**	**0.839**	**0.953**	**0.922**	**1**						
	p	**0.033**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.000**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**						
plNC8-IF	**r**	**0.383**	**0.420**	**0.663**	**0.409**	**0.835**	**0.833**	**0.836**	**0.813**	**0.846**	**0.886**	**0.687**	**0.826**	**0.726**	**1**					
	p	**0.026**	**0.013**	**< 0.0001**	**0.016**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**					
plNC8-HK	**r**	**0.384**	**0.425**	**0.665**	**0.414**	**0.837**	**0.835**	**0.838**	**0.816**	**0.848**	**0.887**	**0.691**	**0.828**	**0.730**	**1.000**	**1**				
	p	**0.025**	**0.012**	**< 0.0001**	**0.015**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**				
plnD	**r**	**0.398**	**0.414**	**0.669**	**0.403**	**0.847**	**0.845**	**0.848**	**0.825**	**0.844**	**0.890**	**0.691**	**0.832**	**0.731**	**0.998**	**0.998**	**1**			
	p	**0.020**	**0.015**	**< 0.0001**	**0.018**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**			
plnM	**r**	**0.425**	**0.643**	**0.827**	**0.631**	**0.930**	**0.929**	**0.932**	**0.943**	**0.731**	**0.888**	**0.943**	**0.966**	**0.907**	**0.819**	**0.822**	**0.827**	**1**		
	p	**0.012**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**		
plnI	**r**	**0.338**	**0.788**	**0.886**	**0.783**	**0.891**	**0.891**	**0.893**	**0.914**	**0.705**	**0.884**	**0.987**	**0.958**	**0.934**	**0.716**	**0.719**	**0.718**	**0.946**	**1**	
	p	**0.051**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**	
plnG	**r**	0.232	**0.806**	**0.914**	**0.816**	**0.799**	**0.798**	**0.797**	**0.839**	**0.578**	**0.770**	**0.893**	**0.881**	**0.880**	**0.634**	**0.637**	**0.642**	**0.842**	**0.878**	**1**
	p	0.187	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.000**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0**

The relative transcription and the change in plantaricin activity (ΔAU) were calculated using the data from each strain after growth in WFE with initial pH value 5 at 20 ºC for 21 h. Statistically significant correlations ($p < 0.05$) are presented in bold.

Table S7.4. Pearson product moment (r) correlations between the relative transcription of the 3 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 and plantaricin activity.

Variables		ΔAU	*pln423* (*plαA*)	*plαB*	*plαD*
ΔAU	**r**	**1**			
	p	**0**			
***pln423* (*plαA*)**	**r**	**-0.528**	**1**		
	p	**< 0.0001**	**0**		
plαB	**r**	**-0.529**	**0.999**	**1**	
	p	**< 0.0001**	**< 0.0001**	**0**	
plαD	r	0.166	-0.216	-0.223	**1**
	p	0.177	0.077	0.067	**0**

Statistically significant correlations ($p < 0.05$) are presented in bold.