



Lactiplantibacillus plantarum IBB082 inoculation shapes microbial succession, metabolite profile and immunomodulatory potential in traditional short-term Romanian cucumber fermentations

José Ángel Salas-Millan^{a,*,1} , Mihai-Alexandru Tonciulescu^{b,c,1} , Todor T. Koev^d ,
Alise J. Ponsero^e , Ignacio Quevedo-Romero^{b,2} , Covadonga Valle Alcaraz^{b,2} ,
Pierric L.D. Chuppa^{b,2} , Caridad Soto^{b,2} , Fernando Ródenas Pérez Cuadrado^{b,2} ,
Nieves García-Lorca^{b,f,2} , Alberto Gila-Navarro^{f,2} , Poorvaja Muley^a , Leilei Yu^g , David Baker^e ,
Andrea Telatin^e , Iulia-Roxana Angelescu^h , Alfredo Palop^{b,f} , Arjan Narbad^e ,
Silvia Melgar^a , Enriqueta Garcia-Gutierrez^{b,f,**}

^a APC Microbiome Ireland, University College Cork, Cork, T12 YT20, Ireland

^b Department of Agronomic Engineering, Technical University of Cartagena, Member of European University of Technology EUT+, Paseo Alfonso XIII, 48, Cartagena, 30203, Spain

^c Department of Food Engineering, Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, Bucharest, 011464, Romania

^d School of Chemistry, Pharmacy and Pharmacology, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

^e Quadram Institute Bioscience, 16, Norwich, NR4 7UQ, UK

^f Instituto de Biotecnología Vegetal, Technical University of Cartagena, Member of European University of Technology EUT+, Cartagena, 30202, Spain

^g School of Food Science and Engineering, Hainan University, Haikou, Hainan, China

^h Institute of Biology Bucharest of the Romanian Academy, 296 Splaiul Independentei, Bucharest, 060031, Romania

ARTICLE INFO

Keywords:

Lactic acid bacteria (LAB)
Lactiplantibacillus plantarum
Romanian cucumber
Fermentation
Metabolomics
Antioxidant
Food safety
Pathogens
Immunoregulatory

ABSTRACT

Romanian fermented cucumbers are traditionally produced by spontaneous lactic acid fermentation at household level and may represent valuable sources of beneficial lactic acid bacteria (LAB) and bioactive metabolites. However, their microbial composition, functional properties, and safety under in-house conditions remain poorly characterised. In this study, Romanian-style fermented cucumbers were produced over the lowest range of typical household fermentation, a two-week period, using either spontaneous fermentation or inoculation with *Lactiplantibacillus plantarum* IBB082. Microbial community dynamics were characterised by 16S rRNA gene amplicon sequencing, while fermentation-derived metabolites were identified by untargeted analytical assays. Total phenolic compounds and antioxidant capacity were also evaluated. Spontaneous fermentations showed greater microbial variability, whereas *Lactiplantibacillus*-inoculated fermentations exhibited faster LAB dominance and more consistent acidification. Moreover, fermentation in the presence of *L. plantarum* IBB082 was associated with distinct metabolite profiles and enhanced antioxidant capacity. Additionally, both fermentation strategies activated NF-κB in IL-1β-stimulated intestinal epithelial cells, suggesting a potential immunomodulatory response. These results were then compared with those of a commercial product. To our knowledge, this is the first study integrating metataxonomic, metabolomic, immunoregulatory and molecular safety profiling in short-term household-style Romanian cucumber fermentations, providing a comprehensive framework for the characterisation and quality control of traditional artisanal fermented foods.

* Corresponding author.

** Corresponding author. Department of Agronomic Engineering, Technical University of Cartagena, Member of European University of Technology EUT+, Paseo Alfonso XIII, 48, 30203, Cartagena, Spain.

E-mail addresses: Jose.SalasMillan@teagasc.ie (J.Á. Salas-Millan), enriqueta.garcia@upct.es (E. Garcia-Gutierrez).

¹ These authors contributed equally.

² These authors contributed equally.

<https://doi.org/10.1016/j.fbio.2026.109620>

Received 2 May 2026; Received in revised form 17 July 2026; Accepted 28 July 2026

Available online 29 July 2026

2212-4292/© 2026 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Romanian fermented cucumbers (“castraveți murăți”) are traditional side dishes widely consumed in Eastern Europe and represent an important source of fermented vegetables in the local diet (Mattalia et al., 2020). They are typically produced at a household level by spontaneous lactic fermentation of fresh cucumbers in salt brine, without heat treatment (Mattalia et al., 2020). As such, these products may act as natural sources of live lactic acid bacteria (LAB), fermentation-derived metabolites and antioxidant compounds (Thiruvengadam & Chung, 2015). However, despite their widespread consumption, the functional properties and microbiological safety of in-house fermented cucumbers remain insufficiently characterised.

In Romania, in-house brined cucumbers and mixed vegetable “murături” are commonly prepared seasonally by immersing cucumbers together with spices in saline brine and allowing the fermentation to proceed at ambient temperature for 2 to 4 weeks (Mattalia et al., 2020). Acidification and product stabilisation depend on indigenous microbiota and naturally occurring LAB, which drive carbohydrate fermentation and the development of the characteristic sour flavour and texture (Dupas de Matos et al., 2019). While artisanal preparation is deeply embedded in culinary tradition, household fermentations are inherently variable (Salas-Millán et al., 2024). Differences in raw materials, salt concentration, temperature, and hygiene conditions may affect fermentation kinetics, microbial succession, metabolite production, and the potential survival of spoilage organisms or pathogens (Tamang et al., 2020).

Cucumber fermentation is generally dominated by LAB, particularly *Lactobacillus* and related genera, which produce lactic acid, lower the pH, inhibit competing microorganisms, and contribute to sensory quality (Ahmed et al., 2021; Yang et al., 2025). Previous studies have shown that the use of selected LAB starter cultures in fermented cucumbers and other pickled vegetables can improve process consistency, enhance sensory attributes, and promote functional and probiotic-associated properties such as antioxidant activity, nitrite degradation and antimicrobial activity against foodborne pathogens (Ahmed et al., 2021; Yang et al., 2025). These findings suggest that traditionally fermented cucumbers could serve as carriers of beneficial microorganisms, provided their safety is ensured (Grosu-Tudor & Zamfir, 2013).

Despite the long-standing tradition of Romanian brined cucumbers and their widespread household consumption, their microbiological safety and functional properties remain poorly characterised under real production conditions. This is particularly relevant given that household fermentations are inherently variable and conducted without quality controls, creating conditions where microbial succession may be unpredictable and pathogen survival cannot be excluded (Tamang et al., 2020). Moreover, while starter cultures have been explored in industrial pickle production, their potential to improve consistency, safety and functional quality in short-term, artisanal fermentations, such as those typically practised in Romanian households, has not been systematically evaluated. Understanding whether the introduction of a well-characterised LAB strain can steer fermentation dynamics toward more predictable and functionally superior outcomes, without compromising the artisanal nature of the product, represents a question with both scientific and public health relevance. The present study addresses this gap by characterising Romanian-style cucumber fermentations conducted in-house over a two-week period, the lower bound of typical household fermentation, under spontaneous conditions or following inoculation with *Lactiplantibacillus plantarum* IBB082 as a single-strain starter culture. By integrating 16S rRNA gene amplicon sequencing, NMR-based metabolomics, antioxidant capacity assays, anti-inflammatory bioactivity assay on intestinal epithelial cells, and digital PCR-based pathogen detection, this study provides, to our knowledge, the first comprehensive characterisation of the microbiological, metabolic and functional properties of short-term in-house

Romanian cucumber fermentations. Our findings contribute to a broader understanding of the safety and functional potential of traditional Eastern European fermented vegetables, and offer a scientific basis for the rational use of starter cultures in artisanal food production.

2. Methods

2.1. In-house fermentations of Romanian cucumbers

Traditional style cucumber fermentations were conducted under controlled conditions at the Department of Microbiology, Institute of Biology Bucharest (Romanian Academy). Fresh cucumbers and all plant ingredients were purchased from the local market “Piata Progresul” in Bucharest, on the 27th June 2025 and processed immediately.

The starter culture used for inoculated fermentations was *L. plantarum* IBB082, an isolate from the culture collection of the Institute of Biology Bucharest (Bucharest, Romania) and was originally isolated from a sample of traditionally homemade *borș* from the North-Eastern region of Romania (Botoșani county) and stored at -80°C in MRS supplemented with 25% glycerol (Grosu-Tudor et al., 2019). The strain was revived in De Man-Rogosa-Sharp (MRS) broth and on MRS agar (Oxoid) at 30°C for 24 h. Biomass obtained from liquid culture was harvested by centrifugation and washed twice with sterile physiological saline (20 mL per wash). The final suspension volume was adjusted to 20 mL.

A total of 24 glass jars (250 mL volume) were prepared, each containing 100 g of cucumbers, 25 g garlic, 2 g mustard seeds, 2 dill inflorescences, and 10 peppercorns (Fig. 1). The brine was prepared using 3 L water, 100 g non-iodised coarse salt, 30 g sugar, bay leaves, celery leaves, sour cherry leaves, and dill. Twelve jars were inoculated with 1 mL of *L. plantarum* IBB082 suspension per 100 mL liquid (starter fermentation), while the remaining twelve jars were left uninoculated to allow spontaneous fermentation. All jars were sealed and incubated at room temperature ($23 \pm 2^{\circ}\text{C}$).

Sampling was performed at four different time points: 24 h, 48 h, 1 week and 2 weeks. At each sampling point, 45 mL of brine and small cucumber fragments were collected aseptically from selected jars. Samples were transferred to sterile 50 mL centrifuge tubes and stored at -20°C until further analysis.

Commercial cucumbers were included as a real-world reference point to contextualise the microbiological and metabolic profiles of the in-house fermented products, rather than as a directly comparable experimental control. Full manufacturing parameters for the commercial products were not available, as these are proprietary.

2.2. DNA extraction and amplicon sequencing

DNA was extracted using a Maxwell® Robot (Promega, UK) with Maxwell® RSC PureFood GMO and Authentication Kit AS1600 (Promega, UK) following manufacturer's protocols. DNA concentration was measured using Qbit (Thermo Fisher Scientific, UK) and normalised to 5 ng/μL with EB (10 mM Tris-HCl). A PCR master mix was made up using 10 μL KAPA 2G Fast Hot Start Ready Mix (Merck, UK), 0.1 μL 100 μM of forward and reverse primers, and 7.8 μL PCR grade water per sample. 18 μL master mix were added to each well to be used in a 96-well plate followed by 2 μL of DNA and mixed. Specific PCR conditions were 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by a final 72°C for 5 min. For the second PCR 10 μL KAPA 2G Fast Hot Start Ready Mix (Merck, UK) and 8 μL PCR grade water were mixed per sample and added to a 96 well plate. 1 μL of 10 μM 8 bp Unique Dual Indexes were added to each well. Finally, 1 μL of PCR 1 was transferred into the PCR 2 master mix plate. The second PCR was run using 95°C for 5 min, 10 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by a final 72°C for 5 min. Final libraries were quantified using Qubit and equimolar pooled together. A single 0.7X SPRI clean-up using sample purification beads (Illumina® DNA Prep, (M)

Tagmentation (96 Samples, IPB), USA) was performed on the pool. A final Qubit and sizing on a D5000 Screen Tape using the Agilent TapeStation 4200 was done to calculate the final library pool molarity. The pool was run at a final concentration of 750 pM on an Illumina NextSeq 2000 instrument using a NextSeq 2000 P1 XLEAP-SBS Reagent Kit (600 Cycles) following the Illumina recommended loading recommendations which included a 20% PhiX spike in.

2.3. Bioinformatics analyses

Quality control and primer removal were performed on the raw sequences using FastQC v0.11.9 (<https://github.com/s-andrews/FastQC/releases/tag/v0.11.9>) and Cutadapt v3.4 (<https://github.com/marcelm/cutadapt/releases/tag/v3.4>). Reads without primers were discarded and polyG tails were trimmed. The sequencing reads were processed using the R package Dada2 v1.30.0 for quality filtering, chimera detection and taxonomic annotation (Callahan et al., 2016). The low quality reads were trimmed and filtered using the following parameters: $truncLen=c(270,250)$, $minLen=250$, $maxN=0$, $maxEE=c(2,2)$, $truncQ=5$. Error models were corrected using a custom Loess function to take into account for the binned quality score used by the sequencing platform. After merging, potential chimeras were removed using the 'consensus' method implemented in Dada2. After completing each stage, the readings are filtered and reduced (Fig. S1). On average, 75% of the raw reads were retained after the filtering, merging and chimera removal steps.

The taxonomic annotation of the ASVs was performed against the SILVA v138.2 database (available at <https://zenodo.org/records/4587955>), using the assignTaxonomy function (minimum bootstrapping support threshold of 50) which implements a naive Bayesian classifier method provided by the Ribosomal Database Project (Wang et al., 2007). Singleton ASVs were excluded, as well as ASVs classified as mitochondrial ($n = 10$ ASV) or chloroplastic ($n = 19$ ASV) or left unclassified at the order level, leaving 991 ASV, 908 (92%) of them classified at the genus level (Fig. S2). Genus-level resolution is the standard output of V3-V4 16S rRNA gene amplicon sequencing, as the inherent sequence similarity within this hypervariable region is insufficient to reliably discriminate between closely related species. Species-level resolution would require full-length 16S sequencing or shotgun metagenomics. The extraction blank had a low sequencing depth (1600 reads), and the 2 ASVs detected in the blanks with more than 100 reads were excluded from the samples. Two of the three commercial controls had a sequencing depth below 20k reads and were

excluded from the downstream analysis. The fermentation negative control had a sequencing depth of 33k reads, consistent with the absence of a fermentation process in the sample and was therefore retained as a baseline comparison.

2.4. Culture-based detection of *Salmonella* spp and *Listeria monocytogenes*

Salmonella spp. and *L. monocytogenes* were selected as target pathogens, given their status as mandatory microbiological criteria for ready-to-eat foods under European food safety legislation (European Commission Regulation No 2073/2005), and their recognition by the European Food Safety Authority (EFSA) as primary hazards of concern in fermented vegetable products (EFSA BIOHAZ Panel, 2018; EFSA & ECDC, 2023). The presence of *L. monocytogenes* and *Salmonella* spp. was evaluated by culture-based methods in accordance with the reference methods established under European Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Detection of *L. monocytogenes* was performed following ISO 11290-1:2017, primary enriched in half-Fraser broth ($25 \text{ h} \pm 1 \text{ h}$ at 30°C), and doing a secondary enrichment in Fraser broth (Scharlab, Spain) ($24 \text{ h} \pm 2 \text{ h}$ at 37°C), followed by an isolation on Agar Listeria according to Ottaviani and Agosti (ALOA) (Scharlab, Spain). Confirmation of presumptive colonies was carried out by appropriate biochemical tests. Detection of *Salmonella* spp. was performed following ISO 6579-1:2017, involving pre-enrichment in buffered peptone water (Scharlab, Spain) (BPW; $18 \text{ h} \pm 2 \text{ h}$ at 37°C), selective enrichment in Rappaport-Vassiliadis soya broth (RVS) (Scharlab, Spain) and Muller-Kauffmann tetrathionate/novobiocin broth (MKTn) (Scharlab, Spain), and plating on xylose lysine deoxycholate (XLD) agar (Scharlab, Spain).

2.5. Pathogen identification via dPCR

Digital polymerase chain reaction (dPCR) was used for their detection and absolute quantification, using primers previously described for distinctively identify them. More specifically, we used the *invA* gene (*Salmonella* spp.) with sequences forward (*invA*-119-F): GTG AAA TTA TCG CCA CGT TCG GG and reverse (*invA*-119-R): TCA TCG CAC CGT CAA AGG AAC (Li & Chen, 2013) and the *hlyA* gene (*L. monocytogenes*), with sequences forward: GCA ACG CAA TCC GAA AAA and reverse: GCT GCA CTT GGA GAC TAA (Liu et al., 2012). DNA extracted from the samples was used as template for dPCR analysis. DNA from *Salmonella*

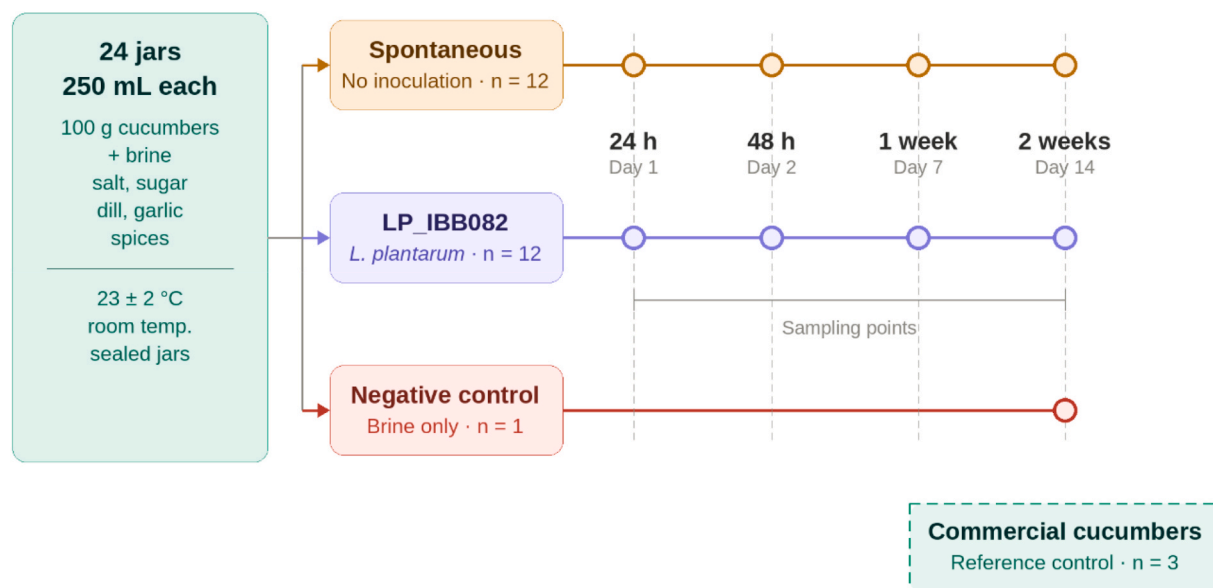


Fig. 1. Processing of fermented cucumber using spontaneous and *L. plantarum* (LP_IBB082) styles.

Typhimurium strain 14028S and *L. monocytogenes* strain EDGe were used as positive controls respectively. dPCR reactions were prepared using the QIAcuity® EvaGreen PCR Master Mix (QIAGEN, Hilden, Germany). Each 12 µL reaction consisted of 5 µL of EvaGreen PCR Master Mix, 1 µL of DNA template, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), and nuclease-free water up to volume. Reaction mixtures were transferred to QIAcuity Nanoplates 8.5K, 96-well format, and partitioning and amplification were carried out using the QIAcuity Digital PCR System (QIAGEN, Germany) according to the manufacturer's instructions. A standard priming profile was applied to seal the partitions, followed by thermocycling under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and annealing/extension at the assay-specific annealing temperature for 30 s, and extension at 72 °C for 2 min. A final extension step was performed at 72 °C for 2 min.

Following amplification, fluorescence imaging was conducted in the green detection channel, corresponding to EvaGreen fluorescence (excitation at 488 nm and emission at 520 nm), using an exposure time of 400 ms and a gain of 4. Images of all wells were acquired for downstream analysis. Each sample was analysed in two technical replicates. A no-template control (NTC) was included on each nanoplate to monitor potential contamination and validate the assay performance. Absolute quantification of target DNA was performed using the QIAcuity Software Suite version 2.1.7.182 (QIAGEN, Germany). Target concentrations were calculated based on the proportion of positive and negative partitions using Poisson statistics and expressed as copies per µL of reaction. Quantification was considered valid only when at least one-third of the total accepted partitions were negative. For each sample, the mean value of copies/µL obtained from the two technical replicates was calculated and results reported as absolute DNA copy numbers. As DNA-based detection was employed, results represent total detectable DNA and do not differentiate between viable and non-viable cells.

2.6. Metabolomics analyses

Aliquots of 400 µL of the brine of the fermented cucumbers were added to 100 µL of metabolomics buffer (Koev et al., 2022), vortexed and placed directly in NMR tubes (Wilmad® 528-PP). All samples were run on a Bruker Avance Neo NMR spectrometer, operating at ¹H frequency of 500.14 MHz, equipped with an iProbe. Spectra recorded were obtained using Bruker's 'zgpgpewg' pulse sequence for optimal solvent suppression with minimal residual artefacts. All other parameters were: ¹H $\pi/2$ rf pulse of 10 µs, a minimum of 256 scans, spectral width of 8000 Hz, acquisition time of 4 s, and relaxation delay of 10 s. Spectra were apodized at 0.3 Hz, fitted and analysed using NMR Suite Processor v8.41 (Chenomx®, Edmonton, Canada) for metabolomic quantification.

2.7. Total phenolic content (TPC) and antioxidant capacity

Total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC) were determined using a Multiscan plate reader (Tecan Infinite M200, Männedorf, Switzerland). The TEAC assay was performed using the ABTS radical cation reagent (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) (Sigma-Aldrich, St. Louis, MO, USA). Measurements were conducted as described previously (Salas-Millán et al., 2024). The analysis was performed on the supernatant obtained from the cucumber fermentation process. Thus, appropriate dilutions were prepared using Milli-Q water. Results were expressed as mg gallic acid equivalents (GAE) for TPC, and mmol Trolox equivalents (TE) per 100 mL of fermented supernatant.

2.8. HCT116-Dual Reporter cells assess the impact of the brine on viability, cytokine-induced NF-κB pathway activation and IL-8 secretion

The human intestinal epithelial reporter cell line, HCT116-Dual™, was purchased from InvivoGen (Toulouse, France). Cells were grown in

Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Missouri, USA), supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (Sigma-Aldrich), 10 µg/mL of blasticidin, 100 µg/mL Zeocin®, and 100 µg/mL of Normocin™ (InvivoGen) at 37 °C, in a humidified atmosphere of 5% CO₂ and 95% air, as previously reported (Kulecka et al., 2025). To study the anti-inflammatory potential, cells were pre-treated for 24 h with 10% of cucumbers covering brine, followed by stimulation with human recombinant (hr)-IL-1β at 100 pg/mL for 24 h. Untreated cells served as negative controls. Cells between passages 6 and 14 were used in this study. The experiments were performed in technical triplicates using samples from three independent fermentation experiments.

To assess cell viability, SYTOX green stain and lactate dehydrogenase (LDH) release assay was utilised. The cells were stained with 1 µM SYTOX™ Green stain (ThermoFisher, Massachusetts, USA) for 20 min at 37 °C, followed by fluorescence reading at 485 nm excitation and 530 nm emission using a Synergy 2 multimode microplate reader (Biotek, Winooski, VT, USA). LDH release was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay as per manufacturer's instructions (Promega, Wisconsin, US). Cells treated with 1% Triton X-100 lysis buffer for 45 min served as a positive control of reduced cell viability.

The activation of the NF-κB pathway was quantified by using the Quanti-Blue™ assay (InvivoGen, Toulouse, France) following the manufacturer's instructions. Briefly, cell culture supernatant from the different treatments was mixed with Quanti-Blue reagent and incubated at 37 °C for 1 h followed by absorbance reading at 630 nm using Synergy 2 multimode microplate reader (Biotek, Winooski, VT, USA). Cell culture supernatants were collected and stored at −80 °C until analysis of Interleukin-8 (IL-8) secretion.

2.9. Enzyme-Linked Immuno Sorbent Assay (ELISA)

Interleukin-8 levels were measured by ELISA in cell culture supernatants collected from HCT116-Dual™ cells after the different treatments using the Human IL-8/CXCL8 DuoSet ELISA kit (DY208, R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's instructions. Sample dilutions were adjusted to ensure that absorbance values fell within the linear range of the IL-8 standard curve.

2.10. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM) unless otherwise stated. Multiple *t*-test, and two-way analysis of variance (ANOVA) were used, followed by Bonferroni post hoc tests for pairwise mean comparisons. A *p*-value of <0.05 was considered statistically significant and physicochemical, metabolomics and inflammatory response analyses were performed using Prism (GraphPad Software, San Diego, California, USA). Furthermore, for metabolomic analysis, metabolites concentration was normalised by sum, and it was used Pareto scaling. A hierarchical heat map of features from all fermentation styles and commercial was generated. A fold change of 2 and a false discovery rate (FDR) of 0.05 were used to identify differences in metabolomic profiles between the spontaneous and LP_ IB082-inoculated fermented cucumbers at 24 h, 48 h, 1 week and 2 weeks, using MetaboAnalyst 6.0 (www.metaboanalyst.ca).

2.11. Integration of amplicon and metabolomic data

Integration was restricted to the balanced set of samples for which both datasets were available giving 24 paired samples. The commercial product and the unfermented control were excluded.

For the amplicon layer, ASV counts were aggregated at genus level ('aggregate_taxa', microbiome package v1.28.0) and converted to relative abundances. Between-sample dissimilarities were computed using the Bray-Curtis index ('vegdist', vegan v2.7-3). For the metabolite layer,

concentrations were sum-normalised per sample and Pareto-scaled.

Concordance between the two configurations was assessed by Procrustes rotation, with the amplicon ordination as target, and tested using PROTEST ('protest', vegan) with 9999 permutations. The first two axes of each layer were retained for the primary test, matching the plotted ordinations; robustness was verified across two to four axes. The analysis was applied to the pooled set ($n = 24$) and separately within each fermentation condition ($n = 12$).

Because sampling points within a replicate series are not independent, and because both layers change directionally over fermentation, an unrestricted permutation null can attribute shared temporal trends to cross-layer concordance. Within the spontaneous fermentation, PROTEST was therefore repeated with permutations restricted to within sampling-point blocks ('how', permute package v0.9.10).

Pairwise associations were then examined between genus abundances and the metabolites found to be differentially abundant at any sampling point (Supplementary Tables S1–S4; adjusted $p < 0.05$; $n = 14$ compounds). Genus abundances were centred log-ratio transformed to account for compositionality, and genera with a mean relative

abundance below 1% across the fermentation set were excluded, retaining 13 genera. Spearman rank correlations were computed for all 182 genus-metabolite pairs, pooled and within each condition, with Benjamini-Hochberg correction applied within each panel. Correlations within the spontaneous fermentation were additionally re-tested against the sampling-point-blocked null described above.

3. Results and discussion

3.1. Inoculation with *L. plantarum* IBB082 drives immediate *Lactiplantibacillus* dominance, while spontaneous fermentation undergoes a prolonged heterogeneous succession

To contextualise the microbial dynamics observed during fermentation, the uninoculated, unfermented cucumber brine (control) was characterised as baseline. The control community was highly diverse (observed ASVs: 249; Shannon index: 4.44; Pielou's evenness: 0.804) and dominated by environmental and plant-associated taxa, with no detectable *Lactiplantibacillus*. This confirms that LAB dominance

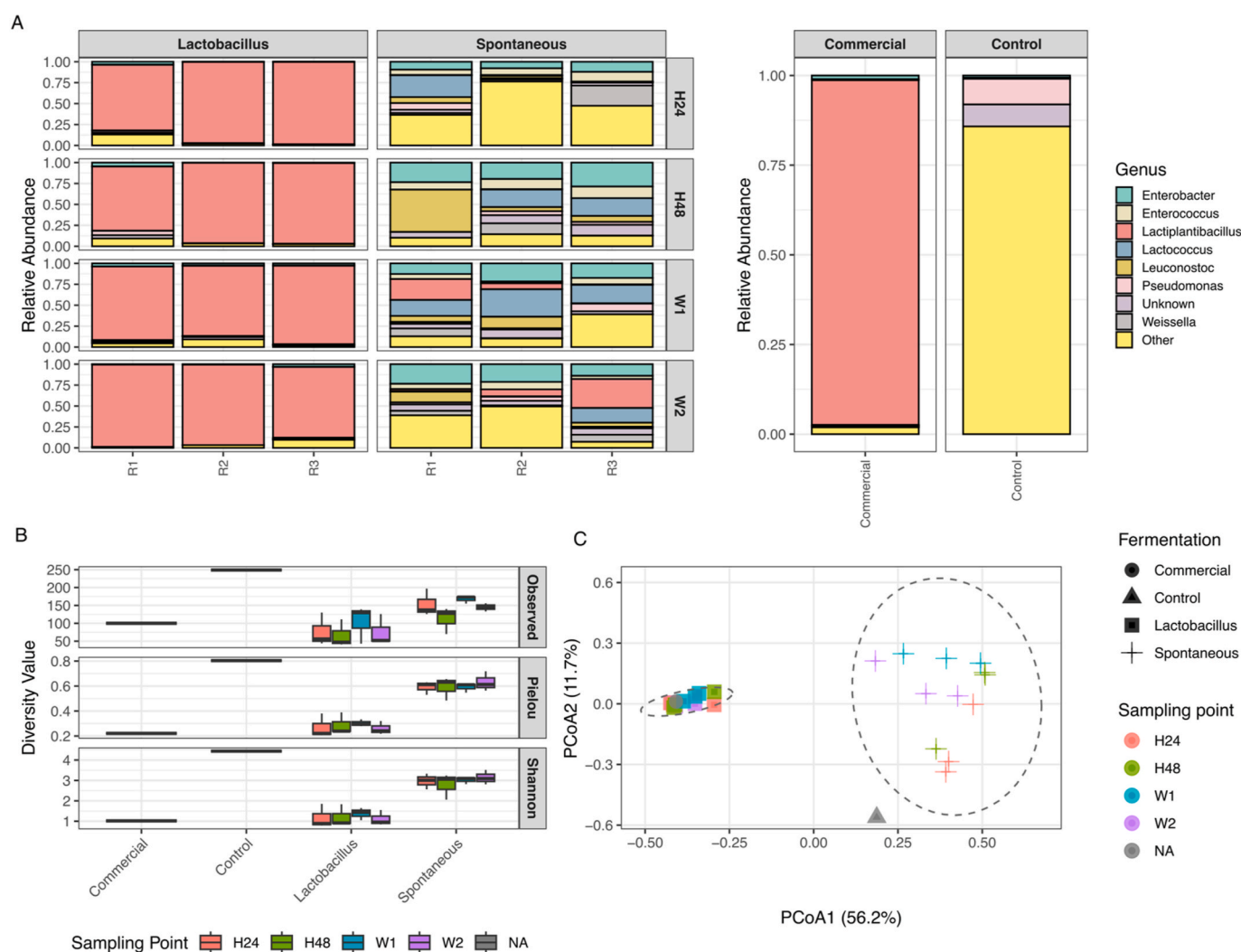


Fig. 2. Microbial community characterisation of Romanian-style cucumber fermentations over two weeks. (A) Genus-level relative abundance of bacterial communities in spontaneous and *LP*_IBB082-inoculated fermentations (left panel) and commercial and unfermented control samples (right panel) at 24 h (day 1), 48 h (day 2), week 1 (day 7) and week 2 (day 14). Taxa present at less than 5% relative abundance in fewer than 10% of samples were aggregated into the "Other" category. Each bar represents an individual replicate ($n = 3$ for spontaneous and *LP*_IBB082; $n = 1$ for commercial and control). (B) Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities computed from genus-level relative abundances. Each point represents one sample, coloured by time point and shaped by fermentation type. Dashed ellipses represent the 95% confidence interval for each fermentation group. (C) Alpha diversity assessed using three indices: observed ASVs (richness), Shannon diversity, and Pielou's evenness. Boxplots show the distribution across replicates ($n = 3$) for each fermentation type and time point. Commercial and control samples are shown as single points ($n = 1$).

observed in subsequent fermentations arose through fermentation-driven selection rather than being present in the initial matrix.

The two fermentation strategies produced strikingly different community trajectories from the outset (Fig. 2A). In LP_ IBB082-inoculated fermentations consistently showed low diversity across all time points (Shannon index range: 1.12–1.38; observed ASVs range: 66–104; Pielou's evenness range: 0.260–0.303), comparable to the commercial product (Shannon: 1.02; observed ASVs: 100; Pielou's evenness: 0.221). Spontaneous fermentations maintained substantially higher diversity throughout (Shannon index range: 2.79–3.14; observed ASVs range: 113–167; Pielou's evenness range: 0.591–0.632), with no sign of convergence toward LAB dominance by day 14. The reduction in diversity associated with LP_ IBB082 inoculation suggests that the starter culture not only accelerates fermentation but also constrains the range of coexisting taxa, which may improve process robustness despite reducing community complexity. Similar patterns have been reported in model and industrial fermentations, where starter cultures produce more consistent microbial trajectories compared to spontaneous processes, which remain more susceptible to variation driven by raw material and environmental heterogeneity (H. Liang, Chen, et al., 2018).

Beta diversity analysis (Fig. 2B) reinforced these patterns. PCoA based on Bray-Curtis dissimilarities explained 56.2% and 11.7% of total variance on axes 1 and 2, respectively. The primary axis of variation (PCoA1) separated the LP_ IBB082-inoculated and commercial samples from the spontaneous fermentations, indicating that the overall community composition of inoculated fermentations closely resembled that of the commercial product throughout the entire process. LP_ IBB082-inoculated samples formed a tight, stable cluster with minimal displacement along both axes, consistent with the compositional stability described above. In contrast, spontaneous fermentation samples were broadly dispersed, with temporal progression captured along PCoA2, reflecting the ongoing community succession over time. Notably, spontaneous fermentation samples remained clearly distinct from both the inoculated and commercial clusters even at day 14, indicating that two weeks of spontaneous fermentation was insufficient to produce a community structure comparable to either LP_ IBB082 inoculation or commercial fermentation. Taken together, the meta-taxonomic data demonstrate that inoculation with LP_ IBB082 steers fermentation toward a predictable, commercially-comparable outcome from the earliest time point, while spontaneous fermentation retains a heterogeneous and variable community structure that reflects the continued influence of the native microbiota and environmental conditions.

3.2. Absence of *L. monocytogenes* and *Salmonella* spp. in fermented cucumber samples by culture-based and dPCR analyses

The presence of *L. monocytogenes* and *Salmonella* spp. in fermented cucumbers samples was evaluated using both culture-based microbiological methods and dPCR. Using the ISO reference methods for microbiological analysis, *L. monocytogenes* and *Salmonella* spp. were not detected in any of the analysed samples (data not shown). Culture-based methods remain the reference standard for food safety assessment, as they demonstrate the absence of viable pathogenic organisms.

Consistent with the microbiological results, dPCR analysis targeting the *hlyA* gene for *L. monocytogenes* and the *invA* gene for *Salmonella* spp. identification yielded no positive partitions in the samples, with positive detection in positive controls, while negative controls remained free of detectable signal. These results indicate the absence of detectable pathogenic DNA in the analysed samples.

The negative results obtained by both approaches strengthen confidence in the microbiological safety of the analysed products. The absence of *L. monocytogenes* and *Salmonella* spp. is consistent with the characteristics of fermented cucumber, such as low pH and the presence of organic acids, that are unfavourable for the survival and growth of these pathogens, even as early as time 24 h. However, the consistency of

the results across all time points suggest that there were no pathogenic contaminants of these species in the initial preparations. Together, these findings indicate that the analysed fermented cucumber samples complied with food safety criteria for these pathogens at all stages of the analyses.

3.3. *L. plantarum* IBB082 determines the metabolic profile of the cucumber fermentation process

The metabolomic patterns were first explored using a heatmap, which revealed differences in metabolite abundance across groups and time points (Fig. 3A). To further assess overall variation and sample clustering, principal component analysis (PCA) was performed (Fig. 3B), showing distinct grouping patterns and differences in metabolomic variability between commercial cucumbers, and spontaneous and LP_ IBB082-inoculated fermentations. The spontaneous fermentation samples showed greater dispersion in the PCA score plot, indicating higher variability in the metabolome both across and within time points. By contrast, the inoculated group formed a more compact cluster, suggesting a more homogeneous metabolic profile and reduced variability among samples from the same time point. These results suggest that spontaneous fermentation involves a less predictable metabolic evolution, whereas LP_ IBB082 inoculation leads to a more consistent metabolic response under controlled fermentation conditions (Xu et al., 2024).

Differentially abundant metabolites were identified using a volcano plot analysis (Fig. 3C–F), which highlighted the compounds that had changed significantly between the spontaneous and LP_ IBB082-inoculated fermentations. Tables S1–S4 also show the fold change and p-adjust values for the different features of LP_ IBB082-inoculated and spontaneous fermentation styles. Only a limited number of metabolites differed between the two fermentation strategies at each time point. This indicates that despite the differences in microbial composition between spontaneous fermentation and LP_ IBB082 inoculation (Fig. 2A), only a small subset of features differed significantly at each stage of fermentation. The significantly altered metabolites were represented as bar graphs (Fig. 4) to display their development over time during the fermentation process and to compare trends between spontaneous and LP_ IBB082 fermentations.

Lactate was one of the main metabolites found in the covering brine of cucumbers using both fermentation strategies. However, it became prevalent earlier in the LP_ IBB082 fermentation process. This was an expected result, as inoculation with a LAB introduces a dominant microbial agent at the beginning of the process, as demonstrated by the metataxonomic analysis (Fig. 2). Consequently, lactic acid fermentation and the associated production of lactate were established quickly during fermentation. By contrast, 2-hydroxyisobutyrate increased rapidly during spontaneous fermentation than during LP_ IBB082 fermentation, demonstrating significant differences between the two fermentation strategies at all time points. This suggests that certain species of bacteria may produce higher amounts of these metabolites through spontaneous fermentation. Additionally, both fermentation styles reached a plateau and stabilisation from 7 to 14 days of fermentation without any further significant differences on 2-hydroxyisobutyrate levels. On the other hand, the levels of the metabolites O-acetylcarnitine and N-carbamoyl- β -alanine increased significantly after one day of spontaneous fermentation. In contrast, their production was delayed during LP_ IBB082 fermentation, except on the second day, after which no significant differences were observed between the two fermentation strategies for the duration of the fermentation process.

As shown in the volcano plot when comparing LP_ IBB082 and spontaneous fermentation (Fig. 3D), formate was the only metabolite that decreased in LP_ IBB082 fermentation relative to spontaneous fermentation after 2 days. This organic acid exhibited higher concentrations in spontaneous fermentation at all time points compared with its inoculated counterpart, decreasing from 0.41 mg/mL on day 1 to

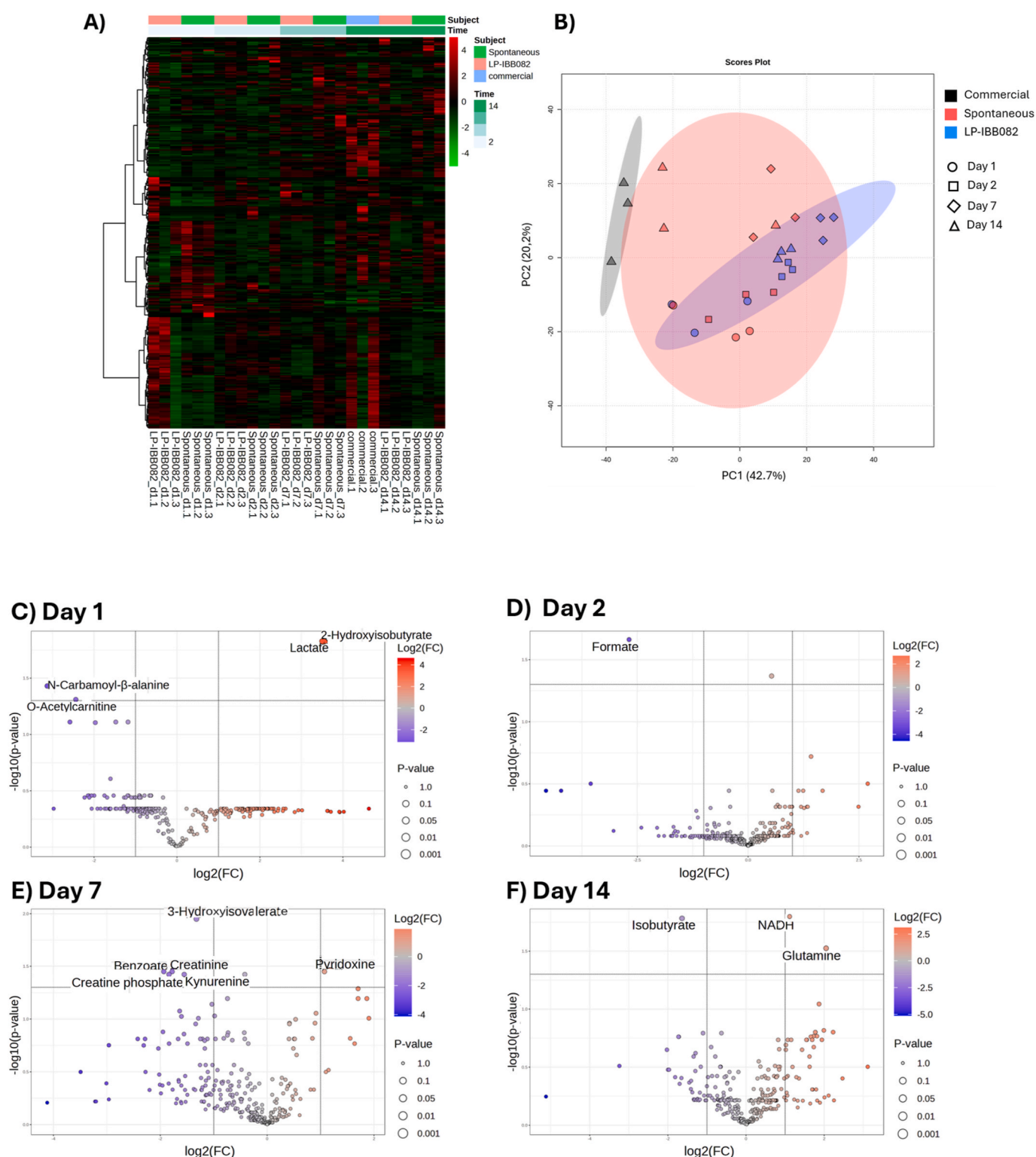


Fig. 3. Heatmap (A) showing the relative abundance of metabolites across the samples. Values were normalised to the mean sum and Pareto scaled and are displayed using a colour scale representing low to high abundance. Metabolites were grouped according to similarity patterns to highlight differences in metabolic profiles. Principal component analysis (PCA) (B) shows sample distribution based on the first two principal components (PC1 and PC2) of metabolite profiles. The percentage of variance explained by each component is indicated on the axes. Clustering patterns reflect similarities in the overall composition of metabolites among samples. Volcano plots (C-F) show significantly differences in metabolites at each fermentation time point (1, 2 7 and 14 days). Dots colour indicates fold change magnitude and dots sizes reflects statistical significance.

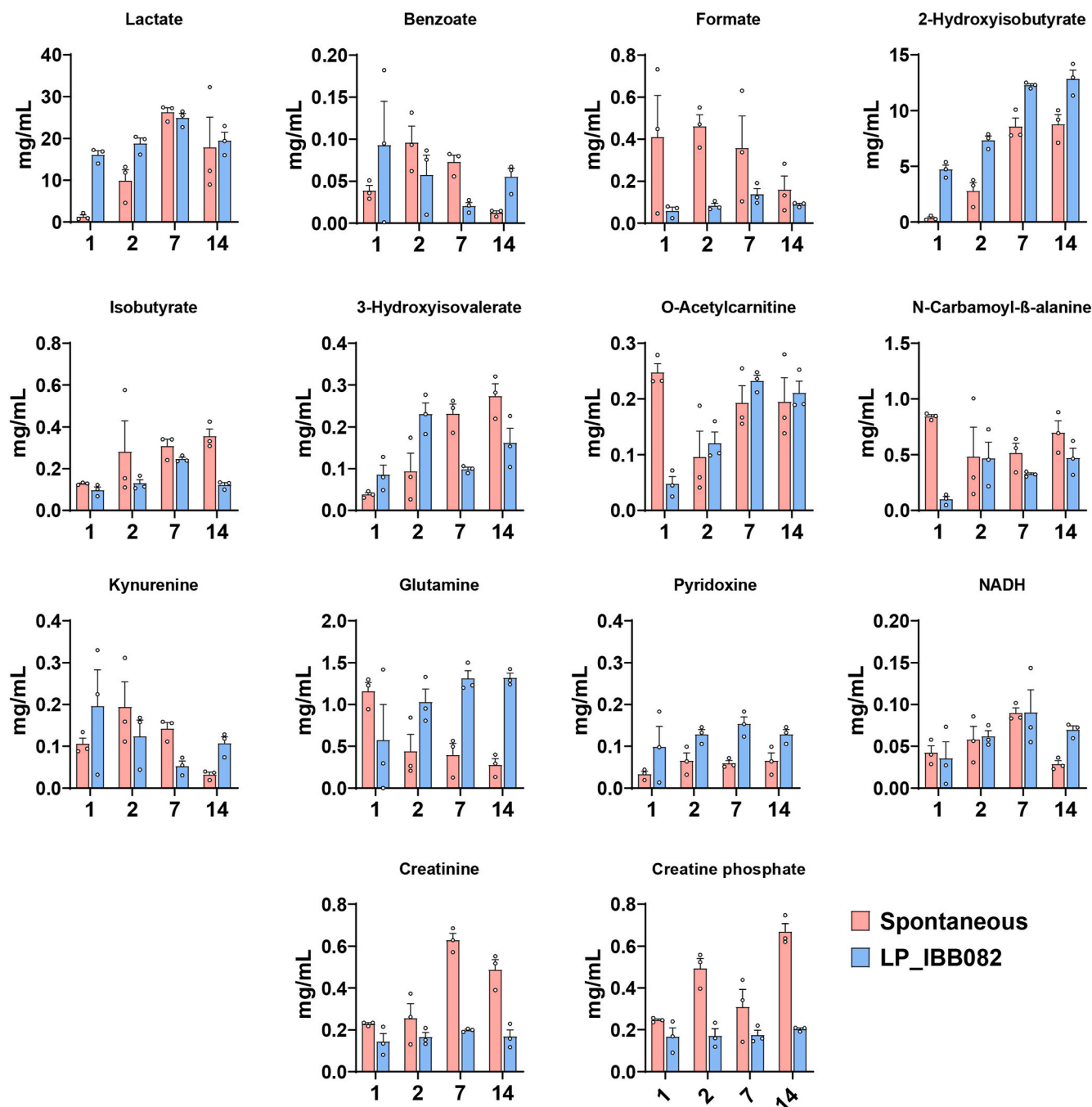


Fig. 4. Differential metabolite analysis between groups across time points. Bar plots (E) show the concentration of selected metabolites (mg/mL) in the Spontaneous and *L. plantarum* IBB082 (LP_IBB082) groups at days 1, 2, 7, and 14. Data are presented as mean $\pm$ SEM ($n = 3$), with individual values shown as dots.

0.159 mg/mL after 14 days. These trends are consistent with the taxonomic composition, particularly the abundance of the genus *Enterobacter*. These bacteria are known to perform mixed-acid fermentation, producing metabolites such as lactate, acetate, ethanol, formate and succinate during metabolism (Kammel et al., 2024). Therefore, the higher formate levels observed in spontaneous fermentation align with the greater abundance of *Enterobacter* detected in this fermentation style compared to LP_IBB082 inoculation throughout the process.

Significant differences were observed between the spontaneous fermentation and the fermentation inoculated with LP_IBB082 after seven days (Fig. 3E). At this time point, five metabolites were less abundant and one more abundant significantly in the inoculated fermentation compared with the spontaneous process. Among these, the

concentration of 3-hydroxyisovalerate increased progressively during spontaneous fermentation, reaching 0.27 mg/mL by day 14. In contrast, its concentration in LP_IBB082 fermentation increased only until day 2, after which it declined to 0.1 mg/mL, with only a slight recovery to 0.16 mg/mL after 14 days of fermentation. 3-Hydroxyisovalerate and 2-hydroxyisobutyrate belong to the leucine and valine degradation pathway, also known as branched-chain amino acid metabolism. Bacteria usually metabolise these amino acids when preferred carbon sources, such as sugars, are depleted (Díaz-Pérez et al., 2015). This is consistent with the sugar quantification results, which showed a significant decrease by day 2 in spontaneous fermentation (Fig. S3). Accordingly, fructose, maltose and sucrose levels were lower in spontaneous fermentation than in the inoculated process, suggesting that the

microorganisms had shifted towards alternative carbon sources. Interestingly, an increase in arginine-derived metabolites, including creatinine and creatine phosphate, was observed exclusively in spontaneous fermentation. In contrast, LP_IBB082-inoculated fermentation maintained consistently low levels of these compounds (<0.2 mg/mL) throughout the bioprocess. These results suggest that the microbial consortium present in spontaneous fermentation exhibits distinct metabolic features for accessing alternative nitrogen and carbon sources. Another metabolite found at higher concentrations in spontaneous fermentation was kynurenine. However, we can observe a time-dependent consumption or degradation of this tryptophan-derived metabolite, with LP_IBB082 fermentation showing faster depletion (Fig. 4). By contrast, LP_IBB082 inoculation promoted the production of pyridoxine. This metabolite, also known as vitamin B6, is produced by several *L. plantarum* strains (Lee et al., 2023). This finding highlights the potentially beneficial aspect of LP_IBB082 fermentation due to its ability to increase the concentration of functional compounds that are relevant to human health, particularly related to the nervous and immune systems (Zhao et al., 2026).

As a final stage, after 14 days of fermentation, glutamine and NADH were more significantly abundant, and isobutyrate was less significantly abundant (Table S4) in LP_IBB082 fermentation compared with spontaneous fermentation. Isobutyrate continued to increase in the spontaneous process, following a trend similar to other branched-chain amino acid-related metabolites such as 3-hydroxyisovalerate and 2-hydroxyisobutyrate. In contrast, glutamine showed opposite trends between the

two fermentation styles: its concentration decreased during spontaneous fermentation, whereas it increased under LP_IBB082 fermentation. *L. plantarum* is known to produce glutamine (Botta et al., 2017), which has been associated with improved glucose homeostasis and reduced inflammation in *in vivo* studies (T. Liang et al., 2025).

Overall, the fermentation strategy and style had a significant impact on the metabolite evolution of cucumbers during processing. LP_IBB082 inoculation produced a more consistent and uniform metabolomic profile, whereas spontaneous fermentation resulted in greater variability and metabolic dispersion. Although only a limited number of metabolites differed significantly, these variations revealed distinct metabolic pathways between the two fermentation styles. Spontaneous fermentation was associated with mixed-acid and branched-chain amino acid metabolism, whereas LP_IBB082 inoculation promoted earlier lactate production and increased the levels of functional metabolites, such as pyridoxine and glutamine.

Procrustes analysis indicated significant global concordance between community structure and metabolite profiles across the fermentation set ($r = 0.43$, $p = 0.013$; stable across two to four ordination axes; Fig. S4A). This concordance reflected the separation of inoculated from spontaneous fermentations recovered by both layers rather than sample-level correspondence: it was not retained within the LP_IBB082 fermentation ($r = 0.47$, $p = 0.13$), and within the spontaneous fermentation the apparent concordance ($r = 0.69$, $p = 0.003$) did not persist when permutations were restricted within sampling points ($p = 0.65$), indicating parallel temporal trajectories of the two layers.

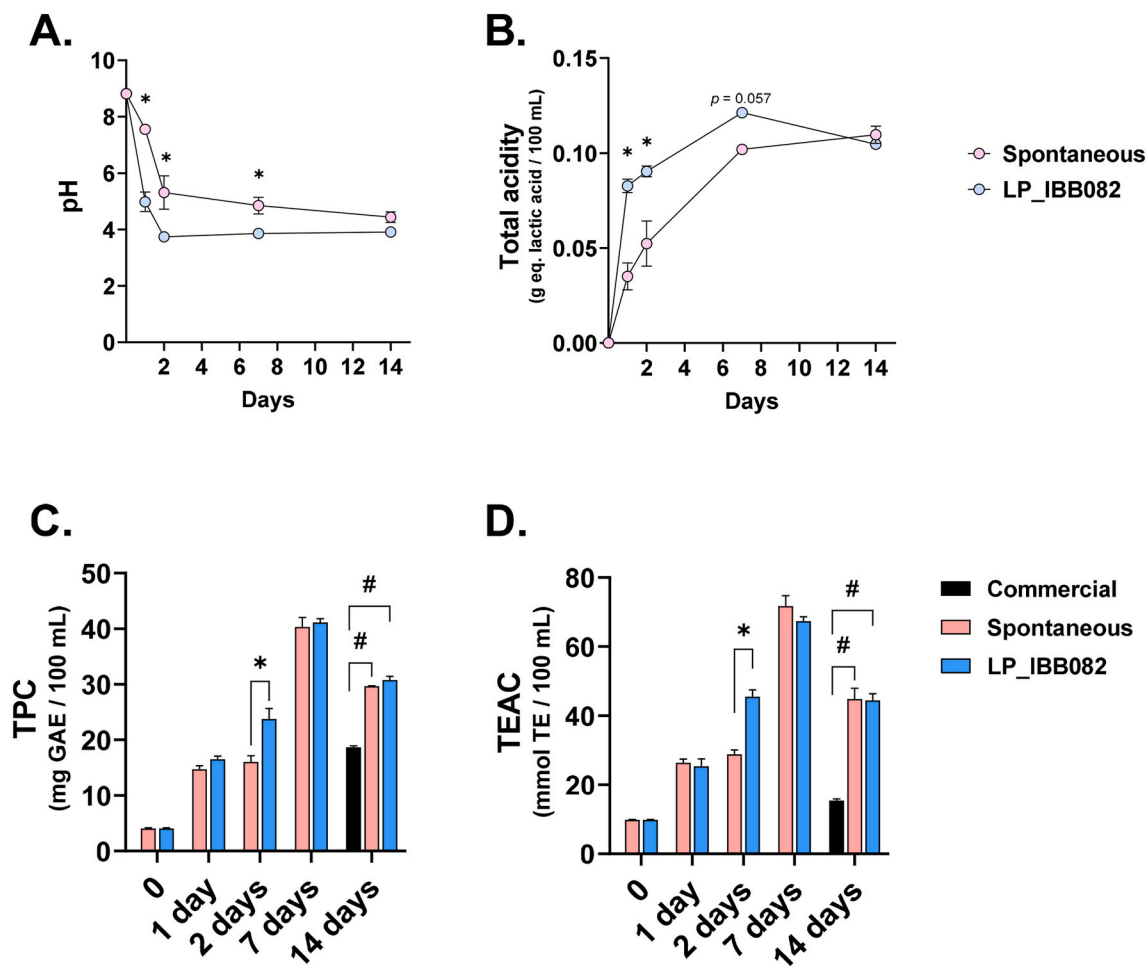


Fig. 5. Trends of pH (A) and total acidity (B), quantification of total phenolic content (TPC, C) and Trolox equivalent antioxidant capacity (TEAC, D) in the spontaneous and the *L. plantarum* IBB082 (LP_IBB082) - inoculated fermentations with over 14 days. Dots and bars represent mean $\pm$ SEM (N = 3). Asterisk (*) indicates significant differences between fermentation types (spontaneous vs. LP_IBB082), (two-way ANOVA with Tukey's post hoc test, $p < 0.05$).

Consistently, of the 42 genus-metabolite correlations significant in the pooled analysis (Fig. S4B), none were significant within the LP_IBB082 fermentation, and the nine significant within the spontaneous fermentation did not survive sampling-point-blocked testing. Genus-level taxonomic resolution, together with the absence of a direct relationship between relative abundance and metabolic activity, likely precludes resolution of individual taxon-metabolite couplings at this sample size.

3.4. *L. plantarum* IBB082 accelerates acidity and phytochemical extraction from cucumbers compared to spontaneous fermentation

Fig. 5A and B shows the trends in pH drop and total acidity, expressed as the equivalent of lactic acid, during the fermentation process for the spontaneous and LP_IBB082-inoculated strategy. The LP_IBB082 inoculation eases the drop in pH and increases acidity faster than the spontaneous fermentation, facilitating the acidification process and increasing the safety of the bioprocess (Salas-Millán et al., 2022). Slower acidification kinetics, as observed in spontaneous fermentations, represent a recognised risk factor for product instability, as the delayed establishment of inhibitory pH values creates a window of opportunity for the proliferation of spoilage-associated microorganisms prior to LAB dominance (Tamang et al., 2020).

The TPC and TEAC values are shown in Fig. 5C and D. The initial brine values were approximately 4 mg/100 mL due to the extraction of phenolic compounds during preparation, which could have promoted the release of these phytochemicals into the brine (Vergara-Salinas et al., 2012). After spontaneous fermentation and inoculation with *L. plantarum* IBB082, TPC increased in a time-dependent manner, reaching a maximum after 7 days, followed by a 25% decrease after 14 days. There were no significant differences between the two fermentations strategies, except after two days of fermentation. At this point, the LP_IBB082 inoculated fermentation resulted in significantly higher TPC values compared to spontaneous fermentation. These results suggest that LP_IBB082 enhances the extractability of polyphenols more efficiently than spontaneous fermentation. It is known that *L. plantarum* produces feruloyl esterase (Esteban-Torres et al., 2013) and β -glucosidase (Yu et al., 2025), enzymes facilitating the release of polyphenols from plant cell walls and non-extractable polyphenols (Zhang et al., 2023). In contrast, the heterogeneity of spontaneous fermentation may reduce the efficiency of enzyme production, consequently decreasing polyphenol extractability, especially when another genus is prevalent, such as *Lactococcus*, *Leuconostoc* and *Weissella* (Fig. 1A).

The antioxidant capacity of the cucumbers followed the same trend as the TPC values. Furthermore, fermentation inoculated with LP_IBB082 significantly increased the antioxidant capacity after two days compared to spontaneous fermentation. The antioxidant capacity continued to increase for the first seven days, dropping by around 35% after 14 days of fermentation, with no significant differences between the two strategies.

3.5. Immunomodulatory potential of cucumbers covering brine in spontaneous and LP_IBB082 fermentations

The HCT116 Dual Reporter NF- κ B cell model is a valuable tool for evaluating the inflammatory response at the human intestinal epithelium level (InvivoGen, 2026), which acts as the first barrier against dietary compounds and metabolites derived from the microbiota (Guzman et al., 2013). NF- κ B pathway activation represents a central node in the regulation of the innate immune response, controlling the expression of pro-inflammatory cytokines such as IL-6, IL-8 and TNF- α , in macrophages and chemokines such as IL-8, CXCL1, CCL2, CCL20 in epithelial cells as well as other mediators involved in intestinal homeostasis and chronic inflammatory diseases (Liu et al., 2017; Wullaert et al., 2011).

The concentration of covering brine (5 to 20%) for pre-treatment of HCT116-Dual cells was determined based on cell viability as the primary

selection criterion. The 20% dilution significantly reduced cell viability, when using Sytox-Green™ and LDH assays, whereas the 5% and 10% dilutions had no significant effect compared to cells cultured in medium alone (Fig. S5). Based on these results, a 10% dilution of fermented cucumber brine was selected for subsequent analyses. Under basal conditions, epithelial cells exposed to the covering brine exhibited increased NF- κ B activation at all time points (Fig. 6A). However, this activation decreased significantly after 14 days of fermentation, compared to the initial time point. This indicates a time-dependent reduction in the inflammatory reactivity of the extracts, for both fermentation styles. In contrast, an increase in IL-8 levels was detected in spontaneous-fermented cucumbers after 14 days, while IL-8 secretion remained stable from day 1 to day 14 in samples fermented with LP_IBB082. This suggests that fermentation with LP_IBB082 regulates the IL-8 secretion and, potentially, the inflammatory response.

Under IL-1 β -induced inflammatory conditions, covering brine from both spontaneous and LP_IBB082-inoculated fermentations was found to significantly reduce NF- κ B activation compared to the IL-1 β -stimulated control at all evaluated time points. This reduction suggests that fermentation-derived metabolites may contribute to the mitigation of IL-1 β -induced inflammatory responses. However, this anti-inflammatory response was not consistently reflected in IL-8 secretion. In cells treated with brine from spontaneous fermentation, IL-8 levels decreased after 7 days, followed by an increase after 14 days of fermentation. This pattern mirrors the evolution of creatinine concentration (Fig. 4), which peaked on day 7 of spontaneous fermentation and then declined by day 14. Previous studies using LPS-activated RAW 264.7 macrophages showed that creatinine reduces NF- κ B nuclear expression, supporting the potential involvement of creatinine in modulating inflammation (Riesberg et al., 2018). Taken together, these observations imply that specific fermentation-derived metabolites may influence inflammatory pathways in a time- and condition-dependent manner. The differential behaviour of NF- κ B activation and IL-8 secretion observed in this study likely reflects the distinct regulatory mechanisms governing these two readouts rather than an inconsistency in the data. Although NF- κ B is a major driver of IL-8 transcription, IL-8 production is regulated by additional pathways, including p38 Mitogen-Activated Protein Kinase (p38 MAPK), Extracellular signal-Regulated Kinase/c-Jun N-terminal Kinase-dependent Activator Protein 1 (ERK/JNK-dependent AP-1), CCAAT/Enhancer-Binding Protein beta (C/EBP β), cAMP Response Element-Binding protein (CREB), and Signal Transducer and Activator of Transcription 3 (STAT3), which can act independently of or alongside NF- κ B (Bezzi et al., 2011; Khanjani et al., 2012). Furthermore, p38 MAPK contributes to post-transcriptional regulation by stabilising IL-8 mRNA, allowing IL-8 secretion to persist despite altered NF- κ B activity (Hoffmann et al., 2002; Parhar et al., 2003). The observed divergence between NF- κ B activation and IL-8 secretion is therefore consistent with a multifaceted immunomodulatory effect of fermentation-derived metabolites acting through both NF- κ B-dependent and NF- κ B-independent mechanisms.

The present study assessed the bioactivity of fermented cucumber brines directly on intestinal epithelial cells, without previous simulated gastrointestinal digestion (Brodtkorb et al., 2019). This approach was considered appropriate given that the test material was an aqueous fermentation brine containing small, water-soluble metabolites that are inherently bioaccessible without requiring enzymatic liberation from a solid food matrix. Furthermore, it has been demonstrated that INFOGEST digests contain bile salts and digestive enzymes at concentrations that, although physiologically relevant, are harmful to epithelial cell lines, and that no consensus protocol currently exists to render such digests biocompatible for downstream cell culture assays (Kondrashina et al., 2024). The present findings therefore represent an initial characterisation of the bioactive potential of the fermented brine under direct contact screening conditions and should not be extrapolated to physiologically relevant effects prior to gastrointestinal transit. Future studies employing more physiologically relevant gut models, including

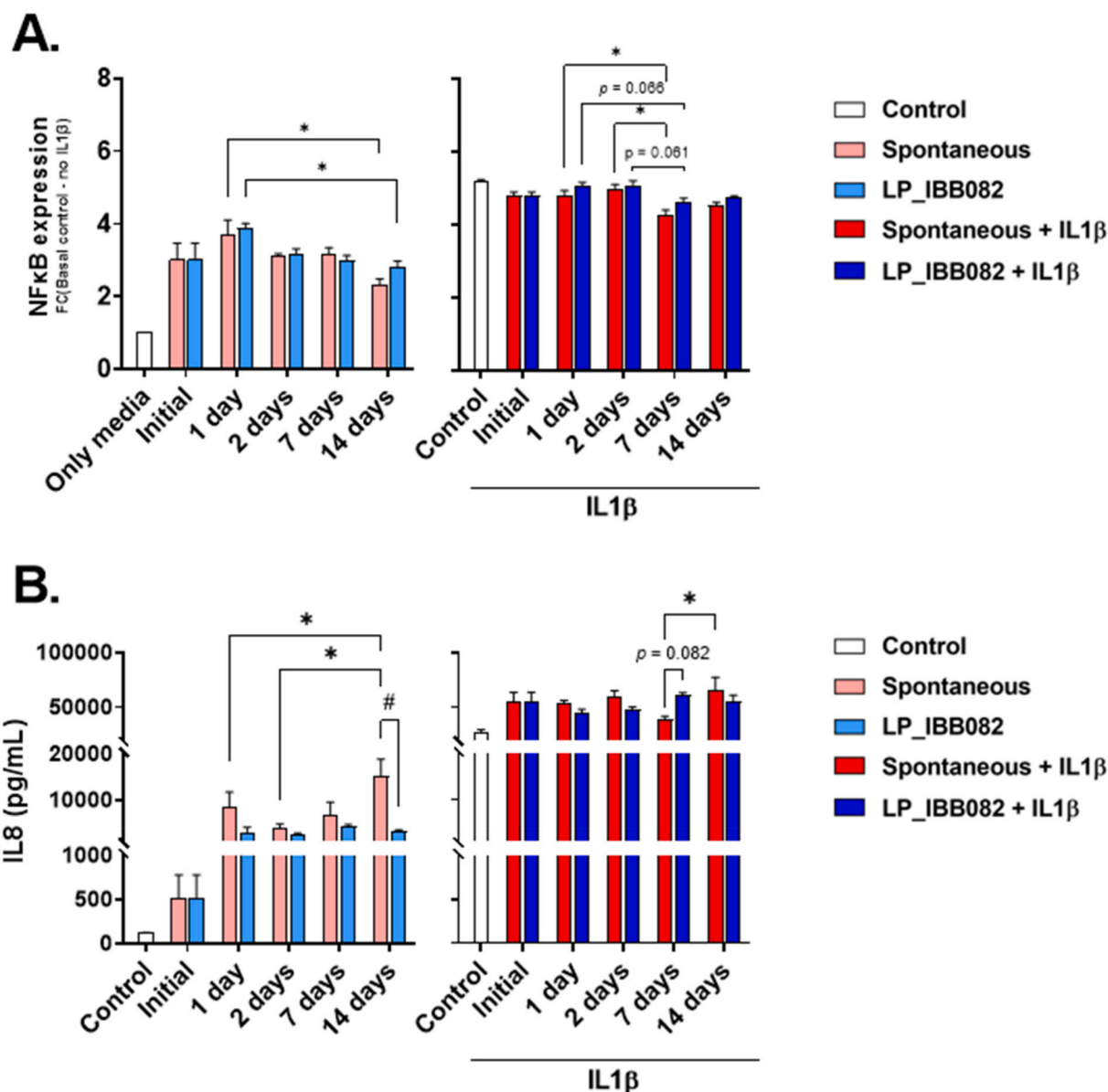


Fig. 6. Anti-inflammatory potential of cucumber covering brine evaluated in HCT116-Dual reporter cells. NF-κB pathway activation (A) and IL-8 secretion by ELISA (B) were assessed after a 24 h pre-treatment of epithelial cells with fermented liquid collected at initial time and after 1, 2, 7, and 14 days of fermentation, followed by an additional 24 h incubation under basal conditions (no IL-1β) or in the presence of the pro-inflammatory cytokine IL-1β (0.1 ng/mL). Initial covering brine was included as a fermentation control (Initial). Cells treated with IL-1β alone (control + IL-1β) were used as a positive control for NF-κB activation and IL-8 secretion. Data in (A) is presented as fold change (FC) against control basal condition (no IL-1β). Bars represent mean $\pm$ SEM (N = 3). Asterisk (*) indicates significant differences between fermentation time points for each fermentation types, and # indicates significant differences between fermentation types (spontaneous vs. LP_IBB082) (two-way ANOVA with Bonferroni's post hoc test, $p < 0.05$).

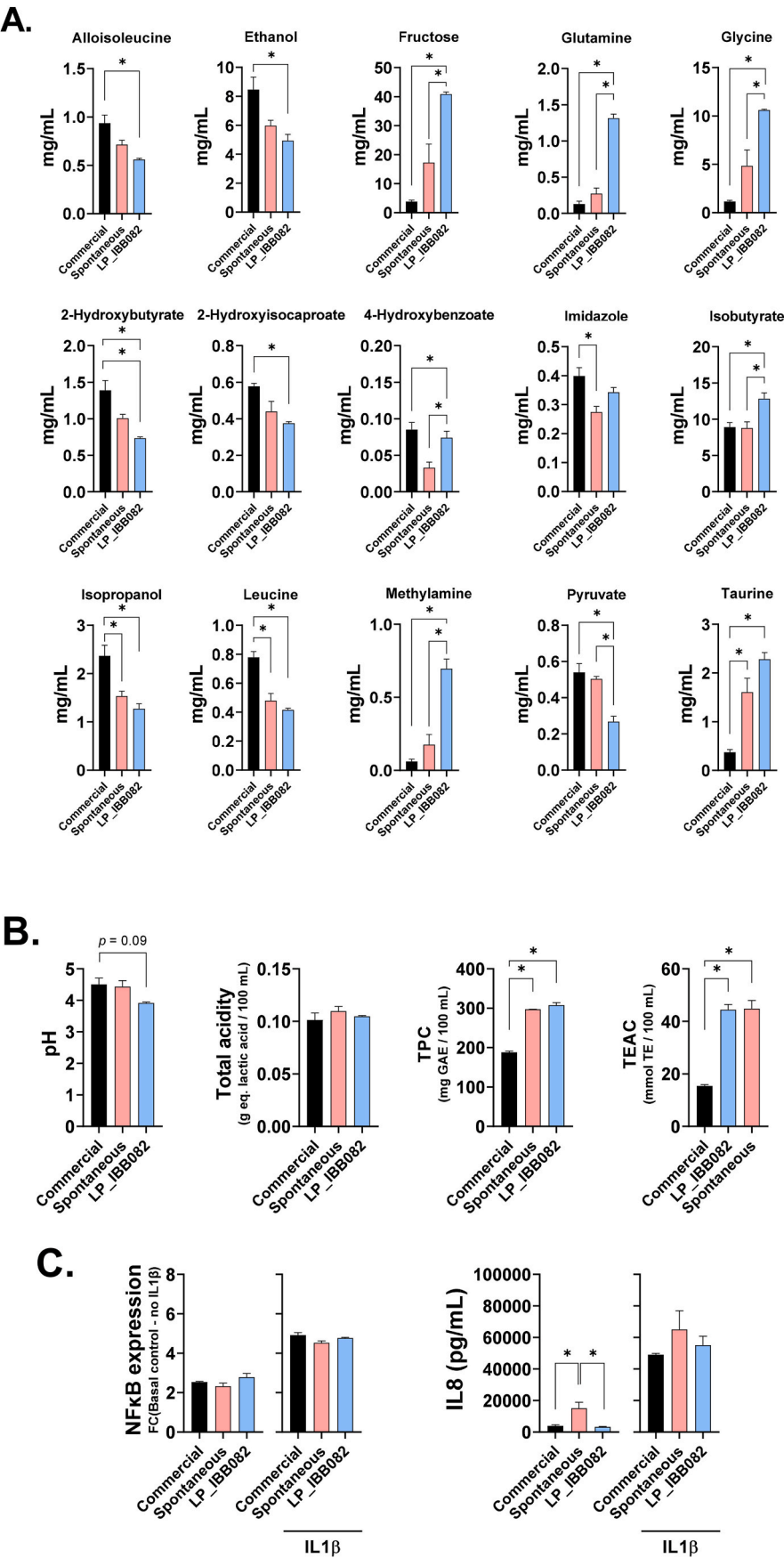
Caco-2/HT-29-MTX co-culture systems, intestinal organoids, and ultimately human dietary intervention trials, will be necessary to validate these findings in a physiological context.

3.6. Metabolomic and physicochemical differences between spontaneous, *L. plantarum* IBB082-Inoculated, and commercial cucumbers

Genus-level composition profiles revealed that inoculated fermentations converged towards a near-monoculture dominated by lactic acid bacteria, closely resembling the microbial composition observed in commercial samples (Fig. 2A). Likewise, alpha and beta diversity (Fig. 2B and C) analysis revealed that inoculated fermentations clustered more closely with commercial samples.

In terms of metabolomic analysis (Fig. 7A), it showed significant

differences between fermented cucumbers (spontaneous and LP_IBB082-inoculated) and commercial cucumbers (Table S5). Several metabolites were significantly altered because of fermentation style (one-way ANOVA, $p < 0.05$). Generally, commercial cucumbers were more similar to spontaneously fermented samples than to LP_IBB082-inoculated cucumbers, consistent with the shared spontaneous nature of these processes. Compared to LP_IBB082-inoculated samples, commercial cucumbers exhibited a marked depletion of simple sugars, such as fructose, as well as amino acids, including glutamine, glycine and taurine, and amine compounds, such as methylamine. A similar trend was observed in the spontaneously fermented cucumbers, which also exhibited lower levels of these metabolites and differed significantly from the LP_IBB082-inoculated cucumbers. By contrast, other fermentation-derived metabolites, including allose, ethanol, 2-



(caption on next page)

Fig. 7. Metabolomic and physicochemical differences between spontaneous and LP_IBB082-inoculated fermentations compared with commercial fermented cucumber. Significantly different metabolites are shown in figure A (one-way ANOVA with Tukey's post hoc test, FDR-adjusted $p < 0.05$). Physicochemical parameters, including pH and total acidity, as well as total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC), were analysed in fermented cucumber brine from spontaneous and LP_IBB082 fermentations and compared with commercial samples. Antioxidant bioactivity was also evaluated by assess NF κ B expression and IL8 secretion in HCT116 Dual cell line. Cells were assessed after a 24 h pre-treatment of epithelial cells with fermented liquid collected at 14 days of fermentation from spontaneous and LP_IBB082-inoculated fermentation, and from commercial brine, followed by an additional 24 h incubation under basal conditions without (w/o) IL-1 β ; or in the presence of the pro-inflammatory cytokine IL-1 β (0.1 ng/mL) (C). Asterisks (*) indicate significant differences between fermentation treatments (spontaneous and LP_IBB082), and commercial fermented cucumber (one-way ANOVA with Tukey's post hoc test, $p < 0.05$).

hydroxybutyrate, 2-hydroxyisocaproate, isopropanol, leucine and pyruvate, were present at higher levels in both spontaneous fermentation and commercial cucumbers than in LP_IBB082-inoculated samples. These results suggest that spontaneous fermentation and commercial products have closer metabolic alignments, likely reflecting similar patterns of substrate utilisation and by-product formation during microbial metabolism.

The physicochemical analysis (Fig. 7B) further highlighted these differences. Although there were no significant differences in pH and total acidity between fermented and commercial cucumbers, the TPC and TEAC were significantly higher in the spontaneous and LP_IBB082-inoculated fermented cucumbers. However, these differences lay on the differences of the initial matrix used, where was the same from the fermented cucumbers in this work than the commercial ones.

Finally, the bioactivity assays (Fig. 7C) revealed clear differences between fermented and commercial cucumbers. Under basal conditions, NF- κ B activation was comparable across all treatments, with no significant differences detected nor under IL-1 β -induced inflammatory conditions. However, the most significant differences were showed in the secretion of IL-8 chemokine after the pretreatments with cucumbers supernatants. Commercial cucumbers and LP_IBB082-inoculated cucumbers showed lower IL8 secretion compared to spontaneous fermentation and, therefore, a lower inflammation reactivity on HCT116 Dual cells after the treatment with cucumbers brines. On the other hand, no differences were found when HCT116 Dual cells were IL1 β -stimulated.

Overall, these results demonstrate that fermentation substantially altered the metabolomic profile and modulates inflammatory responses, regardless of whether it is spontaneous or inoculated, in comparison to commercial cucumbers.

4. Conclusions and future prospects

The present study shows that *L. plantarum* IBB082 inoculation steers traditional Romanian cucumber fermentations towards more predictable microbial and metabolic outcomes, impacting functional quality and immunomodulatory bioactivity. Notably, the microbial variability associated with spontaneous fermentation might represent a source of chemical and biological diversity that contributes to the sensory complexity and functional characteristics of traditional products. Therefore, our work reinforces the need to take into consideration the trade-off between process standardisation and the preservation of artisanal diversity in fermentation design strategies.

From a safety perspective, the absence of *L. monocytogenes* and *Salmonella* spp., the two pathogens subject to mandatory microbiological criteria for ready-to-eat foods under EU legislation, under both fermentation conditions is encouraging; however, these results were obtained under controlled laboratory settings and should not be extrapolated to genuine household environments, where temperature, raw material quality and hygiene conditions are less predictable. Larger field studies sampling real uncontrolled household fermentations should be considered before extending broader safety conclusions. Additionally, dedicated monitoring of sugar consumption kinetics and viable LAB counts throughout fermentation would complement the culture-independent approaches employed here and provide a more complete picture of fermentation progression in future studies.

Although the observed immunomodulatory effect is encouraging, the

underlying mechanisms remain unresolved. The differential IL-8 secretion observed between fermentation types suggests that distinct fermentation-derived metabolites or microbial products, may be driving these responses. However, these findings are currently limited to one epithelial cell model and two inflammatory read-outs (NF κ B activation and IL-8 secretion), restricting broader interpretation. Expanding the analysis to include a wider range of inflammatory markers across both epithelial and immune cells would provide a more comprehensive evaluation of host-fermentation interactions. Moreover, integrating targeted metabolite profiling with advanced, physiologically relevant gut models, alongside human dietary intervention trials, will be essential to establish causal relationships between fermentation-derived compounds and host inflammatory responses.

Overall, this work highlights the underexplored potential of traditional Eastern European fermented vegetables as sources of functionally characterised LAB strains and bioactive compounds. Sensory characterisation of these fermentations, including odour, acidity, crispness, colour and overall acceptability, represents a valuable complementary line of investigation not addressed in the present study. Future studies incorporating consumer sensory panels would provide a more complete evaluation of the artisanal quality of these products. Finally, the feasibility of using single-strain starters such as *L. plantarum* IBB082 to improve the safety and consistency of household fermentations in low-resource settings, without requiring industrial infrastructure, represents a relevant avenue for future investigation with potential public health implications.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Sequencing data are publicly available in ENA (PRJEB112055), and associated metadata are provided in Supplemental File 1.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used Claude.ai in order to generate the Graphical abstract and Fig. 1. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Funding

The work and staff (JASM and SM) at APC Microbiome Ireland were supported by a research center grant funded by Science Foundation Ireland (SFI/12/RC/2273_P2). TTK is funded through the UKRI Future Leaders Fellowship awarded to Dr Matthew Wallace (MR/T044020/1 and MR/Z000114/1). AJP acknowledges the support of the Biotechnology and Biological Sciences Research Council (BBSRC); this research was funded by the BBSRC Institute Strategic Programme Food

Microbiome and Health BB/X011054/1; the BBSRC Core Capability Grant BB/CCG2260/1. AGN thanks the Spanish Ministry of Science, Innovation and Universities for his FPU21/0230. IRA is funded by RO1567-IBB11/2026 from the Institute of Biology Bucharest of the Romanian Academy. This research was partly funded with project PID2023-149211OB-C31 by the Spanish Ministry of Science, Innovation and Universities (MCIU) and the Spanish Agency for Research (AEI) for AP and EGG. EGG is also funded by a Beatriz Galindo scholarship from the Spanish Ministry of Universities (BG22/00060).

CRedit authorship contribution statement

José Ángel Salas-Millan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Mihai-Alexandru Tonciulescu:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Todor T. Koev:** Funding acquisition, Investigation, Methodology, Writing – review & editing. **Alise J. Ponsero:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Ignacio Quevedo-Romero:** Formal analysis, Writing – review & editing. **Covadonga Valle Alcaraz:** Investigation, Writing – review & editing. **Pierric L.D. Chuppa:** Investigation, Writing – review & editing. **Caridad Soto:** Investigation, Writing – review & editing. **Fernando Ródenas Pérez Cuadrado:** Investigation, Writing – review & editing. **Nieves García-Lorca:** Investigation, Methodology, Supervision, Writing – review & editing. **Alberto Gila-Navarro:** Investigation, Methodology, Supervision, Writing – review & editing. **Poorvaja Muley:** Investigation, Methodology. **Leilei Yu:** Formal analysis, Supervision, Writing – review & editing. **David Baker:** Investigation, Methodology, Writing – review & editing. **Andrea Telatin:** Funding acquisition, Supervision, Writing – review & editing. **Iulia-Roxana Angelescu:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Alfredo Palop:** Funding acquisition, Supervision, Writing – review & editing. **Arjan Narbad:** Funding acquisition, Supervision, Writing – review & editing. **Silvia Melgar:** Funding acquisition, Supervision, Validation, Writing – review & editing. **Enriqueta García-Gutierrez:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Emilia Bujnowska for laboratory assistance. TTK is grateful for the use of the UEA Faculty of Science NMR facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fbio.2026.109620>.

Data availability

Our data is deposited publicly at ENA.

References

Ahmed, S., Ashraf, F., Tariq, M., & Zaidi, A. (2021). Aggrandizement of fermented cucumber through the action of autochthonous probiotic cum starter strains of

- Lactiplantibacillus plantarum and Pediococcus pentosaceus. *Annals of Microbiology*, 71(1), 33. <https://doi.org/10.1186/S13213-021-01645-5>, 2021 71:1.
- Bezzetti, V., Borgatti, M., Finotti, A., et al. (2011). Mapping the transcriptional machinery of the IL-8 gene in human bronchial epithelial cells. *Journal of Immunology*, 187(11), 6069–6081. <https://doi.org/10.4049/jimmunol.1100821>
- Botta, C., Acquadro, A., Greppi, A., Barchi, L., Bertolino, M., Coccolin, L., & Rantsiou, K. (2017). Genomic assessment in *Lactobacillus plantarum* links the butyrogenic pathway with glutamine metabolism. *Scientific Reports* 2017, 7(1), Article 15975. <https://doi.org/10.1038/s41598-017-16186-8>, 7(1).
- Brodtkorb, A., Egger, L., Alminger, M., et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, 14, 991–1014. <https://doi.org/10.1038/s41596-018-0119-1>
- Callahan, B., McMurdie, P., Rosen, M., et al. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*, 13, 581–583. <https://doi.org/10.1038/nmeth.3869>
- Díaz-Pérez, A. L., Díaz-Pérez, C., & Campos-García, J. (2015). Bacterial l-leucine catabolism as a source of secondary metabolites. *Reviews in Environmental Science and Bio/Technology*, 15(1), 1–29. <https://doi.org/10.1007/s11157-015-9385-3>, 2015 15: 1.
- Dupas de Matos, A., Marangon, M., Magli, M., Cianciabella, M., Predieri, S., Curioni, A., & Vincenzi, S. (2019). Sensory characterization of cucumbers pickled with verjuice as novel acidifying agent. *Food Chemistry*, 286, 78–86. <https://doi.org/10.1016/J.FOODCHEM.2019.01.216>
- European Food Safety Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC). (2023). The European Union One Health 2022 Zoonoses Report. *EFSA Journal*, 21(12), Article e08442. <https://doi.org/10.2903/j.efsa.2023.8442>
- EFSA Panel on Biological Hazards (BIOHAZ). (2018). *Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU. *EFSA Journal*, 16(1), Article e05134. <https://doi.org/10.2903/j.efsa.2018.5134>
- Esteban-Torres, M., Reverón, I., Manchón, J. M., De las Rivas, B., & Muñoz, R. (2013). Characterization of a feruloyl esterase from *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, 79(17), 5130–5136. <https://doi.org/10.1128/AEM.01523-13/ASSET/DFB27BD3-6F81-4EDE-AB81-7EE098729F66/ASSETS/GRAPHIC/ZAM9991046280005.JPEG>
- Grosu-Tudor, S. S., Stefan, I. R., Stancu, M. M., Cornea, C. P., De Vuyst, L., & Zamfir, M. (2019). Microbial and nutritional characteristics of fermented wheat bran in traditional Romanian borș production. *Romanian Biotechnology Letters*, 24, 440–447.
- Grosu-Tudor, S. S., & Zamfir, M. (2013). Functional properties of lactic acid bacteria isolated from Romanian fermented vegetables. *Food Biotechnology*, 27(3), 235–248. <https://doi.org/10.1080/08905436.2013.8111082>
- Guzman, J. R., Conlin, V. S., & Jobin, C. (2013). Diet, microbiome, and the intestinal epithelium: An essential triumvirate? *BioMed Research International*, 2013(1), Article 425146. <https://doi.org/10.1155/2013/425146>
- Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., & Kracht, M. (2002). Multiple control of interleukin-8 gene expression. *Journal of Leukocyte Biology*, 72(5), 847–855.
- InvivoGen. (2026). HCT116-Dual™ cells | NF-κB & IRF Reporter Cells | InvivoGen. <https://www.invivogen.com/hct116-dual>.
- Kammel, M., Erdmann, C., & Sawers, R. G. (2024). The formate-hydrogen axis and its impact on the physiology of enterobacterial fermentation. *Advances in Microbial Physiology*, 84, 51–82. <https://doi.org/10.1016/bs.amps.2024.02.002>
- Khanjani, S., Terzidou, V., Johnson, M. R., & Bennett, P. R. (2012). NF-κB and AP-1 drive human myometrial IL8 expression. *Mediators of Inflammation*, 2012, Article 504952. <https://doi.org/10.1155/2012/504952>
- Koev, T. T., Harris, H. C., Kiamehr, S., Khimiyak, Y. Z., & Warren, F. J. (2022). Starch hydrogels as targeted colonic drug delivery vehicles. *Carbohydrate Polymers*, 289, Article 119413. <https://doi.org/10.1016/j.carbpol.2022.119413>
- Kondrashina, A., Arranz, E., Cilla, A., Faria, M. A., Santos-Hernández, M., Miralles, B., ... Giblin, L. (2024). Coupling in vitro food digestion with in vitro epithelial absorption; recommendations for biocompatibility. *Critical Reviews in Food Science and Nutrition*, 64(26), 9618–9636. <https://doi.org/10.1080/10408398.2023.2214628>
- Kulecka, M., O'Sullivan, J., Fitzgerald, R., Velikonja, A., Huseyin, C. E., Laserna-Mendieta, E. J., Ruiz-Limón, P., Eckenberger, J., Vidal-Marín, M., Truppel, B. A., Singh, R., Naik, S., Croft, N. M., Temko, A., Zomer, A., MacSharry, J., Melgar, S., Deb, P., Sanderson, I. R., & Claesson, M. J. (2025). Combining mucosal microbiome and host multi-omics data shows prognostic potential in paediatric ulcerative colitis. *Nature Communications*, 16(1), 7157. <https://doi.org/10.1038/s41467-025-62533-z>, 2025 16:1.
- Lee, Y., Jaikwang, N., Kim, S. K., Jeong, J., Sukhoom, A., Kim, J. H., & Kim, W. (2023). Characterization of a potential probiotic *Lactiplantibacillus plantarum* LRCC5310 by comparative genomic analysis and its vitamin B6 production ability. *Journal of Microbiology and Biotechnology*, 33(5), 644–655. <https://doi.org/10.4014/jmb.2211.11016>
- Li, B., & Chen, J. Q. (2013). Development of a sensitive and specific qPCR assay in conjunction with propidium monoazide for enhanced detection of live *Salmonella* spp. in food. *BMC Microbiology*, 13, 273. <https://doi.org/10.1186/1471-2180-13-273>
- Liang, H., Chen, H., Zhang, W., Yu, C., Ji, C., & Lin, X. (2018a). Investigation on microbial diversity of industrial Zhacai paocai during fermentation using high-throughput sequencing and their functional characterization. *LWT*, 91, 460–466. <https://doi.org/10.1016/J.LWT.2018.01.088>
- Liang, H., Yin, L., Zhang, Y., Chang, C., & Zhang, W. (2018b). Dynamics and diversity of a microbial community during the fermentation of industrialized Qingcai paocai, a traditional Chinese fermented vegetable food, as assessed by Illumina MiSeq sequencing, DGGE and qPCR assay. *Annals of Microbiology* 2018, 68(2), 111–122. <https://doi.org/10.1007/S13213-017-1321-Z>, 68(2).

- Liang, T., Jiang, T., Liang, Z., Chen, Y., Chen, T., Dong, B., Xie, X., Gu, B., & Wu, Q. (2025). Lactiplantibacillus plantarum strain 84-3-derived l-glutamine ameliorates glucose homeostasis via AMPK/PPAR γ signaling pathway activation in type 2 diabetes. *Metabolism*, 172, Article 156357. <https://doi.org/10.1016/j.metabol.2025.156357>
- Liu, P., Mizue, H., Fujihara, K., Kobayashi, H., Kamikado, H., Tanaka, T., ... Miyamoto, T. (2012). A new rapid real-time PCR method for detection of *Listeria monocytogenes* targeting the hlyA gene. *Food Science and Technology Research*, 18(1), 47–57. <https://doi.org/10.3136/fstr.18.47>
- Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF- κ B signaling in inflammation. *Signal Transduction and Targeted Therapy*, 2(1), Article 17023. <https://doi.org/10.1038/SIGTRANS.2017.23;SUBJMETA>
- Mattalia, G., Stryamets, N., Pieroni, A., et al. (2020). Knowledge transmission patterns at the border: ethnobotany of Hutsuls living in the Carpathian Mountains of Bukovina (SW Ukraine and NE Romania). *J Ethnobiology Ethnomedicine*, 16, 41. <https://doi.org/10.1186/s13002-020-00391-3>
- Parhar, K., Ray, A., Steinbrecher, U., Nelson, C., & Salh, B. (2003). The p38 mitogen-activated protein kinase regulates interleukin-1 β -induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells. *Immunology*, 108(4), 502–512. <https://doi.org/10.1046/j.1365-2567.2003.01603.x>
- Riesberg, L. A., McDonald, T. L., Wang, Y., Chen, X. M., Holzmer, S. W., Tracy, S. M., & Drescher, K. M. (2018). Creatinine downregulates TNF- α in macrophage and T cell lines. *Cytokine*, 110, 29–38. <https://doi.org/10.1016/J.CYTO.2018.04.021>
- Salas-Millán, J.Á., Aznar, A., Conesa, E., Conesa-Bueno, A., & Aguayo, E. (2022). Functional food obtained from fermentation of broccoli by-products (stalk): Metagenomics profile and glucosinolate and phenolic compounds characterization by LC-ESI-QqQ-MS/MS. *LWT*, 169, Article 113915. <https://doi.org/10.1016/j.lwt.2022.113915>
- Salas-Millán, J.Á., Conesa-Bueno, A., & Aguayo, E. (2024). A novel antidiabetic lactofermented beverage from agro-industrial waste (broccoli leaves): Process optimisation, phytochemical characterisation, and shelf-life through thermal treatment and high hydrostatic pressure. *Food Bioscience*, 59, Article 103999. <https://doi.org/10.1016/J.FBIO.2024.103999>
- Tamang, J. P., Cotter, P. D., Endo, A., Han, N. S., Kort, R., Liu, S. Q., Mayo, B., Westerik, N., & Hutkins, R. (2020). Fermented foods in a global age: East meets West. *Comprehensive Reviews in Food Science and Food Safety*, 19(1), 184–217. <https://doi.org/10.1111/1541-4337.12520>
- Thiruvengadam, M., & Chung, I. M. (2015). Phenolic compound production and biological activities from in vitro regenerated plants of gherkin (*Cucumis anguria* L.). *Electronic Journal of Biotechnology*, 18(4), 295–301. <https://doi.org/10.1016/J.EJBT.2015.05.005>
- Vergara-Salinas, J. R., Pérez-Jiménez, J., Torres, J. L., Agosin, E., & Pérez-Correa, J. R. (2012). Effects of temperature and time on polyphenolic content and antioxidant activity in the pressurized hot water extraction of deodorized thyme (*Thymus vulgaris*). *Journal of Agricultural and Food Chemistry*, 60(44), 10920–10929. <https://doi.org/10.1021/jf3027759>
- Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73(16). <https://doi.org/10.1128/AEM.00062-07>, 5261–7.
- Wullaert, A., Bonnet, M., & Pasparakis, M. (2011). NF- κ B in the regulation of epithelial homeostasis and inflammation. *Cell Research*, 21, 146–158. <https://doi.org/10.1038/cr.2010.175>
- Xu, J., Peng, S., Xiong, Y., Zheng, Z., Liu, M., Xu, J., Chen, W., Liu, M., Kong, J., Wang, C., Wang, Y., Rao, L., Zhao, L., & Liao, X. (2024). A review on fermented vegetables: Microbial community and potential upgrading strategy via inoculated fermentation. *Comprehensive Reviews in Food Science and Food Safety*, 23(3), Article e13362. <https://doi.org/10.1111/1541-4337.13362>
- Yang, Y., Xu, Y., He, X., Guo, M., Chen, J., Luo, L., & Xiang, J. (2025). Characterizations of lactic acid bacteria derived from pickles and the effects of fermentation on phenolic compounds in peony flowers. *Food Chemistry X*, 27, Article 102430. <https://doi.org/10.1016/J.FOCHX.2025.102430>
- Yu, Q., Li, J., Bai, J., Huang, M., He, Y., Zhao, Y., & Xiao, X. (2025). Enzymatic mechanism of a β -Glucosidase from *Lactiplantibacillus plantarum* Dy-1 with potential applications in the release of bound phenolics in fermentation barley. *Journal of Agricultural and Food Chemistry*, 73, 4173. <https://doi.org/10.1021/ACS.JAFC.4C09865>
- Zhang, J., Xia, N., Teng, J., Wei, B., & Huang, L. (2023). Effect of lactic acid bacteria fermentation on extractable and non-extractable polyphenols of soybean milk: Influence of β -glucosidase and okara. *Food Bioscience*, 56, Article 103326. <https://doi.org/10.1016/J.FBIO.2023.103326>
- Zhao, Y., Liu, X., Li, R., Xiao, H., & Zhao, T. (2026). Vitamin B6 nutrition, metabolism, and the relationship of diseases: Current concepts and future research. *Journal of Future Foods*, 6(5), 765–779. <https://doi.org/10.1016/j.jfutfo.2024.12.007>