



Integrated Genome-Based and In Vivo Safety Evaluation of *Lactiplantibacillus plantarum* M2, an Isolate Obtained from the Amazonian Fruit *Humiria balsamifera*

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Abstract

Lactiplantibacillus plantarum M2, previously isolated from the Amazonian fruit *Humiria balsamifera*, has shown a promising probiotic profile. This study performed an integrated safety evaluation combining genome-informed analyses with a 28-day repeated-dose oral toxicity assessment in a murine model. In silico genome screening revealed no detectable signatures associated with pathogenicity, virulence, or acquired antimicrobial resistance. Functional marker profiling and comparative analyses across reference strains further indicated that M2 harbors a conserved repertoire of genomic features associated with probiotic-relevant functions, including potential traits related to gastrointestinal survival, adhesion, oxidative stress response, and biofilm formation, with specific enrichment in substrate utilization pathways associated with niche adaptation. In vivo, repeated oral administration of *L. plantarum* M2 to Swiss mice did not result in mortality, adverse clinical signs, or treatment-related alterations in body weight trajectories in either sex, despite the expected variation over time (females: time effect $p=0.0018$; males: time effect $p<0.0001$). Mean daily food intake was higher in probiotic-treated animals in both sexes (females: mean difference = 3.54 g/day, $p=0.0016$; males: mean difference = 2.84 g/day, $p=0.0008$). A reduction in alanine aminotransferase levels was observed in females (49.40 ± 6.18 U/L in controls vs. 42.33 ± 3.67 U/L in treated animals, $p=0.0428$), without associated histopathological alterations in the liver. Taken together, these findings indicate that *L. plantarum* M2 does not elicit detectable adverse effects under the experimental conditions employed and provide a preclinical safety framework supporting its further investigation for oral applications.

Keywords Probiotics · *Lactiplantibacillus plantarum* · Safety assessment · Genomic analysis · Amazonian biodiversity

Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host [1, 2]. Their beneficial effects include the modulation of gut microbiota composition, enhancement of intestinal barrier function, and protection against gastrointestinal pathogens [3]. Probiotics have also been associated with the prevention or mitigation of conditions such as diabetes, obesity, metabolic syndrome, irritable bowel syndrome, and lactose intolerance [4–7].

Lactic-acid bacteria (LAB) remain among the most commonly used probiotic organisms in food and health-related applications, especially those from the lactobacilli group [8,

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9]. This taxon was recently revised, leading to the redistribution of species and the establishment of multiple genera, including 23 newly proposed genera [10]. *Lactiplantibacillus plantarum*, in particular, has been repeatedly associated with survival under gastrointestinal stress and with antagonism against enteric pathogens, supporting its frequent selection as a probiotic species [11–13].

Previous studies have consistently demonstrated the safety of *L. plantarum* strains in preclinical and clinical settings, including repeated-dose toxicity assessments showing no evidence of adverse effects [14–16]. Despite this generally recognized safety profile, concerns remain regarding the potential role of mobile genetic elements, such as plasmids and prophages, in the dissemination of antimicrobial resistance (AMR), highlighting the importance of comprehensive genomic screening in probiotic safety assessment [17–19].

In our previous work, we reported the isolation of *L. plantarum* M2 obtained from the spontaneous fermentation of *Humiria balsamifera*, an underexplored Amazonian fruit. This isolate was selected based on a combination of functional and genomic features, including strong contact-dependent antagonism, in vivo protective effects against *Salmonella enterica* and *Escherichia coli* in an infection model using *Tenebrio molitor* larvae [20]. These protective effects align with recent evidence highlighting probiotic-mediated regulation of gut microbiota composition, modulation of inflammatory cytokines, and maintenance of intestinal barrier integrity during *Salmonella* infections [21].

Molecular characterization indicated that M2 has a 3.40 Mb draft genome with no detectable acquired antimicrobial resistance or virulence genes. Antimicrobial susceptibility testing indicated that M2 is sensitive to several clinically relevant antibiotics, while resistance to vancomycin and gentamicin was observed, consistent with intrinsic resistance commonly described in LAB. M2 encodes two plantaricin gene clusters and two antimicrobial peptides, along with a CAZymes repertoire dominated by glycosyl hydrolases (GH) and glycosyltransferases (GT) [20]. These characteristics position M2 as a promising candidate among plant-associated *L. plantarum* strains for further safety evaluation.

To further advance the characterization of the biotechnological potential of strain M2 and its prospective application as an oral probiotic, preclinical evaluation of safety in mammalian hosts is required, together with a more detailed assessment of genome features related to genetic stability and potential horizontal gene transfer (HGT) risks. This requirement is consistent with current probiotic regulatory frameworks, which emphasize genome-informed safety assessment, including the absence of transferable AMR

determinants and the evaluation of mobile genetic elements, as recommended by agencies such as EFSA [22].

Accordingly, we hypothesized that *L. plantarum* M2 presents a genomic and toxicological profile consistent with a low-risk probiotic candidate suitable for oral exposure. The present study performed an integrated safety evaluation combining genome-based risk profiling with a repeated-dose oral administration of *L. plantarum* M2 in a murine model. This integrated approach contributes to a more comprehensive preclinical safety evaluation and provides a basis for the further investigation of M2 in food and health-related applications.

Materials and Methods

Microorganism Storage and Culture

L. plantarum M2 is maintained in the culture collection of the Laboratory of Microbial Pathogenicity of CEUMA University (UNICEUMA), where it is preserved at -80°C in appropriate cryoprotective medium. For the experiments performed in this study, the strain was reactivated by cultivation in de Man, Rogosa and Sharpe (MRS) broth at 37°C for 24–48 h before the assays.

Genome Assembly Quality Assessment

The whole-genome sequence of *L. plantarum* M2 (GenBank accession: JBQOUW0000000000; BioProject: PRJNA1303982) was subjected to additional quality assessment using CheckM2 (Galaxy implementation, version 1.1.0+galaxy0) under default settings. Genome completeness and contamination estimates were used as complementary indicators of assembly quality prior to downstream annotation and comparative analyses.

A confirmatory in silico screening for acquired AMR genes was performed using ResFinder 4.0 with identity and minimum length thresholds of 90% and 60%, respectively. In the context of the present study, this analysis was used to complement the previous genome-based safety assessment of strain M2.

Genome Annotation and Subsystem Analysis

Following this, the genome was analyzed using the Rapid Annotation using Subsystem Technology (RAST) server (v2.0) under default parameters. Functional categorization was performed in the SEED Viewer environment to obtain an overview of major metabolic subsystems. Loci initially assigned by RAST to the “Defense and Virulence”

category were subjected to manual curation. This process included sequence similarity searches against the NCBI non-redundant (nr) database and conserved domain analysis using CD-Search. Assignments were re-evaluated qualitatively based on congruence between homolog annotations, conserved domain content, and known physiological roles in LAB.

Comparative Genomics and Phylogenomic Analysis

For comparative analyses, a panel of ten *L. plantarum* genomes was assembled, comprising strain M2 and reference strains (WCFS1, ATCC 14917, DSMZ 20174, JDM1, and 299v) as well as emerging plant-associated isolates (Z45, NCU116, ST III, and 6Y-15). All genomes included in the comparative analyses were re-annotated using Bakta Web (version 1.12.0; <https://bakta.computational.bio/>) [23], under default parameters, to ensure a standardized annotation framework before the analyses.

Whole-genome-based phylogenetic placement was inferred using genome sequences deposited in GenBank, and the phylogenomic tree was generated using the TYGS server (<https://tygs.dsmz.de>) [24]. Orthologous protein clusters were identified using OrthoVenn3 (<https://orthovenn3.bioinfotoolkits.net>) with default parameters. Core, accessory, and strain-specific clusters (singletons) were defined based on ortholog presence-absence patterns across the analyzed genome set.

Integrated Genomic Profiling for Functional and Safety Assessment

Genome-informed functional profiling and biosafety screening were performed using a custom Python-based computational suite developed for this study [25] (available at <https://github.com/luiscnascimentoasilva/LAB-Genomics-Suite>). The pipeline performed a rule-based scan of predicted proteomes to detect genetic markers associated with probiotic-relevant functions, using gene names, KEGG orthology identifiers, PFAM domains, and curated regular expressions as evidence. A locus was considered positive for a given marker when at least one curated rule matched its annotation evidence. To ensure biological accuracy and reduce redundancy, multiple annotation matches mapping to the same coding sequence were collapsed and counted once per locus within each functional category.

The functional categories included (i) gastrointestinal survival, including bile salt hydrolase (BSH) and acid tolerance via the F1F0-ATPase complex; (ii) intestinal adhesion and colonization, including sortase-anchored surface proteins

and mucin-binding domains (MucBP, WxL, LysM); (iii) oxidative and industrial stress resilience, including antioxidant enzymes and exopolysaccharide (EPS)/biofilm-associated determinants; and (iv) metabolism and intermicrobial competition, including prebiotic utilization pathways, glycerophosphodiester uptake (*ugp/glpQ*), GABA production, folate biosynthesis, and antimicrobial systems. Grouped multiprotein systems were scored only when a minimum number of distinct markers required for functionality were detected (e.g., ≥ 3 for *atp_operon*; ≥ 2 for PTS modules, *gad_operon*, and *ugp_operon*). Marker counts were normalized per 1,000 coding sequences (CDS) to account for genome size variation. Comparative visualization was performed using hierarchically clustered heatmaps, and global functional dissimilarity among strains was assessed using Principal Coordinates Analysis (PCoA) based on Bray-Curtis distance.

In the *in silico* detection of canonical biogenic amine-related loci, the genomes were screened using curated rules targeting histamine (*hdcA*, *hdcB*, *hdcP*), tyramine (*tdcA/tyrDC*, *tyrP*), putrescine/agmatine (*odc*, *aguA*, *aguB*, *aguC*, *aguD*), and cadaverine (*cadA*). Hits were searched across gene names, product annotations, and database cross-references. To reduce false positives, ambiguous annotations unrelated to aminobiogenic pathways were excluded by conservative blacklist filters, and putrescine/agmatine calls based on the *agu* system were only classified as pathway-supported when at least two distinct *agu* markers were detected in the same genome. The ornithine decarboxylase gene (*odc*) was treated as a canonical marker when present. Biogenic amine evidence was summarized into four levels: absent, single-marker hit, pathway-supported, and canonical marker. The genomes of selected *Levilactobacillus brevis* strains (CRAI, KB290, and TMW1.2108), *Enterococcus faecalis* JH2-2, *Lentilactobacillus parabuchneri* FAM21731, and *Escherichia coli* K12 were included as positive controls to validate the sensitivity of the workflow for putrescine/agmatine-associated loci.

Mobilome and Genome Defense Analyses

Plasmid sequences were reconstructed from the draft genome assembly using MOB-recon implemented in the Galaxy platform [26]. Circular plasmid maps were generated and visually inspected using Proksee (<https://proksee.ca/>), integrating functional annotation using the Bakta pipeline (v1.8.2). Prophage-associated regions were identified using PHASTEST 3.0 (<https://phastest.ca/>) [27], under default settings. Genomic islands (GIs) were predicted using IslandViewer 4 (<https://www.pathogenomics.sfu.ca/i>

slandviewer/), integrating IslandPick, IslandPath-DIMOB, and SIGI-HMM methods. CRISPR-associated loci were detected using CRISPRFinder [28], under default settings, with particular attention to the presence of Cas proteins involved in interference against foreign nucleic acids.

Genome-Informed Pathogenicity Prediction

Genome-based pathogenicity estimation was performed using PathogenFinder 2 (v. 0.6.0; <https://genepi.food.dtu.dk/pathogenfinder>), under default settings [29]. The analysis was used as a similarity-based screen to identify matches to pathogenic or non-pathogenic reference genomes and to estimate the probability of pathogenicity. Results were interpreted as supportive evidence and integrated with mobilome analyses and in vivo safety data.

Animals and Ethical Considerations

This project was approved by the Animal Research Ethics Committee of UNICEUMA (CEUA: 052301/24). Swiss mice of both sexes (8–10 weeks old) were obtained from the Universidade CEUMA animal facility, where they were housed and monitored throughout the experiment under standard conditions in a clean environment (23 ± 2 °C) with controlled humidity and light/dark cycle. Animals had free access to standard rodent chow and water (*ad libitum*). A total of 24 mice were used for the safety assessment. Animals were allocated according to the assays performed and grouped by experiment, as described in the following sections.

Repeated Dose Oral Toxicity Assay

A total of 24 animals were used (12 males and 12 females), with six animals per sex per group ($n = 6/\text{sex}/\text{group}$). This sample size is consistent with commonly adopted designs in exploratory repeated-dose toxicity studies in murine models, allowing the detection of overt toxicological effects while minimizing animal use in accordance with ethical principles. Animal experiments were designed and reported in accordance with the ARRIVE guidelines. The 28-day repeated-dose oral toxicity protocol was adapted from the OECD Test Guideline 407, tailored for probiotic safety assessment as recommended by EFSA frameworks. Male and female mice were randomized separately into treatment groups using simple random allocation, with six animals per sex per group: (i) GO (negative control): treated daily with 100 μL of 0.9% NaCl by oral gavage; (ii) G1 (M2): treated daily with 100 μL of freshly prepared *L. plantarum* suspension (1×10^8 CFU/mL) by oral gavage. The administered dose (1×10^8 CFU/mL, 100 μL per day) was selected based

on ranges commonly employed in preclinical evaluations of probiotic strains in murine models [12, 30].

Body weight was measured daily, and food and water intake were recorded at 2 and 4 weeks. On day 29, mice were euthanized. Animals were monitored for clinical signs, including eye appearance, respiration, coat condition, locomotion, and body weight. The specific growth rate (SGR) was expressed as the mean weekly weight gain (g) and calculated as $\text{SGR} (\%) = [(W - W_0)/W_0] \times 100$, where W is the body weight at the defined time points and W_0 is the body weight on day 0.

Evaluation of Hematological and Biochemical Parameters

Hematological assessments (erythrogram and leukogram) were performed on blood smears, with cell counts obtained using a Neubauer chamber. Biochemical test results were also evaluated using the automated analyzer Audimax Evolution. Biochemical analyses included key hepatic markers (ALT, AST, bilirubin), renal markers (urea and creatinine), and the lipid profile (total cholesterol, HDL, LDL, VLDL, and triglycerides).

Histopathological Analysis

A gross necropsy was performed to evaluate external and internal macroscopic findings. After euthanasia, the heart, liver, spleen, kidneys, stomach, small intestine, and large intestine were carefully collected. Samples were fixed in 10% (v/v) formaldehyde prepared in 0.01 M PBS (pH 7.2) for a maximum of 48 h and then transferred to 70% (v/v) ethanol. Intestinal fragments were dehydrated through increasing ethanol concentrations, cleared in xylene, and embedded in paraffin. After microtomy (5 μm sections), slides were stained with hematoxylin and eosin (H&E). To ensure objective evaluation, the histopathological examination was performed in a blinded manner by a trained observer. A minimum of three sections per organ per animal were analyzed to assess architectural integrity and potential tissue injury. No semi-quantitative scoring system was applied due to the complete absence of baseline lesions across groups. Sections were analyzed, and representative fields were photographed at 40 $\times$ magnification using a Nikon Eclipse Si trinocular light microscope (Nikon Instruments Inc., Tokyo, Japan) equipped with a Prime Life Science Corp digital imaging system.

Statistical Analysis

Results were expressed as mean $\pm$ standard deviation (SD). Data normality was assessed using the Shapiro-Wilk test.

Body weight trajectories were analyzed separately for males and females using a mixed-effects model (REML), with time and treatment as fixed factors and subject as a random effect. The Geisser–Greenhouse correction was applied when sphericity could not be assumed. Food intake was compared between groups within each sex using the paired Student's t-test. Biochemical and hematological variables were analyzed separately by sex after assessment of normality using unpaired Student's t-test for variables meeting normality assumptions or Mann–Whitney U test for those that did not meet normality assumptions. Exact p-values are reported where applicable, and statistical significance was set at $P < 0.05$. All analyses were performed using GraphPad Prism (version 10.1).

Results

Genome Subsystem Annotation and Curated Intrinsic Tolerance-Related Features of *L. plantarum* M2

In the present study, the whole-genome assembly of *L. plantarum* M2, previously reported as a 3.40 Mb sequence [20], was subjected to reconstruction using MOB-recon to better resolve its chromosomal and extrachromosomal architecture. This analysis revealed that M2 has a 3,287,257 bp chromosome and three distinct plasmids: AE887 (9,679 bp), AB384 (19,586 bp), and AB794 (86,660 bp). The genome assembly was additionally evaluated using CheckM2 and showed high quality, with an estimated completeness of 100.0% and contamination of 0.79%.

Confirmatory screening with ResFinder 4.0 did not identify acquired antimicrobial resistance genes. However, annotation via Bakta identified the *vanZ* marker, and subsequent specialized analysis using CARD RGI (v6.0.5) detected the presence of *vanH* and *vanY* genes. These markers were localized within the chromosomal DNA (Fig. 1A). This chromosomal localization, coupled with the absence of acquired determinants in ResFinder, confirms that the vancomycin resistance in M2 is a non-transmissible, intrinsic trait typical of the *Lactiplantibacillus* genus.

The RAST analysis (Table S1) identified genes involved in carbohydrate and protein metabolisms as the categories with higher gene counts, as well as functions related to DNA metabolism and cofactors/vitamins. Initially, 35 *loci* were identified within the category “Defense and Virulence”; however, manual inspection indicated that nine of these were housekeeping genes (e.g., SSU and LSU ribosomal proteins) misclassified due to sequence similarity with mycobacterial proteins. The remaining *loci* corresponded to intrinsic stress-tolerance traits frequently observed in LAB,

including metal homeostasis, efflux-associated systems, and bile salts hydrolase-related sequences (Table S2). Additionally, prophage-associated genes were detected, indicating exposure to mobile genetic elements. The detection of genetic determinants for stress response, cell wall and capsule formation, and membrane transport is consistent with features commonly described in *L. plantarum* strains and may contribute to environmental adaptation and host interaction.

Phylogenetic Analysis and Orthologous Cluster Distribution of *L. plantarum* M2

Whole-genome-based phylogenetic analysis placed *L. plantarum* M2 within the Lactobacillaceae clade and confirmed its close relationship with other representative *L. plantarum* reference strains (Fig. 1A).

To further understand the genomic features of *L. plantarum* M2, a comparative analysis of orthologous protein clusters was performed using OrthoVenn3 across M2 and nine-genome panel, including commercial reference strains and plant-associated isolates. Across this global comparison, M2 contained 3,250 predicted proteins distributed into 2,975 orthologous clusters and 203 singleton proteins. The overall cluster distribution across the ten genomes is summarized in Fig. 2A, which shows the most frequent orthologous cluster intersections among the analyzed strains.

A five-way Venn diagram comparing M2 with WCFS1, JDM1, ATCC 14,917, and DSMZ 20,174 is represented in Fig. 2B. In this focused comparison, M2 showed a large fraction of orthologous clusters with the reference strains and some clusters that remained exclusive to M2. This pattern is consistent with the presence of an accessory gene fraction in M2, which is expected for *L. plantarum*, and provides a basis for subsequent functional interpretation. A summary of proteome-level orthologous cluster statistics for the ten *L. plantarum* genomes is provided in Table S3.

Functional Marker Profiling and Comparative Clustering of Probiotic-Associated Pathways

The comparative analysis of the functional proteome using a rule-based marker scan and an operon-completeness filter indicated that the M2 strain shares a similar marker profile with the other nine *L. plantarum* strains analyzed (Fig. 3). Marker counts were normalized per 1,000 CDS to reduce bias due to genome-size variation. Regarding gastrointestinal survival, the M2 strain presented a complete gene set required for the F1F0-ATPase complex (4 hits; 1.241 per 1,000 CDS) and harbored a functional Bile Salt Hydrolase (BSH) hit (0.310 per 1,000 CDS). This profile is consistent with established reference strains such as WCFS1 and

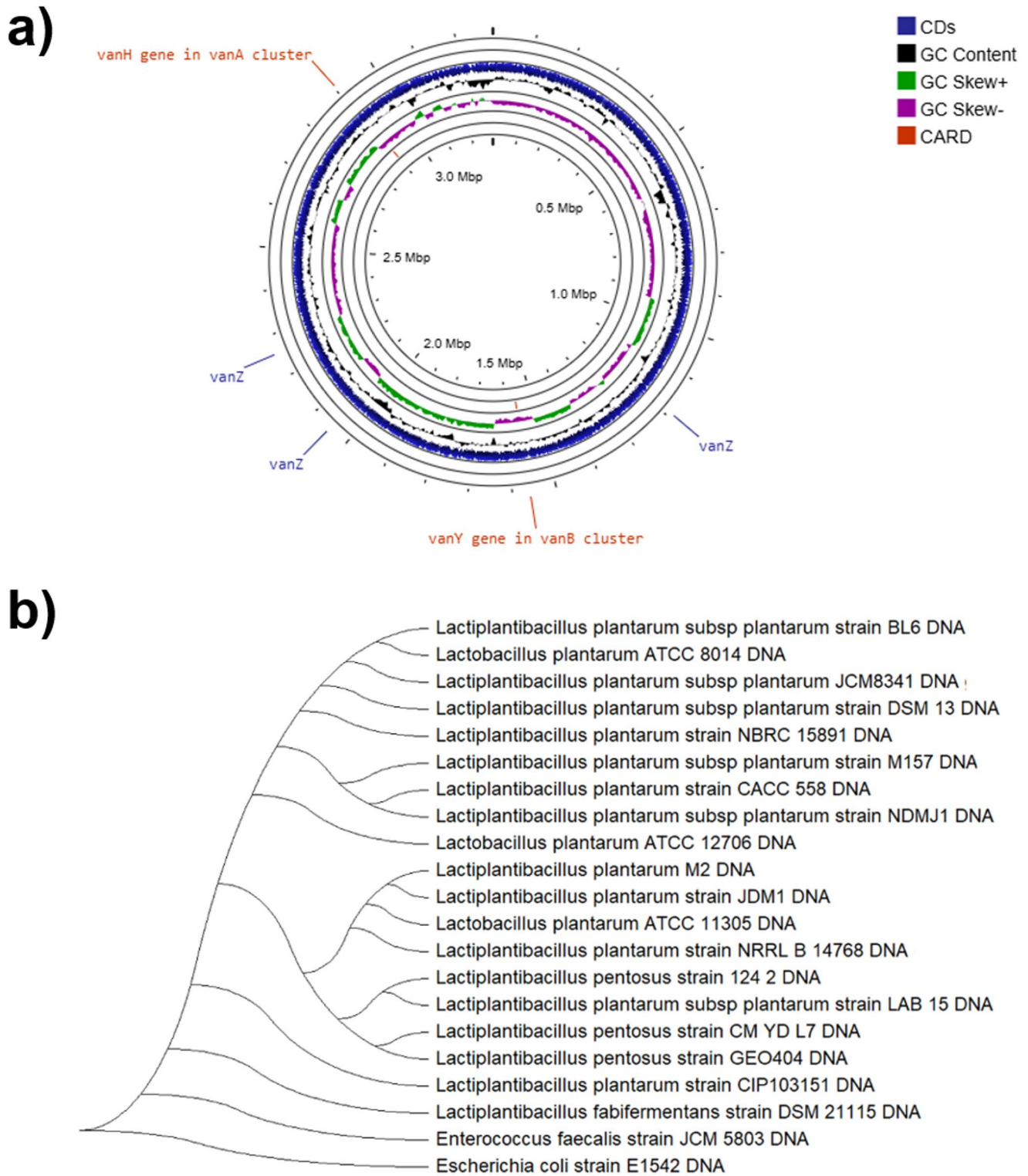
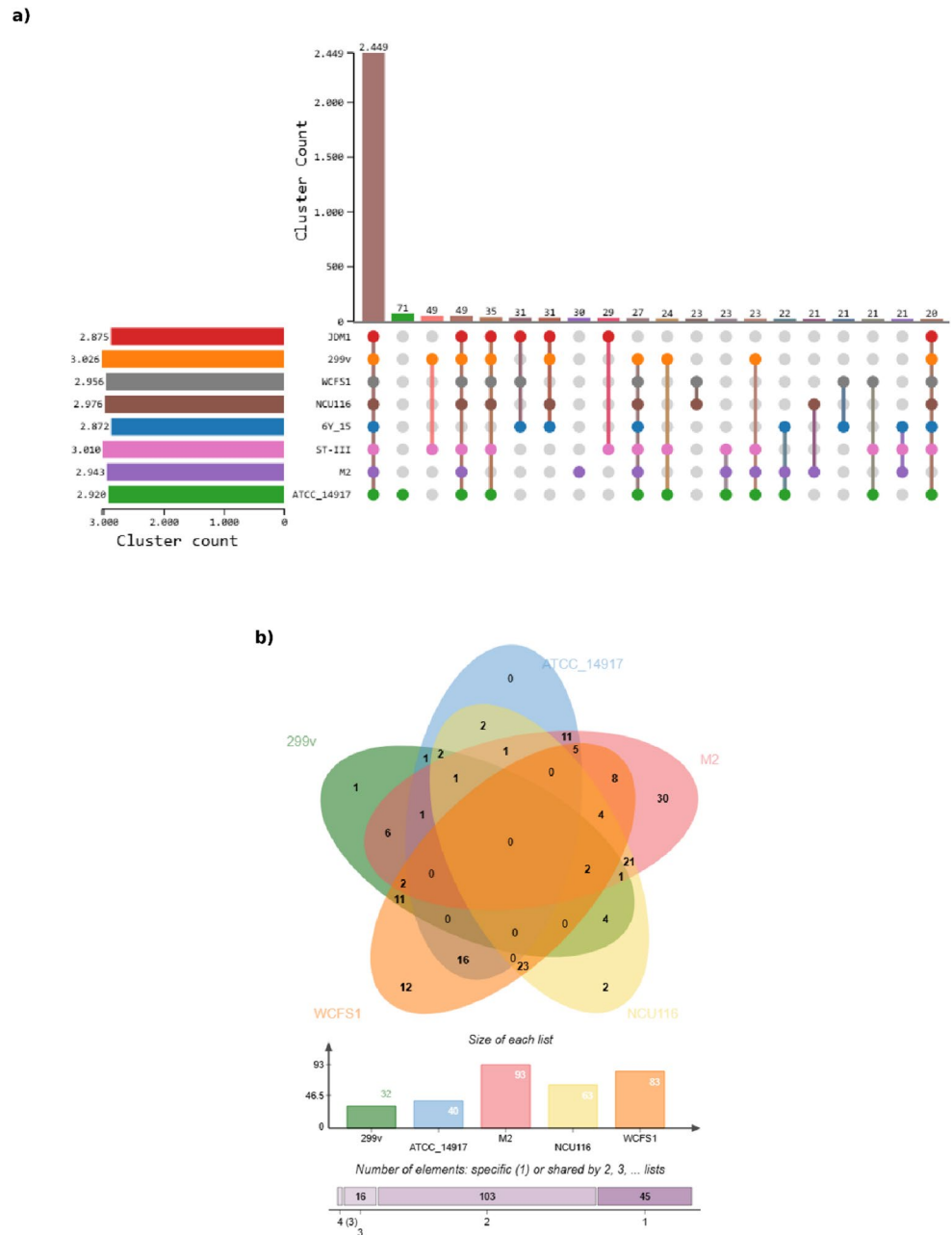


Fig. 1 Chromosomal architecture and phylogenomic placement of *Lactiplantibacillus plantarum* M2. **(A)** Circular map of the M2 chromosome. Tracks from outside to inside: (1) forward-strand CDS, (2) reverse-strand CDS, (3) GC content (black), and (4) GC skew (green/purple). The labels indicate the chromosomal localization of intrinsic vancomycin resistance-related markers identified through integrated

annotation (*vanZ* by Bakta; *vanH* and *vanY* by CARD RGI). The map was generated using Proksee. **(B)** Whole-genome-based phylogenetic tree showing the relationship of strain M2 with representative *L. plantarum* reference strains and related species. Bootstrap values are indicated at the nodes

Fig. 2 Comparative analysis of orthologous protein clusters across *Lactiplantibacillus plantarum* genomes. **(A)** Summary of orthologous cluster intersections across ten *L. plantarum* proteomes. Only the 20 largest intersections are shown. **(B)** Five-way Venn comparison highlighting shared and restricted orthologous clusters among M2 and four reference strains (WCFS1, JDM1, ATCC 14917, and DSMZ 20174)



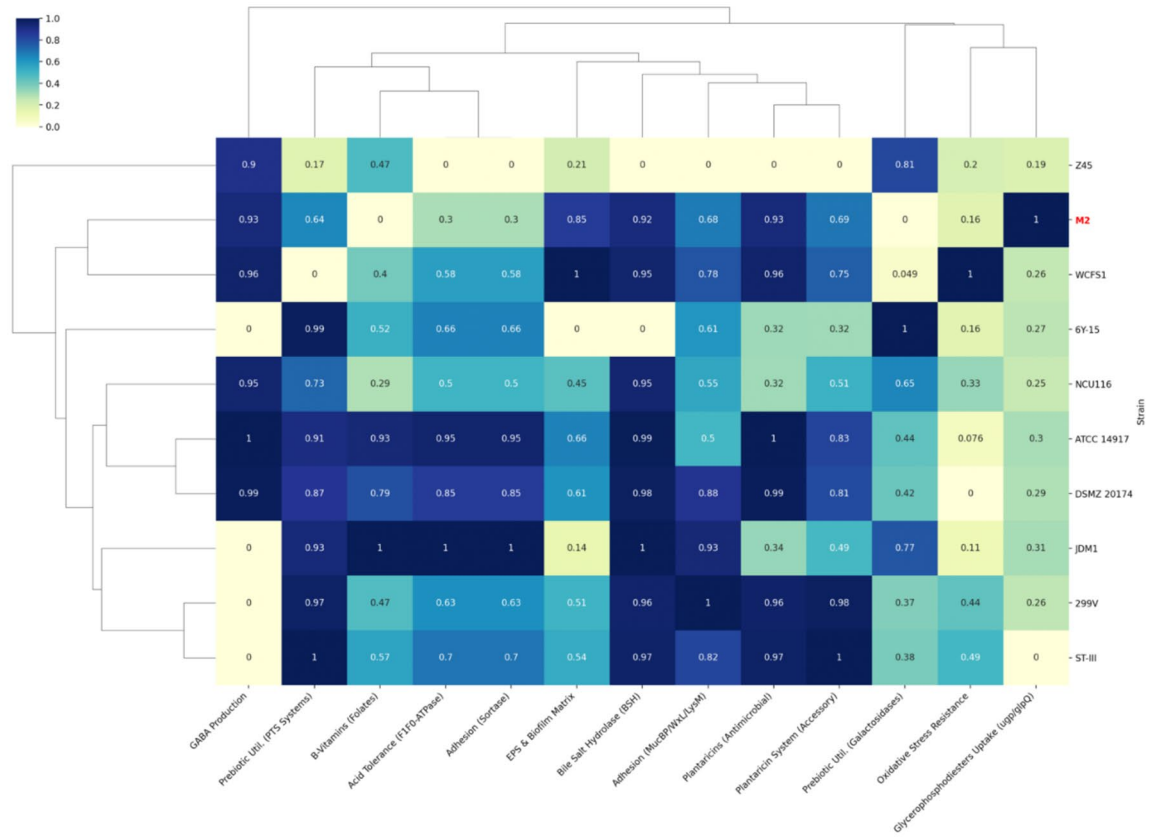
ATCC 14917 (Fig. 3A). Regarding GABA production, M2 harbored a specific genetic marker (0.310 per 1,000 CDS), a trait shared with five other strains in the panel (ATCC 14917, DSMZ 20174, NCU116, WCFS1, and Z45). However, the presence of a single locus suggests a partial or non-canonical configuration compared to reference strains that harbor multiple components of this system.

Markers associated with B-vitamin (folate) biosynthesis exhibited minimal variation across the panel, with counts ranging from 4.032 to 4.343 per 1,000 CDS. In the antimicrobial category, M2 harbored 1.861 plantaricin-related hits per 1,000 CDS. This value was numerically similar to those observed in reference and commercial strains, such as

WCFS1 (1.922), 299v (1.933), and ATCC 14917 (2.005), while exceeding the counts recorded for JDM1 (0.672), 6Y-15 (0.647), NCU116 (0.635), and Z45 (0.000). For adhesion-related features, M2 showed a marker frequency comparable to the panel, including Sortase A and multiple cell wall-associated domains captured by MucBP/WxL/LysM rules (4.96 per 1,000 CDS).

M2 also displayed 24 antioxidant-related hits (7.44 per 1,000 CDS) and reached the second highest count for EPS/biofilm matrix determinants in the panel (9 hits; 2.79 per 1,000 CDS), close to the count observed in the reference WCFS1 (2.88 per 1,000 CDS). Regarding carbohydrate metabolism, PTS system markers were detected in M2 at a

a)



b)

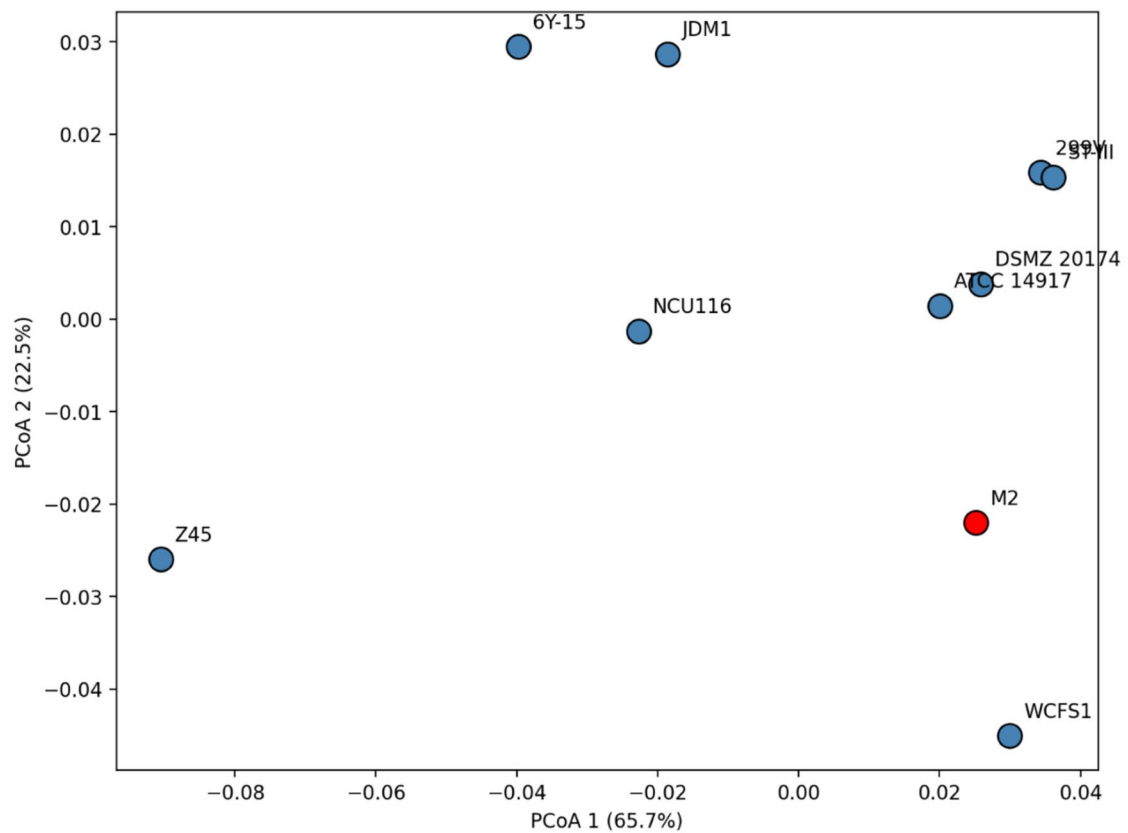


Fig. 3 Genome-informed functional marker profiling of *Lactiplantibacillus plantarum* M2 compared with reference and plant-associated strains. **(A)** Hierarchically clustered heatmap presenting the relative distribution of curated probiotic-relevant functional genetic markers across a ten-strain panel. The color scale (inset) ranges from light yellow (relative absence/low abundance) to dark blue (relative genomic enrichment per category), utilizing a standard scale based on marker counts normalized per 1,000 coding sequences (CDS). **(B)** Principal Coordinates Analysis (PCoA) plot based on Bray-Curtis dissimilarity matrices computed from normalized functional marker counts. Percentages on axes indicate the explained variance for PCoA 1 and PCoA 2. Strain M2 is highlighted as a red circle

frequency of 5.893 per 1,000 CDS. Conversely, for prebiotic utilization via galactosidases, M2 presented the lowest normalized count in the panel (1.551 per 1,000 CDS). The most significant relative enrichment in M2 was the glycerophosphodiester uptake module (*ugp/glpQ*), with 7 hits (2.17 per 1,000 CDS), whereas all other strains in the panel harbored 3 to 4 hits (maximum of 1.343 per 1,000 CDS).

In the Bray–Curtis PCoA (Fig. 3B), which explained 88.2% of the total functional variance (PC1: 65.7%; PC2: 22.5%), M2 is positioned within the functional space defined by the reference strains, showing high proximity to ATCC 14917 and DSMZ 20174, which underscores its genetic potential for probiotic applications.

Canonical Biogenic Amine-Related Loci

No retained canonical or pathway-supported biogenic amine-related loci were detected across the *L. plantarum* panel under the conservative screening criteria adopted in this study. In contrast, the positive-control *L. brevis* strains showed a consistent putrescine/agmatine-associated module composed of *aguA*, *aguB*, *aguC*, and *aguD*, while two control genomes additionally carried *odc* as a canonical marker (Figure S1).

Mobilome Composition and Genome Defense Features Relevant to Safety Assessment

Plasmid Repertoire and Structural Diversity

As mentioned above, plasmid reconstruction using MOB-recon identified three extrachromosomal elements in the M2 genome, corresponding to distinct putative plasmids designated as AE887, AB384, and AB794. These replicons varied markedly in size and gene content, indicating a heterogeneous plasmid repertoire (Figure S2). No canonical antimicrobial resistance genes, virulence determinants, or intact prophage regions were identified across the plasmid sequences, supporting a non-pathogenic and accessory role of these elements.

AE887 (Figure S2) was predicted as a compact, non-mobilizable element of approximately 9,679 bp encoding 9

CDS. Its genetic backbone seems to be dedicated to transcriptional regulation and DNA processing, encoding a SpoVT-AbrB domain-containing protein, an mRNA interferase, and two HTH cro/C1-type domain-containing proteins. It also encodes a YkuD/YxeA family protein, a Tyr recombinase domain-containing protein, suggesting a specialized role in DNA integration or maintenance.

AB384 (19,586 bp; 20 CDS) is also a non-mobilizable plasmid but exhibits a repertoire geared toward ecological adaptation (Figure S3). Its key features include a four-component ABC transport system, a glycerophosphodiester phosphodiesterase, and lysostaphin. The stability and plasticity markers are represented by DNA invertase, a BRCT domain protein, and an IS30-family transposase remnant. In contrast, AB794 is a large (86,660 bp, 95 CDS) mobilizable plasmid, and it carries genes for horizontal transfer (*trbC* and TraG-D-C) and stable inheritance (ParA and a Phd/YefM toxin-antitoxin system) (Figure S4). It also encodes determinants for stress resilience (universal stress protein and MntH divalent cation transporter), nutrient uptake (ABC and MFS transporters), and genome plasticity (multiple transposases, DNA topoisomerase, and repair ATPases), complemented by several regulatory ncRNAs.

Prophage Prediction

PHASTEST analysis identified a single intact prophage region in the M2 genome, spanning approximately 58.9 kb between positions 116,979 and 175,964 bp (Fig. 4). The region received the maximum completeness score (150) and contained 58 predicted proteins, of which 46 were classified as phage-hit proteins and 11 as hypothetical proteins, with only one protein annotated as bacterial. Phage-related and hypothetical proteins accounted for 98.2% of the region, supporting its classification as an intact prophage-derived locus. Attachment sites were detected at both ends of the region, with the sequence TGAAAT AACTCATT found at *attL* and *attR*. The most frequently matched phage was PHAGE_Lactob_Sha1_NC_019489, represented by 17 hit genes.

The region displayed a typical prophage-like modular organization, including lysis-associated genes (holin and endolysin), structural genes related to tail, tail fiber, base-plate, capsid, portal, and terminase proteins, as well as regulatory and recombination-associated proteins such as RinA-like activator, HNH nuclease, anti-repressor, and site-specific recombinase. These features are consistent with prophage-derived regions commonly observed in *L. plantarum* genomes, and similar intact prophage regions were detected in the genomes of 299v, JDM1, ATCC 14,917, and DSMZ 20,174 (Table S4).

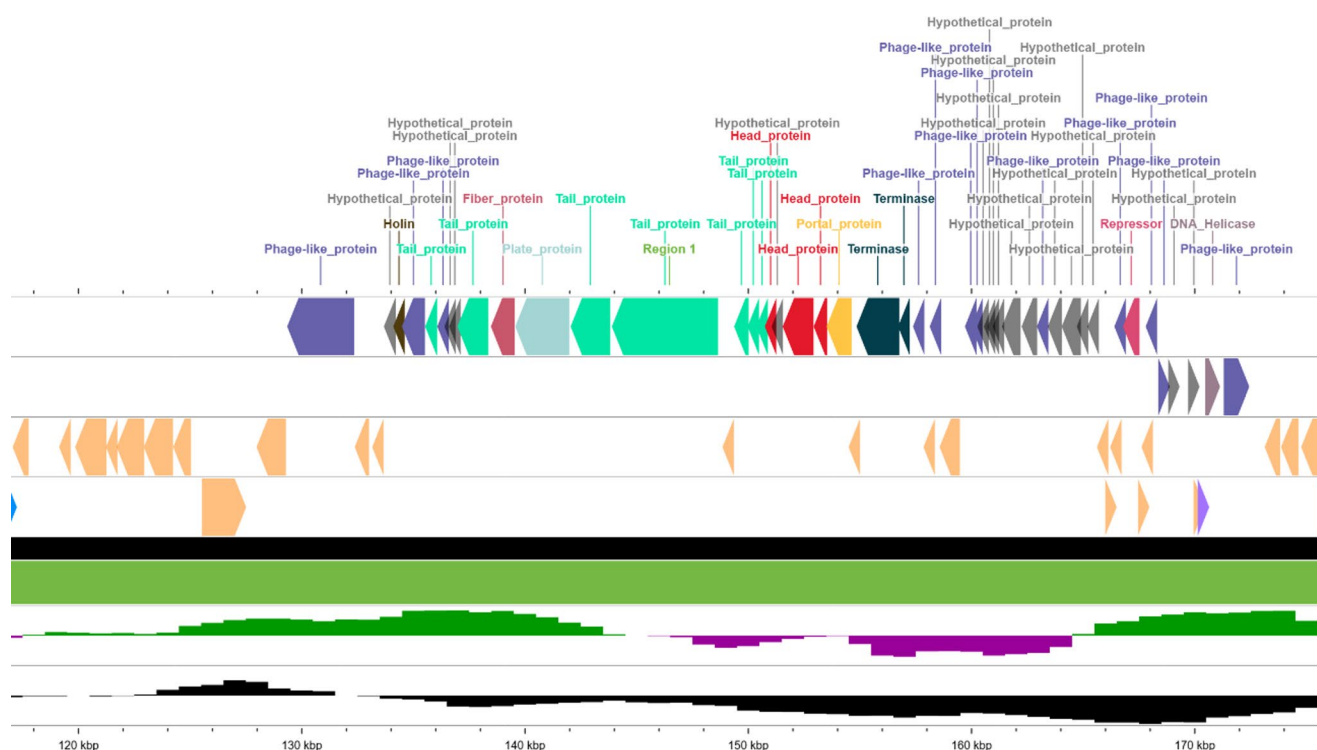


Fig. 4 Organization of the intact prophage region predicted in the *Lactiplantibacillus plantarum* M2 genome by PHASTEST. The prophage spans approximately 58.9 kb (116,979–175,964 bp) and comprises 58 predicted proteins, including 46 phage-hit proteins and 11 hypo-

thetical proteins. The region contains structural, regulatory, packaging, and DNA-processing modules typical of prophage-derived loci and is flanked by *attL/attR* attachment sites

Genomic Island Distribution and Horizontal Gene Transfer-Associated Signatures

Integrated prediction using IslandViewer4 identified 32 putative GI intervals distributed across the M2 genome (Fig. 5 and Table S5). These regions ranged from 4.2 to 72.1 kb and collectively contained 533 GI-associated gene entries. No predicted GI corresponded to a recognized pathogenicity island or was enriched in canonical virulence determinants. Hypothetical proteins (22.70%), phage/prophage-associated proteins (21.95%), and mobile element/HGT-related functions (10.88%) together accounted for 55.33% of the annotated entries within the predicted islands. Additional genes encoded transporters, surface-associated proteins, bacteriocin-related factors, and other accessory functions that may contribute to ecological adaptation. This GI landscape of M2 is consistent with an accessory genome shaped by mobile genetic elements and horizontally acquired DNA rather than by pathogenicity-related islands.

CRISPR-Associated Locus Detection and In Silico Defense-Related Indicators

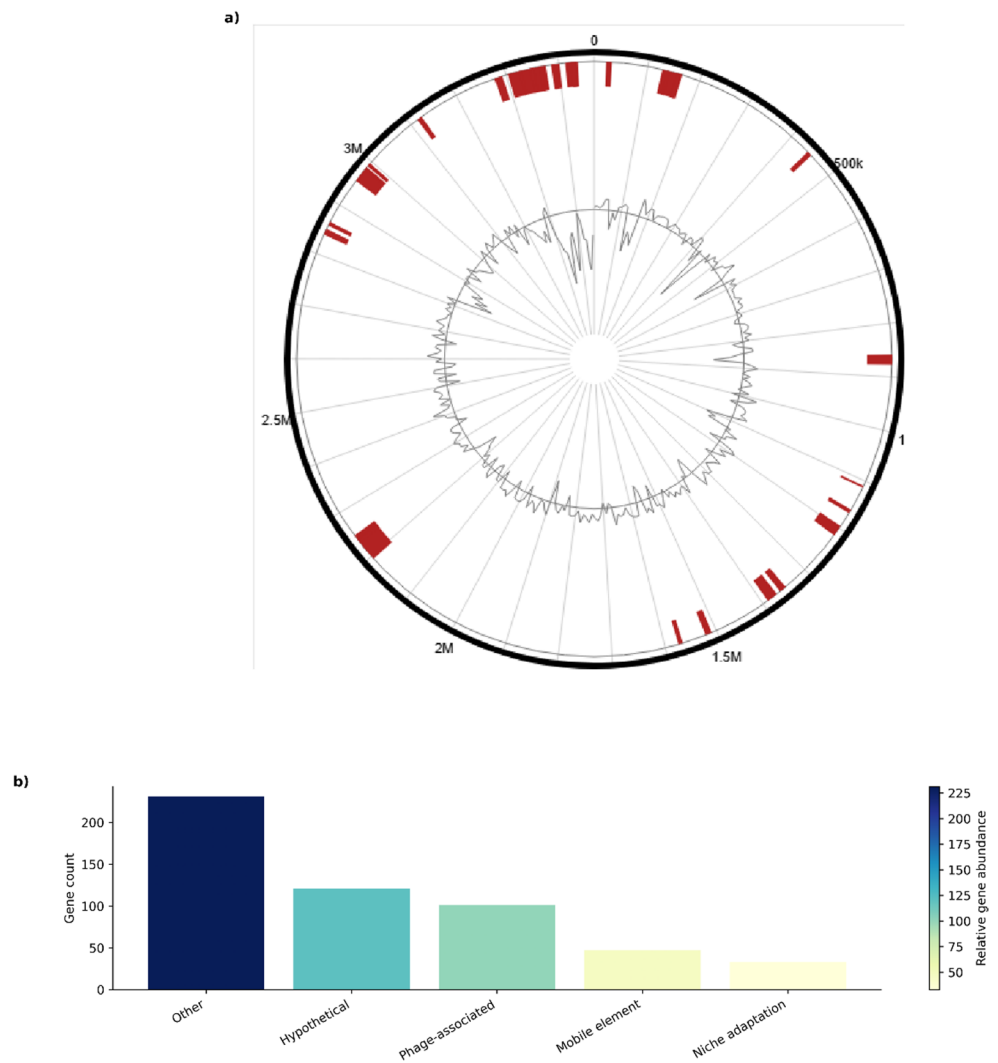
One CRISPR-associated locus was identified in the *L. plantarum* M2 genome (nt 12,040–13,383), including a

Cas3-encoding gene consistent with a type I CRISPR–Cas system. Cas3 is a helicase–nuclease involved in interference against invading nucleic acids, and its presence is consistent with the presence of a genome-encoded defense feature against foreign genetic elements. This protein is involved in protecting the bacterium against bacteriophages, as it functions as a DNA helicase–nuclease that degrades invading viral genetic material [31]. The presence of this locus is indicative of a defense feature. In the context of genome-informed risk profiling, the coexistence of a CRISPR-associated locus with a limited prophage signal suggests a potential genome-encoded defense mechanism against mobile genetic elements. However, in vitro validation of CRISPR activity would be required to confirm a phenotype of reduced foreign DNA acquisition. Therefore, this result is used as supportive context alongside the mobilome analyses and the in vivo safety endpoints reported below.

Genome-Informed Pathogenicity Estimation of *L. plantarum* M2

A genome-informed screen using PathogenFinder was performed to estimate whether *L. plantarum* M2 shows sequence patterns found in human pathogenic bacteria. The predicted probability of pathogenicity was very low

Fig. 5 Genomic island prediction in the *Lactiplantibacillus plantarum* M2 genome. **(a)** Circular representation of the chromosome showing genomic islands predicted by at least one prediction method. **(b)** Functional classification of genes located within genomic islands, with colors reflecting relative gene abundance. The genome was aligned against the reference genome *L. plantarum* WCFS1



(0.19%), with no matches reported to virulence-associated gene signatures. In contrast, 17 hits were assigned to non-pathogenic bacteria, all classified within lactobacilli, with functions corresponding to metabolic or stress-related genes frequently found in *L. plantarum* isolates. This result is consistent with a low-risk genomic profile. The complete list of PathogenFinder hits is provided as Supplementary Table S6.

Body Weight Trajectories and Food Intake Patterns Following Oral Administration of *L. plantarum* M2

The body weight of mice was daily measured during the 28-day oral toxicity assay and separately analyzed for males and females using a mixed-effects model. In female mice, body weight changed significantly over time ($p=0.0018$), but no significant treatment effect ($p=0.8915$) or time $\times$ treatment interaction ($p=0.1576$) was detected (Fig. 6A). Similarly, a significant effect of time was observed in the body weight of male mice ($p<0.0001$), whereas neither the

treatment effect ($p=0.0911$) nor the time $\times$ treatment interaction ($p=0.7247$) was significant (Fig. 6B).

Daily food intake showed expected fluctuations throughout the experimental period (Fig. 6C and D) and was consistently higher in probiotic-treated groups of both sexes. In the paired day-by-day comparison, the mean daily intake was higher in probiotic-treated females than in controls (mean difference=3.54 g/day, $p=0.0016$) and also higher in probiotic-treated males (mean difference=2.84 g/day, $p=0.0008$).

Serum Biochemistry and Hematological Parameters Following Repeated Oral Administration of *Lactiplantibacillus plantarum* M2

Serum biochemical and hematological parameters (Table 1) were evaluated after 28 days of oral administration to assess potential systemic toxicity in female and male SWISS mice. In females, a statistically significant reduction in

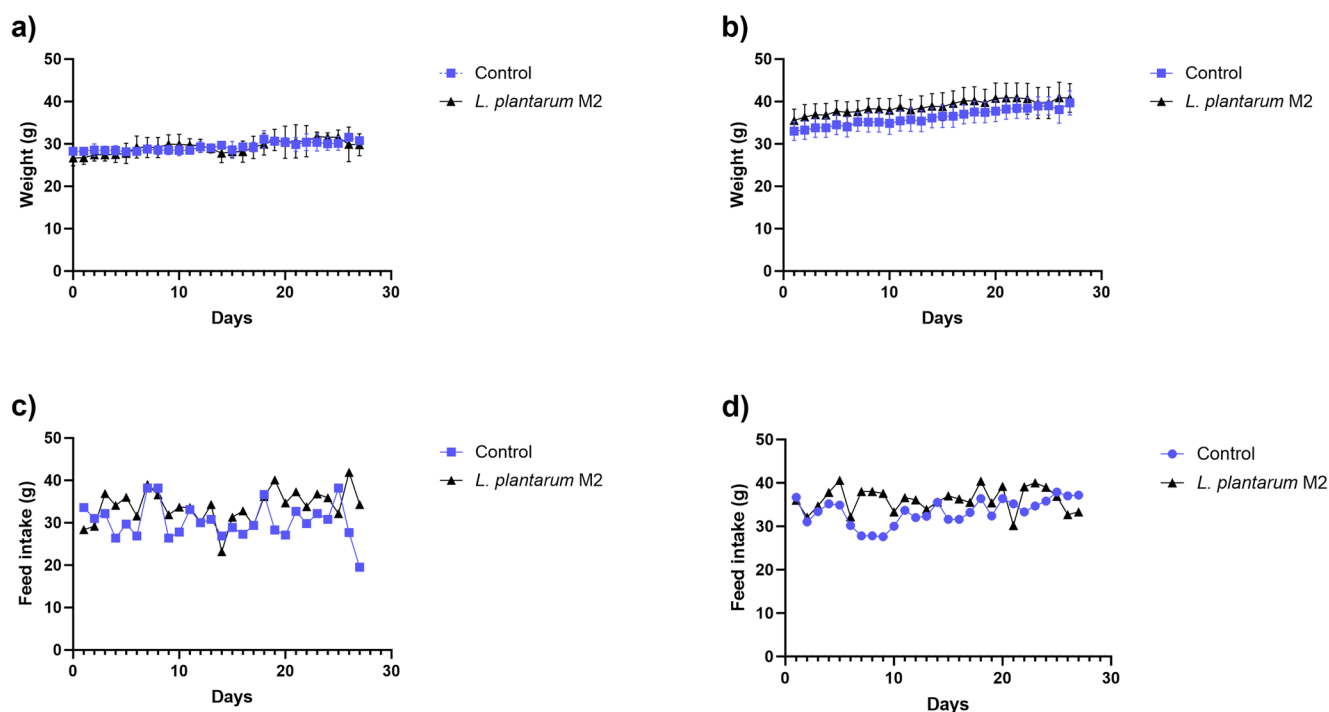


Fig. 6 Body weight trajectories and food intake patterns during repeated oral administration of *Lactiplantibacillus plantarum* M2. (A) Body weight progression in female mice; (B) mean daily food intake

in female mice; (C) body weight progression in male mice; (D) mean daily food intake in male mice. Data are expressed as mean $\pm$ SEM ($n=6$ /group/sex). Statistical significance: $p<0.01$, $p<0.001$

Table 1 Serum biochemical and hematological parameters following 28-day oral administration of *Lactiplantibacillus plantarum* M2

Parameter	Female			Male		
	Control	<i>L. plantarum</i> M2	<i>p</i> -value	Control	<i>L. plantarum</i> M2	<i>p</i> -value
Alanine aminotransferase - ALT (U/L)	49.40 $\pm$ 6.18	42.33 $\pm$ 3.67	0.0428 ¹	49.00 $\pm$ 5.29	55.50 $\pm$ 7.3	0.1647 ¹
Aspartate aminotransferase - AST (U/L)	92.20 $\pm$ 39.50	80.67 $\pm$ 21.14	0.4589 ²	70.20 $\pm$ 8.58	78.50 $\pm$ 4.79	0.1298 ¹
Urea (mg/dL)	80.40 $\pm$ 7.09	80.33 $\pm$ 9.48	0.8779 ¹	69.00 $\pm$ 3.08	67.75 $\pm$ 2.50	0.4191 ¹
Creatinine (mg/dL)	0.11 $\pm$ 0.05	0.12 $\pm$ 0.02	0.1104 ²	0.05 $\pm$ 0.03	0.07 $\pm$ 0.03	0.3730 ²
Total cholesterol (mg/dL)	81.40 $\pm$ 5.85	72.83 $\pm$ 9.17	0.1059 ¹	118.4 $\pm$ 10.90	106.8 $\pm$ 13.99	0.2014 ¹
HDL cholesterol (mg/dL)	52.80 $\pm$ 6.38	48.83 $\pm$ 5.91	0.3127 ¹	86.00 $\pm$ 4.12	78.25 $\pm$ 6.89	0.0794 ²
LDL cholesterol (mg/dL)	15.80 $\pm$ 3.70	20.83 $\pm$ 3.71	0.0516 ¹	21.00 $\pm$ 12.10	25.50 $\pm$ 7.93	0.5441 ¹
Triglycerides (mg/dL)	191.8 $\pm$ 26.45	213.3 $\pm$ 7.06	0.085 ¹	236.6 $\pm$ 88.07	270.8 $\pm$ 66.77	0.5430 ¹
Total leukocytes ($\times 10^3/\mu\text{L}$)	4.86 $\pm$ 0.35	4.85 $\pm$ 0.40	0.9410 ¹	4.25 $\pm$ 0.32	4.26 $\pm$ 0.36	0.9628 ¹
Neutrophils ($\times 10^3/\mu\text{L}$)	1.41 $\pm$ 0.06	1.42 $\pm$ 0.09	>0.9999 ²	1.16 $\pm$ 0.12	1.16 $\pm$ 0.13	>0.999 ¹
Lymphocytes ($\times 10^3/\mu\text{L}$)	6.77 $\pm$ 0.33	6.76 $\pm$ 0.25	0.9322 ¹	7.65 $\pm$ 0.37	7.47 $\pm$ 0.38	0.4748 ¹
Monocytes ($\times 10^2/\mu\text{L}$)	2.77 $\pm$ 0.29	2.60 $\pm$ 0.64	0.5667 ¹	2.40 $\pm$ 0.36	2.37 $\pm$ 0.40	0.8953 ¹
Eosinophils ($\times 10^2/\mu\text{L}$)	1.16 $\pm$ 0.12	1.16 $\pm$ 0.12	>0.999 ¹	0.84 $\pm$ 0.17	0.95 $\pm$ 0.13	0.2925 ¹

Data are expressed as mean $\pm$ SD ($n=6$ /group/sex). ¹Unpaired Student's *t*-test; ²Mann–Whitney U test

* $p<0.05$ vs. control

alanine aminotransferase (ALT) levels was observed in the probiotic-treated group compared with controls, with mean values decreasing from 49 U/L to 43 U/L ($p<0.05$). No significant differences were detected between female groups for other hepatic (AST), renal (urea, creatinine), or lipid profile parameters. In male mice, none of the evaluated biochemical parameters differed significantly between probiotic-treated and control groups. Lipid profile components,

including total cholesterol, HDL, LDL, and triglycerides, remained comparable between groups in both sexes.

Hematological analysis revealed no statistically significant differences between probiotic-treated and control animals in either females or males. Total leukocyte counts and differential leukocyte profiles (neutrophils, eosinophils, lymphocytes, and monocytes) remained within comparable ranges across all experimental groups.

Histopathological Analysis Following Repeated Oral Administration of *Lactiplantibacillus plantarum* M2

Histopathological examination of the heart, liver, spleen, kidneys, stomach, small intestine, and large intestine revealed no treatment-related microscopic lesions in any experimental group (Fig. 7). Across all evaluated organs, tissue architecture was preserved, with no evidence of inflammatory infiltrates, necrosis, hemorrhage/congestion, edema, or mucosal/epithelial injury. These findings reinforce the absence of target-organ toxicity associated with oral exposure to *L. plantarum* M2 under the conditions tested, supporting its histological safety profile.

Discussion

The present study integrates genome analyses with a 28-day repeated oral toxicity assessment to evaluate the safety of *L. plantarum* M2 for oral application. This combined approach aligns with current recommendations for probiotic characterization, which emphasize the need to couple *in silico* screening for potential risk determinants with *in vivo* confirmation of the absence of adverse effects [14, 17].

The subsystem annotation and comparative analyses indicate that *L. plantarum* M2 exhibits features commonly reported for the species. The metabolic plasticity is a hallmark of environmental adaptation that enables survival across diverse ecological niches [32]. The absence of canonical virulence determinants, together with the predominance of housekeeping functions and intrinsic tolerance-related loci within categories initially flagged as “defense and virulence,” is consistent with observations in other lactic acid bacteria and highlights the importance of manual curation in genome-based safety assessments [33–37].

The relative enrichment of the *ugp/glpQ* module in strain M2 suggests a potential capacity to utilize glycerophosphodiester-derived substrates. The genomic enrichment of this module is compatible with a theoretical capacity for niche adaptation under conditions where such substrates are available, though future colonization and transcriptomic assays are necessary to validate this hypothesis [38, 39]. This is particularly relevant considering the profound influence of microbial fermentation properties and specific substrate utilization capacities on overall gut microbiota dynamics [40]. Similarly, the phylogenetic placement of M2 within the *L. plantarum* clade and its orthologous cluster distribution relative to reference strains support its classification as a typical member of this species [10].

Mobilome analysis further contextualizes the M2 genomic plasticity. The identification of a heterogeneous plasmid repertoire comprising three extrachromosomal

elements (AE887, AB384, and AB794), together with a limited prophage signal and multiple predicted genomic islands lacking pathogenicity-associated signatures. This pattern is consistent with mobilome data reported for *L. plantarum* and is best interpreted as a signature of past exposure to mobile genetic elements and ecological fitness rather than an indicator of virulence [11–13, 41, 42]. Another important finding is that the majority of M2 singletons are localized within the chromosome, a feature that warrants further experimental investigation to elucidate the biological roles of these strain-specific genes [43].

The coexistence of these multiple mobile genetic elements with a CRISPR-associated locus encoding Cas3 suggests the presence of genome-encoded defense mechanisms against foreign DNA, which may be associated with limiting the acquisition of exogenous genetic elements [31], although this effect remains to be validated. These features are consistent with a low-risk mobilome architecture frequently reported for *L. plantarum* strains used in food and health-related contexts [44].

The genome-informed pathogenicity screen is consistent with this interpretation [29]. The very low predicted probability of pathogenicity (0.19%), together with the absence of similarity to known virulence-associated gene signatures and the exclusive association of detected hits with non-pathogenic lactobacilli, is consistent with a low-risk genomic background [35]. Such similarity-based predictions are not intended to replace phenotypic testing; however, when interpreted alongside mobilome analyses, they provide additional evidence consistent with the suitability of M2 for oral exposure.

In vivo data corroborate the genomic findings since repeated oral administration of *L. plantarum* M2 for 28 days did not result in mortality, clinical signs, or alterations in body weight trajectories in either sex. Although an increase in food intake was observed in probiotic-treated groups of both sexes in day-by-day comparisons, this change occurred in the absence of concomitant weight gain. The phenomenon of increased feed intake without weight gain is biologically intriguing and has been documented in other probiotic interventions. This effect may be linked to microbiota-mediated metabolic modulation, where probiotic strains influence energy homeostasis or satiety signaling through the gut-brain axis [45, 46]. Furthermore, alterations in nutrient absorption efficiency or the production of specific microbial metabolites, such as short-chain fatty acids (SCFAs), could theoretically increase energy expenditure, thereby offsetting the higher caloric intake [47]. However, as the present study was not designed to investigate these specific pathways, future research integrating metabolomic and microbiota composition analyses is necessary to elucidate these host-microbe interactions. Such multi-omics approaches have

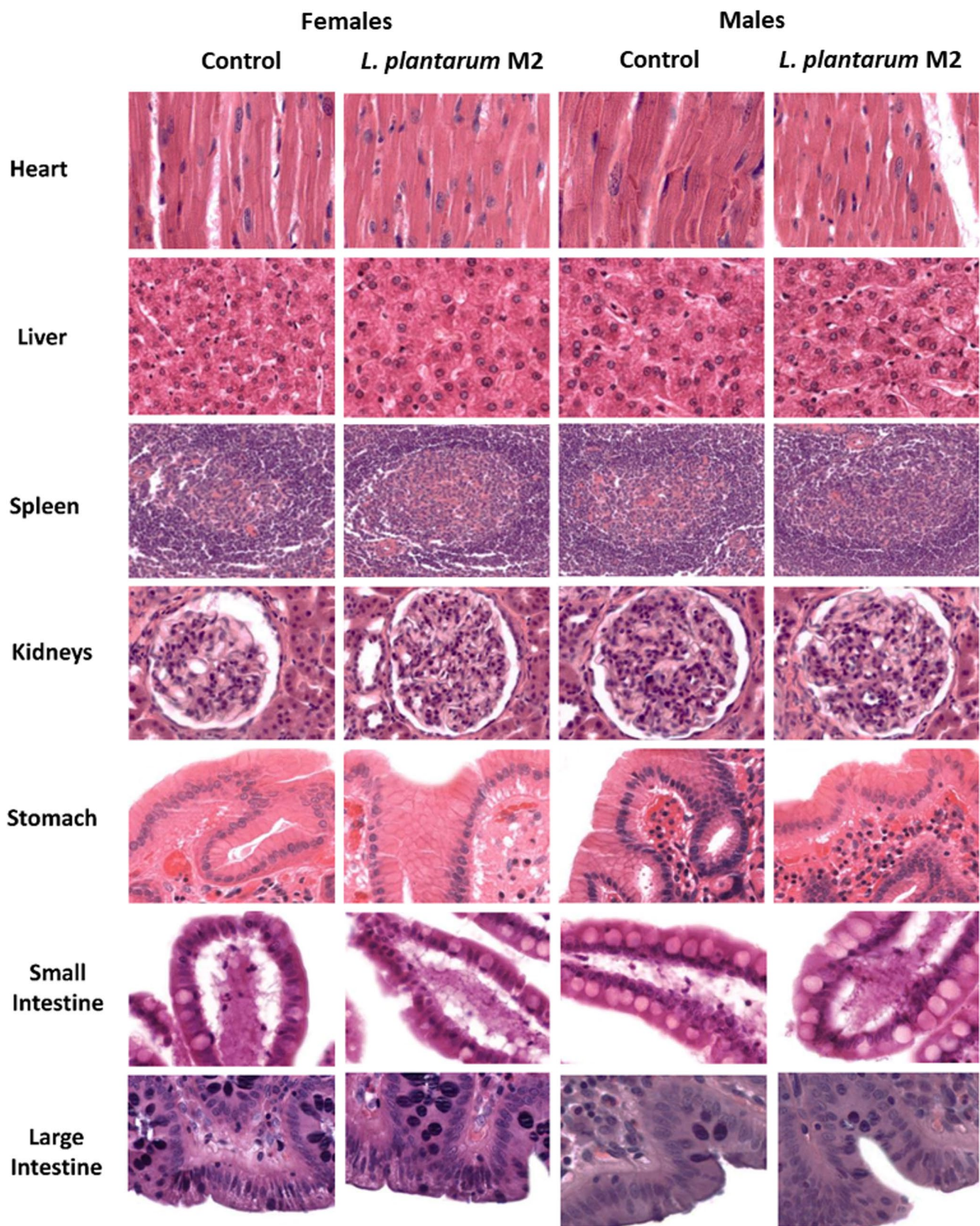


Fig. 7 Representative histopathological evaluation of major organs following repeated oral administration of *Lactiplantibacillus plantarum* M2 ($n=6/\text{group/sex}$)

become crucial for comprehensively decoding probiotic effects on host immunity and metabolic homeostasis [48].

Clinical pathology parameters remained largely unchanged between control and treated groups, except for a significant reduction in ALT levels in females. Importantly, this change was not accompanied by histopathological alterations in the liver, suggesting that it does not reflect hepatocellular injury under the conditions tested. Nevertheless, in the absence of complementary mechanistic evidence, the biological significance of this isolated reduction remains unclear and is not interpreted here as a definitive health benefit. Comparable findings, including preserved organ morphology and unaltered clinical pathology profiles following repeated oral exposure, have been reported in other safety assessments of *L. plantarum* strains [14, 35, 44, 49].

Histopathological evaluation provides a structural counterpart to the clinical pathology data and represents a critical endpoint in toxicological assessment. Preservation of tissue architecture across highly responsive target organs, particularly the liver, kidneys, and gastrointestinal tract, indicates that *L. plantarum* M2 did not induce detectable target-organ toxicity in this 28-day model. The integrity of gastric and intestinal mucosa is of particular relevance for probiotic safety, as these tissues constitute the primary interface between the host and administered microorganisms [50–52]. The absence of epithelial disruption or inflammatory injury is consistent with the low-risk genomic profile observed for M2 and with reports describing similar outcomes for other *L. plantarum* strains.

Some limitations of the present study must be acknowledged. First, although the study design included six animals per sex per group, consistent with exploratory preclinical safety studies, the overall sample size may limit the statistical power to detect subtle or low-frequency toxicological effects. Second, while previous phenotypic testing indicated a susceptibility profile consistent with intrinsic resistance to vancomycin and gentamicin in *L. plantarum* M2, the use of agar diffusion methods does not provide quantitative thresholds required for regulatory classification. Therefore, standardized MIC determination according to EFSA guidelines was not performed and remains necessary. Third, the functional implications of the described genomic features were inferred predictively and lack validation through targeted experimental approaches. Fourth, the absence of microbiota and transcriptomic analyses limits mechanistic interpretation of the observed increase in food intake. Finally, longer-term exposure studies are required to address chronic use scenarios.

Despite these limitations, the integration of genome-based screening with in vivo toxicological endpoints provides a conservative basis for the safety evaluation of *L. plantarum* M2. The convergence of these findings indicates

that *L. plantarum* M2 does not elicit detectable adverse effects under the experimental conditions employed. While the present study does not address long-term exposure or specific efficacy endpoints, it establishes a conservative safety framework that supports further investigation of M2 in food or health-related applications, in line with established practices for probiotic development.

Conclusion

This study combined genome-informed analyses with a 28-day repeated oral toxicity assessment to evaluate the safety of *L. plantarum* M2 for oral application. The absence of detectable adverse effects in vivo, together with a low-risk genomic profile, is consistent with the conclusion that M2 is compatible with oral exposure under the experimental conditions employed. The data provide a conservative safety framework for further investigation of M2 in food-related applications, in line with established practices for probiotic development.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12602-026-11111-w>.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Institutional Review Board Statement This project was approved by the Animal Research Ethics Committee of Universidade CEUMA (CEUA: 052301/24).

Competing interests The authors declare no competing interests.

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