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Selection of lactic acid bacteria as biopreservation agents and optimization of their mode of application for the control of *Listeria monocytogenes* in ready-to-eat cooked meat products

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ABSTRACT

In order to meet consumers' demands for more natural foods and to find new methods to control foodborne pathogens in them, research is currently being focused on alternative preservation approaches, such as biopreservation with lactic acid bacteria (LAB). Here, a collection of lactic acid bacteria (LAB) isolates was characterized to identify potential biopreservative agents. Six isolates (one *Lactococcus lactis*, one *Lacticaseibacillus paracasei* and four *Lactiplantibacillus plantarum*) were selected based on their antimicrobial activity in in vitro assays. Whole genome sequencing showed that none of the six LAB isolates carried known virulence factors or acquired antimicrobial resistance genes, and that the *L. lactis* isolate was potentially a nisin Z producer. Growth of *L. monocytogenes* was successfully limited by *L. lactis* ULE383, *L. paracasei* ULE721 and *L. plantarum* ULE1599 throughout the shelf-life of cooked ham, meatloaf and roasted pork shoulder. These LAB isolates were also applied individually or as a cocktail at different inoculum concentrations (4, 6 and 8 $\log_{10}$ CFU/g) in challenge test studies involving cooked ham, showing a stronger anti-Listerial activity when a cocktail was used at 8 $\log_{10}$ CFU/g. Thus, a reduction of up to ~5.0 $\log_{10}$ CFU/g in *L. monocytogenes* growth potential was attained in cooked ham packaged under vacuum, modified atmosphere packaging or vacuum followed by high pressure processing (HPP). Only minor changes in color and texture were induced, although there was a significant acidification of the product when the LAB cultures were applied. Remarkably, this acidification was delayed when HPP was applied to the LAB inoculated batches. Metataxonomic analyses showed that the LAB cocktail was able to grow in the cooked ham and outcompete the indigenous microbiota, including spoilage microorganisms such as *Brochothrix*. Moreover, none of the batches were considered unacceptable in a sensory evaluation.

Overall, this study shows the favourable antilisterial activity of the cocktail of LAB employed, with the combination of HPP and LAB achieving a complete inhibition of the pathogen with no detrimental effects in physico-chemical or sensorial evaluations, highlighting the usefulness of biopreservation approaches involving LAB for enhancing the safety of cooked meat products.

Abbreviations: NC, non-inoculated negative control; PC, positive control, only inoculated with *L. monocytogenes*; LAB, samples inoculated only with lactic acid bacteria; V, samples packaged under vacuum; MAP, samples packaged under modified atmosphere packaging; HPP, samples packaged under vacuum and treated with high pressure processing.

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1. Introduction

Foodborne outbreaks caused by consumption of contaminated meat and meat products occur recurrently, with important negative impacts for public health and food business operators (Omer et al., 2018). The bacteria responsible for the five more frequent zoonoses associated with food consumption are *Campylobacter jejuni/coli*, *Salmonella enterica*, Shiga toxin-producing *Escherichia coli*, *Yersinia enterocolitica* and *Listeria monocytogenes*, commonly associated with consumption of meat and meat products. Among these foodborne pathogens, *L. monocytogenes* is a cause of major concern as it has the highest fatality rate (13.7 % in Europe) (EFSA and ECDC, 2022). This pathogen can be a biosafety concern, as it is frequently isolated from food contact surfaces such as tables, trays, knifes, conveyor belts, slicers and other industrial surfaces, which demonstrates its ability to adhere, colonize and survive in processing environments in biofilm state (Alvarez-Molina et al., 2021; Bolocan et al., 2015). In addition, some *L. monocytogenes* lineages show tolerance to different adverse environmental conditions prevailing along the food chain, such as refrigeration temperatures, disinfectants or acids, which are common control strategies (Hingston et al., 2017).

One of the most notable listeriosis outbreaks during the last decade, reported in Spain in August 2019, was linked to the consumption of a ready-to-eat (RTE) cooked meat product and involved 227 confirmed cases (WHO, 2019). Those RTE meat products that are minimally processed or not subjected to thermal inactivation, or that have long shelf-life allowing psychrotrophic bacteria to multiply at cold temperatures, are of special concern. In meat products, the risk of *L. monocytogenes* contamination is affected by multiple factors, such as the cross-contamination of the product during production operations, like stuffing, slicing or packaging, or the absence of growth inhibitors in the formulation. Indeed, retail-sliced deli meats present higher risk than pre-packaged sliced deli meats, while the risk is reduced if growth inhibitors are used in the formulation of cooked meat products (EFSA, 2018a). Different bacterial growth inhibitors, such as organic acids (sorbic, lactic and acetic acids), nitrites or sulphites, are commonly employed in RTE meat products with this aim (Luchansky et al., 2023; Punia Bangar et al., 2022). Although these additives are very efficient and are considered safe, some of them are under rigorous evaluation due to possible health risk concerns under some circumstances (Flores and Toldrá, 2021). This, together with the increased demand by consumers for more natural and minimally processed products, is driving researchers to look at new alternatives to preserve foods whilst ensuring quality and safety. Most of such research initiatives are being currently focused on the discovery and/or characterization of agents which control the growth of *L. monocytogenes* and other relevant foodborne pathogens (Martín et al., 2022; Serra-Castelló et al., 2022).

The use of lactic acid bacteria (LAB) as protective cultures in food has received wide research attention in the last few decades, being the main topic of several original research studies and review articles (Barcenilla et al., 2022; Martín et al., 2022; Ramaroson et al., 2018; Vieco-Saiz et al., 2019). Most LAB are Generally Recognized As Safe (GRAS) and/or have Qualified Presumption of Safety (QPS) status as they have been traditionally employed in fermentation processes (EFSA, 2023). These include representative species from *Carnobacterium*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus* or the former *Lactobacillus* genus, recently reclassified into twenty-five new genera (EFSA, 2022; Zheng et al., 2020). Some of these LAB display promising antimicrobial properties, being able to inhibit the growth of undesirable microorganisms either by direct competition or by production of antimicrobial peptides (e.g., bacteriocins) or other substances with antimicrobial properties (Di Gioia et al., 2016). Yet, LAB are scarcely employed in meat products other than in fermented foods.

Bacteriocins have been widely studied as food biopreservatives (Field et al., 2018; Soltani et al., 2021). These peptidic compounds are ribosomally synthesized by bacteria and can inhibit different pathogens, such as *E. coli* (Arief et al., 2012), *L. monocytogenes* (Balay et al., 2017; Ruiz et al., 2010), *Salmonella Typhimurium*, or *Staphylococcus aureus* (Chakchouk-Mtibaa et al., 2017), depending on their specific antimicrobial spectrum. They can be applied to food either through direct addition as purified or semi-purified compounds (Yildirim et al., 2016), or can be produced in situ by the producing strain(s) used as food culture (s) (Hu et al., 2019). Nevertheless, to date, the only purified bacteriocin allowed as an additive in the European Union is nisin (E234) (European Parliament, 2008). In addition, pediocin PA-1 is commercialized as a crude extract obtained following fermentation by the producing strain (Back et al., 2016), and colicins and salmocins have received favourable regulatory reports by the FDA (FDA, n.d.; Hahn-Löbmann et al., 2019). Their sometimes narrow spectrum of activity, uneven distribution in or binding to the food matrix, susceptibility to inactivation by proteolytic enzymes, or the possible development of bacterial resistance to them are among the main current limitations to the use of bacteriocins as biopreservation agents in the food industry (Soltani et al., 2021).

The aims of this study were to identify LAB with promising antimicrobial activities and to develop novel strategies for the biopreservation of RTE cooked meat products based on their efficacy as protective cultures. To achieve this objective, a collection of 479 isolates of LAB was evaluated to detect strains capable of inhibiting the growth of *L. monocytogenes* and *E. coli* in culture media. We selected six strains that were further characterized through whole genome sequencing (WGS) and tested for the inhibition of *L. monocytogenes* growth in three different RTE cooked meat products. The most promising isolates were further assessed, applied individually or as a strain cocktail, at different inoculation levels, in exhaustive challenge test studies with cooked ham, where the growth potential of *L. monocytogenes*, some physicochemical and sensory characteristics of the product, as well as the microbiota profile, were monitored throughout the shelf-life under defined conditions of storage and commercialization.

2. Materials and methods

2.1. Lactic acid bacteria collection

A LAB collection comprising 479 isolates obtained in a previous research project where environmental samplings were performed in >30 Spanish processing plants (unpublished data), mainly from the meat and dairy sector, was used. They had been recovered from food- and non-food contact surfaces, such as tables, equipment, trays, drains, floors, and walls from different areas of the facilities (processing, cold storage, ripening and packaging areas) by using HydraSponge sterile swabs (3 M, USA). They had been enriched at 30 °C for 18–24 h in 100 mL Buffered Peptone Water (BPW, Merck, Germany) and subsequently plated on De Man, Rogosa and Sharpe (MRS, Merck) agar plates incubated at 30 °C for 72 h under anaerobic conditions (Anaerocult A, Merck).

A presumptive identity confirmation relying on their protein profile was obtained by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS, Microflex LT model, Bruker-Daltonics, USA). Briefly, each isolate was inoculated on MRS agar plates and after its growth for 24 h at 37 °C one single colony was spread on a well of the MSP96 Bruker steel plate. Afterwards, 1 μL of matrix (containing α-cyano-4-hydroxycinnamic acid, tri-fluoroacetic-acid (TFA) and acetonitrile) was added to the well and left to dry for 5 min in a laminar flow hood. For calibration, one well of the plate was spotted with the Bruker BTS standard. The MALDI Biotyper software (Bruker Daltonics) was employed for spectra interpretation and identity assignment.

A schematic figure showing the full workflow is shown in Fig. 1.

2.2. In vitro assays for antimicrobial activity assessment

The antimicrobial activity of 164 LAB strains was assessed using spot-on-lawn and wells-in-agar assays with *L. monocytogenes* CECT 911 (serovar 1/2c) and *E. coli* CECT 515 (serotype O1:K1(L1):H7) as

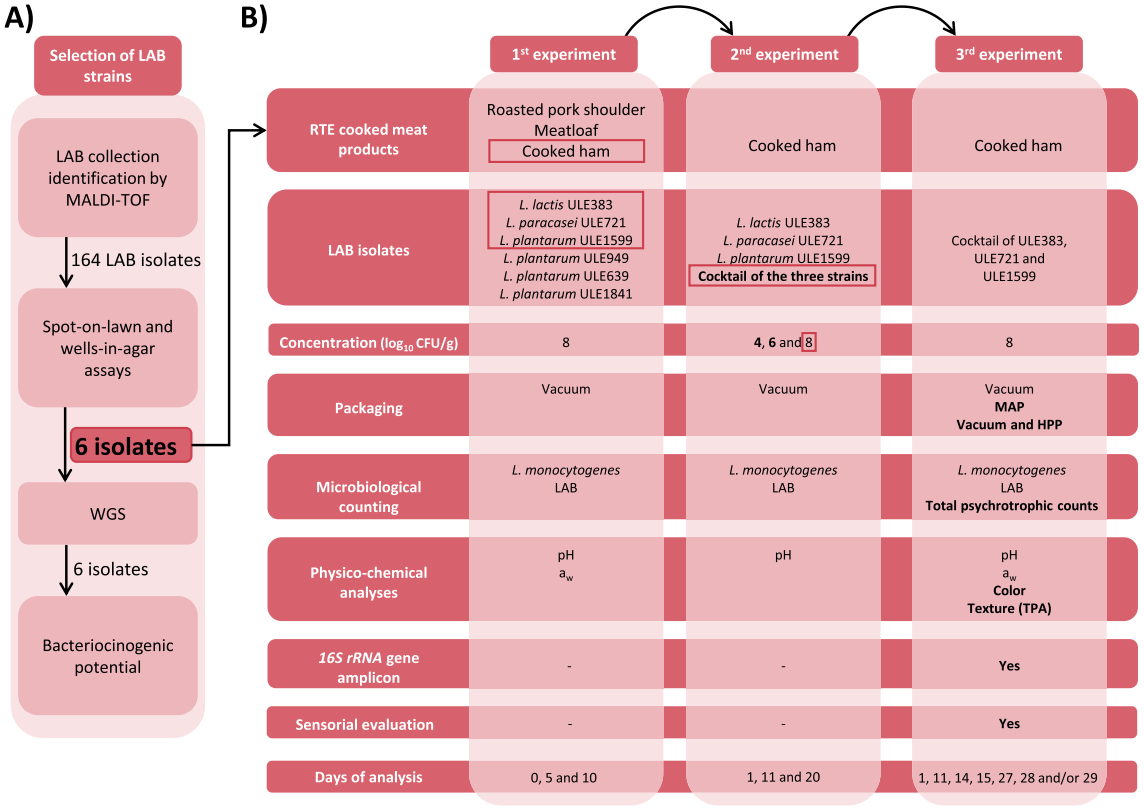

Fig. 1. Design of the study. A) Identity confirmation of the LAB strain collection, in vitro characterization of the isolates of interest, and subsequent WGS characterization of the six selected LAB strains; B) Experiments performed in different RTE meat products with the addition of the selected LAB strains. Those conditions selected to be used in follow-up challenge test experiments are highlighted with red boxes, while bold text indicates new analyses undertaken.

indicator strains, both obtained from the Spanish Type Culture Collection (*Colección Española de Cultivos Tipo - CECT*).

For spot-on-lawn assays, LAB strains were grown in MRS broth and incubated at 30 °C for 24 h under anaerobiosis. Then, 2 μL of the bacterial suspensions were spotted onto MRS agar plates in triplicate. After 24 h of anaerobic incubation at 30 °C, the grown bacteria were inactivated through exposure of the lid-opened inverted agar plates to filter paper discs saturated with chloroform for 20 min. The plates were then overlaid with approximately 20 mL of Brain Heart Infusion broth (BHI, Merck) supplemented with 0.75 % agar and previously inoculated with 200 μL of an overnight culture of the *L. monocytogenes* or *E. coli* target strain, grown at 37 °C in BHI and Luria-Bertani (LB, Merck) respectively, to reach a concentration of approximately 8 $\log_{10}$ CFU/mL. After aerobic incubation at 37 °C for 24 h, the plates were examined to identify and measure growth inhibition zones surrounding each spot (Hoover and Harlander, 1993; Leite et al., 2015).

For the wells-in-agar assays, LAB previously confirmed as having antagonistic activity in the spot-on-lawn assays were grown in MRS broth as earlier described. After 24 h of anaerobic incubation, the bacterial suspensions were centrifuged for 10 min at 7000 xg and the cell-free-supernatants (CFS) were collected. Meanwhile, molten (48 °C) BHI agar was inoculated with an overnight culture of the *L. monocytogenes* or *E. coli* target strain to reach a concentration of 8 $\log_{10}$ CFU/mL, and poured into Petri dishes which were air-dried for 30 min. Wells were made onto the agar plates using a Durham tube and 50 μL of the CFS were added to the corresponding wells in triplicate. A period of 2 h at room temperature was used for a better diffusion to occur. Finally, the plates were incubated at 37 °C for 24 h. The diameters of the growth inhibition zones were expressed as arbitrary units. One arbitrary unit (AU) of antimicrobial activity was defined as equal to 1 mm of diameter. In both assays, a *L. lactis* strain with previously observed antagonistic activity against the target bacteria was used as a positive control and non-inoculated MRS broth was also employed as a negative control.

2.3. Characterization of LAB by whole genome sequencing

DNA from six selected LAB strains, namely ULE383, ULE639, ULE721, ULE949, ULE1599 and ULE1841, was extracted using the DNeasy® PowerSoil® Pro Kit (Qiagen, Netherlands) following the manufacturer's instructions, but with a final elution with 25 μL of Solution C6 (10 mM Tris) in order to increase DNA concentration. The final DNA concentration was measured with a Qubit fluorometer using the dsDNA HS assay kit (Invitrogen, Thermo Fisher Scientific, USA).

The 150 bp paired-end libraries were prepared with the extracted DNA using the Nextera XT DNA Library Preparation kit (reference guide 15,031,942 v03) (Illumina Inc., San Diego, CA, USA) at Macrogen Inc. (Seoul, Korea). Sequencing was performed on an Illumina NovaSeq6000 platform following standard Illumina sequencing protocols.

2.3.1. Filtering and assembly

Filtering of raw reads was performed with TrimGalore (https://github.com/FelixKrueger/TrimGalore) using *–stringency 5 –length 75 –quality 20 –max_n 2 –trim-n* parameters. Genome assembly was performed using SPAdes v3.15.2 (Prjibelski et al., 2020) with k-mer lengths of 55, 75 and 97. The quality of the genomes was checked with CheckM v1.1.3 (https://github.com/Ecogenomics/CheckM).

2.3.2. Taxonomic assignment

The genomes were taxonomically assigned by GTDB-Tk v1.7.0 (Chaumeil et al., 2020) with default parameters. To complement the results, dRep v 2.6.2 (Olm et al., 2017) was employed to elaborate a phylogenetic tree based on ANI distances (95 %) using reference genomes from the National Center for Biotechnology Information (NCBI -

https://www.ncbi.nlm.nih.gov/).

2.3.3. Detection of antimicrobial resistance, virulence genes, plasmids and other mobile genetic elements

Two different databases were used to detect known antimicrobial resistance genes (ARG). The web-server ResFinder v4.1 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017) from the Centre for Genomic Epidemiology (CGE) was firstly employed with cut-offs of 70 % percentage of identity (ID) and 60 % length of coverage. Secondly, the genome sequences were aligned against the protein sequences from ARG included in Resistance Gene Identifier (RGI) v6.0.1 using the default parameters and the Perfect, Strict and Loose hits criteria from the Comprehensive Antibiotic Resistance Database (CARD) v3.2.6 (Alcock et al., 2023). Only hits with >70 % ID were retained for analysis.

The presence of genes coding for known virulence factors was assessed by using VirulenceFinder v2.0.3 (Camacho et al., 2009; Joensen et al., 2014; Tetzschner et al., 2020) from the CGE with the cut-offs of 85 % ID and 60 % length of coverage.

Detection of plasmids was achieved via two tools from the CGE: PlasmidFinder v2.0.1 (Camacho et al., 2009; Carattoli et al., 2014), with 70 % ID and 60 % length of coverage, and MobileElementFinder v1.0.3 (Johansson et al., 2021). Insertion sequences or transposons were also analysed with MobileElementFinder.

2.3.4. Bacteriocin production prediction

The potential of each strain to produce bacteriocins or ribosomally synthesized and post-translationally modified peptides (RiPPS) was also determined. For that purpose, the genome fasta file of each of the six sequenced strains was uploaded to the webserver BAGEL4 (Van Heel et al., 2018). A comparison of the predicted nisin Z with a previously described nisin Z was performed at amino acid level using BLASTP (Mulders et al., 1991).

2.4. Assessment of the bacteriocinogenic potential of the strains

The six selected LAB isolates (ULE383, ULE639, ULE721, ULE949, ULE1599 and ULE1841) were grown in MRS broth as previously described and the bacterial suspensions were centrifuged at 16,000 ×*g* for 2 min. A culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 was also prepared in MRS and incubated overnight at 37 °C as the target microorganism. Then, 20 mL of molten MRS agar was inoculated with 100 μL of the target overnight culture, and solidified in a petri dish. Afterwards, wells were made in the agar and 50 μL of LAB CFS, obtained as earlier described, were added to each well. Finally, the plates were incubated at 37 °C for 24–48 h and examined for inhibition zones.

2.4.1. Confirmation of nisin Z production

To confirm that *L. lactis* ULE383 produced nisin Z, a single colony of the strain from a GM17 (Oxoid, Ireland) plate was inoculated into 5 mL of GM17 broth. Following overnight incubation at 30 °C this culture was used to inoculate 250 mL of Tryptone Yeast (TY) broth (Merck, Ireland) which was again incubated overnight at 30 °C. The fully grown culture was centrifuged at 8000 ×*g* for 20 min at 10 °C and the CFS was passed through an Econo column (BioRad, UK) containing 10 g Amberlite XAD16N (Phenomenex, UK) hydrophobic interaction beads. The column was washed with 100 mL 30 % ethanol and antimicrobial activity eluted in 100 mL 70 % propan-2-ol containing 0.1 % TFA (IPA).

The IPA eluent was removed using rotary evaporation and the concentrated sample applied to a 12 mL, 2 g Strata–E C18 SPE column (Phenomenex, UK) pre-equilibrated with methanol (Merck, Ireland) and distilled water. The column was washed with 12 mL 25 % ethanol and antimicrobial activity eluted with 12 mL IPA. All eluents were assayed in a *L. bulgaricus* LMG 6901 indicator plate as described before. The IPA was removed from the C18 SPE eluent by rotary evaporation and the resulting sample applied to the analytical Jupiter Proteo C12 RP-HPLC column (250 × 4.6 mm, 4 μm, 90 Å) running a 25–45 % acetonitrile, 0.1 % TFA gradient where mobile phase A is 0.1 % TFA and mobile phase B is 100 % acetonitrile 0.1 % TFA. Fractions were collected at 1 min intervals and assessed for antimicrobial activity. Active fractions were assessed for the presence of the nisin Z mass (3330 Da) by MALDI-TOF MS spectrometry (Bruker Ultraflex, Bremen, Germany) in positive ion reflectron mode.

2.5. Evaluation of the biopreservative potential of six LAB isolates in RTE cooked meat products

2.5.1. Sample preparation

Three different RTE cooked meat products were used to test the anti-Listerial activity of the six selected LAB strains (ULE383, ULE639, ULE721, ULE949, ULE1599 and ULE1841) in food, i.e., cooked ham, meatloaf and roasted pork shoulder. The sliced cooked ham was obtained from a local supermarket and the meatloaf and roasted pork shoulder from a meat producer after a maximum of 48 h from production. The ingredients of the products were: ham (85 %), water, salt, dextrose, E-451i, E-407, sodium ascorbate, spices, aroma and sodium nitrite for the cooked ham; pork (95 %), salt, dextrose, E-451i, E-250, spices, aroma and E-306 for roasted pork shoulder; and pork (85 %), lard, salt, wine, spices, E-262i, E-320, flavor enhancers and aroma for meatloaf. These latter meat products were aseptically cut to obtain slices of approximately 12 g and 1 mm thick. Challenge tests were performed using a cocktail of two *L. monocytogenes* strains, to account for variations in growth between strains. The cocktail comprised the *L. monocytogenes* strain previously used for the in vitro antimicrobial assays (CECT 911) and a *L. monocytogenes* strain isolated by Alvarez-Molina et al. (2021) from the processing environment of a meat industry (Lm-970).

The preparation and inoculation of samples with the LAB isolates and/or the *L. monocytogenes* strain cocktail were carried out in accordance with the "EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods" (ANSES, 2021). Briefly, LAB strains were grown individually in MRS broth at 30 °C for 48 h under anaerobiosis. *L. monocytogenes* strains were grown individually in BHI broth at 37 °C for 15–18 h to reach the early stationary phase and then a fresh culture was prepared by adding 0.1 mL of the previous subculture to 9 mL of BHI and incubating at 7 °C for 7 days in order to adapt the cells to the food storage temperature conditions. To prepare the cocktail with the two *L. monocytogenes* strains, equal volumes of each culture were mixed. LAB strains and the *L. monocytogenes* cocktail were centrifuged at 3000 x*g* for 10 min at 20 °C and the pellets were resuspended in sterile saline water (0.95 % NaCl).

The RTE meat samples (~12 g) were inoculated with the LAB and target *L. monocytogenes* cocktail on the surface by homogenously spreading the prepared cultures to obtain a concentration of approximately 8 and 2 $\log_{10}$ CFU/g for LAB and *L. monocytogenes*, respectively. The total volume added did not exceed 1 % of the mass of the samples, in order not to alter the initial physico-chemical properties. Four batches of samples were prepared: (i) non-inoculated negative control (NC); (ii) positive control, only inoculated with *L. monocytogenes* (PC); (iii) inoculated only with LAB (LAB); and (iv) inoculated with both LAB and *L. monocytogenes*. Finally, the samples were vacuum packaged (25 mbar) in 30 μm polyamide – 130 μm polyethylene bags (30 $cm^3/(mm^2 \cdot 24\ h \cdot bar)$ permeability to oxygen) (Pargon, Spain) and stored at 7 °C for 7 days, followed by 3 days at 12 °C. The experiment was performed in triplicate.

2.5.2. Microbiological and physico-chemical analyses

Microbiological and physico-chemical (pH and a_w) analyses of cooked ham, meatloaf and roasted pork shoulder were performed on days 0, 5 and 10 of storage. For microbiological analyses, the whole samples (12 g) were mixed with 108 mL of 0.1 % BPW in sterile filter bags (15–23 cm, Whirl-pack, USA) and homogenized in a stomacher (IUL Instruments, Spain) at full speed for 4 min. The suspension was decimally diluted in BPW and counts of *L. monocytogenes* and LAB

determined after spread plating on Agar Listeria Ottavani and Agosti (ALOA, VWR) plates and MRS agar plates, respectively. MRS agar plates were incubated at 30 °C for 48 h under anaerobiosis, while ALOA agar plates were incubated at 37 °C for 48 h. The growth potential of *L. monocytogenes* was determined by calculating the difference between the counts ($\log_{10}$ CFU/g) at day 5 or 10 and those at day 0.

pH was determined using a pH-meter (VioLab, XS Instruments, Italy) after homogenizing the samples with distilled water in a 50:50 w/w mixture. The pH electrode was calibrated using buffered solutions with pH 4.0 and pH 7.0 and rinsed with deionized water. Water activity (a_w) was measured using a Decagon CX-2 hygrometer (Decagon Devices Inc., USA). All measurements were done in triplicate.

2.6. Assessment of the biopreservative potential of three selected LAB strains at different concentrations in cooked ham

2.6.1. Sample preparation

To carry out these trials, fresh cooked ham (< 2 days after production) was purchased from a local butchery and sliced under aseptic conditions in the laboratory to obtain slices of 20 g. The ingredients were pork, water, salt, dextrose, E-451, skimmed milk powder, E-331iii, E-621, aroma, E-301, E-250, E-120 and soy protein. Each slice was superficially inoculated as previously described individually with the three LAB strains that showed the most promising results in the preceding experiments, or with a cocktail of the three strains. The two strains cocktail of *L. monocytogenes* was also superficially inoculated as above described. The challenge test was carried out under three different LAB concentrations, 4, 6 and 8 $\log_{10}$ CFU/g. The samples were vacuum packaged and incubated for 13 days at 7 °C, followed by 7 days at 12 °C. The same four batches as previously described were used.

2.6.2. Microbiological and pH analyses

Microbiological and pH analyses were performed on days 1, 11 and 20 of storage. For microbiological analyses, samples were firstly diluted and homogenized with 180 mL of 0.1 % BPW following the procedure previously described. Serial decimal dilutions were performed and plated on MRS and ALOA agar for enumeration of LAB and *L. monocytogenes*, respectively. Incubation of MRS and ALOA plates, as well as pH determinations were done as described in the previous section. All measurements were done in triplicate.

2.7. Detailed evaluation through challenge testing of the biopreservative potential of a three-strains LAB cocktail inoculated at a concentration of 8 $\log_{10}$ CFU/g in cooked ham packaged under different conditions

2.7.1. Sample preparation

Here, the objective was to evaluate the effect of the inoculation with the three-strain LAB cocktail at a concentration of 8 $\log_{10}$ CFU/g on the microbiota, pH, a_w, color, texture and sensorial characteristics of cooked ham under three different packaging conditions. The cooked ham was prepared as previously described, but only the cocktail of LAB at 8 $\log_{10}$ CFU/g was included in the design. The packaging conditions were: vacuum packaging (25 mbar), modified atmosphere packaging (MAP) (20 % CO_2–80 % N_2), and vacuum packaging (25 mbar) followed by High Pressure Processing (HPP) at 500 MPa for 3 min at 15 °C (Hiperbaric, Burgos, Spain). The samples were stored at 7 °C for 19 days, followed by 10 days at 12 °C.

2.7.2. Microbiological and physico-chemical analyses

Microbiological and physico-chemical (pH and a_w) analyses were performed in triplicate, as previously described, on days 1, 11, 20 and 29 of storage. MRS, ALOA and Plate Count Agar (PCA, Merck) were employed for the enumeration of LAB, *L. monocytogenes* and total psychrotrophic bacteria, respectively. Incubation of MRS and ALOA plates was done as above described, while PCA plates were incubated for 10 days at 7 °C.

2.7.3. Color and texture evaluation

The surface color of cooked ham was measured with a CM-5/CR-5 Konica Minolta device (Illuminant D65, 8 mm aperture and 10° standard observer) on day 1, 14 and 27 of the shelf-life. CIE lightness (L^*), *chroma* (C^*) and *hue* (h^*) values were recorded. Total color difference (TCD) was calculated as $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$.

The TA-XT2i Texture Analyzer (Stable Micro Systems, UK) was employed for Texture Profile Analysis (TPA). Samples (1 × 1 × 1 cm) were allowed to equilibrate to room temperature and axially compressed in a two-cycle compression with a 40 mm circular flat probe to 60 % the height of the sample at a cross speed of 5 mm/s. Texture parameters included hardness, cohesiveness, resilience, elasticity, gumminess and chewiness. Nine replicates at each sampling point were conducted for color and texture determination.

2.7.4. Sensorial analysis

Sensory analysis was conducted in a single session with 52 panelists. Six sample types were included in this analysis: NC cooked ham packaged under vacuum, MAP or HPP, and LAB-inoculated cooked ham packaged under vacuum, MAP or HPP. The samples were transferred to a transparent plastic plate with lid after 14 days storage at 7 °C, assigned a three-digit identifier and served randomly. A ranking test with the six samples was performed to independently evaluate: visual appearance as darkness/lightness, acid-related odor intensity, acid taste intensity and global pleasantness. Concurrently, a consumer acceptance test was conducted. Those samples ranked with the lowest value (1) were the lightest (color), the least acid (odor and flavor) or the least acceptable (global) and those with the highest value (6) were the darkest (color), the most acid (odor and flavor) or the most acceptable (global). Consumer acceptance was expressed as frequency, referring to the percentage of panelists that assigned the odor of each sample to the categories pleasant, unpleasant, or not rated.

2.7.5. Microbiota profiling through 16S rRNA gene amplicon sequencing

Total DNA was extracted from samples at days 1, 15 and 28 of storage. From each sample, 10 g were homogenized with 90 mL Phosphate Buffer Saline (PBS, Sigma-Aldrich) in a stomacher (IUL Instruments) at maximum speed for 2 min. The homogenized samples were centrifuged at 5000 x*g* for 15 min at room temperature. DNA was isolated from the cell pellets with the DNeasy® PowerSoil® Pro Kit (Qiagen) following the manufacturer's specifications. The ZymoBIOMICS Microbial Community Standard (Zymo Research, USA) was used as a positive control.

The Illumina Miseq platform was used to sequence 16S rRNA gene amplicons with 300 bp pair-end sequencing. The primers used were S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Carrasco et al., 2020), which amplify the V3-V4 hypervariable regions. PCR amplification, preparation of Miseq libraries and sequencing were performed at the sequencing platform of *Centro de Investigación Biomédica de La Rioja* (CIBIR), Spain.

For bioinformatics analysis and data processing, raw reads were processed using DADA2 v1.8.0 (Callahan et al., 2016) following the authors' tutorial. Firstly, *cutadapt* (Martin, 2011) was used to remove primer sequences, and ambiguous bases were detached using *truncLen = c(275,250)*. Chimeras were removed and an Amplicon Sequence Variant (ASV) table was obtained by comparing the clean sequences with the reference RDP database version 18 (Ribosomal Database Project) by using the *assignTaxonomy* command in DADA2 (Cole et al., 2014). Alpha-diversity indexes were calculated using the *specnumber* and *diversity* commands from vegan whereas beta-diversity analyses were performed using Bray Curtis dissimilarity distances with the *cmdscale* command. Plots were produced using *ggplot2*. All analyses and plots were carried out in *RStudio version 4.0.2*.

2.8. Statistical analyses

Data from microbiological, physico-chemical, color, texture and sensory ranking tests were statistically analysed using one way ANOVA with a linear model regression using the *lm* function in *RStudio version 4.0.2*. Data from the frequency results of the sensory analysis were analysed with the statistical tables for estimating significance in paired-preference tests (Roessler et al., 1978). In metataxonomic analyses, significant differences in alpha-diversity indices were determined using the Wilcoxon test by using the *compare means* command from *ggpubr*. Significant differences in the beta-diversity analyses were determined through an analysis of dissimilarity test (ADONIS), using the *adonis* command from *vegan*.

2.9. Accession numbers

Raw reads from WGS of the six isolates and *16S rRNA* gene amplicon sequencing of the cooked ham have been deposited at the Nacional Centre for Biotechnology Information under the Bioproject ID PRJNA941229.

3. Results and discussion

3.1. Characterization of the LAB culture collection

The 479 presumptive LAB isolates, which had been isolated from food processing environments of meat (66 % samples), dairy (32 % samples) and honey (2 % samples) industries, were characterized through MALDI-TOF MS for identity confirmation. In total, 34.2 % of the isolates were identified as LAB of interest based on the scope of this study, which included isolates from *Lactococcus* ($n = 109$), the former *Lactobacillus* genus ($n = 48$), *Leuconostoc* ($n = 5$) and *Pediococcus* ($n = 2$) (Supplementary file 1), genera previously associated in the literature with bacteriocin production (da Costa et al., 2019; Skariyachan and Govindarajan, 2019) or with promising activities as biopreservative agents (Mayo and Flórez, 2020). Other LAB were excluded from subsequent analyses (i.e., *Enterococcus* and *Weisella*) as they are not included in the QPS list (EFSA, 2022). Among the selected LAB of interest, at species level, the most frequent identification was *L. lactis* (52.7 %), followed by *L. plantarum* (17.4 %) and *Lactococcus garvieae* (12.6 %). It must be taken into account that the non-selective enrichment step could have favour the detection of some particular aciduric LAB over the rest. Nearly all of the identifications were of high confidence, considering the score values (ranging from 2 to 3) obtained with the MALDI Biotyper. However, 3 strains (one identified as *L. lactis* and two as *L. garvieae*) yielded a low-confidence identification (scores between 1.70 and 1.99), and another 3 strains could not be identified through MADI-TOF MS, either because no peaks were detected or because no reliable organism identification was achieved, which could be due to missing species in the library. Nevertheless, these isolates were included for further analysis.

3.2. Screening of antimicrobial activity

All the selected LAB isolates were further subjected to antimicrobial activity assays using two foodborne pathogens, i.e., *L. monocytogenes* and *E. coli*, as target microorganisms (Supplementary file 1).

The antagonistic assays revealed that a total of 73 and 71 LAB strains inhibited to some extent in the spot-on-lawn assay the growth of *L. monocytogenes* and *E. coli*, respectively, whereas in the wells-in-agar test, only 17 and 44 LAB strains, respectively, produced inhibition halos (Supplementary file 2).

Interestingly, while similar antimicrobial activities against *L. monocytogenes* CECT 911 and *E. coli* CECT 515 were found on the spot-on-lawn assay, in the wells-in-agar test *E. coli* was generally more susceptible than *L. monocytogenes*, with various LAB isolates showing activity only against this Gram-negative bacterium. This finding does not agree with results from other research studies where Gram-positive bacteria were reported as more sensitive to the antimicrobials produced by LAB than Gram-negative bacteria, which have an outer cell membrane that protects the cell against antimicrobial agents and other compounds such as antibiotics (Gupta, 2011; Kim et al., 2022; Sewify et al., 2017). A possible explanation for the increased vulnerability of *E. coli* might be that the presence of organic acids produced by the LAB strains, such as lactic acid, and the decrease in pH, could destabilize and permeabilize the outer membrane, favouring the antimicrobial activity to take place (Alakomi et al., 2000; Chen et al., 2022). In addition, some authors have also reported that some bacteriocins can have a wide antimicrobial spectrum, being also active against Gram-negative bacteria due to their specific binding to a membrane protein that can act as a bacteriocin-specific receptor (Acuña et al., 2012).

The growth inhibition halos obtained varied in size and shape. Most inhibition zones showed a fuzzy edge, while others showed clear and sharp edges. Minimum halo sizes were of 5 AU in the spot-on-lawn assay and 6 AU in the wells-in-agar assay. The biggest inhibition areas, of up to 30 AU, were achieved for some *L. plantarum* strains in the spot-on-lawn test against both pathogens. Halos obtained in the wells-in-agar assay were smaller, of up to 12 AU in the case of a *L. plantarum* strain against *E. coli* and 17 AU for a *L. lactis* strain against *L. monocytogenes*. The mean diameters of the inhibition zones were of 12.6 ± 4.2 and 16.4 ± 5.7 AU in the spot-on-lawn test and 11.1 ± 2.5 and 10.0 ± 0.8 AU in the wells-in-agar assay for *L. monocytogenes* and *E. coli*, respectively. All the results obtained in both assays for each LAB strain and target microorganism are shown in Supplementary file 1.

Considering the results obtained, 6 LAB isolates (namely, ULE383, ULE639, ULE721, ULE949, ULE1599 and ULE1841) showing the most robust antimicrobial activity (i.e., strains that showed inhibition zones in both the spot-on-lawn and wells-in-agar tests against both target microorganisms, or that showed AUs > 12 for any of the wells-in-agar and AUs > 20 for any of the spot-on-lawn tests) were selected to further explore their application as biopreservation agents in meat products.

3.3. Characterization through whole genome sequencing

WGS and tailored bioinformatics analyses showed the genomic features of the 6 selected strains (Table 1). The predicted number of genes in each genome ranged from 3253 to 3442 for the isolates from the former *Lactobacillus* genus, while the *L. lactis* genome harbored 2376 predicted genes, similar to the results described by other authors (Goel et al., 2020; Mataragas, 2020; Wels et al., 2019).

To confirm the taxonomic assignation for each bacterial genome, the GTDB-Tk database was used. The best match obtained for each of the isolates, with the corresponding Average Nucleotide Identity (ANI) scores, was: *L. lactis* for ULE383 (97.26 % ANI), *L. paracasei* for ULE721 (98.2 % ANI), and *L. plantarum* for ULE639, ULE949, ULE1599 and ULE1841 (ANI ranging from 98.76 % to 98.85 %). These assignations agreed with the results obtained through MALDI-TOF MS, except for ULE1599, strain for which no peaks were detected in the MALDI-TOF MS analysis. Moreover, a phylogenetic tree based on the whole genome sequences was generated (Fig. 2), which corroborated the results obtained in GTDB-Tk.

Generally, members of *Lactococcus* and the former *Lactobacillus* genus are considered to be non-pathogenic and are widely used as starter cultures in fermentation processes or as probiotics. Indeed, a wide variety of species from these taxa have QPS or GRAS status. No virulence genes were detected for any of the six LAB isolates. However, LAB isolates can carry antimicrobial resistance genes (Campedelli et al., 2019). In the European Union, any strain intended to be used for human or animal consumption must be free of antimicrobial resistance determinants (EFSA, 2018b). The analysis of the genomes with the ResFinder database only rendered a resistance-related gene, encoding for the CIpL protein, involved in resistance to heat (98 % ID), which was

Table 1
Data obtained from the whole genome sequencing analysis of the six selected LAB isolates with most promising antimicrobial activities.

	ULE383	ULE639	ULE721	ULE949	ULE1599	ULE1841
Taxonomic identification	*Lactococcus lactis*	*Lactiplantibacillus plantarum*	*Lacticaseibacillus paracasei*	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
Genome length (bp)	2,392,345	3,398,951	3,135,190	3,398,196	3,400,408	3,428,239
GC (%)	34,92	44,34	46,27	44,34	44,34	44,25
Number of predicted genes	2376	3413	3253	3405	3420	3442
Antibiotic resistance genes Resfinder	None	*ClpL* (98 % ID)	None	*ClpL* (98 % ID)	*ClpL* (98 % ID)	*ClpL* (98 % ID)
Antibiotic resistance genes CARD	*lmrD* (loose, 99.85 % ID)	None	None	None	None	None
Virulence factors (Virulence finder)	None	None	None	None	None	none
Plasmids (PlasmidFinder and MGE)	None	pLBUC03 (88.48 % ID), pCIS4 (97.16 % ID), LBPp6 (100 % ID), LBPp1 (90.53 % ID)	LSEI_A15 (92.1 % ID)	pLBUC03 (88.48 % ID), pCIS4 (97.16 % ID), LBPp6 (100 % ID), LBPp1 (90.53 % ID)	pLBUC03 (88.48 % ID), pCIS4 (97.16 % ID), LBPp6 (100 % ID), LBPp1 (90.53 % ID)	pR18 (92.17 % ID), LBPp6 (100 % ID), pLBUC03 (83.75 % ID), LBPp1 (99.43 % ID)
Insertion sequences and transposons (MGE)	None	ISP1 (99.86 % ID), ISLhe30 (94.81 % ID), ISLpl1 (94.64 % ID), ISS1N (98.64 % ID)	None	ISP1 (99.86 % ID), ISLhe30 (94.81 % ID), ISLpl1 (94.64 % ID), ISSN1 (98.64 % ID)	ISP1 (99.86 % ID), ISLhe30 (94.81 % ID), ISLpl1 (94.64 %ID), ISS1N (98.64 % ID)	ISP2 (98.66 % ID), ISLhe30 (94.72 % ID), ISLpl3 (99.53 % ID)
Bacteriocin gene cluster (BAGEL4)	Nisin Z	None	None	None	None	None

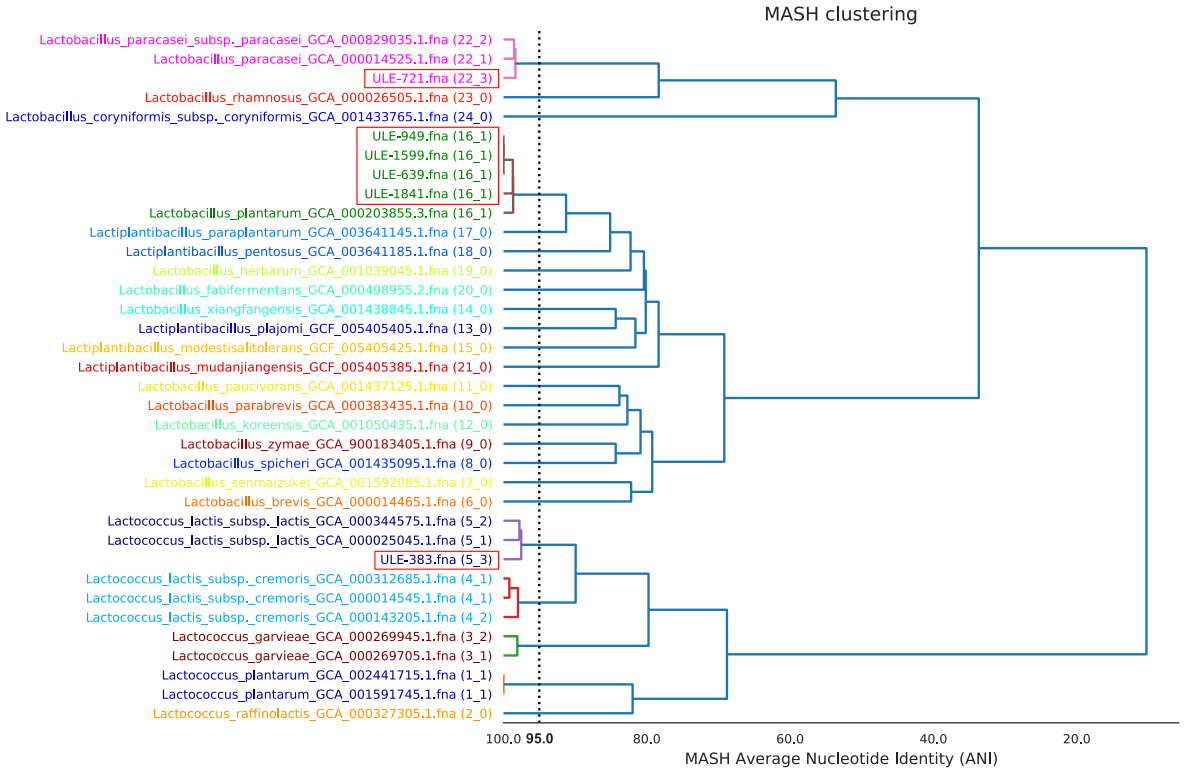

Fig. 2. Phylogenetic tree performed in a pair-wise manner using dRep v.2.6.2 showing the Mash clustering. The six LAB isolates selected are highlighted in red boxes. The dashed line delimits the 95 % Mash Average Nucleotide Identity (ANI).

detected in the four *L. plantarum* strains, i.e., ULE639, ULE949, ULE1599 and ULE1841. Okoye et al. (2022) also found the same heat resistance gene in another *L. plantarum* strain. On the other hand, CARD analysis predicted with 99.85 % identity and 100 % length coverage that ULE383 carries a lincosamide antibiotic resistance gene (*lmrD*). This gene has been previously reported in other *L. lactis* strains (Belén Flórez et al., 2006; Lubelski et al., 2006, 2004). However, *lmrD* needs to form an heterodimer with the subunit *lmrC* to have activity as ATP-binding antibiotic efflux pump (Lubelski et al., 2004), and *lmrC* was not present in the genome of *L. lactis* ULE383.

It has been earlier reported that some LAB strains commonly harbor plasmids carrying genes with technological and metabolic capabilities such as bacteriocin production or phage resistance (Stefanovic and McAuliffe, 2018). The genomes of the five strains assigned to the former *Lactobacillus* genus were found to contain at least one contig showing homology to plasmids previously described, while the genome of *L. lactis* ULE383 did not contain any (Table 1).

With regard to the results obtained with BAGEL4, the only predicted bacteriocinogenic strain was *L. lactis* ULE383, with a predicted peptide sequence consistent with nisin Z (Supplementary file 3). Nisin Z is a closely related nisin A variant, with just one amino acid difference (Mulders et al., 1991). Supplementary file 3 shows the genetic

organization of the nisin Z gene cluster, comprising genes encoding the core peptide, several modification enzymes, immunity, transport and regulatory proteins.

3.4. Bacteriocinogenic potential of the strains

To assess the bacteriocinogenic potential of the six LAB strains, *L. bulgaricus* LMG 6901 was used as indicator microorganism. *L. lactis* ULE383 was the only strain that showed inhibition halos after 24 and 48 h of incubation, with 19 AU and 20 AU of activity, respectively (Fig. 3A). In addition, these inhibitory activities were maintained when *L. lactis* ULE383 was grown at temperatures between 25 and 37 °C for 24 to 72 h. No inhibition halos were observed with the rest of LAB strains against *L. bulgaricus* LMG 6901 under any of the tested scenarios. Hence, *L. lactis* ULE383 was the only strain selected for the detailed assessment of its bacteriocinogenic potential.

The antimicrobial metabolite was purified from CFS using C18 Solid Phase Extraction and reversed phase HPLC (Fig. 3Bi). MALDI TOF MS analysis of the most active HPLC fraction corresponding to the peak eluting at 21 min, where nisin Z typically elutes (Fig. 3Bii), showed the predicted nisin Z mass (i.e., 3330 Da) (Fig. 3Biii), suggesting that *L. lactis* ULE383 is a bacteriocinogenic strain producing nisin Z.

3.5. Biopreservative potential of the six selected LAB strains in RTE cooked meat products

In order to evaluate the potential of the selected LAB strains as biopreservation agents targeting *L. monocytogenes* in RTE cooked meat products, artificially spiked samples of cooked ham, meatloaf and roasted pork shoulder were vacuum-packaged and stored. Microbiological analyses and measurements of pH and a_w were performed at days 0, 5 and 10 during storage of samples.

All six LAB strains were able to adapt to each food matrix, reaching counts ~1.3 $\log_{10}$ CFU/g higher than those observed at day 0 (~7.6$\log_{10}$ CFU/g) (Fig. 4A). In cooked ham, LAB strains showed a progressive growth during the storage, while in meatloaf and roasted pork shoulder no additional LAB growth was observed from day 5 to day 10 of storage, when even a slight decrease in counts was found, except for one isolate in pork shoulder. These differences might be due to the specific composition of each product. For instance, meatloaf and roasted pork shoulder are richer in fat, thus, the hydrophobic fraction can interfere with LAB growth (Macieira et al., 2018). In this regard, research is being conducted by various investigation groups to ensure the viability of LAB and/or their metabolites in different food matrices or polymers in order to carry out their desired antimicrobial actions (Castellano et al., 2017; Ghabraie et al., 2016; Xie et al., 2018).

In cooked ham the pH decrease was quicker in the presence of LAB compared to that observed for samples with no LAB strains added (Fig. 4B). In addition, there was a sharper acidification in this product than in roasted pork shoulder and meatloaf. The decreasing trend in pH was maintained in cooked ham up to day 10 of storage, when the lowest pH value of 4.7 was observed, whereas in roasted pork shoulder pH values remained stable at 5.6–5.9 from day 5 to day 10. Meatloaf showed quite stable pH values, between 6.1 and 6.5, along the whole storage period.

The a_w of the RTE cooked meat samples (Supplementary file 4A)

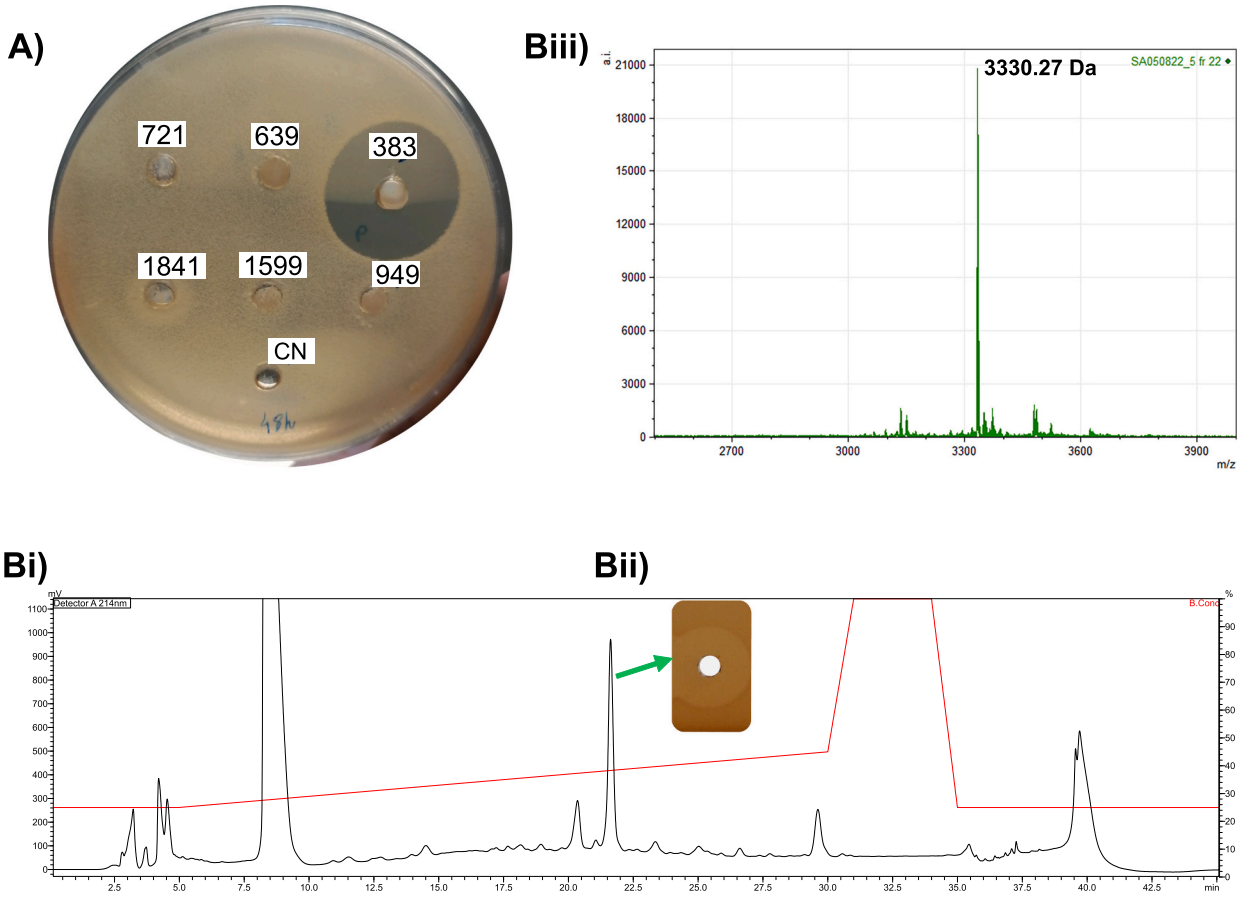

Fig. 3. Assessment of the bacteriocinogenic potential of the six selected LAB strains. A) Results of the wells-in-agar assay for the six LAB strains, showing the growth inhibition zone of *L. lactis* ULE383 against the indicator strain *L. bulgaricus* LMG 6901; B) Purification of nisin Z from *L. lactis* ULE383: Bi) RP-HPLC chromatogram, Bii) zone of inhibition of an aliquot of the fraction corresponding to the peak eluting at 21 min and Biii) Mass spectrum of the active fraction, showing a peak with a mass of 3330 Da, corresponding to nisin Z.

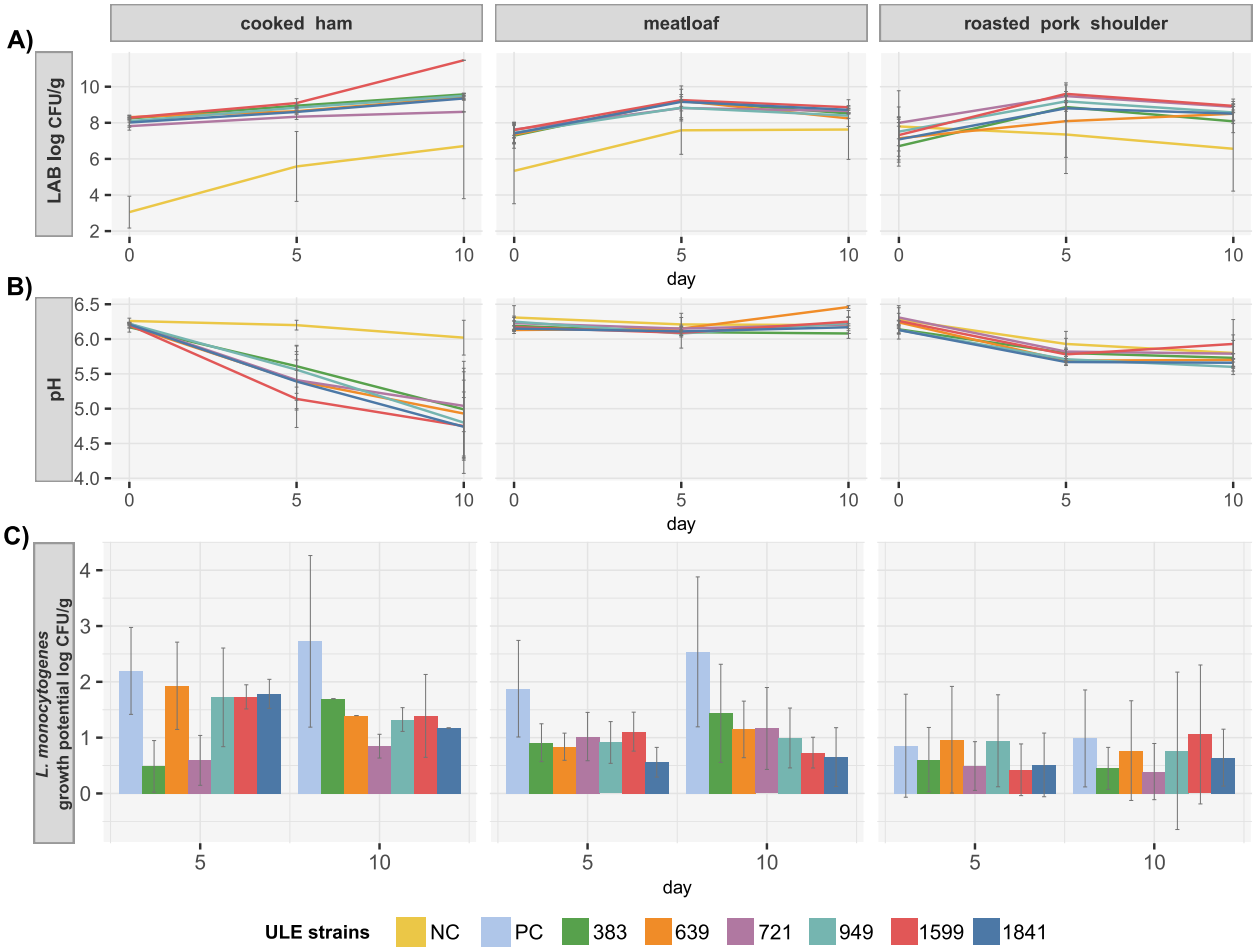

Fig. 4. Microbiological counts of LAB (A), pH evolution (B) and *L. monocytogenes* growth potential (C) in the presence of the six selected LAB strains (ULE383, ULE639, ULE721, ULE949, ULE1599 and ULE1841) in cooked ham, meatloaf and roasted pork shoulder. The growth potential (C) represents the difference in counts ($\log_{10}$ CFU/g) of *L. monocytogenes* at day 5 or 10 of storage with respect to day 0. NC (negative control) are samples without *L. monocytogenes* or LAB added, and PC (positive control) are samples inoculated with *L. monocytogenes* and without LAB added. NC samples were also plated on ALOA, and *L. monocytogenes* was not detected. Results are presented with mean values ± standard deviations.

ranged from 0.969 to 0.995. In general, a_w of meatloaf and roasted pork shoulder samples artificially inoculated with LAB tended to be lower throughout the storage period than those of non-inoculated samples.

Overall, the conditions of pH and a_w prevailing in the three products are favourable to support the growth of *L. monocytogenes*, which has been previously described to grow down to pH 4.1 and a_w 0.90 (İncili et al., 2020). The growth potential of *L. monocytogenes* (difference between the $\log_{10}$ CFU/g at day 5 or day 10, and the $\log_{10}$ CFU/g at day 0) is shown in Fig. 4C. In the positive control samples (inoculated with 2 $\log_{10}$ CFU/g *L. monocytogenes* but not with LAB), the growth potential of *L. monocytogenes* in meatloaf and cooked ham was of around 2 $\log_{10}$ CFU/g, both at day 5 and 10 of storage, while it was of ~1 $\log_{10}$ CFU/g in roasted pork shoulder, which evidences that the three meat products, supported the growth of the pathogen. In the vast majority of the cases, the inoculation of the meat products with the LAB strains helped to control the pathogen's growth to a certain extent. However, the decrease in *L. monocytogenes* growth potential differed among samples and/or strains tested, with the greatest biopreservation effects being consistently observed in cooked ham and the weakest in roasted pork shoulder. The highest reductions in *L. monocytogenes* growth potential (i.e., a decrease of 1.9 $\log_{10}$ CFU/g) were achieved at day 10 in cooked ham with ULE721 and in meatloaf with ULE1841. On the other hand, in roasted pork shoulder, the maximum reduction in growth potential was of 0.6 $\log_{10}$ CFU/g for ULE721 at day 10.

The reductions in growth potential achieved were comparable to those previously reported by other authors in similar meat products using LAB as biopreservatives. However, comparative analyses should be made with caution because the findings in the literature sometimes refer to other food matrices or even different packaging conditions, and, as a result, apparently inconsistent results have been often reported. For instance, Macieira et al. (2018) and Zanette et al. (2015) tested *L. plantarum* in fermented sausages and obtained quite different antimicrobial activities against *L. monocytogenes*. Indeed, whereas Zanette et al. (2015) reported reductions in *L. monocytogenes* populations of 1.7 $\log_{10}$ CFU/g, Macieira et al. (2018) observed no additional inhibition upon addition of *L. plantarum* when compared to the control sample. Hence, there are several parameters that may determine the preservative activity and should be considered, such as type of meat and ingredients used, concentration of the LAB strain and target microorganism, or the influence of the natural microbiota, packaging conditions and storage temperature, among others (Barcenilla et al., 2022).

When comparing the antimicrobial activity of the LAB strains in the meat products with the growth inhibition results obtained on agar media, the strains with better inhibition results in the spot-on-lawn and/or wells-in-agar assays were not always the ones showing the highest reductions in *L. monocytogenes* growth potential in the meat products. These differences can be attributed to the food matrix being a complex ecosystem in which the microbial populations and external factors interact affecting the community structure, unlike in vitro assays, which

are less complex. Also, all the different parameters mentioned above might interfere with the activity of the LAB and/or their metabolites in the meat products.

Considering the results obtained for the six LAB strains in in vitro tests of inhibition and the anti-Listerial activity they showed in the different meat products studied, *L. lactis* ULE383, *L. paracasei* ULE721 and *L. plantarum* ULE1599 were selected for undertaking detailed biopreservation trials in cooked ham.

3.6. Detailed evaluation through challenge tests of the biopreservative potential of three selected LAB strains in cooked ham

3.6.1. Microbiological counts and pH of vacuum packaged cooked ham with the addition of three LAB strains (from L. lactis, L. paracasei, L. plantarum), and a mix of them, at three different concentrations

Firstly, the three selected strains were applied individually or as a cocktail on the surface of sliced cooked ham at three different concentrations in a series of challenge tests at which the inoculated samples were stored under vacuum for 13 days at 7 °C followed by 7 days at 12 °C (Fig. 5). At the three tested concentrations, the inoculated LAB, both individually or as a cocktail, were able to progressively grow throughout all the storage period up to final concentrations of approximately 8–9 $\log_{10}$ CFU/g (Fig. 5A). This confirmed the ability of the LAB strains to adapt and grow in the environment of the food matrix. Remarkably, LAB counts for the negative control samples were of approximately 2 $\log_{10}$ CFU/g at day 1, but progressively increased to also reach around 8 $\log_{10}$ CFU/g on day 20 of storage, attaining similar levels to those observed for the artificially inoculated cooked ham samples.

As shown in Fig. 5B, the pH of cooked ham decreased more rapidly, achieving lower final pH values, as the LAB inoculum concentration increased, especially when the strains were added as a cocktail, reaching, at day 20 of storage, pH values of 4.6. This aspect could be detrimental from a palatability perspective.

The growth potential of *L. monocytogenes* in the samples without LAB added was already of 5 $\log_{10}$ CFU/g after 11 days of storage. The strongest anti-Listerial activity was observed when the three LAB strains were inoculated as a cocktail mix at the highest concentration (8 $\log_{10}$ CFU/g), with an outstanding decrease in the pathogen's growth potential by 4.6 and 4.3 $\log_{10}$ CFU/g after 11 and 20 days of incubation, respectively (Fig. 5C). On the contrary, lower decreases in the growth

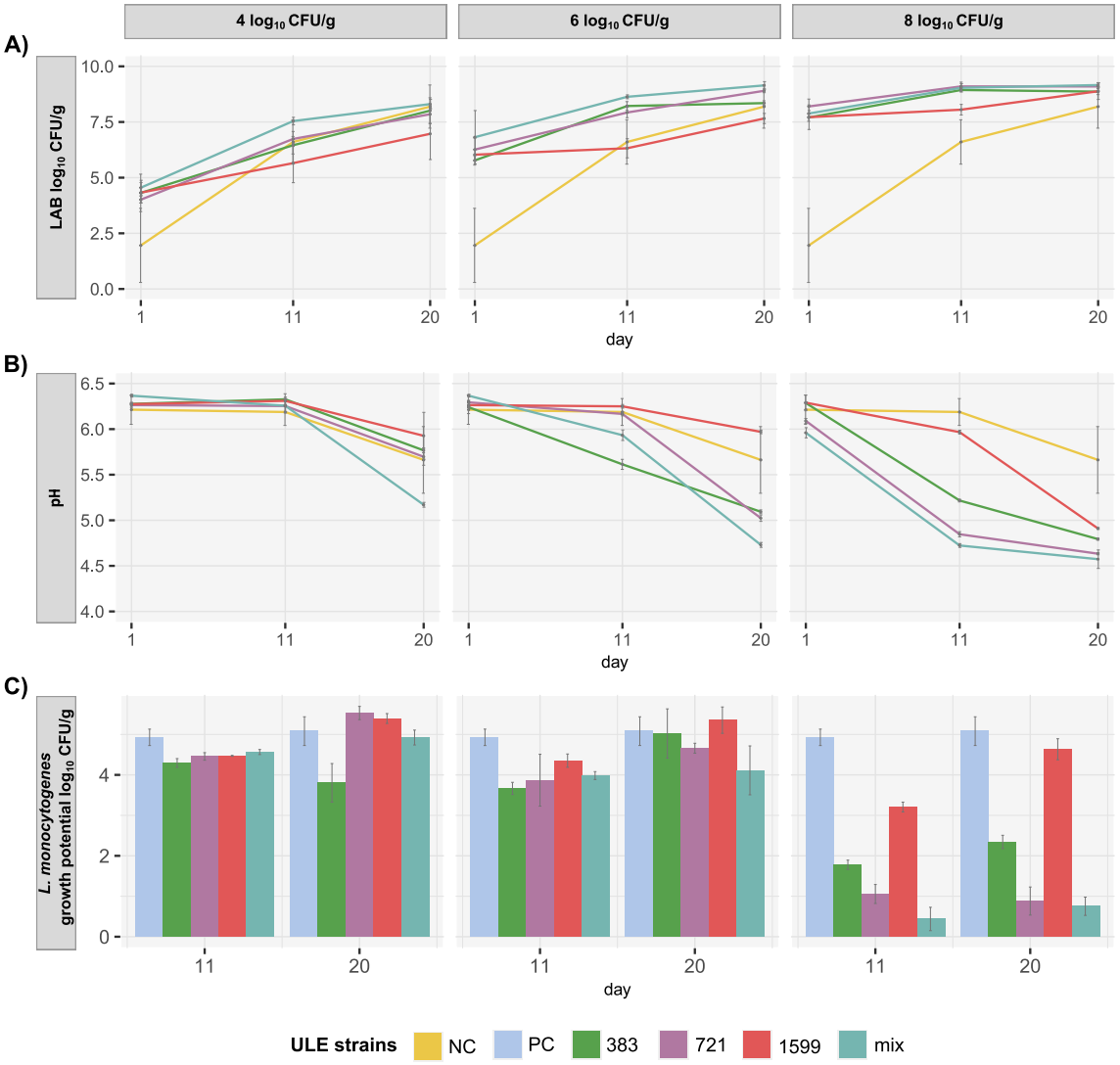

Fig. 5. Evaluation of the application of the three selected LAB strains (ULE383, ULE721, ULE1599), and of a cocktail of the three of them, at different concentrations (4, 6, and 8 $\log_{10}$ CFU/g) in cooked ham. (A) LAB counts; (B) pH evolution at days 1, 11 and 20 of storage; and (C) *L. monocytogenes* growth potential, representing the difference in counts ($\log_{10}$ CFU/g) of *L. monocytogenes* at day 11 and 20 of storage with respect to day 0. NC refers to the cooked ham without LAB or *L. monocytogenes* inoculation and PC refers to the cooked ham with *L. monocytogenes* inoculation. NC samples were also plated on ALOA, and *L. monocytogenes* was not detected. Results are presented with mean values ± standard deviations.

potential of *L. monocytogenes* were obtained when the concentrations of the applied LAB were lower (6 $\log_{10}$ CFU/g and especially 4 $\log_{10}$ CFU/g). In addition, at the highest LAB concentration tested, differences were observed among the individually used strains, with ULE721 being the strain with the highest anti-Listerial activity (growth potential reduction of ~4 vs ~ 3 and 0.4–1.7 $\log_{10}$ CFU/g for ULE383 and ULE1599, respectively).

3.6.2. *Cooked ham with the addition of the LAB cocktail (8 $\log_{10}$ CFU/g) under different packaging conditions: microbiological counts, pH and a_w*

Considering the robust anti-Listerial activity achieved with the three LAB strains cocktail inoculated at 8 $\log_{10}$ CFU/g, a new trial was set up to evaluate the bioprotective effects under different packaging conditions in a more detailed study. In this case, the samples were packaged under (i) vacuum, (ii) modified atmosphere packaging (MAP) conditions, (iii) or vacuum followed by high pressure processing (HPP), with a storage period of 19 days at 7 °C followed by 10 days at 12 °C.

Throughout storage, LAB progressively grew both under MAP and vacuum to reach a final concentration of 9 $\log_{10}$ CFU/g on the last day of storage (Fig. 6A). When the cooked ham was treated by HPP, the inoculated LAB, or any other indigenous LAB present, showed a delay in growth throughout storage by the effect of pressure. A similar effect was observed in previous studies with cooked ham pressurized with 400 and 600 MPa (Han et al., 2011). However, at day 29, LAB counts were not significantly different from those observed for samples stored under vacuum or MAP. Non-inoculated control samples showed lower LAB counts at the beginning of the storage period than their counterparts inoculated with the LAB cocktail, while similar LAB counts were obtained for all sample categories at the end of the storage period.

Total psychrotrophic counts in samples packaged under vacuum or MAP remained at around 8 $\log_{10}$ CFU/g during all storage period, while counts were significantly lower when samples were HPP treated after packaging (Fig. 6B). In addition, psychrotrophic bacterial counts in the negative control samples remained significantly lower than those on the samples with inoculated LAB until day 11. From day 20 onwards, negative control samples packaged under vacuum or MAP had similar counts to those from samples with LAB added. On the other hand, total psychrotrophs remained significantly lower in pressurized negative control samples than in the rest of the samples until the end of storage period.

The pH of LAB-inoculated samples decreased notably since day 1 in samples stored under vacuum or MAP, as shown in Fig. 6C. However, on LAB-inoculated pressurized samples, the pH remained stable at values above pH 5.9 up to day 20 of storage, although at the end of the storage period the pH decreased to 4.6, similar to that of the other LAB-inoculated samples. This delay in the pH drop could be attributed to the detrimental effect of pressurization over LAB growth. Similarly, Pavli et al. (2019) obtained significant higher pH values in cooked ham treated with HPP than in untreated samples.

The growth potential of *L. monocytogenes* in the non-inoculated

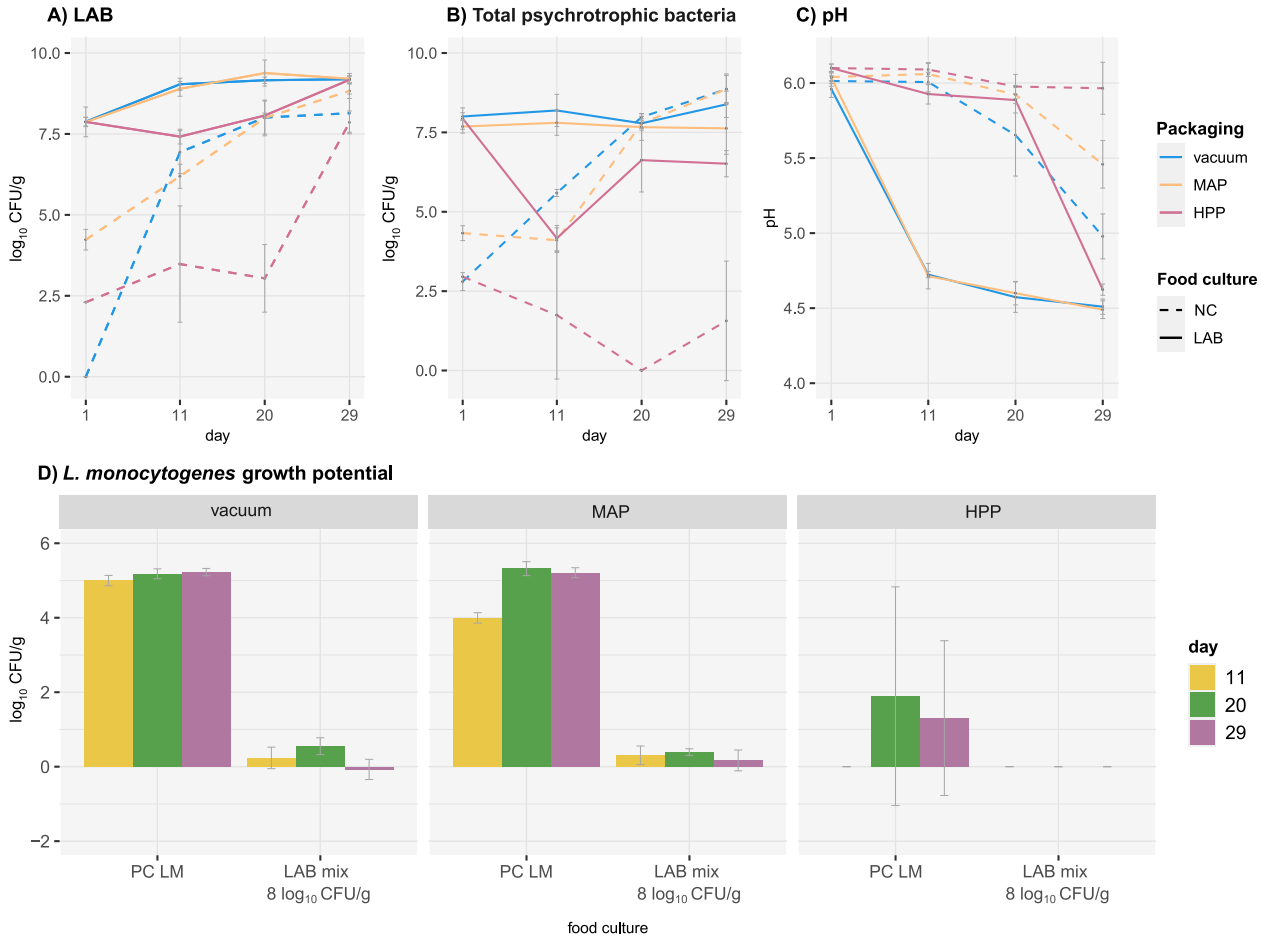

Fig. 6. Application of the cocktail of the LAB strains ULE383, ULE721 and ULE1599 at a concentration of 8 $\log_{10}$ CFU/g in cooked ham under three packaging conditions: vacuum, modified atmosphere packaging (MAP), and vacuum and High Pressure Processing (HPP). A) LAB counts, B) Total psychrotrophic bacterial counts, C) pH and D) *L. monocytogenes* growth potential, determined by calculating the difference between the counts ($\log_{10}$ CFU/g) of *L. monocytogenes* at day 11, 20 and 29 and those at day 0. NC refers to the cooked ham without LAB or *L. monocytogenes* inoculation and PC LM refers to the cooked ham with *L. monocytogenes* inoculation. NC samples were also plated on ALOA, and *L. monocytogenes* was not detected. Results are presented with mean values ± standard deviations.

samples stored under vacuum was approximately 5 $\log_{10}$ CFU/g, whereas, when the LAB strain cocktail was added, *L. monocytogenes* growth potential was <0.6 $\log_{10}$ CFU/g. Similar observations were made in samples under MAP (Fig. 6D). In this case, *L. monocytogenes* showed a growth potential of 5.2 $\log_{10}$ CFU/g at day 29 in non-inoculated samples, while in samples with the LAB cocktail added the growth potential was of 0.3, 0.4 and 0.2 $\log_{10}$ CFU/g at day 11, 20 and 29 of storage, respectively. When the HPP was applied, the growth potential of the pathogen in non-inoculated samples was significantly lower, with counts under the detection limit on day 11 and reaching values of 1.9 and 1.3 $\log_{10}$ CFU/g on day 20 and 29 of storage, respectively. Interestingly, when HPP and the LAB cocktail were applied together, a total inhibition of the pathogen's growth was achieved, which evidences the synergistic effect between both treatments. Likewise, Dučić et al. (2023) reported absence of *L. monocytogenes* and *Salmonella Typhimurium* from day 18 onwards when HPP and LAB were applied in combination in a fermented sausage.

Throughout storage, no relevant differences were observed in a_w among samples, obtaining values between 0.976 and 0.985 (Supplementary file 4B).

3.6.3. *Cooked ham with the addition of the LAB cocktail (8 $\log_{10}$ CFU/g) under different packaging conditions: metataxonomic profile*

The metataxonomic profile of the cooked ham batches throughout storage was assessed through *16S rRNA* gene amplicon sequencing. The main bacterial genera prevailing at the different days of storage (1, 15 and 28) or under the different packaging conditions used (vacuum, MAP, or vacuum + HPP) are shown in Fig. 7. There is a clear differentiation of samples based on the addition of the LAB strain cocktail, with LAB-inoculated samples showing a more homogeneous taxonomic profile. The rather heterogeneous metataxonomic profile observed in non-inoculated samples is reflected in the abundance of various genera such as *Brochothrix*, *Pseudomonas* or *Acinetobacter*, while the three genera included in the LAB cocktail, and especially the genus *Lacticaseibacillus*, clearly dominated in the microbial community of LAB-inoculated samples. The relative abundance of these three LAB genera together in LAB-inoculated samples ranged from 95 to 99.7 %. The high relative abundance of the *Lacticaseibacillus* genus in artificially inoculated batches probably reflects the better acclimatization of the ULE721 strain to the meat product throughout its shelf-life. *Brochothrix* showed a high abundance in samples without LAB inoculation, especially in those packaged under vacuum. Hence, the LAB cocktail also allowed control of the population of spoilage microorganisms in the product. In samples without LAB added and packaged under MAP, *Leuconostoc* and *Carnobacterium* were quite abundant, in agreement with the findings by other authors (Raimondi et al., 2019). In addition, in samples without LAB added, at day 1, the taxonomic profile was similar regardless of the packaging condition used. However, the bacterial communities under MAP or vacuum rapidly evolved on the following days of shelf-life to be mainly dominated by just 2–4 genera. On the contrary, HPP-treated samples maintained a quite stable profile from day 1 to 15 of shelf-life, while on day 28 they were dominated by the spore-forming genus *Sporosarcina*, together with a wide range of minority members from diverse genera. Chaillou et al. (2022) also found major changes in the growth dynamics of the microbiota of ham after HPP treatment.

As shown in Fig. 8, the artificial inoculation with the LAB cocktail explained 74.47 % of the variation observed in the Bray-Curtis beta diversity distance matrix, which showed the marked impact of the use of the LAB mix, which prevailed over the background microbiota of the cooked ham. A more homogeneous taxonomic profile was observed among samples with inoculated LAB compared to samples without LAB added (Fig. 8A). Considering the large differences observed between batches with and without the LAB cocktail added, beta-diversity analyses were performed also separately for both sample categories (Fig. 8B, C). Significant differences were found among samples from different storage days in both sample categories ($P = 0.005$ and $P = 0.001$ for samples with LAB or without LAB added, respectively). In samples without LAB added, the bacterial community evolved to a more heterogeneous profile at the end of storage in comparison to day 1. Regarding the effect of packaging method, there were also significant differences among them in both sample categories, although they were more evident in samples without LAB added ($P = 0.019$ and $P = 0.001$,

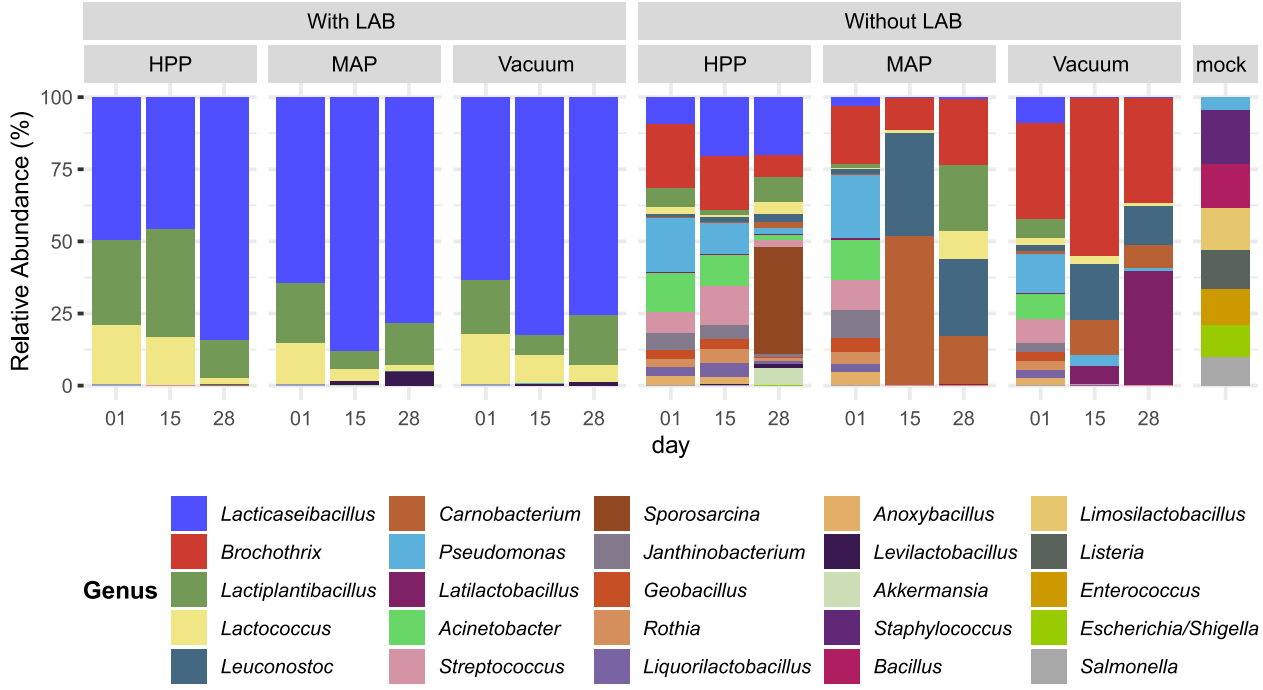

Fig. 7. Relative abundance of the genera found in cooked ham inoculated with a cocktail of the LAB strains ULE383, ULE721 and ULE1599, and in a negative control without inoculation of LAB under three different packaging conditions: vacuum, MAP and vacuum followed by HPP. A mock community was used as a microbial standard for the validation of the sequencing procedure.

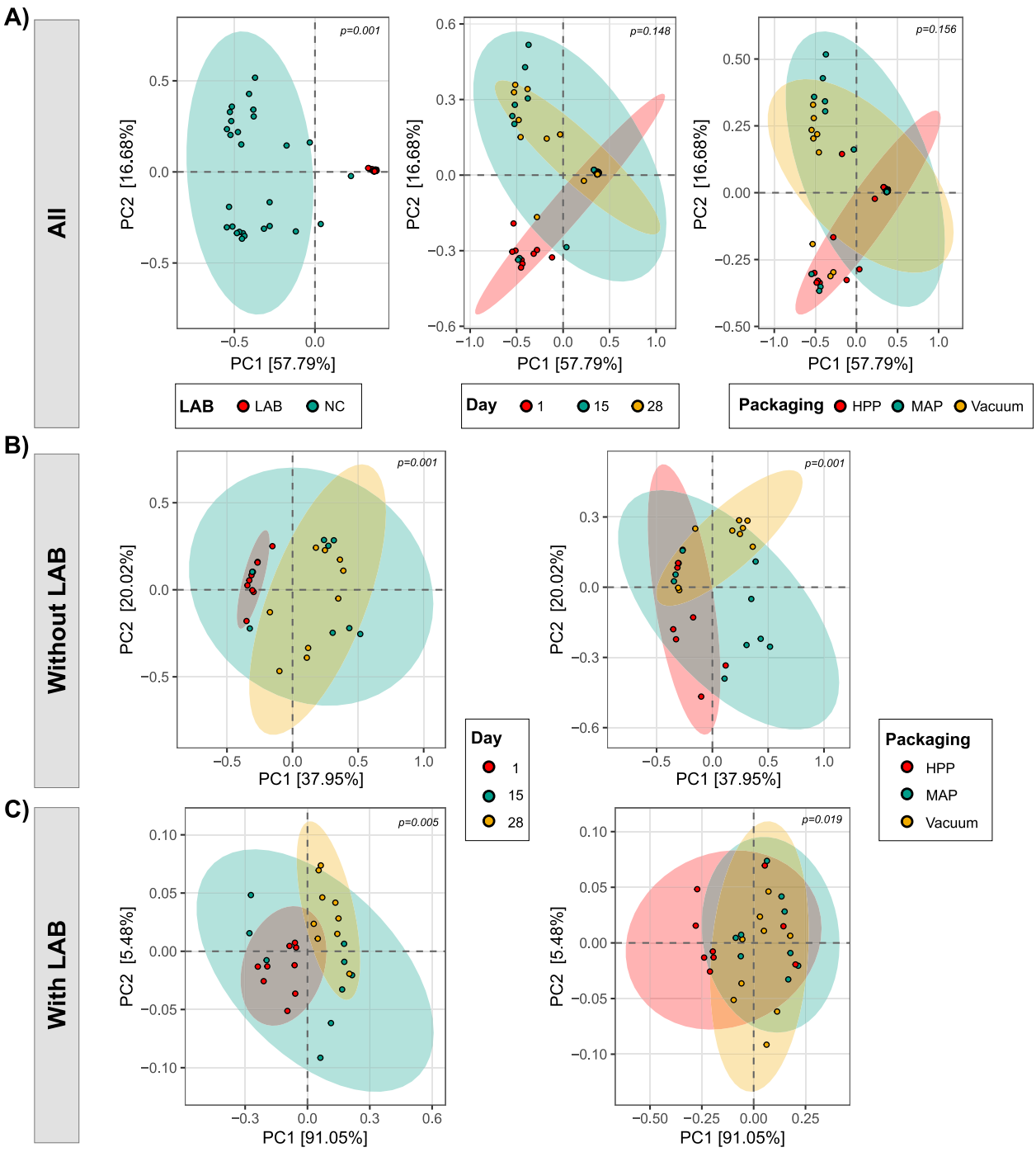

Fig. 8. Principle Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity metrics of the bacterial communities of cooked ham showing differences among storage days and packaging method used. A) All samples; B) Only samples without inoculation of LAB strains; and C) Only samples inoculated with the LAB cocktail.

respectively).

The influence of storage time and type of packaging on the intra-sample alpha diversity is shown in Fig. 9. From 7 to 30 different Amplicon Sequence Variants (ASV) were obtained per sample. Similar alpha-diversity values were observed in a previous study performed by our group in dry fermented sausages with starter cultures added and applying HPP at the early stages of ripening (Dučić et al., 2023). Generally, a higher richness was observed in samples without LAB inoculation. Furthermore, it can be observed that ASV richness progressively and significantly decreased ($P < 0.05$) along storage time in samples with LAB added to reach maximum values of 10 ASV on the last day of storage. In samples without LAB added, there was a statistically significant decrease in richness from day 1 to day 15 or day 28 of storage, but no significant differences were found from day 15 to day 28. The Simpson diversity index provided similar results, showing that samples without LAB added had a higher diversity than samples with the LAB cocktail, where the inoculated strains (and particularly the ULE721 strain) dominated. Moreover, a significantly higher Simpson index was observed at day 1 than at day 28 of storage in both sample categories. This agrees with the results obtained by Raimondi et al. (2019), who observed a more complex community in cooked ham at the start of shelf-life than at the end. The differences among the packaging methods employed were less marked. Thus, only HPP-treated non-LAB inoculated cooked ham samples showed a significantly higher ASV richness than

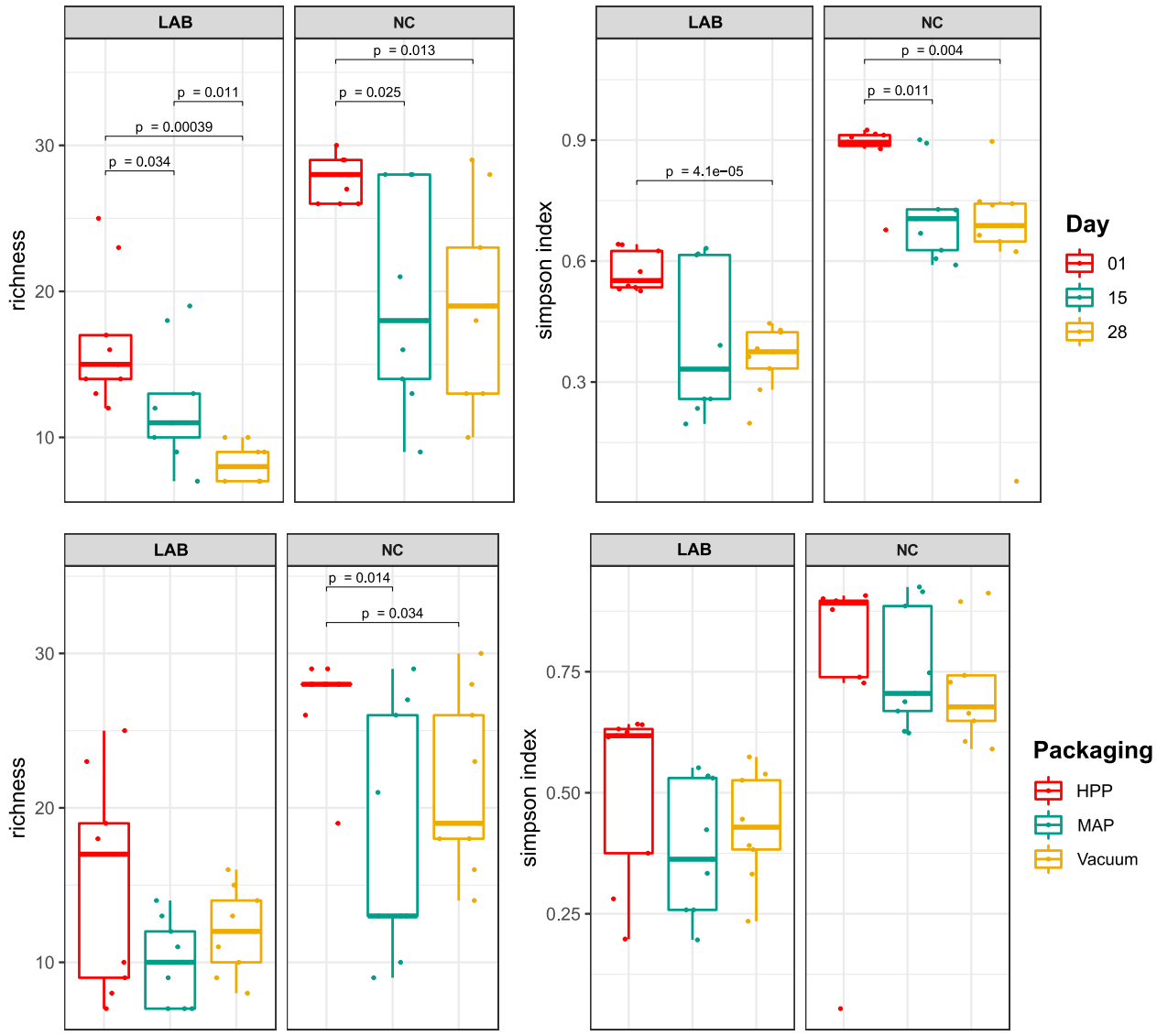

Fig. 9. Alpha-diversity analysis showing the richness and Simpson diversity index of the cooked ham as influenced by storage day or packaging method used.

Table 2
Color parameters (lightness (L*), chroma (C*), hue (h*) and total color difference (TCD)) during storage of cooked ham with (LAB) and without (NC) LAB added packaged under vacuum (V), modified atmosphere packaging (MAP) and vacuum and High-Pressure Processing (HPP).

	*L**			*C**			*h**			TCD (ΔE)
	Day 1	Day 14	Day 27	Day 1	Day 14	Day 27	Day 1	Day 14	Day 27	
V-NC	63.5 ± 0.5b, A	63.4 ± 0.4 a, A	63.6 ± 0.9 ab, A	12.6 ± 0.5 b, A	13.3 ± 0.4 b, B	13.5 ± 0.3 bc, B	37.3 ± 1.4 a, B	35.5 ± 1.2 a, A	36.3 ± 1.5 ab, AB	1.3 ± 0.6 a
V-LAB	63.7 ± 0.7 b, A	63.7 ± 0.7 b, B	64.4 ± 0.7 bc, AB	12.4 ± 0.5 b, A	14.0 ± 0.4 c, B	13.9 ± 0.5 c, B	37.1 ± 1.8 a, B	36.5 ± 1 a, AB	35.6 ± 0.9 a, A	1.9 ± 0.9 ab
HPP-NC	63.5 ± 0.4 ab, A	63.0 ± 0.8 a, A	63.1 ± 0.6 a, A	12.7 ± 0.3 b, A	13.2 ± 0.3 ab, B	13.1 ± 0.5 a, AB	39.0 ± 2.1 a, B	35.9 ± 1.4 a, A	37.3 ± 0.9 ab, AB	1.2 ± 0.6 a
HPP-LAB	62.6 ± 0.8 a, A	64.6 ± 0.6 b, A	63.8 ± 0.7 ab, B	12.0 ± 0.8 ab, A	13.1 ± 0.4 ab, B	14.0 ± 0.4 c, C	37.6 ± 2.2 a, A	36.2 ± 1.4 a, A	36.1 ± 1.6 ab, A	2.6 ± 0.9 bc
MAP-NC	62.9 ± 0.8 ab, A	64.4 ± 0.6 b, B	64.3 ± 1.0 bc, B	11.9 ± 1 ab, A	12.8 ± 0.3 a, B	13.4 ± 0.5 bc, B	38.5 ± 4.1 a, A	37.0 ± 1.8 a, A	37.3 ± 1.5 ab, A	2.5 ± 0.6 b
MAP-LAB	63.2 ± 0.4 ab, A	65.6 ± 0.6 c, B	65.3 ± 0.5 c, B	11.1 ± 0.6 a, A	13.9 ± 0.2 c, B	13.9 ± 0.6 c, B	37.9 ± 1.7 a, A	37.1 ± 1.1 a, A	37.9 ± 1.4 b, A	3.6 ± 0.9 c

Results are presented with mean values ± standard deviations.
a-c: significant differences ($P < 0.05$) among samples in the same day.
A-B: significant differences ($P < 0.05$) among storage times for each sample type.

those counterparts packaged under MAP or vacuum.

3.6.4. Cooked ham with the addition of the LAB cocktail (8 log_{10} CFU/g) under different packaging conditions: color and texture analyses

Color results are summarized in Table 2. It can be observed that on the last day of storage, samples packaged under MAP tended to be lighter than the rest of the samples. In particular, cooked ham samples with LAB added and packaged under MAP, which had the highest lightness values on days 14 and 27 of storage (L values of 65.6 ± 0.6 and 65.3 ± 0.5, respectively). Moreover, in all packaging conditions, the addition of LAB significantly increased the lightness of the samples on day 14, and subtly on day 27.

A rise in *chroma* was generally observed from day 1 to day 14 and was maintained until day 27, meaning that color intensity increased during the first two weeks. This finding indicates that discoloration is not occurring during shelf-life, as was observed in similar studies (Moges Haile et al., 2013). On the other hand, no major differences in *hue* values were observed among samples on the same day, but a slight decrease was detected during storage time. Nonetheless, taking into consideration the total color difference (TCD), the samples that had the highest color change during storage corresponded to those with the LAB cocktail added and packaged under MAP (ΔE = 3.6 ± 0.9). According to Tamm et al. (2016), consumers could be able to detect differences among samples when ΔE is above 2. Hence, considering that threshold, samples under MAP with and without LAB added, and samples treated with HPP and inoculated with LAB, might be differentiated by consumers based on their color.

Results of the TPA are summarized in Table 3, which includes hardness, cohesiveness and elasticity values of the cooked ham during storage. There were no significant differences in hardness regardless of the type of packaging used or of whether LAB were inoculated or not, with mean values of 43.9 ± 3.6 N. On the other hand, samples packaged under MAP showed lower cohesiveness than those under vacuum or subjected to HPP treatment, although this only turned out to be statistically significant for the samples under MAP without LAB inoculation. The fact that the HPP treatments were applied to previously vacuum-sealed samples suggests that vacuum packaging increases the cohesiveness of cooked ham, which is in accordance with the findings by García-Esteban et al. (2004). In addition, it can be observed that cohesiveness and elasticity did not decrease along storage time. No further differences were observed in other TPA parameters, such as resilience, gumminess or chewiness, with mean values of 0.41 ± 0.02, 25.6 ± 3.0 and 24.8 ± 3.1, respectively (data not shown).

3.6.5. Cooked ham with the addition of the LAB cocktail (8 log_{10} CFU/g) under different packaging conditions: sensorial evaluation

In order to evaluate the consumer perception of the product, at day 14, a 52-member panel assessed the cooked ham considering various parameters, including color, odor, flavor, global appearance and whether the ham odor was generally pleasant or not (Table 4). It must be highlighted that no undesirable exudates were visually observed in any of the samples during the entire experiment. LAB-inoculated samples, both stored under vacuum or under MAP, were considered more acid than non-inoculated samples when evaluating taste. This acidity was slightly appreciated also as odor-associated acidity. In addition, when panelists were asked to order the samples from a global point of view, those samples were the ones ranking lowest. These results reflect the pH decrease that occurred in both sample types during the storage period. On the contrary, the pH of samples packaged under HPP did not suffer such a pronounced decrease and was maintained at approximately 5.9 until day 20, and these samples showed a better acceptability by panelists. The samples which were best ranked were those samples without LAB added and packaged under MAP.

When the panelists had to choose which samples were pleasant or unpleasant according to odor, samples with LAB added tended to have higher unpleasant frequency, which can be attributed to the pH drop in the product. However, no statistically significant differences were found among sample categories (Table 4). This led us to conclude that those samples with LAB added, although considered more acid, were still accepted by the consumer. Samples with LAB added and HPP-treated were globally better accepted and had lower unpleasant frequency than other LAB-inoculated samples. This result is in agreement with another study where a HPP-treatment with 400 MPa and the addition of 2560 AU/g of enterocin extended the shelf-life of cooked ham and also improved the sensory profile during the whole storage (Liu et al., 2012).

4. Conclusions

The screening of a wide collection of LAB led to the selection of 6 strains, namely *L. lactis*, *L. plantarum* ($n = 4$) and *L. paracasei*, based on their in vitro antagonistic activity against *L. monocytogenes* and/or *E. coli*. These six LAB strains demonstrated good anti-Listerial activity in three RTE cooked meat products: cooked ham, meatloaf and roasted pork shoulder. Remarkably, *L. lactis* ULE383 was confirmed as a nisin Z producer. The three strains with the most promising activity, namely *L. lactis* ULE383, *L. paracasei* ULE721 and *L. plantarum* ULE1599, were selected for subsequent tests, undertaken individually or as a cocktail, in cooked ham at different inoculum concentrations, obtaining the best anti-Listerial activity with the mix of the three strains at the highest inoculum concentration tested (i.e., 8 log_{10} CFU/g). When testing the

Table 3
Hardness, cohesiveness and elasticity values calculated from the Texture Profile Analysis (TPA) of cooked ham with (LAB) and without (NC) LAB added, packaged under vacuum (V), modified atmosphere packaging (MAP) and vacuum and High-Pressure processing (HPP).

	Hardness (N)			Cohesiveness			Elasticity		
	Day 1	Day 14	Day 27	Day 1	Day 14	Day 27	Day 1	Day 14	Day 27
V-NC	44.1 ± 4.5 a, A	48.7 ± 5.2 a, A	44.4 ± 6.3 a, A	0.58 ± 0.04 bc, A	0.60 ± 0.03 bc, B	0.61 ± 0.03 bc, AB	0.94 ± 0.01 a, A	0.99 ± 0.02 ab, A	0.98 ± 0.02 ab, A
V-LAB	42.0 ± 6.8 a, A	45.8 ± 6.8 a, A	42.5 ± 8.2 a, A	0.58 ± 0.03 bc, A	0.63 ± 0.02 c, A	0.65 ± 0.02 c, A	0.94 ± 0.03 a, A	0.96 ± 0.04 a, B	0.95 ± 0.02 a, B
MAP-NC	35.3 ± 5.7 a, A	42.8 ± 5.5 a, A	42.0 ± 5.3 a, A	0.46 ± 0.05 a, A	0.49 ± 0.05 a, A	0.51 ± 0.06 a, A	0.95 ± 0.02 a, A	0.99 ± 0.03 ab, B	1.00 ± 0.0 b, B
MAP-LAB	39.7 ± 6.9 a, A	49.9 ± 10.3 a, A	46.6 ± 8.1 a, A	0.51 ± 0.03 a, A	0.55 ± 0.02, ab, B	0.55 ± 0.04 a, B	0.96 ± 0.02 a, A	0.98 ± 0.02 ab, A	0.97 ± 0.02 ab, A
HPP-NC	41.9 ± 2.2 a, A	45.8 ± 5.8 a, B	43.9 ± 5.3 a, AB	0.57 ± 0.02 bc, A	0.61 ± 0.06 bc, A	0.61 ± 0.03 bc, A	0.95 ± 0.02 a, A	1.00 ± 0.01 b, B	0.99 ± 0.02 ab, B
HPP-LAB	41.1 ± 9.0 a, A	49.3 ± 5.3 a, A	44.4 ± 7.7 a, A	0.61 ± 0.07 c, A	0.60 ± 0.04 bc, A	0.61 ± 0.04 bc, A	0.95 ± 0.03 a, A	1.00 ± 0.0 ab, B	0.97 ± 0.04 ab, AB

Results are presented with mean values ± standard deviations.
a-c: significant differences ($P < 0.05$) among samples in the same day.
A-B: significant differences ($P < 0.05$) among storage times for each sample type.

Table 4
Results of the sensory tests after 14 days of storage of the cooked ham with (LAB) and without (NC) LAB added, packaged under vacuum (V), modified atmosphere packaging (MAP) and vacuum and High-Pressure processing (HPP).

	Ranking values				Odor (% frequency)		
	Color	Odor	Flavor	Global	Unpleasant	No rated	pleasant
V-NC	2.9 ± 1.6 a	2.7 ± 1.5 a	2.5 ± 1.4 a	3.5 ± 1.8 ab	20.83	37.50	41.67
V-LAB	3.6 ± 1.7 ab	4.1 ± 1.6 cd	4.8 ± 1.4 b	2.7 ± 1.8 a	29.17	35.42	35.42
HPP-NC	3.4 ± 1.5 ab	3.0 ± 1.5 ab	2.9 ± 1.4 a	3.9 ± 1.4 b	18.75	45.83	35.42
HPP-LAB	4.3 ± 1.7 b	3.3 ± 1.6 abc	3.0 ± 1.6 a	3.9 ± 1.4 b	22.92	45.83	31.25
MAP-NC	3.3 ± 1.8 a	3.7 ± 1.8 bcd	3.1 ± 1.3 a	4.1 ± 1.6 b	14.58	41.67	43.75
MAP-LAB	3.5 ± 1.7 ab	4.3 ± 1.7 d	4.7 ± 1.6 b	2.8 ± 1.8 a	33.33	33.33	33.33

Ranking values represent the mean values ± standard deviations obtained in the ranking test (1–6) carried out by the 52 panelists.
Frequency refers to the percentage of panelists that assigned the odor of each sample as pleasant, unpleasant, or not rated.
a-d: significant differences ($P < 0.05$) among sample categories for a given quality attribute.

application of the three strains LAB cocktail under different packaging conditions (vacuum packaging, modified atmosphere packaging, and vacuum packaging followed by HPP), an efficient control of the growth of *L. monocytogenes* was achieved, which was accompanied by a significant acidification of the samples throughout shelf-life, which represents a major challenge for the application of the LAB cultures in practice by meat producers. Remarkably, samples with LAB added and HPP-treated achieved a complete inhibition of the pathogen but the acidification was controlled along shelf-life, which opens new opportunities to the application of the agents in practice. Metataxonomic analyses evidenced that the food cultures applied to cooked ham were able to adapt to the product and dominate over the background microbiota, including spoilage microorganisms. Indeed, a higher richness of bacterial taxa was observed in samples without LAB inoculation. Generally, there were no major changes in color and texture parameters attributed to the different packaging conditions tested or to the addition of LAB. In the sensory evaluation, the pH decrease that occurs in cooked ham artificially inoculated with LAB, both under vacuum and MAP, was noticed by the panelists, although none of the batches were considered unacceptable. Overall, the application of the cocktail of *L. lactis* ULE383, *L. paracasei* ULE721 and *L. plantarum* ULE1599 achieved great results controlling the growth of artificially inoculated *L. monocytogenes* and of some relevant spoilage microorganisms, without causing substantial quality losses. The antilisterial effect observed in the cooked ham might be attributed to different facts (i.e., the acidification throughout shelf life, direct competition with the LAB strains, or production of antimicrobial substances), and further studies are needed in order to ascertain whether the production of nisin Z by *L. lactis* ULE383 played an important role. Moreover, if the cocktail of strains is intended to be used for commercial purposes, a more insightful biochemical characterization of the strains would be warranted and, the application of the LAB at a concentration of 10^8 cfu/g in cooked ham would need to be assessed in detail from a practical point of view, considering also the acceptance by the producers and consumers. Also, it could be important to reduce the acidification effect taking place, as was here accomplished by combining the LAB cocktail with mild HPP treatments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110341.

Declaration of competing interest

None declared.

Data availability

Data will be made available on request.

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