Food Research International 220 (2025) 117039

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Batch-dependent microbiota variation in mixed vegetable fermentations shapes the probiotic potential of autochthonous multifunctional cultures

Cristian Botta *, Daniele Spada, Davide Buzzanca, Negin Seif Zadeh, Dimitra Tsoureki, Federica Biolcati, Ilario Ferrocino, Giuseppe Zeppa, Paola Dolci

Department of Agricultural, Forest and Food Sciences (DiSAFA), University of Torino, Largo Paolo Braccini 2, Grugliasco 10095, TO, Italy

ARTICLE INFO

Keywords:
Handcrafted fermentations
Fermented vegetables
Metataxonomics
Lactic acid bacteria
Multifunctional cultures

ABSTRACT

Fermented vegetables (FVs) are valuable sources of lactic acid bacteria (LAB), which can be employed as functional cultures to enhance the quality, stability and probiotic potential of traditional food products. In this study, the selection of LAB strains with both technological and probiotic properties was supported by a parallel investigation of the microbiota and mycobiota in six FVs mixtures.

Metataxonomic analysis and culture-based isolation were consistent in displaying a succession of LAB species through fermentation. *Leuconostoc* (*Lc.*) *mesenteroides* dominated the early stages, followed by lactobacilli species such as *Latilactobacillus* (*Lat.*) *sakei*, *Levilactobacillus* (*Lev.*) *brevis* and *Lactiplantibacillus* (*Lpb.*) *plantarum* from the middle to the late fermentation stages. The majority of LAB isolates were identified as *Lc. mesenteroides* and *Lpb. plantarum*, with the latter displaying superior probiotic performances *in vitro*, including high tolerance to bile salts and resistance to simulated human gastrointestinal conditions. Differences in microbial composition were observed between the two experimental batches (A and B), regardless of the vegetable composition. Notably, batch A showed more homofermentative conditions across all FVs, as confirmed by the organic acids profile. Remarkably, most of the promising probiotic *Lpb. plantarum* strains were isolated from the homofermentative FVs.

By leveraging a complementary metataxonomic approach, this study demonstrates how batch-to-batch variability can influence the probiotic potential of FVs and guide the selection of functional LAB cultures.

1. Introduction

Since the early Metchnikoff's discoveries, the identification of fermented foods with beneficial impact on human health or their amelioration through the addition of probiotic microorganisms are never-ending research activities for food microbiologists (Gordon, 2016). In this context, fermented vegetables (FVs) offer several nutritional advantages over animal-based fermented products, such as dairy or sausages. Compared to these, FVs have lower lipid content, no cholesterol, and are rich in polyphenols and dietary fibre. Notably, fibres have a proven positive impact on the human gut microbiome. Fibre-degrading bacteria, such as *Prevotella* and members of the Firmicutes phylum, ferment these substrates, producing short-chain fatty acids (SCFAs) in the colon (De Filippis et al., 2015). Among these, butyrate is particularly important for maintaining gut homeostasis, as it regulates inflammation, strengthens the intestinal barrier, reduces oxidative stress, and supports epithelial cell renewal (Annunziata et al., 2020; Botta, Spyridopoulou, et al., 2022; Czarnowski et al., 2024).

Beyond their prebiotic activity, FVs harbour a more complex microbiota compared to animal-based fermented products (Parente et al., 2016). This microbial diversity results in a broader range of potential probiotic strains that may actively participate in fermentation until its completion and, upon consumption, exert beneficial effects in the gut (Ghini et al., 2020). However, successful intestine colonization depends on each strain's ability to survive the harsh conditions of the stomach and small intestine, where acidic pH and digestive enzymes can significantly reduce microbial viability. Therefore, high resistance to gastrointestinal transit is a key characteristic when selecting new probiotic strains from food and other sources (Botta et al., 2014; de Melo Pereira et al., 2018).

The role of fermented vegetables (FVs) as a source of novel probiotic candidates has gained significant attention over the past two decades.

* Corresponding author at: Largo Paolo Braccini, 2, 10095 Grugliasco (TO) /Piazza Torino 3, 12100 Cuneo, CN, Italy.
E-mail address: cristian.botta@unito.it (C. Botta).

https://doi.org/10.1016/j.foodres.2025.117039
Received 24 March 2025; Received in revised form 16 July 2025; Accepted 24 July 2025
Available online 28 July 2025

Numerous studies have characterized the microbiota of various handcrafted vegetable fermentations to identify and isolate microbes with promising probiotic properties, primarily from the lactic acid bacteria (LAB) group. Extensive research has focused on microbial communities in table olives (Botta et al., 2014; Pessione et al., 2015), sauerkraut (Touret et al., 2018), and cabbage-based mixtures of vegetables like kimchi (Hossain et al., 2020; Liang et al., 2022; Seo et al., 2021; Zhao et al., 2023), leading to the isolation of LAB strains with dual functionality as probiotics and starters.

Autochthonous starter LAB have been successfully applied in FV production, including kimchi (Seo et al., 2021), olives (Coimbra-Gomes et al., 2023) and sauerkraut (Beganović et al., 2014), enhancing their technological, sensory, and nutritional properties (C. Garcia et al., 2020; Septembre-Malaterre et al., 2018). The selection of new functional starters typically involves the *in vitro* evaluation of technological traits such as growth performances under varying temperatures, salt and pH resistance, and other fermentation-specific parameters (Franciosa et al., 2022). Additionally, screening for undesirable traits, such as antibiotic resistance, is essential before pilot-scale fermentation trials (de Melo Pereira et al., 2018).

However, the selection of autochthonous LAB starters for specific handcrafted fermentations cannot rely solely on *in vitro* physiological characterization. The complexity and variability of each FV's microbiota must also be considered, as even minor changes in ingredient can influence its composition and the fermentation dynamics (Liu et al., 2023). Moreover, beginning with the same vegetable mixture does not guarantee uniformity in microbial, nutraceutical, or sensory characteristics, as fermentation methods vary widely across regions due to local traditions and cultural influences. Although convenient, the use of commercial starter may instead lead to a loss of product uniqueness (Cardinali, Botta, Harasym, Ferrocino, et al., 2024; Cardinali, Botta, Harasym, Reale, et al., 2024).

A comprehensive understanding of these autochthonous microbial communities requires both culture-dependent techniques and culture-independent methods, such as metataxonomic or metagenomic analyses (Sabater et al., 2021). While these culture-independent approaches can elucidate the microbial succession and identify key species contributing to an optimal final product—thereby guiding the selection of starter strains—few studies have effectively integrated these methods as complementary tools in probiotics isolation and development (Boban et al., 2024; Cardinali, Botta, Harasym, Ferrocino, et al., 2024; Cardinali, Botta, Harasym, Reale, et al., 2024; Liu et al., 2023; Min et al., 2022). Today, the identification of suitable probiotic and technological LAB starters for a given vegetable formulation should be coupled with descriptive metataxonomic analyses to fully capture the complexity of the native microbial ecosystem.

In this frame, we aimed to decipher at which level different vegetable mixtures and batches can shape the initial microbiota and mycobiota composition in handcrafted fermentations of mixed vegetables, as well as how these factors were related to the presence of autochthonous LAB with potential probiotic and technological features.

2. Materials and methods

2.1. Laboratory scale fermentations

Six different handcrafted fermentations of mixed vegetables were conducted, each in two independent batches ($n = 2$): A and B produced in the months of March and April 2019, respectively. The vegetables used were as follows: carrot/yellow pepper (CP); white cabbage/carrot/yellow pepper (WCP); white cabbage (W); red cabbage (R); red cabbage/beetroot/red onion (RBO); beetroot/red onion (BO). All vegetables were supplied on the production day from a local retail centre (Turin, Italy) and selected on the basis of UNECE standards for fruit and vegetables (https://unece.org). Processing was performed at the School for Catering and Hospitality "*G. Colombato*" (Turin, Italy), using the same facilities and tools for both batches. Briefly, vegetables were rinsed in cold tap water, finely chopped into approximately 1 cm^3 pieces, and mixed in the defined proportions shown in **Supplementary Table 1**. For each fermentation, 600 g of the vegetable mixture was transferred into 1.5 L sterile jars containing 150 mL of 12.5 % (*w/v*) NaCl brine, maintaining a 4:1 vegetable-to-brine ratio and achieving a final salt concentration of 2.5 %. The vegetable proportions and brine concentration were based on the guidelines described by Shockey and Shockey (2014). The headspaces of the jars were filled with sterile air bags, and bubblers were used to prevent CO_2 accumulation (Supplementary Fig. 1). Fermentations were carried out in the dark at a constant temperature of 15 °C for 30 days. Samples (70 g of vegetables-brine mixture) were collected on days 1, 7, 15 and 30 (T01, T07, T15, T30). Additionally, fresh vegetable mixtures (70 g) were sampled the day of production (T0).

2.2. Matrices sampling, DNA extraction and amplicon-based sequencing

Total DNA was extracted from samples collected at 0, 1, 7, and 30 days of fermentation using the Master Pure complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) and according to the manufacturer's instructions. DNA quality and concentration was evaluated with NanoDrop spectrophotometer (Thermo Scientific, Milan, Italy). Library of V3-V4 region were constructed from the 16S rRNA gene region of bacterial DNA, while library of 26S rRNA gene of fungal DNA was constructed using MiSeq Reagent Kit V3 (2 × 250 bp; Illumina, San Diego, USA), as previously described (Botta, Franciosa, et al., 2022).

The PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged with sequencing adapters using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions. Sequencing was performed using a MiSeq Illumina instrument (Illumina) with V3 chemistry, which generated 2 × 250 bp paired-end reads. MiSeq Control Software, V2.3.0.3, RTA, v1.18.42.0, and CASAVA, v1.8.2, were used for the base-calling and Illumina barcode demultiplexing processes.

2.3. Bioinformatic analysis

A total of 400,802 and 535,306 raw-reads were produced by 16S and 26S rRNA genes amplicon-based sequencing, respectively. To obtain Amplicon Sequence Variants (ASVs) the raw-reads were analysed with *DADA2* package (Callahan et al., 2016) in R environment (R version 4.3.2; http://www.r-project.org). The pipeline previously described was followed to filter and merge raw-reads (Botta et al., 2021).

Bacteria taxonomy was assigned at the genus level with 99 % sequence similarity through Bayesian classifier method (Wang et al., 2007) by matching bacterial ASVs to the 2024 release (version 138.2) of Silva prokaryotic SSU reference database (https://zenodo.org/records/14169026), with a further check at 100 % of similarity for ASVs assigned to the species level with the *addSpecies* script. Fungi taxonomy was assigned at 99 % sequence similarity an internal database of 26S rRNA genes. The 26S taxonomic assignments were double checked by using BLASTn suite (https://blast.ncbi.nlm.nih.gov), and ASVs were assigned to the species in case of 100 % of similarity to reference sequences. Fungal and bacterial ASVs with uncertain assignment (to the Order rank or lower resolution) or matching (> 99 % similarity) with vegetable genomes were removed from the frequency tables. All samples with less than 1000 reads were removed from the analysis; 313,362 paired-end bacterial reads (12,124 reads/sample) and 402,858 paired-end fungal reads (11,510 reads/sample) were finally used to construct ASVs tables of the two kingdoms.

Fungal and bacterial ASVs were aligned with *DECIPHER* package and two unrooted phylogenetic trees were constructed with *phangorn* package (Schliep, 2011; Wright, 2016). Alpha diversity metrics and weighted UniFrac beta-diversity distance were calculated with *phyloseq* and

picante packages (Kembel et al., 2010; McMurdie & Holmes, 2013): rarefaction limit was set to the lowest number of sequences/samples.

Sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/sra/PRJNA1163264) under the bioproject accession number PRJNA1163264.

2.4. Microbiological analysis and pH

Serial dilutions were set up from homogenized samples collected at all sampling point (10 g in 90 mL Ringer's solution; Oxoid) and microbial counts were performed to enumerate: lactic acid bacteria on De Man, Rogosa and Sharpe (MRS) agar incubated for 48 h at 30 °C; *Staphylococcus* spp. on Mannitol Salt Agar (MSA) incubated at 30 °C for 48 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA) incubated for 24 h at 37 °C; yeasts and moulds on Malt Extract agar (MEA) supplemented with tetracycline (0.05 g/L; Sigma-Aldrich, St. Louis, USA) and incubated at 25 °C for five days. All media and supplements were provided by Biolife s.p.a. (Milan, Italy) unless differently stated. Three technical replicates were analysed for each sampling point for both batches A and B.

The pH of each sample was measured by using a digital pH-meter (Crison, Modena, Italy).

2.5. Isolation and identification of lactic acid bacteria (LAB)

Morphologically distinct colonies were collected from the highest diluted MRS plates across all vegetable mixtures, batches, and time points. At least seven colonies were considered for each sampling point, purified by streaking and checked, through Gram staining and catalase activity. DNA was then extracted from each isolate and normalized at 100 ng/μL (Cocolin et al., 2004). Identification of the isolates was performed with species-specific PCR assays for: *Leuconostoc* (*Lc.*) *mesenteroides* (Cho et al., 2009), *Levilactobacillus* (*Lev.*) *brevis* (Guarneri et al., 2001), *Lactiplantibacillus* (*Lpb.*) *plantarum, Lpb. paraplantarum* and *Lpb. pentosus* (Torriani et al., 2001). All isolates not identified by species-specific PCR assays were subjected to amplification and sequencing of 16S rRNA gene and subsequent sequences alignment with BLASTn suite (https://blast.ncbi.nlm.nih.gov).

2.6. Progressive selection of LAB strains with promising technological and probiotic features

All isolates identified as LAB were subjected to a progressive selection based on their growth/survival phenotypes in stress conditions typically encountered during fermentation and along the transit in the upper human gastrointestinal tract. Antibiotic susceptibility has been tested as well on the final set of selected LAB isolates. All reagents used in these experiments were provided by Sigma-Aldrich (Milan, Italy) unless differently stated.

2.6.1. Growth rate in conditions related to probiotic and technological potentials

Growth capability of the LAB isolates was tested in MRS broth supplemented with 2.5 % of NaCl and 0.2 % of bile salts (Oxoid, Milan, Italy), incubated for 24 h at 15 and 37 °C, respectively. In parallel, the growth rate of the isolates was quantified in MRS broth acidified with HCl to pH 3.0 and pH 2.0, respectively incubated at 15 and 37 °C for 24 h. Briefly, an overnight broth culture (37 °C for 18 h) of each isolate was standardized at defined optical density (OD) 600 nm and inoculated (1:100 *v*/v ratio) in 200 μL of the four modified MRS. Normal MRS broth was used as reference condition. Growth rate was spectrophotometrically measured by optical density (OD) at 600 nm after 24 h of incubation at 37 °C. Relative growth rate was expressed as ratio between treated and reference condition: ($OD_{\text{modified MRS T24}} - OD_{\text{modified MRS T0}}$) / ($OD_{\text{MRS T24}} - OD_{\text{MRS T0}}$).

2.6.2. Simulated digestion

An in vitro process of human digestion has been performed by subjecting LAB isolates to a simulated transit consecutively through stomach (gastric step) and upper intestine (duodenum), as previously described (Botta et al., 2014).

Briefly, each isolate was grown for 24 h at 37 °C, centrifuged and the pellet resuspended in Phosphate Buffered Saline (PBS). Initial concentration was set to 8.5 ± 0.2 Log CFU/mL through an internal calibration curve. Before the simulated digestion, an initial plate count (T0) on MRS agar plates has been performed. Bacterial pellet was collected, resuspended in a synthetic gastric juice (pH 2.00; 0.05 g/L porcine bile, lysozyme 0.1 g/L and 0.0133 g/L of pepsin), and incubated for 2 h at 37 °C in an orbital shaker. After gastric digestion step, the gastric juice was removed, bacterial cells were resuspended in PBS and their enumeration was performed as previously (T1). Bacteria pellets were finally resuspended in simulated intestinal juice, which was formulated using bile (3 g/L, Oxoid) and pancreatin (0.1 g/L) in a buffer solution at pH 8.0 (Bautista-Gallego et al., 2013). After 4 h of incubation at 37 °C, bacterial cells were washed and resuspended in PBS to be enumerated on MRS plates (T2). The survival rate of each isolate, after the gastric step passage (GSP) and after the complete simulated digestion (CSD), have been expressed as difference to the initial count, according to the formula: Δ Log CFU/mL $_{(\text{GSP or CSD})}$ = Log CFU/mL $_{(\text{T1 or T2})}$ – Log CFU/mL $_{\text{T0}}$. The strain *Lpb. plantarum* S2T10D has been used as reference phenotype with high survival rate in simulated digestion processes.

2.6.3. Antibiotic susceptibility

The microdilution method reported by the ISO 10932:2010 standard was used to determine the minimum inhibitory concentrations (MICs) of eight antibiotics: Ampicillin (AMP), Gentamicin (GEN), Kanamycin (KAN), Streptomycin (STR), Erythromycin (ERY), Clindamycin (CLI), Tetracycline (TET) and Chloramphenicol (CHL). Isolates tested were classified after 48 h of incubation as resistant or sensitive according to the defined cut-off values (EFSA, 2012).

2.7. High performance liquid chromatography

Ten millilitres of brine from the samples collected at 1, 7, and 30 days have been analysed using High Performance Liquid Chromatography (HPLC).

The HPLC system (Thermo Electron Corporation, Waltham, MA, USA) was equipped with a SCM 1000 degasser, a P2000 binary gradient pump, a multiple autoinjector (AS3000), a photodiode array (PDA) detector (Thermo Electron Corporation, UV6000LP) and a refractive index detector (RI-150). The mobile phase was 0.013 N H_2SO_4 and the sample injection volume was 20 μL. The isocratic elution method was applied with a flow rate of 0.6 mL. The analysis has been performed using a reverse-phase Aminex HPX-87H column (300 mm* 7.8 mm) equipped with a Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA) working at 65 °C. The data have been elaborated with ChromQuest chromatography data system (ThermoQuest software 5.0, Inc., San Jose, CA, USA), while identification was achieved by comparison with the retention time of authentic standards from Sigma-Aldrich (Milan, Italy).

2.8. Statistical analysis

Statistical analyses and data plotting were performed in R environment (R version 4.3.2; http://www.r-project.org). Normality and homogeneity of metataxonomic, counts, growth parameters and chemical data were checked by means of the Shapiro-Wilk W test and Levene's test, respectively. Variation and differences between multiple groups were assessed with one-way ANOVA (coupled with Tukey's post-hoc test) and Kruskal–Wallis's test (coupled with pairwise Wilcoxon's test) for parametric and not parametric data, respectively. Pairwise comparisons were alternatively performed with Wilcoxon and *t*-tests

according to data normality.

Non-metric Multidimensional Scaling (NMDS) and Principal-coordinate Analysis (PCoA) were used to visualize ASVs distribution and beta-diversity, respectively. Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted on ASVs abundances and weighted UniFrac distance matrices (beta-diversity) to evaluate the effects of each categorical variable (Time, Fermentation, Batch) on the variations in bacterial/fungal communities using the Adonis function based on 999 permutations and Brey-Curtis dissimilarity distances.

Co-exclusion/co-occurrence between bacterial and fungal taxa were calculated with sparCC algorithm run with default parameters and 100 bootstraps in the R package *SpiecEasi* (Friedman & Alm, 2012). Pseudo *P*-values were calculated as the proportion of simulated bootstrapped (pseudo-*P*-values <0.001). Correlation existing between metataxonomic data (Log transformed abundance) and chemical data were assessed by the Spearman's rank correlation (Weiss et al., 2016).

Relative growth rate and survival rate in simulated digestion (Delta Log) were transformed in z-scores on which *P*-values (FDR adjusted) were computed by separate one-tailed z-tests (Taddese et al., 2019). Based on these *P*-values, isolates that were located in the upper 5 % extreme of z-scores distribution (significantly higher than population mean; $P < 0.05$) were selected for further characterization steps.

3. Results

3.1. Culture-independent landscape of microbiota and mycobiota

Due to the limited number of ASVs assigned to bacteria (< 1000 sequences/sample), all samples collected from vegetable mixtures on the day of production (T0), as well as two samples (theses CP and RBO form batch A) collected the first day of fermentation (T01), were excluded from the 16S rDNA amplicons-based analysis. Regarding the fungal community, all samples from T0 and T01 exhibited a high prevalence of contaminant sequences originating from vegetable genomes, with fewer than 1000 sequences per sample assigned to fungi. As a result, only samples collected from the seventh (T07) and thirtieth (T30) day were included in the 26S rDNA amplicons-based analysis of the mycobiota.

Batches A and B were characterized by distinct taxonomic compositions (Fig. 1 **A and B**). Permutational Analysis of Variance

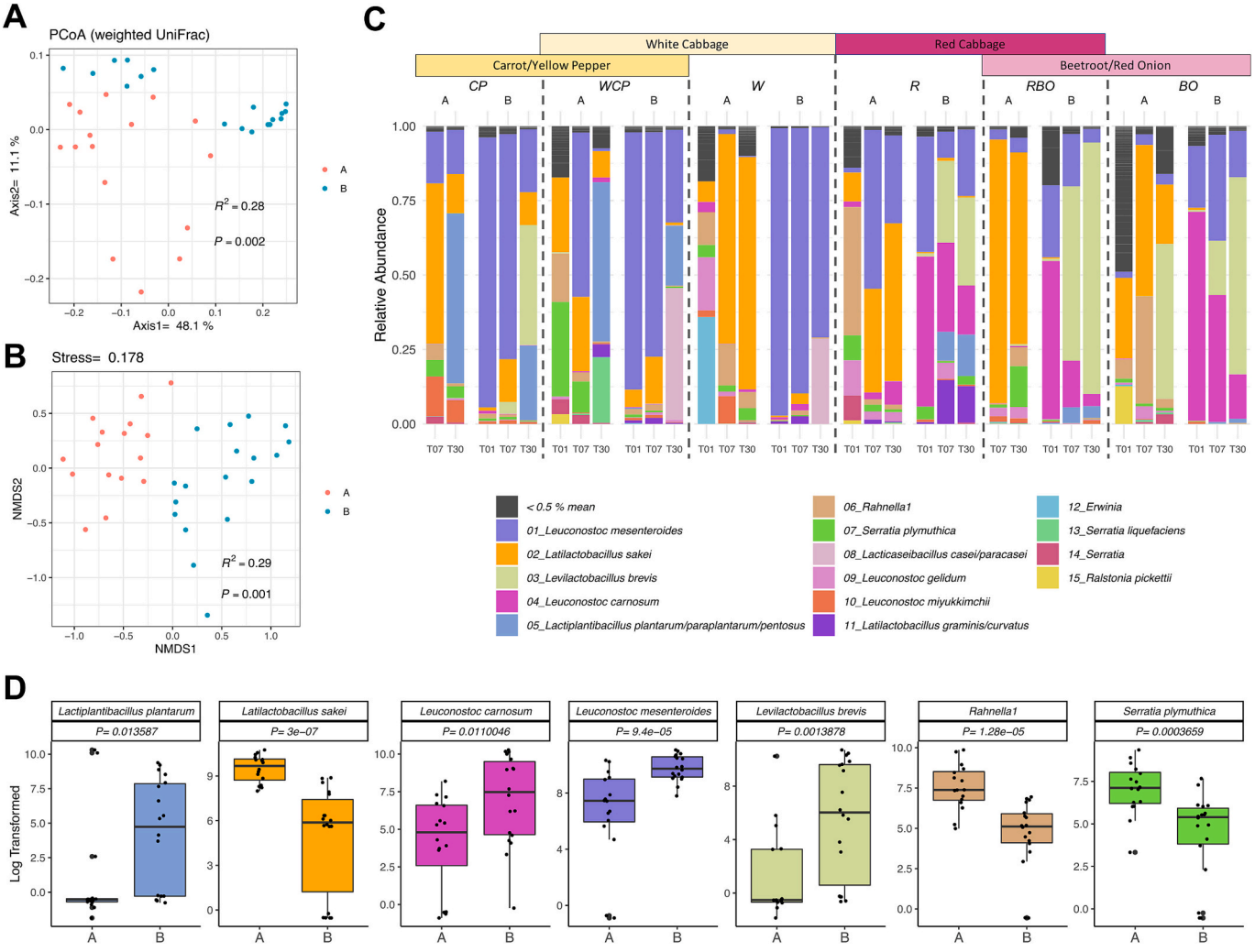

Fig. 1. Microbiota composition and variability.
PCoA **(A)** based on weighted UniFrac distance matrix (β-diversity) and NMDS (**B**) ordination displaying the segregation of the samples in relation to the batch of origin; significance and R^2 of PERMANOVA are reported in each chart ($P < 0.001$ [FDR adjusted], ANOSIM). Stacked bar plots **(C)** showing microbiota composition (relative abundance) at the genus/species taxonomic level and relative colour coding key. Samples are grouped by batch in each fermentation mixture and sequentially displayed according to the time; taxa are sorted in the legend from the most to the least abundant (> 0.5 %). Box plots **(D)** displaying Central Logarithmic Transformed Counts (interquartile range/median) of the core species/genera in the two batches. Significance (Wilcoxon's test; *P*-value [FDR adjusted]) is reported in each chart.

(PERMANOVA) based on weighted UniFrac distances (β-diversity) and species abundances highlighted that the greatest portion of bacterial community variance (R^2 = 0.28 and 0.29 in PCoA and NMDS, respectively) was attributable to differences between batches (A and B), rather than to the type of fermentation mixture (W, R, CP, BO, WCP, RBO) or sampling time. Nevertheless, compositional differences were observed between samples collected during the early stages of fermentation (1st and 7th day) and those from the final fermented product.

As far as the taxonomic composition, the microbiota was dominated by members of *Lactobacillacea* family. Species-level identification was refined through manual alignment at a 100 % of identity threshold, revealing *Lc. mesenteroides*, *Latilactobacillus* (*Lat.*) *sakei*, *Levilactobacillus* (*Lev.*) *brevis*, *Lc. carnosum* and members of *Lpb. plantarum* group as the core microbiota. These taxa collectively accounted for over 50 % of the abundance in most samples (Fig. 1 **C**). Their distribution varied between batches: *Lat. sakei* was dominant in batch A, whereas *Lc. mesenteroides*, *Lev. brevis*, *Lc. carnosum*, and *Lpb. plantarum* were more abundant in B (Fig. 1 **D**). Overall, batch A showed higher bacterial biodiversity (*P* [FDR] < 0.05; data not shown) and a greater presence of Gram-negative species such as *Rahnella* and *Serratia plymuthica*. Moreover, in both batches, *Lpb. plantarum* was more abundant in the final fermented product (30th day) compared to the initial stages of fermentation (*P* [FDR] < 0.05; data not shown).

Similarly, mycobiota composition during the fermentation differed between the two batches, but showed no consistent variation related to the type of vegetable used or between samples taken on the seventh and the thirtieth days (Fig. 2 **A and B**). The core mycobiota encompassed *Saccharomyces* (*S.*) *cerevisiae*, *Hanseniaspora* (*H.*) *meyeri*, *Candida* (*C.*) *sake*, *Debaryomyces* (*D.*) *hansenii* and *H. clermontiae*, which represented the most abundant yeast species and were detected in all six fermentation mixtures and in both batches (Fig. 2 **C**). In terms of fungal distribution, *Pichia* (*P.*) *fermentans* was exclusively found in batch A, which also exhibited higher presence of *Hanseniaspora* species and significantly greater alpha diversity values (*P*[FDR] < 0.05; data not shown). In contrast, batch B had significantly higher abundances of *S. cerevisiae* and *C. sake*, along with *Penicillium* sp., which was the most abundant filamentous fungus (Fig. 2 **D**).

Pairwise co-occurrence and co-exclusion patterns between taxa were partially constrained by the observed batch-related distribution of bacteria and fungi (**Supplementary Table 2**). Moreover, several bacteria and fungi taxa belonging to the same genus or family were co-occurring throughout the dataset.

3.2. Microbiological and chemical-physical dynamics during fermentations

LAB counts and chemical-physical dynamics are displayed in Fig. 3, with data grouped in relation to the batch or fermentations,

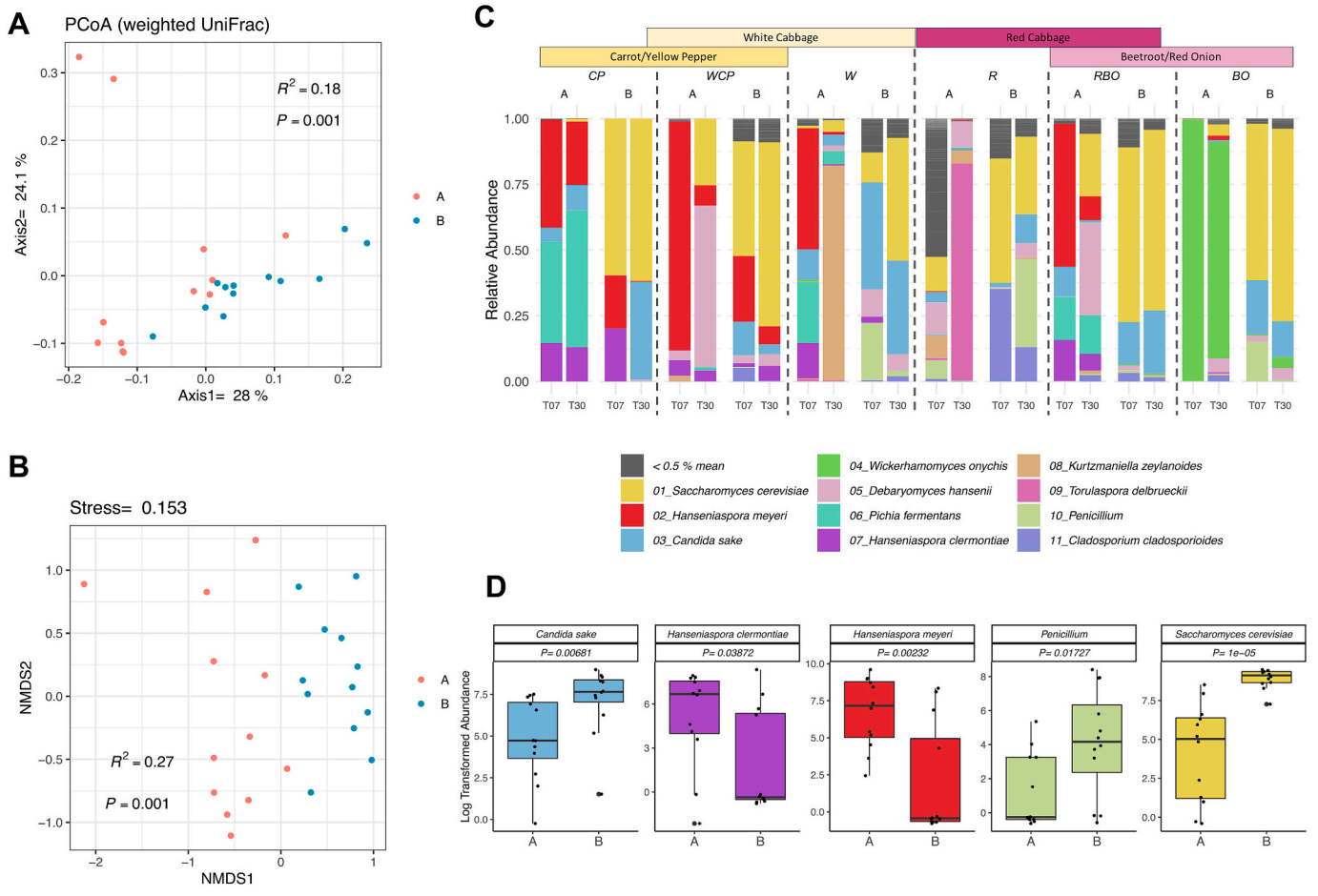

Fig. 2. Mycobiota composition and variability.
PCoA **(A)** based on weighted UniFrac distance matrix (β-diversity) and NMDS (**B**) ordination displaying the segregation of the samples in relation to the batch of origin; significance and R^2 of PERMANOVA are reported in each chart ($P < 0.001$ [FDR adjusted], ANOSIM). Stacked bar plots **(C)** showing mycobiota composition (relative abundance) at the genus/species taxonomic level and relative colour coding key. Samples are grouped by batch in each fermentation mixture and sequentially displayed according to the time; taxa are sorted in the legend from the most to the least abundant (> 0.5 %). Box plots **(D)** displaying Central Logarithmic Transformed Counts (interquartile range/median) of the core species/genera in the two batches. Significance (Wilcoxon's test; *P*-value [FDR adjusted]) is reported in each chart.

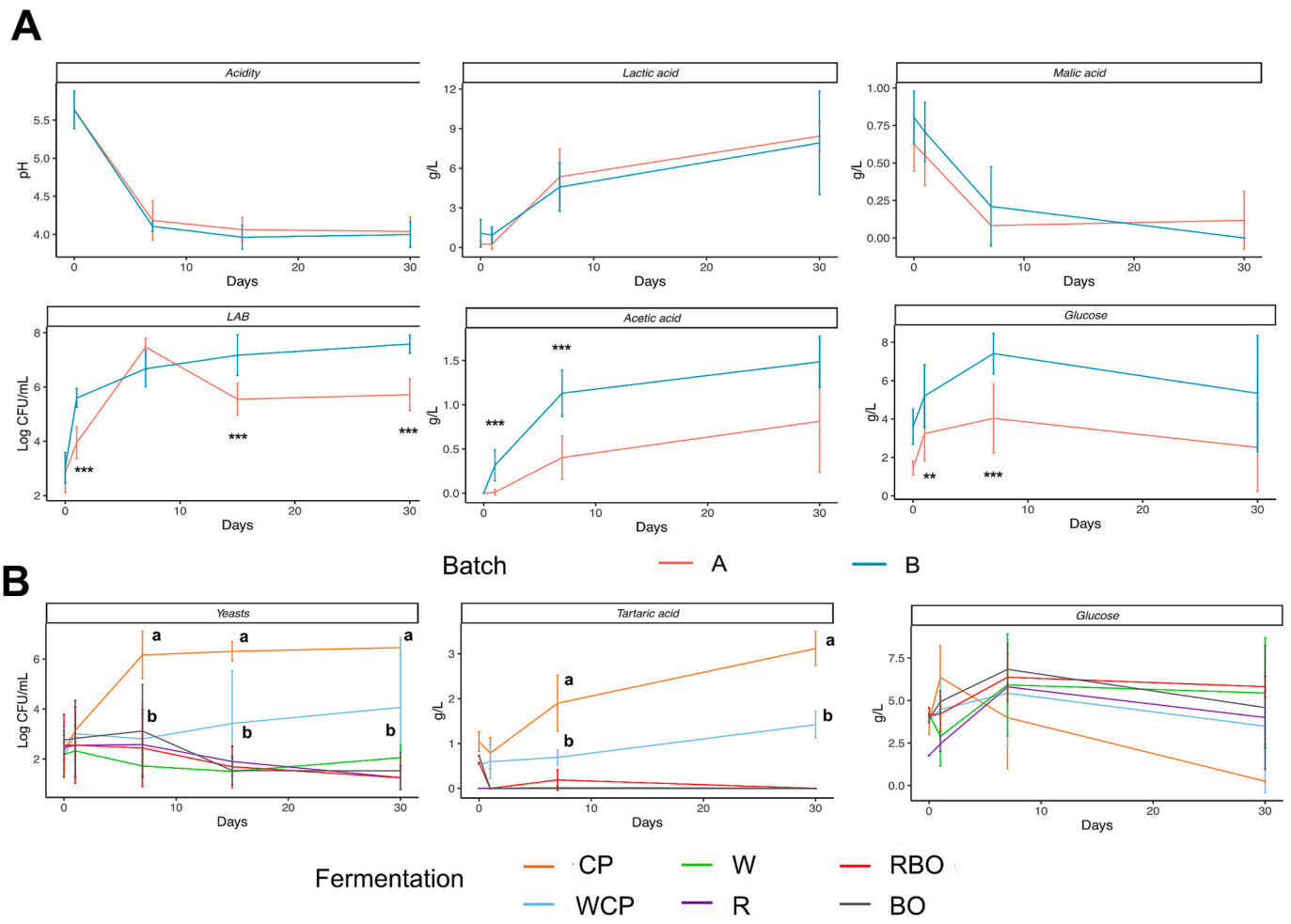

Fig. 3. Microbiological and chemical-physical dynamics during fermentation.
Microbial counts, pH, organic acids and sugars concentrations (mean ± standard deviation) during the thirty days of fermentations grouped in relation to (**A**) the two batches or to (**B**) the fermentation mixtures: carrot/yellow pepper (CP); white cabbage/carrot/yellow pepper (WCP); white cabbage (W); red cabbage (R); red cabbage/beetroot/red onion (RBO); beetroot/red onion (BO). Only dynamics with significant differences between batches, fermentation or along the time are shown; complete dataset and statistics are reported in **Supplementary Table 2 A and B**. Asterisk indicate significant differences between batches at each time point (Pairwise *t*-test; P-value: * ≤0.05; ** ≤0.01, *** ≤0.001), while letters indicate significant differences among fermentation mixtures (ANOVA coupled with Pairwise t-test).

respectively. A strong pH decrease was observed in both batches within the first ten days of fermentation, accompanied by an increase in organic acids concentration (Fig. 3 **A**). In batch B, the increase of acetic acid concentration was significantly faster within the first ten days, which coincided with a more rapid consumption of glucose in the brine (*p-value* < *0.01*). Among the other organic acids detected, production of lactic acid and consumption of malic acid were observed without significant differences between batch A and B (*p-value* > *0.05*). Despite the overall increase in organic acids, glucose was not entirely consumed in most of the fermentations by the end of the process. Unlike the metataxonomics results, the physicochemical parameters varied not only between batches but also among the six fermentation mixtures, indicating an influence of the vegetable composition. Fermentation mixtures containing carrot and peppers (CP, WCP) were characterized by high yeasts presence, complete glucose consumption, and elevated tartaric acid concentration at the end of the process (Fig. 3 **B and Supplementary Table 3**).

Chemical analyses aligned with bacterial growth dynamics, revealing significantly lower LAB loads in batch A compared to batch B (*p-value* < *0.001*). Lactic acid content increased rapidly in both batches, paralleling the rise in LAB counts (Fig. 3 **A**). Other bacteria population dynamics were influenced by chemical and physical characteristics, like the low pH. For instance, *Enterobacteriaceae* and *Staphylococcus* spp. populations were not detected after the first week of fermentation (**Supplementary Table 4**). In contrast, yeast populations remained stable throughout the fermentation process, except in the previously mentioned CP and WCP mixtures.

3.3. Culture-dependent ecology of lactic acid bacteria population and phenotypic characterization

A total of 334 isolates were identified as LAB during the monitored fermentation period, representing the 80 % of the isolates initially collected. The species *Lc. mesenteroides* and *Lpb. plantarum* were the most represented, with 120 and 112 isolates, respectively. These were followed by *Lev. brevis* (43 isolates), *Lat. sakei* (26 isolates), *Lpb. paraplantarum* (20 isolates) and *Lacticaseibacillus* (*Lcb.*) *casei* (11 isolates). Reflecting the batch-related distribution observed in the metataxonomics analysis, *Lat. sakei* was more frequently —or exclusively—isolated in batch A, while *Lev. brevis* and *Lcb. casei* were more frequently isolated in batch B (Fig. 4 **A**). In batch A, the relative presence of *Lc. mesenteroides* and *Lat. sakei* together accounted for over 69 % of the LAB isolates, whereas in batch B, an equivalent portion encompassed *Lpb. plantarum/paraplantarum* and *Lev. brevis*. Regardless of the original

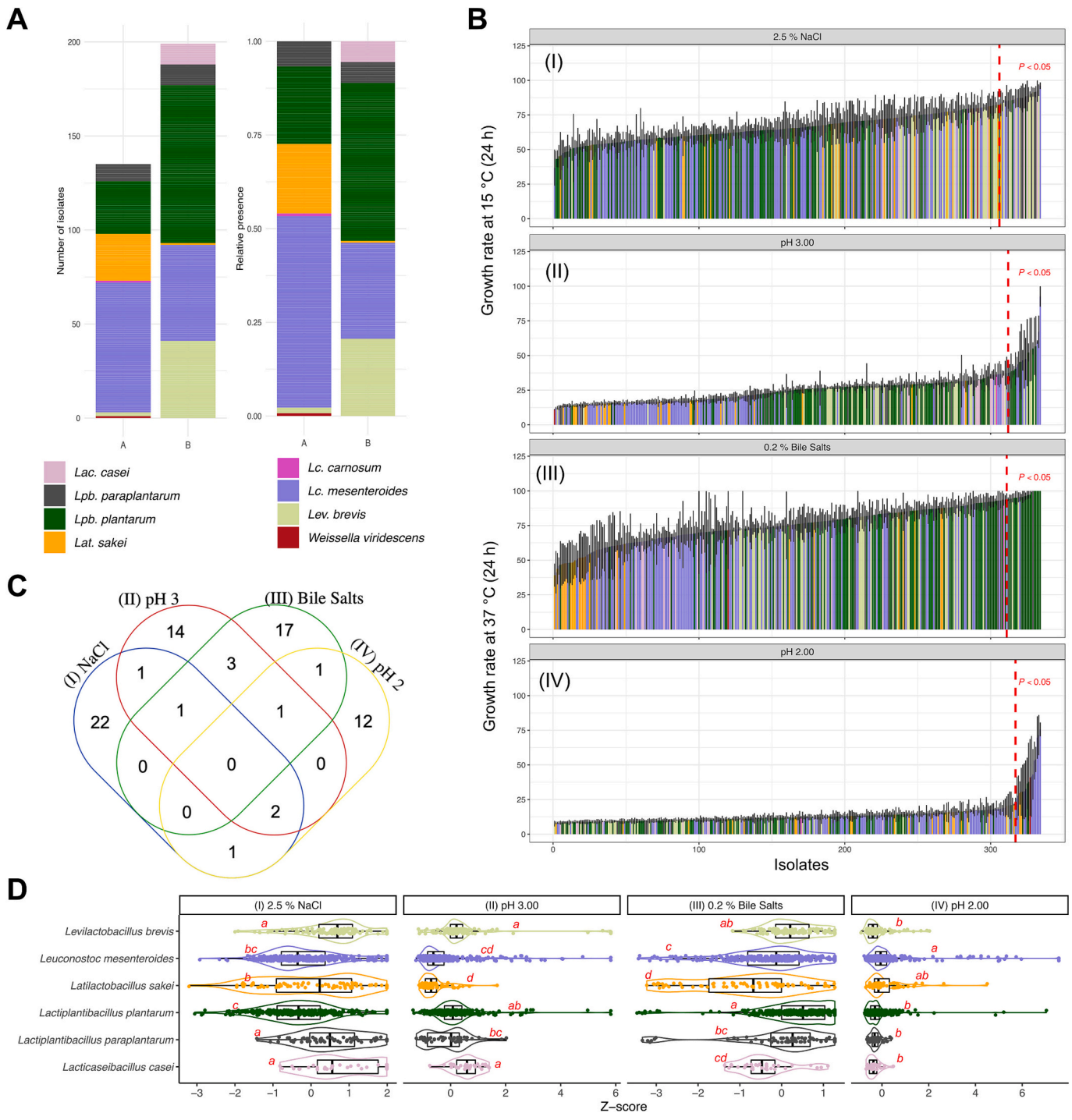

Fig. 4. Culture-dependent composition and phenotypes of the Lactic Acid Bacteria population.
Stacked bar plots (**A**) displaying for each batch the absolute and relative number of LAB isolates. Relative colour coding key of the species assignment refers to all graphs shown in this figure. Bar plots (**B**) of growth rate of each LAB isolate in the incubation/substrate conditions linked to their technological (I, II) and probiotic (III, IV) fitness. In each condition the isolates are sorted following their increasing percentage values (mean ± standard deviation). Growth rate is calculated as: (OD $_{\text{modified MRS T24}}$ – OD $_{\text{modified MRS T0}}$) / (OD $_{\text{MRS T24}}$ - OD $_{\text{MRS T0}}$). Dashed red line segregates isolates with a significantly higher growth rates from the rest of the population (one-tailed z-test; higher than population mean for a *P*-value [FDR] < 0.05). Venn diagram (**C**) displaying the number isolates with significantly higher growth rates in at least one of the four conditions (I, II, III, IV). Comparison (**D**) between growth rates (single observations/interquartile range/median values of data distribution) of six LAB species in the four conditions (I, II, III, IV); different letters (a, b, c, d) highlight significant differences (ANOVA coupled with Tukey's test on z-scores distribution; $P < 0.05$) between species.

batch, *Lpb. plantarum/paraplantarum* were more frequently isolated after the first week of fermentation (70 % of the isolates). Noticeably, *Lc. mesenteroides* was the only LAB species consistently present across both batches and throughout the entire fermentation period, despite the majority of isolates (68 %) being collected within the first seven days.

The growth capability of all LAB isolates was assessed over 24 h under four stress conditions: (I) at 15 °C in presence of 2.5 % of NaCl; (II) at 15 °C and pH 3.00; (III) at 37 °C in the presence of bile salts, and (IV)

at 37 °C and pH 2.00, the latter two simulating the conditions of the upper gastrointestinal tract (Fig. 4 **B**). Compared to salts supplemented media (I, III) and regardless from incubation temperatures, the two low-pH conditions (II, IV) determined a more pronounced growth inhibition and allowed for clearer distinction between isolates with significantly high growth rates and the rest of the population (one-tailed z-test, *P*-value [FDR] < 0.05).

In total, seventy-five LAB isolates showed growth rates exceeding the population mean in at least one of the four conditions tested and were selected for further characterization. Only a few isolates in this group demonstrated high growth performance across multiple stress conditions (Fig. 4 **C**).

When comparing species-level performances (Fig. 4 **D**), *Lc. mesenteroides* and *Lpb. plantarum* showed significantly higher capability to growth at pH 2.00 and in presence of bile salts, respectively (Tukey's test, $P < 0.05$). Moreover, *Lc. mesenteroides* isolates from batch A displayed significantly higher growth rates in presence of NaCl, whereas isolates from batch B showed grater performances in acidified medium (data not shown).

3.4. Simulated digestion and antimicrobial resistance of the selected isolates

The seventy-five selected isolates were subjected to simulated digestion (Fig. 5). At the species level, a significant reduction of LAB viability was observed after the gastric step (GSP), while the subsequent exposure to duodenal conditions did not further decrease their overall survival rate (CSD). Notably, for *Lc. mesenteroides* and *Lpb. paraplantarum*, counts even increased after the CSD phase compared to post-GSP levels (Fig. 5 **A**). Among all species, *Lpb. plantarum* exhibited the greater survival capability after both GSP and CSD (*P*-value <0.001).

Regardless of species, twelve isolates showed significantly higher

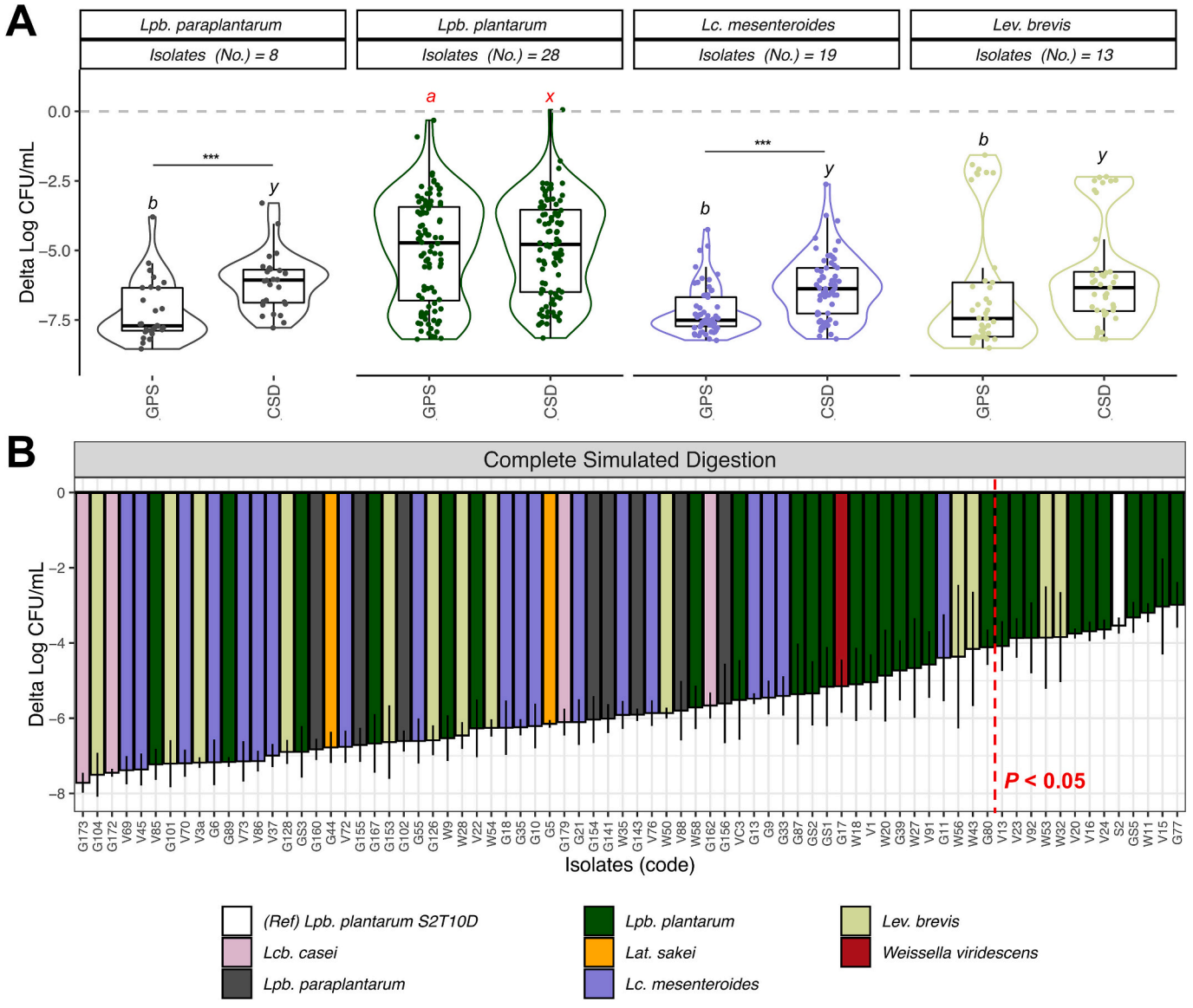

Fig. 5. Survival rate after simulated human digestion.
Comparison (**A**) between survival capability of four species after gastric step passage (GSP) and after complete simulated digestion (CSD). Data are expressed as Delta Log (single observations/interquartile range/median values of data distribution) following the formula: Log CFU/mL $_{(T1 \text{ or } T2)}$ − Log CFU/mL $_{T0}$. Asterisks (***) highlight significant differences between GSP and CSD values (t-test; *P*-value <0.001); different letters highlight significant (ANOVA and Tukey's test; $P < 0.001$) differences among species survival rate after GPS (a, b) and CDS (x, y). Bar plots (**B**) of survival rate after CSD (mean ± standard deviation). Isolates are sorted by increasing survival rate: dashed red line segregates all isolates with a significantly higher survival rates to CSD from the rest of the population (one-tailed z-test; higher than population mean for a *P*-value [FDR] < 0.05). Relative colour coding key of the species assignment refers to all graphs shown in this figure.

survival rates to CSD compared to the rest of the population (one-tailed z-test, *P*-value [FDR] < 0.05; Fig. 5 **B**).

Antibiotic susceptibility test was conducted on these twelve high-survival isolates (Table 1). All ten *Lpb. plantarum* showed antimicrobial resistance to ampicillin while nine and six isolates were resistant to chloramphenicol and tetracycline, respectively. Only one isolate was resistant to kanamycin. Both *Lev. brevis* isolates were found to be resistant to ampicillin, tetracycline and chloramphenicol. Gentamycin, kanamycin, streptomycin, erythromycin and clindamycin were effective against all LAB tested.

4. Discussion

Handcrafted fermentation of fresh vegetables in brine represents one of the oldest and most widespread method to preserve these perishable foods. Their nutraceutical quality and stability may be significantly improved through the use of functional cultures tailored to specific vegetable mixtures and fermentative parameters. To support the selection of such cultures, the fermentation processes of six handcrafted vegetable mixtures were comprehensively characterized across two production batches, aiming to identify the key drivers behind their microbial variability and fermentative dynamics.

Metataxonomic and culture-based analyses suggested a marked influence of the initial microbial contamination, human handling, and processing environment in shaping the FVs microbiota of each batch. In contrast, the specific nutrients released in the brine by the different vegetable mixtures appeared to play comparatively a minor role. Batch-to-batch variability is common in FVs (Huang et al., 2022; Liu et al., 2023; Lu et al., 2023), as well as in other minimally processed foods (Botta et al., 2023; Botta, Franciosa, et al., 2022). Notably, sensory and microbiological characteristics of fermented cabbages may vary significantly even within the same batch of raw vegetable in relation to minimal differences in storage conditions (Min et al., 2022). In this context, it is important to acknowledge that our metataxonomic survey could not capture the original microbiota composition of the raw vegetables, as samples collected on the day of production were excluded due to insufficient microbial sequence data. This limitation is expected, given that freshly washed vegetables typically carry low microbial loads and consequently yield low amounts of microbial DNA (Tommasi et al., 2021). In such samples, plant-derived contaminant amplicons may dominate the sequencing output (Mantegazza et al., 2023). Nevertheless, a comprehensive investigation of contamination pathways in these handcrafted fermentations was beyond the scope of this study, which was primarily focused on the selection of functional microbial cultures.

The pro-technological group of LAB was predominant in these FVs, as commonly observed in lacto-fermented salted cabbage and in fermentations of mixed vegetables. In these products, it determines a fast lactic acid accumulation from the early stages with a consequent pH decrease that leads to a final microbial stability and safety (Aguirre-Garcia et al., 2024; Cardinali, Botta, Harasym, Ferrocino, et al., 2024; Min et al., 2022). Bio-acidification conducted by LAB limits the development of spoilage species of *Enterobacteriaceae* and potentially harmful *Staphylococcus* spp. (Li et al., 2022), which indeed were not enumerated in these products after the first week by microbial culture analyses, and not detected at all by the metataxonomic profiling.

Reflecting changes in overall microbiota composition, the distribution of LAB taxa varied primarily by batch and, to a lesser extent, by fermentation time. Culture-based and metataxonomic analyses largely agreed on LAB distribution and dynamics. This concordance aligns with previous findings in food processing environments and minimally processed foods, where dynamics and spatial distribution of the main bacterial populations were captured similarly by culturing and 16S rRNA-gene based sequencing (Botta et al., 2020; Botta et al., 2024; Botta, Franciosa, et al., 2022). An exception was observed for *Lpb. plantarum*, whose high prevalence from the middle-late fermentation stages was only captured through isolation. This discrepancy likely reflects differences in species cultivability under laboratory conditions, as even slight variations in temperature or growth media composition can preferentially support the proliferation of certain taxa (Walsh et al., 2022).

The high prevalence of *Lc. mesenteroides* during the early fermentative stages has been observed in Sauerkraut (Plengvidhya et al., 2007; Touret et al., 2018) and Kimchi (Ahmadsah et al., 2015), where it may also predominate through the entire fermentation period (Jung et al., 2011). *Lc. mesenteroides* is known to be ubiquitous in plant-based raw materials stored at cold temperature as it is psychotropic. Moreover, it can easily conduct the first fermentation phases of vegetables in brine due to its high salt tolerance (Chun et al., 2017). Later in the vegetable fermentation process other species of *Lactobacillaceae* are usually taking over the dominance (Jung et al., 2011). The development of *Lat. sakei* after the first week of fermentation observed in batch A is characteristic

Table 1
Antibiotic resistance profiles, technological/probiotic phenotypes and origin of selected lactic acid bacteria isolates.

Species	Isolate code	Antibiotic susceptibility								Phenotypes					Origin		
		AMP	GEN	KAN	STR	ERY	CLI	TET	CHL	(I) NaCl	(II) pH 3	(III) Bile	(IV) pH 2	CSD	Batch	Fermentation	Days
Lpb. plantarum	V92	R	S	S	S	S	S	S	S	ns	***	ns	ns	*	A	RBO	30
	V24	R	S	S	S	S	S	S	R	ns	ns	*	ns	**	A	BO	1
	G77	R	S	S	S	S	S	S	R	ns	ns	ns	***	**	A	WCP	30
	V13	R	S	S	S	S	S	S	R	ns	**	*	ns	*	A	R	1
	GS5	R	S	S	S	S	S	R	R	ns	ns	*	ns	**	A	CP	30
	V23	R	S	S	S	S	S	R	R	ns	ns	*	ns	*	A	BO	1
	V20	R	S	S	S	S	S	R	R	ns	ns	*	ns	*	A	RBO	1
	V16	R	S	S	S	S	S	R	R	ns	**	*	**	*	A	RBO	1
	V15	R	S	S	S	S	S	R	R	ns	ns	*	ns	***	A	R	1
	W11	R	S	R	S	S	S	R	R	ns	ns	*	ns	**	B	BO	7
Lev. brevis	W53	R	S	S	S	S	S	R	R	ns	***	ns	ns	*	B	BO	15
	W32	R	S	S	S	S	S	R	R	*	ns	ns	ns	*	B	RBO	7

Isolates tested with microdilution standard method (ISO 10932:2010) were classified as resistant (R) or sensitive (S) to Ampicillin (AMP), Gentamicin (GEN), Kanamycin (KAN), Streptomycin (STR), Erythromycin (ERY), Clindamycin (CLI), Tetracycline (TET) and Chloramphenicol (CHL). EFSA cut-off values were used according to the minimum inhibitory concentrations (MICs) method. Isolation origin, phenotypes in growth rate (pH 2, pH 3, bile salts, NaCl), and survival rate to complete simulated digestion (CSD) are reported.
Significance of higher growth rates or survival to CSD are indicated with asterisks (one-tailed z-test; higher than population mean for a *P*-value [FDR] < 0.05):
*** = $P < 0.001$.
** = $P < 0.01$.
* = $P < 0.05$.
ns = $P > 0.05$.

in Kimchi (Ahmadsah et al., 2015), whereas the predominance of *Lev. brevis* and *Lpb. plantarum* in the mid-to-late stages of fermentations, as seen in batch B, is typical in Sauerkraut (Plengvidhya et al., 2007; Touret et al., 2018). The greater presence of facultative and obligate heterofermentative LAB in batch B is likely linked to the higher production of acetic acid observed in the middle stages of all these fermentations. However, final acidity and lactic acid accumulation in this batch was not lower than batch A, despite heterofermentative LAB are considered less efficient in producing organic acids (Xiong et al., 2016).

Moreover, no deacidification phenomena related to yeasts presence occurred during fermentation (Ferreira & Mendes-Faia, 2019). Yeasts were indeed detected in low amounts, with the exception of mixtures containing carrots (CP, WCP), which showed a greater presence of tartaric acid. Notably, this organic acid cannot be produced through fermentation, and its concentration has been found to increase in shredded carrots during storage, being released by this root vegetable (Bergqvist et al., 2005; Kakiomenou et al., 1996). Since tartaric acid inhibits bacteria but not fungi (Karpiński & Ożarowski, 2024; Liang et al., 2022), its presence in carrot-containing mixtures may have favoured the yeasts growth. However, the overall low yeast counts suggest that this population is unlike to actively influence the final characteristics of these FVs: for instance, reducing anti-nutritional levels of cabbages (Ananda et al., 2019). The composition of the mycobiota here resembled that observed in Kimchi (Maoloni et al., 2020), with *Hanseniaspora* spp. and *S. cerevisiae* being characteristic of batch A and B, respectively.

The systematic and progressive selection of functional LAB cultures with technological and probiotic properties have undoubtedly benefited from the observational metataxonomic analyses. The selected cultures will be tested in FVs with similar ecological profiles to improve their quality stability. However, they could also be successfully employed as starters in other fermentation processes, contributing to improve their nutraceutical characteristics (Botta et al., 2014; Botta et al., 2015). This is particularly true for *Lpb. plantarum*, a natural inhabitant of plant-based foods that can readily adapt to diverse fermentative substrates due to its large and highly flexible genome (Botta et al., 2017; Pasolli et al., 2020). Compared to *Lc. mesenteroides*, *Lat. sakei* and *Lev. brevis*, the *Lpb. plantarum* isolates showed a major capability to grow in cold-acid conditions and in presence of bile salts, as previously reported in a cabbage-based fermentation study (Lee et al., 2016; Szutowska & Gwiazdowska, 2021).

Although LAB isolates of non-intestinal origin exhibit intermediate levels of bile salts hydrolase (BSH) activity (Ru et al., 2019), *Lpb. plantarum* possesses this enzymatic function and is typically characterized by high bile salts tolerance (C. F. Guo et al., 2015). The presumptive BSH activity of *Lpb. plantarum* may also explain the high survival rate of its isolates during the complete simulation of human digestion (CSD), where gastric acidity and digestive enzymes challenged bacterial viability. Resistance to low pH is another strain-specific trait that is not commonly observed in most foodborne LAB (De Albuquerque et al., 2018; E. F. Garcia et al., 2016). When interpreting CDS survival results, it is also important to consider the generally higher survival rate of LAB cultures when incorporated into food matrices, due to the buffering and protective effects of components such as lipids and proteins (Botta et al., 2014, Botta et al., 2015).

The antibiotic resistance of the twelve selected functional strains was assessed as part of their final safety evaluation. Most of the antibiotics tested were effective against *Lpb. plantarum* and *Lev. brevis* isolates, although we observed a generalized resistance to ampicillin, tetracycline and clindamycin. Unlike what was observed in this study, *Lpb. plantarum* isolates from table olive fermentations were resistant to aminoglycoside antibiotics like kanamycin and gentamicin (Botta et al., 2014). Resistance to aminoglycosides is considered an intrinsic feature of *Lactobacillaceae* (Campedelli et al., 2019), which can also acquire resistance towards antibiotics that inhibit synthesis of bacterial wall, such as ampicillin and tetracycline (Zarzecka et al., 2020). In particular, *Lev. brevis* and *Lpb. plantarum* isolates were found to be resistant to ampicillin and tetracycline (Campedelli et al., 2019). Overall, the presence of antibiotic-resistant LAB in FVs is not uncommon (Duche et al., 2023; Haryani et al., 2023). However, this does not pose a direct safety risk unless it is accompanied by antibiotic-resistance genes and mobile genetic elements, which can only be identified through further genome analyses.

Apart from safety considerations, it is noteworthy that nine out of the ten final selected *Lpb. plantarum* strains were isolated from FVs of batch A, which were characterized by more homofermentative environments. In this context, recent findings have shown that *Lpb. plantarum* isolates from homofermentative dairy fermentations exhibit better probiotic performance compared to those isolated from vegetable-based fermentations, which are typically characterized by more heterofermentative activities (S. J. Guo et al., 2024). It can be hypothesized that more stressful conditions present in the FVs of batch B led to a selection of strains with lower probiotic fitness: i.e., minor resistance to bile salts and simulated digestion. Despite its well-known metabolic versatility, *Lpb. plantarum* may be subject to oxidative stress under heterofermentative conditions (Stevens et al., 2023).

However, due to the observational nature of this study, this hypothesis cannot be confirmed without further investigation. We also acknowledge that the limited scope of this study —based on two batches—may not fully reflect the broader microbiota variability present in these FV mixtures. Targeted phenotypic analyses involving a larger number of independent fermentations will be required to validate these findings (Polak-Berecka et al., 2018).

5. Conclusion

To conclude, the metataxonomic survey integrated with culture-based isolation, revealed the succession of LAB taxa driving these fermentations, and demonstrated how even minimal batch-to-batch microbiota variation can significantly affect the isolation of potential probiotic cultures. Notably, the establishment of a heterofermentative microbiota—and a resulting heterofermentative environment—appears to reduce the probiotic potential of LAB isolates, particularly their resistance to digestive enzymes and acidic conditions. These findings underscore the need of carefully considering the fermentative microbial succession of LAB in studies aimed at identifying novel probiotic strains from artisanal fermentations. Among the isolates, *Lpb. plantarum* strains consistently emerged as the most promising candidates for the development of multifunctional starter-probiotic cultures. Further phenotypic and genomic characterizations are now required to fully validate the probiotic and technological effectiveness of these selected strains.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.117039.

CRediT authorship contribution statement

Cristian Botta: Writing – original draft, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Daniele Spada:** Writing – review & editing, Investigation, Conceptualization. **Davide Buzzanca:** Writing – review & editing, Data curation. **Negin Seif Zadeh:** Investigation, Writing – review and editing. **Dimitra Tsoureki:** Writing – review & editing, Investigation. **Federica Biolcati:** Writing – review & editing, Investigation. **Ilario Ferrocino:** Writing – review & editing, Investigation. **Giuseppe Zeppa:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Paola Dolci:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgements

The authors thank all students involved in the experiments: Giulia Basanisi, Martina Genova, Edoardo Gobbato, Elisa Montanari, Chiara Sismondi, Xiaoqing Wang.

Data availability

Links to the data repository and code used in this study are provided in the manuscript. Additional datasets and supporting materials can be made available from the corresponding author on request.

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