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Hemp fermentation enriches acid-resilient *Lacticaseibacillus paracasei* psychobiotic candidates from water kefir while preserving neuroactive potential

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ABSTRACT

Water kefir represents a compelling reservoir of psychobiotic candidates, yet the capacity of individual strains to withstand gastrointestinal stress while retaining neuroactive biosynthetic potential remains poorly understood. Approaches capable of conditioning these microorganisms toward greater resilience may therefore improve their potential as psychobiotic candidates. Here, we investigated whether hemp niche could simultaneously condition kefir-derived psychobiotic candidates toward enhanced gastrointestinal resilience while preserving their neuroactive potential. Dried hemp leaves and flowers were fermented with water kefir consortia under submerged and solid-state conditions. Multi-omics and *in vitro* gastrointestinal assays were used to investigate hemp niche adaptation and its effects on gastrointestinal stress resilience.

Submerged fermentation preferentially supported the persistence of *Lacticaseibacillus paracasei* despite the taxonomic diversity of the kefir consortium. Comparative genomics revealed limited divergence among pre- and post-fermentation isolates, with only subtle variation in the stress-associated chaperone *clpC* and enrichment of a low-abundance lineage carrying a hypothetical DNA-processing gene. Post-fermentation strains exhibited enhanced acid resilience and increased *trp* expression, whereas *gltB* and *gdh* expression was lineage dependent. All strains lacked plasmids and mobile antimicrobial resistance elements. Collectively, hemp functioned as a conditioning niche that enriched resilient *L. paracasei* strains while preserving neuroactive potential and genomic safety.

1. Introduction

Depression is among the leading causes of disability worldwide and is associated with substantial social and economic burdens, including increased healthcare costs, reduced productivity, and high suicide risk (Dinan & Cryan, 2017). Despite the availability of pharmacological and psychological interventions, many patients fail to achieve sustained remission or experience undesirable side effects, highlighting the urgent need for alternative treatment strategies. The growing recognition of the microbiota-gut-brain axis has positioned the gut microbiota as a promising target for novel interventions in mental health disorders, including depression and anxiety (Binda et al., 2024; Kyei-Baffour et al., 2025; Sisubalan et al., 2026). Those microorganisms that confer such mental health benefits through interactions with the microbiota-gut-brain axis are so called psychobiotics (Binda et al., 2024; Cryan et al., 2019). Several psychobiotic strains have demonstrated the capacity to modulate host neuroactive compound production, regulate inflammation, and improve symptoms of depression, supporting their potential as adjunctive therapies for neuropsychiatric disorders (Binda et al., 2024; Cryan et al., 2019; Dinan et al., 2013). Among fermented foods, water kefir has emerged as a valuable reservoir of potential psychobiotic candidates owing to its rich microbial diversity and reported benefits for gut and mental health (Bakırhan et al., 2025; de Almeida et al., 2025). Its growing popularity as a dairy-free functional beverage has paralleled a

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rapidly expanding global kefir market, valued at approximately USD 1.3 billion in 2024 and projected to reach USD 1.7 billion by 2030 (de Souza et al., 2024; Grand View Research, 2024). Against this backdrop, improving the functional attributes of water kefir microbial consortia represents a promising strategy for developing next-generation probiotics and functional foods. The relatively modest survival of water kefir isolate *Lacticaseibacillus paracasei* CT12 during simulated gastric (57%) and intestinal digestion (40%) suggests that some water kefir-derived strains may have limited intrinsic adaptation to gastrointestinal stress (Romero-Luna et al., 2020). Approaches capable of conditioning these microorganisms toward greater resilience may therefore improve their potential as next generation psychobiotic candidates. Gastrointestinal transit represents a major bottleneck in probiotic functionality, as exposure to gastric acidity, digestive enzymes, and bile salts can substantially reduce microbial viability and consequently limit functional efficacy *in vivo* (Han et al., 2021). *Cannabis sativa* (industrial hemp) contains diverse bioactive constituents, including cannabinoids and phenolic compounds that can influence microbial growth and metabolism (Andre et al., 2016). Such environments may therefore act as ecological conditioning niches, favoring the persistence of strains with enhanced functional traits (Al Ubeed et al., 2023). Considering that survival of kefir bacteria would require adaption and/or even evolution when cultured on a new substrate, we hypothesized that hemp fermentation with Tibetan water kefir bacteria could drive adaptive changes, allowing these bacteria to metabolize hemp-derived compounds. More importantly, we proposed that this environmental adaptation could enhance traits linked to gastrointestinal resilience while preserving their neuroactive potential. Consequently, we fermented dried hemp leaves and flowers using water kefir microbial consortium and investigated the microorganisms recovered following fermentation. We integrated metagenomics, phylogenetics, comparative genomics, targeted gene expression and *in vitro* gastrointestinal assays to investigate whether the phytochemical-rich hemp niche could condition kefir-derived bacteria toward improved gastrointestinal resilience without compromising their neuroactive biosynthetic potential.

2. Materials and methods

2.1. Hemp leaves and flowers preparation

Hemp leaves and flowers were collected from the Lithuanian Research Centre for Agriculture and Forestry (LAMMC), Akademija, Kedainiai district, in Lithuania. Leaves and flowers were washed with sterile water to remove surface dirt before drying at room temperature for seven days. Dried samples were crushed into coarse powder using a knife mill (Restch GM 300) and 0.5 kg of sample obtained and stored in airtight glass jars at 4°C pending further use.

2.2. Water kefir grain starter culture preparation

Whole Tibetan kefir grains were obtained from the Lithuanian Research Centre for Agriculture and Forestry (LAMMC), Akademija, Kėdainiai District, Lithuania, which sourced the grains from a local producer (Herbioloom, MB, Kaunas, Lithuania). The kefir grain starter culture was prepared based on (Gul et al., 2015) with modifications. Briefly, kefir grains were washed with sterile water and soaked at 5% (w/v) into sterile water for three days with glucose supplementation (2% w/v). The grains were separated by filtration through a plastic sieve (previously sterilized by immersion in ethanol and then washed with sterile water) to obtain the kefir starter culture used for inoculation.

2.3. Hemp fermentation

Dried hemp leaves and flowers were fermented either under solid-state or submerged conditions. Solid-state fermentation was prepared at substrate-to-liquid ratio of 1:1 (w/w) whereas submerged fermentation was prepared at substrate-to-liquid ratio of 1:40 (w/w), corresponding to one-part dried hemp to forty parts distilled water, respectively. The hemp samples were inoculated with 5% (v/v) water kefir culture based on an inoculation volume pilot study. Prior to inoculation with kefir culture, hemp samples were autoclaved at 121°C for 30 min at 15 psi. Each sample was supplemented with 2% (w/v) glucose (Sigma-Aldrich; ≥99.0% purity) to aid initial survival of bacteria. Fermentation was allowed to occur for 14 days in an incubator (Orbicult IBS-NR-25-8) at 37°C and pH of samples monitored every other day.

2.4. Isolation of bacterial strains

Bacterial strains were isolated from fermented hemp samples and kefir grain culture according to (Teneva-Angelova & Beshkova, 2016) with slight modifications. Briefly, the samples (1 mL) were transferred into De Man, Rogosa, and Sharpe (MRS) or glucose yeast calcium carbonate (GYC) (Thermo Scientific Oxoid, dehydrated) broth (9 ml) with the addition of 100 μg/mL of cycloheximide (Sigma-Aldrich; ≥ 94.0% purity) to the hemp samples to prevent fungal growth and to select for acid producing bacteria. Tubes were incubated for 48 h at 37°C. Serial dilutions of sample broth were made with peptone water (0.1%) (Millipore, Sigma-Aldrich) and the dilutions plated on MRS or GYC agar plates by spreading. Plates were incubated at 37°C for 48 to 72 h to obtain single bacteria colonies. The colonies were randomly selected based on sizes, colour and appearance, purified by repeated streaking on fresh MRS or GYC agar plates. Pure single colonies were cultured in MRS or GYC broth overnight, 500 μL of overnight culture pipetted into 500 μL glycerol (Sigma-Aldrich; ≥99.96% purity) in Eppendorf tubes and stored at −80°C pending further experiments.

2.5. DNA extraction

DNA was extracted from bacterial isolates using the quick-DNA bacterial microprep kit (Zymo Research, Catalog No. D6007) according to the manufacturer's instructions. Briefly, 20 mg (wet weight) of bacterial cells were resuspended in 200 μL PBS, transferred to a bashing bead lysis tube, and mixed with 750 μL bashing bead buffer. Samples were lysed by bead beating using next advance bullet blender at maximum speed for 15 min, followed by centrifugation (Eppendorf, 5420) at 10,000 × g for 1 min. Four hundred microliters (400 μL) of the supernatant was filtered by centrifugation at 8000 × g for 1 min using a spin filter. Genomic lysis buffer (1200 μL) was added to the filtrate and mixture loaded onto a solid-phase extraction column in two successive steps of 800 μL each and centrifuged at 10,000 × g for 1 min. The column was washed with 200 μL DNA pre-wash buffer and 500 μL g-DNA wash buffer, each followed by centrifugation at 10,000 × g for 1 min. DNA was eluted by adding 20 μL DNA elution buffer directly to the column matrix, incubating for 1 min, and centrifuging at 10,000 × g for 30 s. The DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis).

2.6. Metagenomic sequencing and analysis of Tibetan water kefir grains

Frozen Tibetan Kefir grain samples were put on dry ice and sent to Novogene Company (Cambridge, UK) for sequencing. Briefly, shotgun metagenomic sequencing was performed using the Illumina NovaSeq [6000/X Plus] platform. Genomic DNA was extracted and quality-controlled via 1% agarose gel electrophoresis and Qubit® 2.0 Fluorometer quantification. Sequencing libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit, where samples were fragmented to an insert size of 350 bp, end-polished, A-tailed, and ligated with Illumina adapters. Following PCR enrichment and purification, library quality was validated using an Agilent 2100 Bioanalyzer. The libraries were sequenced using a paired-end 150 bp (PE150) strategy, and raw reads were filtered to remove adapters, low-quality bases, and reads

with > 10 N bases to ensure high-quality clean data for downstream analysis.

Raw Illumina short read files received from Novogene Company (FastQ files) were initially assessed for reads quality using FastQC tool (Wingett & Andrews, 2018) before assembly and identification. Read trimming, cleaning, and filtering were performed using the JGI-QC pipeline, PRINSEQ, and Trimmomatic, respectively (Bolger et al., 2014). High-quality reads were assembled into contigs using metaSPAdes (Prjibelski et al., 2020), IDBA (Peng et al., 2011) and MEGAHIT (Li et al., 2016) and the assembler that gave high quality contigs selected for genome binning. Binning was performed using three independent binning pipelines (Concoct, Bat2 and MaxBin) followed by bin refinement and optimization using DAS Tool to generate high-quality metagenomes. Assembly quality was evaluated with CheckM (Chklovski et al., 2023) and genomes with high completeness level and low contamination retained. The assembled contigs were taxonomically classified with GTDB-Tk (Parks et al., 2026).

2.7. Whole genome sequencing and analysis of bacterial isolates

Pure extracted DNA samples were sent to Novogene Company Limited (Cambridge, UK) for whole genome sequencing. Briefly, sequencing libraries were prepared using standard Illumina protocols, including fragmentation to 350 bp, end repair, A-tailing, adapter ligation, and PCR amplification, and sequenced as paired-end reads (2 × 150 bp) on an Illumina platform (Daliri et al., 2023; Megur et al., 2024; Tadesse et al., 2025). Raw reads were subjected to quality control and adapter trimming prior to downstream genomic analyses. Raw Illumina short read files received from Novogene Company (FastQ files) were initially assessed for reads quality using FastQC tool (Wingett & Andrews, 2018) before assembly and identification. Read trimming, cleaning, and filtering were performed using the JGI-QC pipeline, PRINSEQ, and Trimmomatic, respectively (Bolger et al., 2014). High-quality reads were assembled into contigs using SPAdes (Prjibelski et al., 2020) and assembly quality evaluated with CheckM (Chklovski et al., 2023). The assembled contigs were taxonomically classified with GTDB-Tk (Parks et al., 2026).

2.8. Functional annotation and gene predictions

All high-quality genomes were functionally annotated using RASTtk within the U.S. Department of Energy Systems Biology Knowledgebase platform, KBase (Chivian et al., 2023) unless otherwise stated. Protein coding genes were predicted and annotated against curated subsystem databases, enabling consistent functional comparison across all genomes. Annotated genomes were used as the basis for downstream analyses of genes and genome comparisons. Pangenome circle plot (version 1.6.0) and OrthoVenn 3 (version 3.0) was used to generate graphical comparison of pangenomes and orthologous gene cluster outputs (Sun et al., 2023).

2.9. GIT simulated stress tolerance tests

2.9.1. Acid tolerance assessment

Acid tolerance was evaluated as described by (Oh et al., 2018). Briefly, frozen isolates were thawed, and a loopful of each culture was revived in MRS broth overnight at 37°C. Following revival, 1 mL of each culture was centrifuged at 10,000 × g for 10 min, and the supernatant was discarded. Cell pellets were resuspended in 1 mL of phosphate buffer saline (RMBio; 1X; pH 7.2) to determine viable counts at time 0 h, and in 1 mL of acidified sterile phosphate buffer saline adjusted to pH 2.5 to assess acid tolerance. The percentage survival of bacterial cells following acid exposure was calculated by comparing colony forming units (CFU/mL) at 4 h to those at 0 h using equation (1).

$$\text{Percentage survival} = \frac{(\text{CFU of viable cells survived})}{(\text{CFU of initial cells inoculated})} \text{X } 100 \quad \text{equation 1}$$

2.9.2. Pepsin tolerance assessment

Pepsin tolerance was evaluated as described by (Oh et al., 2018). Briefly, loopful of thawed cultures were transferred into MRS broth for revival, at 37°C overnight. A pepsin (Sigma-Aldrich; 30 units/mg) solution was prepared by dissolving 3 mg of pepsin in 1 mL of physiological saline solution (0.85% NaCl), with the pH adjusted to 2.0. For the assay, 990 μL of the acidified pepsin saline solution was inoculated with 10 μL (1% v/v) of overnight cultures. Immediately after inoculation, serial dilutions were prepared in peptone water (0.1%) and plated in replicates on MRS agar to determine viable counts at time 0 h. The remaining inoculated tubes were incubated at 37°C for 4 h, after which serial dilutions were prepared and plated in replicates on MRS agar to assess survival at 4 h. The percentage survival of bacterial cells following pepsin exposure was calculated using equation (1).

2.9.3. Bile salts and pancreatin tolerance assessment

Bile salts and pancreatin tolerance were evaluated as described by (Megur et al., 2026). Briefly, loopful of thawed cultures were transferred into MRS broth for revival, at 37°C overnight. A simulated intestinal solution was prepared by dissolving bile salts (3 mg; Sigma-Aldrich) and pancreatin (1 mg; Sigma-Aldrich) in 1 mL of physiological saline solution (0.85% NaCl), with the pH adjusted to 8. For the assay, 1 mL of saline solution (pH 8.0) containing bile salts and pancreatin was inoculated with 10 μL (1% v/v) of overnight culture, and serial dilutions were immediately prepared in peptone water (0.1%) and plated in replicates on MRS agar to determine viable counts at time 0 h. The remaining inoculated tubes were incubated at 37°C for 6 h, after which serial dilutions were prepared and plated to assess survival at 6 h. The percentage survival of bacterial cells following bile salts and pancreatin exposure was calculated using equation (1).

2.10. Gene expression analyses

2.10.1. RNA extraction

Bacterial cultures were grown to mid-exponential phase (OD600 = 0.5), harvested by centrifugation, and immediately processed for RNA extraction. Total RNA was extracted from cell pellets using the ZymoBIOMICS™ MagBead RNA Kit (R2137, Zymo Research, USA) according to the manufacturer's instructions. Briefly, cells were mechanically lysed by bead beating, and total RNA was purified by magnetic bead-based extraction followed by DNase treatment to remove residual genomic DNA. RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis) prior to cDNA synthesis.

2.10.2. cDNA synthesis and qPCR analyses

First-strand cDNA was synthesized from 100 ng of total RNA in a 20 μL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Reverse transcription was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Quantitative PCR (qPCR) was performed on a QuantStudio™ Real-Time PCR System (Thermo Fisher Scientific, USA) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA). Each 20 μL reaction contained SYBR Green Master Mix, cDNA template, and gene-specific primers at a final concentration of 400 nM. Amplification conditions consisted of UDG activation at 50°C for 2 min, polymerase activation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, after which melt-curve analysis was performed to verify amplification specificity. Expression of genes associated with neuroactive compound biosynthesis, including tryptophan synthase (*trp*), glutamate synthase (*gltB*), glutamate dehydrogenase (*gdh*),

and pyruvate dehydrogenase complex (*pdc*), was normalized against the housekeeping gene *gyrB*. Two biological replicates and three technical replicates were analyzed for each sample, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.11. Statistical analysis

Quantitative data were analyzed using GraphPad Prism version 10.4.2 (GraphPad Software, San Diego, USA). Descriptive statistics were

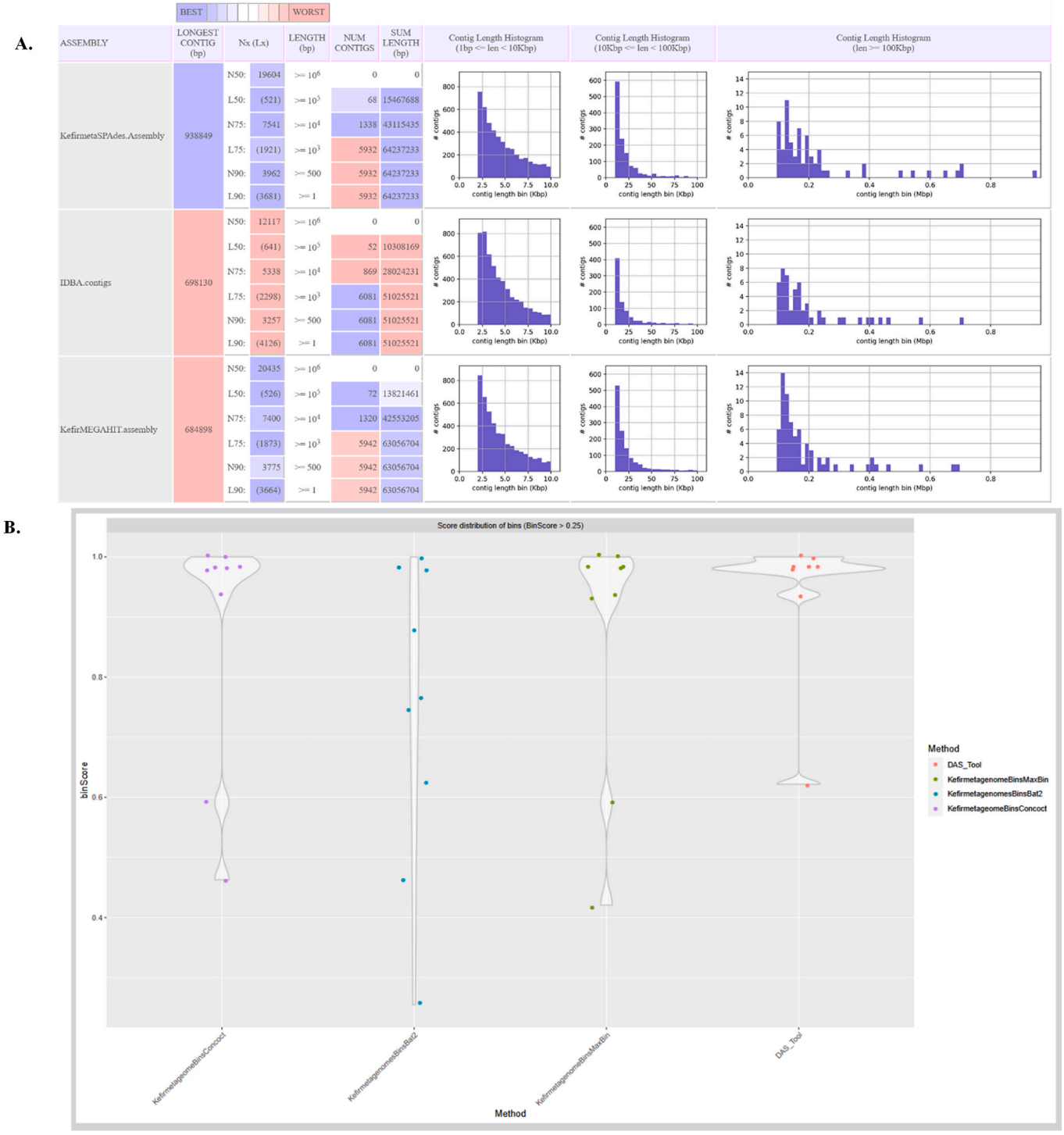

Fig. 1. Optimization of metagenome assembly and genome binning for reconstruction of the water kefir microbial consortium. (A) Comparison of three *de novo* metagenome assemblers (SPAdes, IDBA and MEGAHIT) using assembly quality metrics, including contig statistics, N50, assembly completeness and contig-length distribution. Heat maps summarize the relative performance of each assembler, with blue indicating best and red indicating worst assembly performance for the corresponding metric. (B) Genome binning of the SPAdes assembly using three complementary binning algorithms: CONCOCT (violet), MetaBAT2 (blue) and MaxBin2 (green). Each coloured block represents a genome bin reconstructed by the respective algorithm. Bin quality was evaluated using DAS Tool, where the distribution of bin scores is shown for all bins with scores $\geq$0.25, and higher scores indicate greater bin quality and confidence. DAS Tool (red) integrates the outputs of the three binning algorithms, selects the highest-quality non-redundant bins, and optimizes the final metagenome-assembled genomes (MAGs). All seven refined MAGs selected for downstream analyses had bin scores >0.80, indicating high-confidence genome reconstruction.

mainly presented as mean values ± standard error of the mean (SEM), or as percentages, as appropriate. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to assess significance ($p < 0.05$) between measures. Graphical representations of data were generated using GraphPad Prism or appropriate tools and platforms as described in methods.

3. Results and discussions

3.1. Microbial composition of water kefir culture

According to the manufacturer, the whole Tibetan kefir grains used to prepare the water kefir culture comprise a symbiotic consortium of lactic acid bacteria, including *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus kefiri* and *Leuconostoc mesenteroides*. Consequently, the microbial composition of the water kefir consortium used in this study was independently characterized by shotgun metagenomic sequencing. Accurate metagenomic reconstruction is fundamental for reliable taxonomic and comparative genomic analyses. Since assembly quality directly influences genome recovery and downstream biological interpretation, we first optimized the metagenomic assembly and binning workflow before characterizing the water kefir consortium. The metagenomic shotgun profiling of kefir culture using SPAdes, IDBA and MEGAHIT assemblers showed that SPAdes was the optimal assembler for complete contiguous metagenome assembling evidenced by the highest N50 and longest contig lengths while maintaining comparable assembly completeness (Fig. 1A). Genome binning of SPAdes assembly using three separate binning pipelines, followed by bin refinement resulted in seven high-quality metagenomes (Fig. 1B). Taxonomic analyses revealed two dominant bacterial families within the kefir consortia namely *Lactobacillaceae* and *Acetobacteriaceae*. Members of the *Lactobacillaceae* family included *Leuconostoc pseudomesenteroides*, *Lentilactobacillus kefiri*, *Lacticaseibacillus paracasei*, and *Lactobacillus kefiranofaciens* (Fig. 2A) whereas those of the *Acetobacteriaceae* family comprised *Gluconobacter* spp, *Acetobacter fabarum*, and *Acetobacter persici* (Fig. 2B). This taxonomic structure is consistent with the well-established kefir microbial ecosystem, where lactic acid bacteria and acetic acid bacteria coexist in a metabolically cooperative consortium (Xia et al., 2022).

3.2. Submerged fermentation of hemp promotes L. paracasei activity

Submerged fermentation of hemp leaves and flowers supported acid producing microbial activity better than solid-state fermentation and this is evident by the progressive decrease in pH over the 14-day fermentation period (Fig. 2C). The pH decreased from 7.61 to 4.60 by

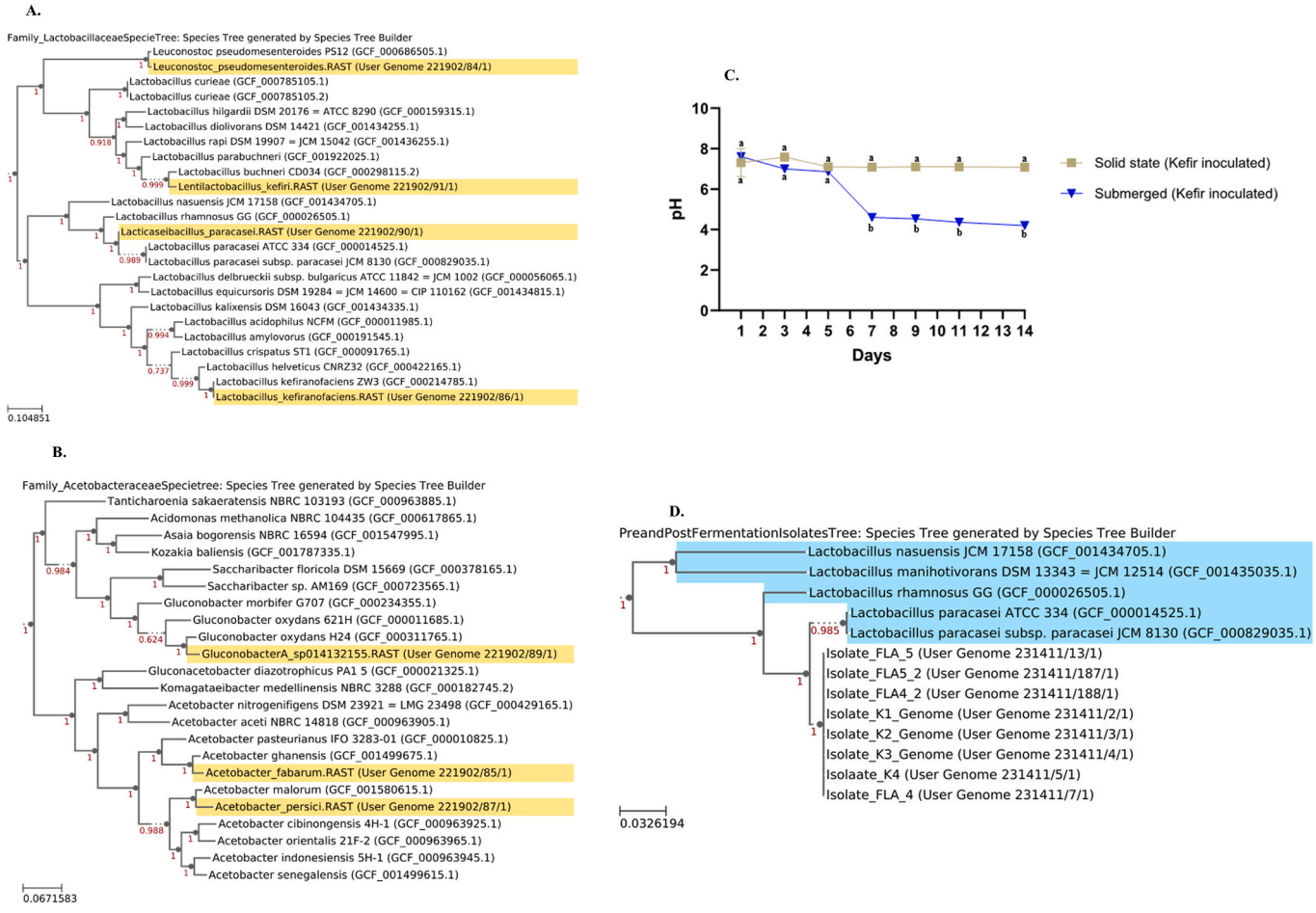

Fig. 2. Taxonomic composition of the water kefir consortium and microbial activity during hemp fermentation. (A) Phylogenomic tree of metagenome-assembled genomes (MAGs) assigned to the family Lactobacillaceae reconstructed from the water kefir consortium. Yellow-highlighted branches indicate the MAGs recovered in this study, while non-highlighted branches represent closely related reference genomes used for phylogenetic placement. (B) Phylogenomic tree of MAGs assigned to the family Acetobacteraceae reconstructed from the water kefir consortium. Yellow-highlighted branches indicate the MAGs recovered in this study whereas non-highlighted branches represent reference genomes. (C) Changes in pH during the 14-day submerged and solid-state hemp fermentation. Error bars represent mean ± SD (n = 3). Different letters indicate significant differences between time points ($p < 0.05$). (D) Whole-genome phylogenetic analysis of *Lacticaseibacillus paracasei* isolates recovered from the water kefir consortium (K1 - K4) and following submerged hemp fermentation (FLA4 and FLA5). Post-fermentation isolates clustered within the *L. paracasei* clade and were selected for subsequent comparative genomic analyses.

day 7 and remained stable till day 14. This reduction in pH is indicative of organic acids production, consistent with lactic acid bacterial activity commonly observed during submerged plant fermentations (Yu et al., 2020). Similar observations were reported by A'yuni et al. (2025), who showed that hemp's rich phytochemical environment can promote lactic acid bacterial growth. Hemp is also known to contain alkaloids that confer slightly alkaline environment (A'yuni et al., 2025), and this may account for the slow reduction in pH observed from day 1 to 5. In contrast, solid-state fermentation did not result in measurable pH changes, indicating limited acid producing microbial activity. This is likely due to reduced water availability, which constrains microbial growth and metabolite diffusion, particularly for lactic acid bacteria that require sufficient moisture for carbohydrate fermentation (Yeboah et al., 2023).

Despite the taxonomic diversity of the water kefir consortium, only *Lacticaseibacillus paracasei* was recovered following submerged hemp fermentation (Fig. 2D). The absence of growth on YGC agar further indicates that the hemp niche selectively enriched *L. paracasei* and did not support the persistence of acetic acid bacteria. The antimicrobial phytochemical richness of hemp likely imposed a strong ecological filter that selectively enriched *L. paracasei*, a species renowned for its ecological versatility and resilience to diverse environmental stresses (Hill et al., 2018; Andre et al., 2016). The selective persistence of this species prompted a comparative analysis of orthologous gene clusters to

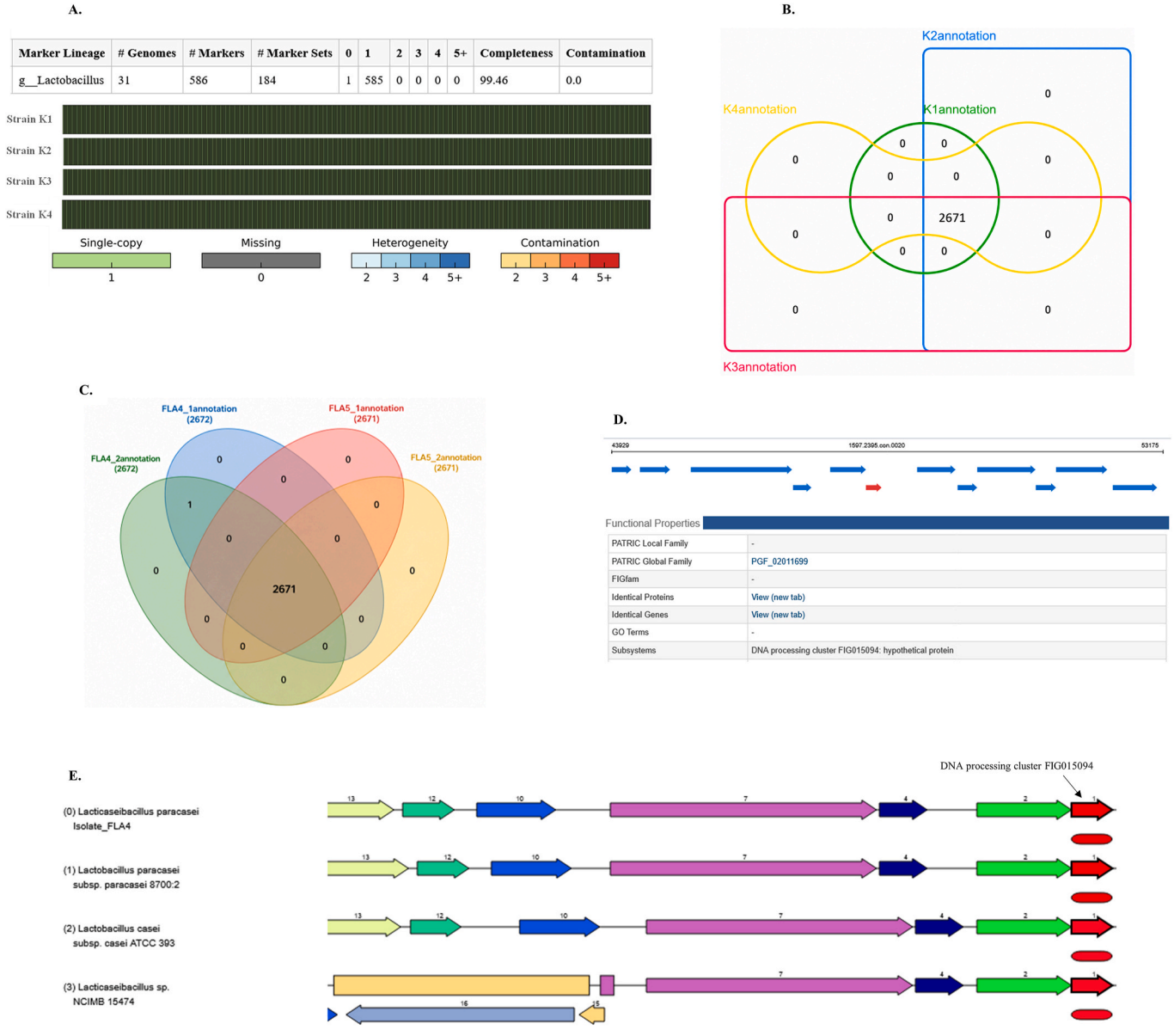

Fig. 3. Comparative genomics showing the enrichment of a low abundance *Lacticaseibacillus paracasei* lineage following hemp fermentation. (A) Genome quality assessment of four *L. paracasei* isolates (K1 - K4) recovered from the water kefir consortium. Bars summarize lineage-specific marker gene analysis, where green; indicates single-copy marker genes, blue; duplicated marker genes, grey; missing marker genes and red; contaminated marker genes. All four genomes exhibited >99% completeness, with all marker genes present as single copies and no detectable missing or contaminating markers, confirming high-quality genome assemblies. (B) Orthologous gene cluster comparison among the four kefir isolates (K1 - K4). Each shape represents the orthologous gene repertoire of an individual isolate, with overlapping regions indicating shared orthologous gene clusters and non-overlapping regions representing isolate-specific genes. (C) Orthologous gene cluster comparison of post-fermentation isolates recovered from two independent fermentation events. The FLA5 lineage retained the same 2671 orthologous gene clusters as the kefir strains, whereas the FLA4 lineage contained one additional orthologous gene cluster. (D) Functional annotation of the lineage-specific orthologous gene identified in the FLA4 lineage, predicted as a hypothetical protein associated with DNA processing. (E) Comparative synteny analysis demonstrating conservation of the DNA-processing gene cluster in FLA4 and publicly available Lacticaseibacillus/Lactobacillus genomes.

identify genomic determinants of this ecological resilience.

3.3. Hemp niche enriched low abundance L. paracasei lineages

To ensure that subsequent comparative genomic analyses were not confounded by assembly artifacts, four independent *L. paracasei* isolates recovered from the kefir consortium were sequenced and compared. The identical completeness, lack of contamination, and shared orthologous gene repertoire among all four kefir isolates (Fig. 3A and B) support the robustness of the assembly pipeline and indicate minimal assembly bias. Following hemp fermentation, two *L. paracasei* genomic profiles were recovered (Fig. 3C). Whereas the FLA5 lineage retained the same orthologous gene complement as the kefir isolates, the FLA4 lineage contained a single additional hypothetical gene predicted to be involved in DNA processing (Fig. 3D). Although acquisition of this gene during fermentation cannot be completely excluded, its presence in publicly available *L. paracasei* genomes (Fig. 3E) suggests that the FLA4 lineage likely existed within the kefir consortium at low abundance and was selectively enriched by the hemp niche. Such enrichment of rare microbial lineages following environmental perturbation is well documented and forms the basis of the microbial 'rare biosphere' concept (Jousset et al., 2017; Lynch & Neufeld, 2015). These findings suggest that the hemp environment selectively enriched pre-existing *L. paracasei* lineages rather than driving extensive genome remodeling.

3.4. Impact of hemp fermentation on native stress-associated molecular chaperones

To determine the impact of fermentation on *Lacticaseibacillus paracasei* stress tolerance, nine genes encoding molecular chaperones known to maintain proteostasis under environmental stress were analyzed. These systems are critical for maintaining proteostasis under stress by refolding or degrading damaged proteins (Kathiriya et al., 2023). Phylogenetic comparison of the nine stress-associated molecular chaperones revealed that the corresponding genes from kefir strains (represented by K4) and post-fermentation isolates clustered almost identically, indicating a high degree of sequence conservation across lineages (Fig. 4A). The only exception was the ATP-dependent ClpC protease, where the FLA4 lineage formed a short but distinct branch from its corresponding kefir strain (K4), suggesting subtle sequence divergence. Nucleotide alignment of the clpC gene between kefir strain K4 and FLA4 revealed a single nucleotide substitution (Fig. 4C). This mutation was supported by a Phred quality score of 33 at the variant position, corresponding to a base calling accuracy of approximately 99.95%. A nucleic acid base substitution from adenine (A) to guanine (G) was observed in clpC resulting in an asparagine (N) to aspartate (D) at position 35 in the open reading frame (Fig. 4C). Despite this amino acid substitution, AlphaFold3 predicted nearly identical three-dimensional structures for the ClpC proteins of K4 and FLA4, with pLDDT values exceeding 90 and a pTM score of 0.74 (Fig. 4B), indicating preservation of the overall protein fold (Guo et al., 2022; Weeratunga et al., 2024).

3.5. Hemp fermentation enhances acid resilience in Lacticaseibacillus paracasei

The gastrointestinal tract represents a major barrier to probiotic functionality, as exposure to gastric acidity, digestive enzymes, and bile salts can substantially reduce microbial viability (de Melo Pereira et al., 2018; Papadimitriou et al., 2016). Consistent with this, microorganisms isolated from water kefir have been reported to exhibit relatively low gastrointestinal resilience (Romero-Luna et al., 2020). Contrary, the kefir-derived *Lacticaseibacillus paracasei* and post fermentation lineages examined in this study displayed appreciable tolerance to all simulated gastrointestinal stresses, with survival rates ranging from 85.83 to 98.20% under acid stress, 93.32 - 97.73% following pepsin exposure, and 90.64 - 93.04% after bile salts and pancreatin treatment (Fig. 5B). This observation highlight the strain-dependent nature of

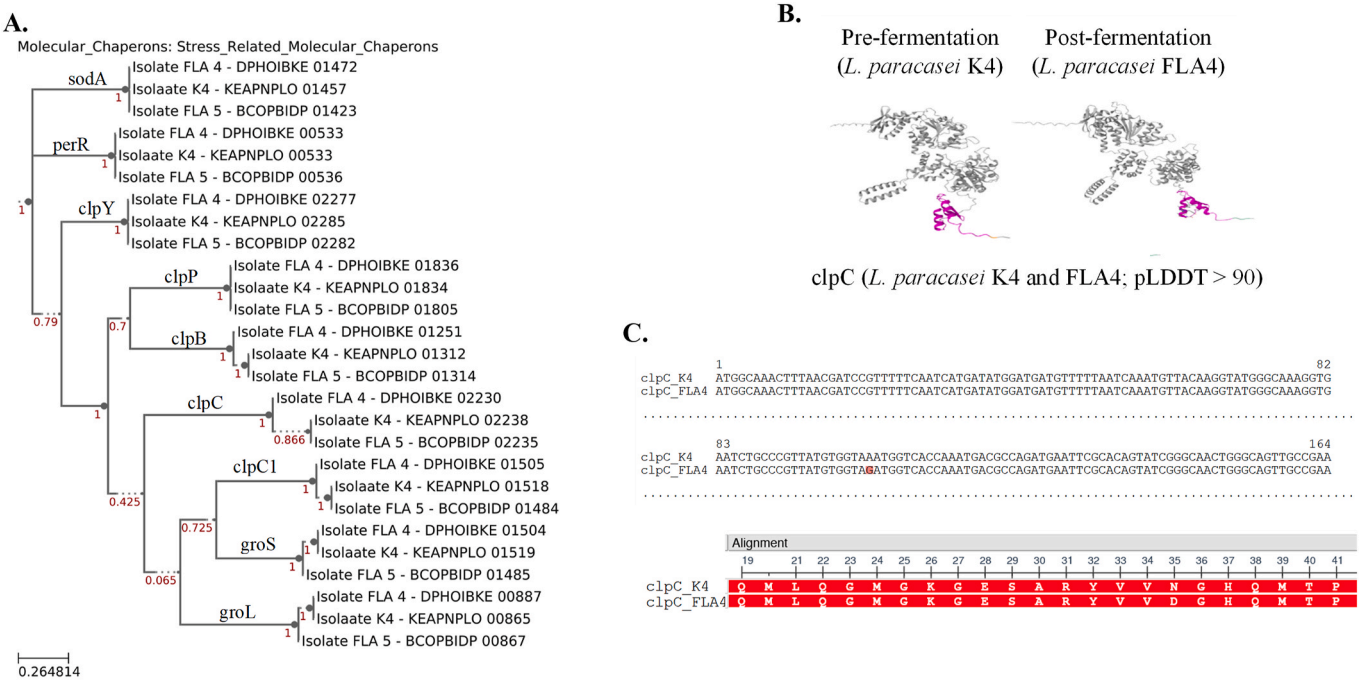

Fig. 4. Conservation of stress-response genes and validation of a lineage-specific *clpC* variant following hemp fermentation. (A) Phylogenetic analysis of major stress-associated molecular chaperones and oxidative stress response proteins (*groEL, groES, clpB, clpP, clpY, clpC, clpC1, perR* and *sodA*) in kefir-derived and post-fermentation *Lacticaseibacillus paracasei* isolates, demonstrating a highly conserved stress-response repertoire across all strains. (B) Predicted three-dimensional structures of the ClpC protein from the pre-fermentation strain K4 and post-fermentation strain FLA4 generated using AlphaFold3. Both proteins exhibited highly similar structural conformations with high prediction confidence (pLDDT > 90). (C) Nucleotide sequence alignment of the *clpC* gene between K4 and FLA4 showing a single nucleotide substitution. The variant was supported by a Phred quality score of 33, confirming a high-confidence base call.

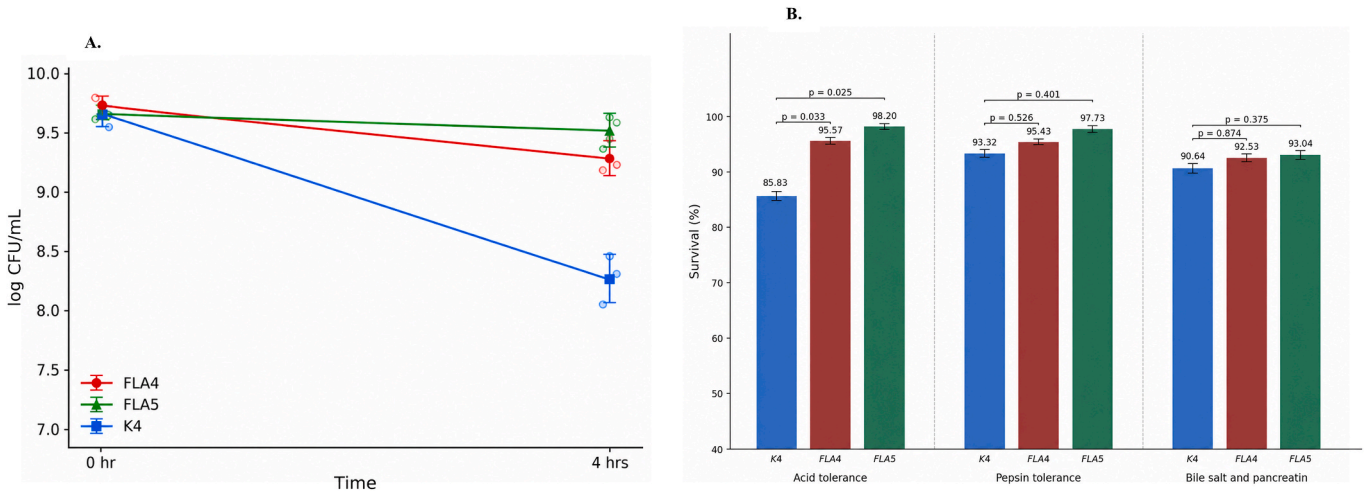

Fig. 5. Hemp fermentation enhances acid resilience of kefir-derived *Lacticaseibacillus paracasei*. (A) Survival line plot of the pre-fermentation kefir strain (K4) and post-fermentation lineages (FLA4 and FLA5) following exposure to simulated gastric acid (pH 2.5) for 4 h. (B) Survival of K4, FLA4, and FLA5 under simulated gastrointestinal stress conditions, including gastric acid, pepsin, bile salts and pancreatin. Hemp fermentation significantly improved acid tolerance in FLA4 ($p = 0.033$) and FLA5 ($p = 0.025$) relative to K4. Data are presented as mean ± SEM from three independent replicates. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, with $p < 0.05$ considered significant.

gastrointestinal resilience within water kefir communities. Notably, hemp exposure significantly enhanced acid tolerance. After 4 h of acid exposure, kefir strains (K4) exhibited a reduction in viability, declining from approximately 9.7 to 8.2 log CFU/mL (85.83% survival), whereas post fermentation lineages (FLA4 and FLA5) showed only modest reductions to approximately 9.3 and 9.5 log CFU/mL, retaining significantly higher viabilities of 95.57% ($p = 0.033$) and 98.20% ($p = 0.025$), respectively (Fig. 5A and B). Although tolerance to pepsin, bile salts and pancreatin was consistently higher in the post-fermentation lineages, these increases did not reach statistical significance ($p = 0.526$ and 0.401 for pepsin; $p = 0.874$ and 0.375 for bile salts and pancreatin). These findings suggest that the hemp niche specifically conditioned responses associated with acid stress rather than broadly improving gastrointestinal stress tolerance.

3.6. *Hemp fermentation preserves the transcriptional potential for neuroactive compound biosynthesis*

Gene mapping revealed that kefir *L. paracasei* strains possessed genes involved in neuroactive compound biosynthesis, including the complete tryptophan biosynthetic pathway, glutamate metabolism genes (*gltB* and *gdh*), and genes encoding the pyruvate dehydrogenase complex, *pdc* (Fig. 6A). Of particular interest was the presence of the complete *de novo* tryptophan biosynthetic pathway, as tryptophan serves as the precursor

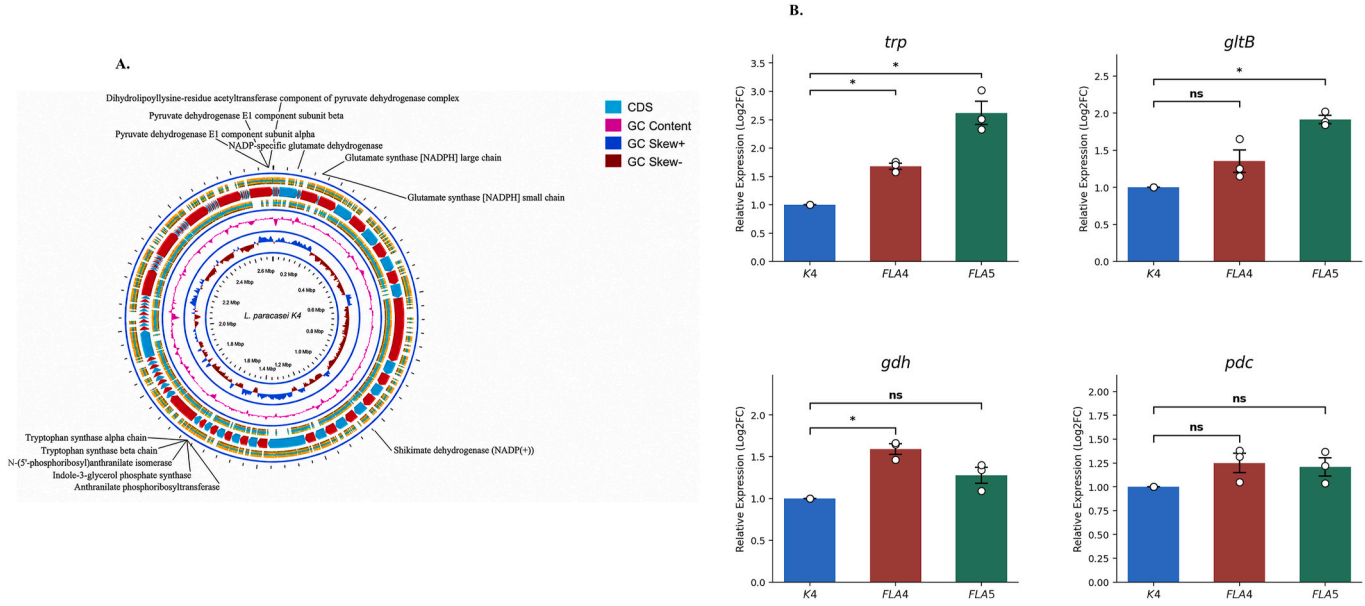

Fig. 6. Neuroactive biosynthetic genes and their expression in pre- and post-fermentation *Lacticaseibacillus paracasei*. (A) Circular genome map showing genes involved in neuroactive compound biosynthesis, including the tryptophan biosynthetic pathway (*trp*), glutamate metabolism (*gltB* and *gdh*), and the pyruvate dehydrogenase complex (*pdc*). Concentric rings represent coding sequences (CDS), GC content, and GC skew. (B) Relative expression of *trp*, *gltB*, *gdh*, and *pdc* in the pre-fermentation strain (K4) and post-fermentation lineages (FLA4 and FLA5). Expression was normalized to the housekeeping gene *gyrB*. Data are presented as mean ± SEM from three biological replicates (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$); ns, not significant.

for serotonin, a neurotransmitter central to mood regulation and depression pathophysiology (Agus et al., 2018; O'Mahony et al., 2015). However, the mere presence of biosynthetic genes does not necessarily reflect their functional activity, necessitating transcriptional assessment of these pathways.

Consistent with the preservation of neuroactive potential, both post-fermentation lineages exhibited significantly higher *trp* expression than the kefir strain, with approximately 1.7-fold and 2.6-fold increases in FLA4 and FLA5, respectively (Fig. 6B). These findings suggest that exposure to the hemp niche did not compromise, and may have enhanced, the transcriptional potential for *de novo* tryptophan biosynthesis. Since microbial tryptophan metabolism can influence host serotonin production through the microbiota-gut-brain axis, enrichment of strains with elevated *trp* expression may be advantageous for psychobiotic functionality (Agus et al., 2018; Clarke et al., 2013). In contrast, expression of genes associated with glutamate metabolism was lineage dependent. While FLA5 exhibited significantly higher *gltB* expression than kefir strain, *gdh* expression was significantly elevated only in FLA4, suggesting distinct transcriptional responses among the post-fermentation lineages. The lack of significant difference for *pdc* expression among strains further indicates that core central metabolic functions remained largely conserved following hemp fermentation.

3.7. Genomic evidence supports the safety of post-fermentation *Lacticaseibacillus paracasei*

Genomic safety is a fundamental prerequisite for the development of next-generation probiotics, particularly because probiotic microorganisms may serve as reservoir for transmissible antimicrobial resistance determinants (Botthoulath et al., 2025). Screening of the post-fermentation *L. paracasei* genomes identified only a single hit associated with tolerance to quaternary ammonium compounds (Fig. 7A). Importantly, this determinant was chromosomally encoded, showed no association with transposable elements, and did not harbour plasmids (Fig. 7B). Collectively, these findings indicate a low likelihood of horizontal transfer of this resistance determinant. The observed genomic architecture is consistent with previous reports that members of the *L. paracasei* predominantly harbour intrinsic, non-transferable resistance determinants that do not constitute a significant safety concern (Campedelli et al., 2019). Thus, despite exposure to the phytochemically complex hemp niche, the post-fermentation *L. paracasei* strains retained a favourable genomic safety profile, supporting their further evaluation as potential psychobiotic candidates.

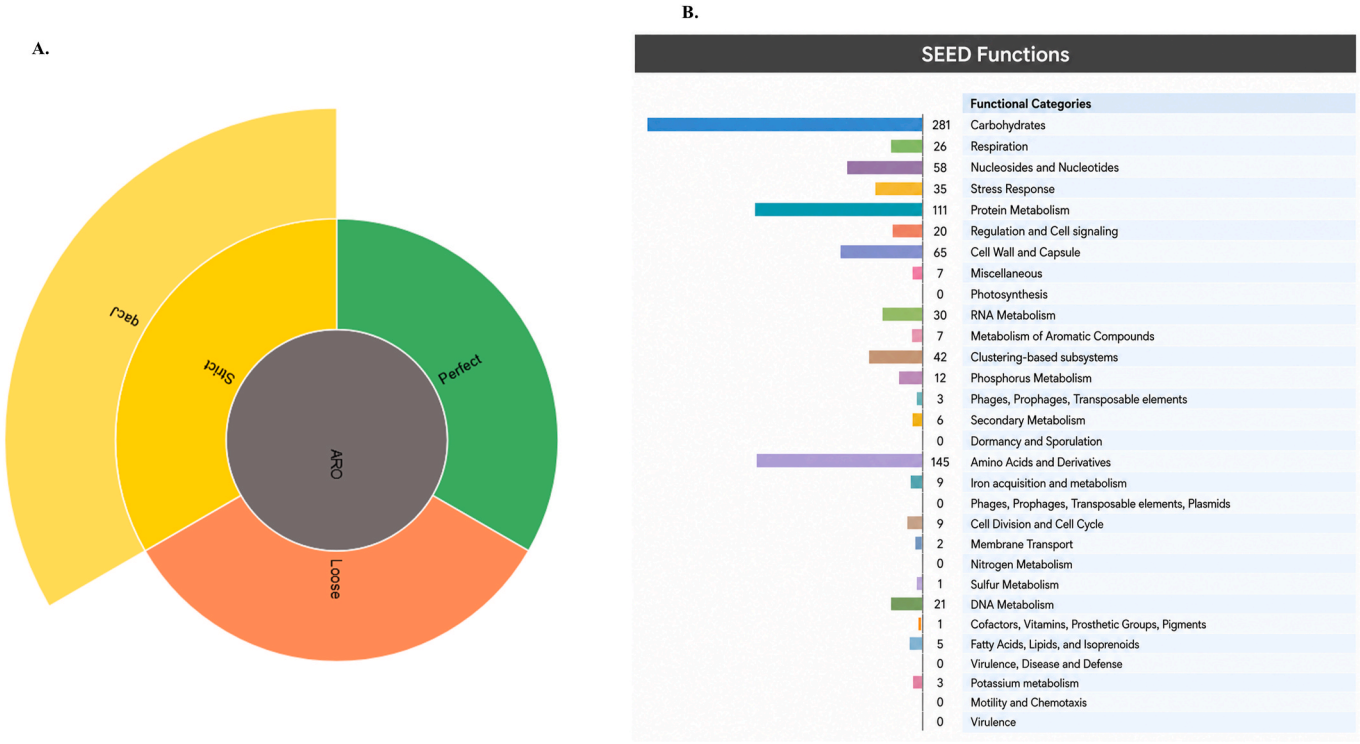

Fig. 7. Genomic safety assessment of post-fermentation *Lacticaseibacillus paracasei*. (A) Classification of antimicrobial resistance genes identified in the post-fermentation *Lacticaseibacillus paracasei* genomes based on the Comprehensive Antibiotic Resistance Database (CARD). Only chromosomally encoded genes associated with quaternary ammonium compound tolerance were detected. (B) Functional annotation of the post-fermentation genomes using the RAST SEED subsystem database, showing the distribution of predicted genes across major metabolic and cellular functional categories. No virulence-associated genes or plasmids were identified.

4. Conclusion

Our findings demonstrate that the hemp niche acts as a selective ecological filter that preferentially enriches resilient *Lacticaseibacillus paracasei* populations from the water kefir microbiome, including low abundance lineages that become detectable under altered environmental conditions. Exposure to the hemp niche enhanced acid resilience while preserving the genomic and transcriptional potential for neuroactive compound biosynthesis, particularly *de novo* tryptophan synthesis. Importantly, post-fermentation strains retained a favourable genomic safety profile, characterized by the absence of plasmids and mobile antimicrobial resistance determinants. These findings highlight hemp fermentation as a promising strategy for conditioning and enriching resilient psychobiotic candidates with potential applications in microbiota-gut-brain axis interventions. Future work will focus on harnessing this platform to enhance neuroactive functionality by steering microbial metabolism toward increased tryptophan and downstream indole derivative production.

Moreover, our findings have important implications for the water kefir industry, where there is increasing demand for scientifically validated functional cultures with defined health-promoting properties. Rather than introducing exogenous probiotic strains, our results demonstrate that targeted substrate fermentation can selectively enrich naturally occurring *L. paracasei* lineages already present within complex kefir microbial consortia. This approach provides a simple and potentially scalable strategy for improving the functional composition of starter cultures while preserving the ecological integrity of traditional fermentations. Such precision-guided enrichment could support the development of standardized water kefir products with improved consistency and reproducibility.

CRediT authorship contribution statement

Vincent Owusu Kyei-Baffour: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Paulina Štreimikytė:** Writing – review & editing, Resources, Conceptualization. **Eglė Lastauskienė:** Writing – review & editing, Supervision, Resources. **Aurelijus Burokas:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Eric Banan-Mwine Daliri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interests

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.

Data availability

Metagenome assembly and annotation pipelines, as well as comparative genomics analyses performed in KBase, are available at the provided narrative links respectively. (https://narrative.kbase.us/narrative/221902; https://narrative.kbase.us/narrative/231411). Raw metagenomic and whole-genome sequencing data, along with associated metadata, have been deposited in the European Nucleotide Archive under accession number PRJEB111860.

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