Physiological and genomic characterization of *Lactiplantibacillus plantarum* isolated from *Indri indri* in Madagascar

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Abstract

Aims. *Indri indri* is a lemur of Madagascar which is Critically Endangered. The analysis of the microbial ecology of the intestine offers tools to improve conservation efforts. This study aimed to achieve a functional genomic analysis of three *Lp. plantarum* isolates from indris.

Methods and Results. Samples were obtained from 18 indri; 3 isolates of *Lp. plantarum* were obtained from 2 individuals. The three isolates were closely related to each other, with fewer than 10 single nucleotide polymorphisms, suggesting that the two individuals shared diet-associated microbes. The genomes of the three isolates were compared to 96 reference strains of *Lp. plantarum*. The three isolates of *Lp. plantarum* were not phenotypically resistant to antibiotics but shared all 17 genes related to antimicrobial resistance that are part of the core genome of *Lp. plantarum*. Genomes of the three indri isolates of *Lp. plantarum* also encoded for the 6 core genome genes coding for enzymes related to metabolism of hydroxybenzoic and hydroxycinnamic acids. The phenotype for metabolism of hydroxycinnamic acids by indri isolates of *Lp. plantarum* matched the genotype.

Conclusions. Multiple antimicrobial resistance genes and gene coding for metabolism of phenolic compounds were identified in the genomes of the indri isolates, suggesting that *Lp. plantarum* maintains antimicrobial resistance in defense of antimicrobial plant secondary pathogens and that their metabolism by intestinal bacteria aids digestion of plant material by primate hosts.

Significance and Impact of Study: Better knowledge of the microbial composition of wild primates may inform and improve *ex situ* conservation efforts.

Keywords: *Indri indri*; lemur, *Lactobacillus, Lactiplantibacillus plantarum*; comparative genomics; sugar fermentation; phenolic compounds, ferulic acid, polyphenols, antimicrobial resistance.

Introduction

Indri indri is one of the largest extant living lemurs of Madagascar. *I. indri* is classified as a Critically Endangered species by the International Union for Conservation of Nature Red List of Threatened Species (King *et al.*, 2023). This species has not yet been bred under human care, suggesting that behavioral and/or ecological factors for successful *ex-situ* conservation protocols remain unknown. Indris are strictly folivorous and have morphological specialization for the consumption and digestion of leaves. The species shows a preference for immature leaves with a reduced emphasis on seeds, whole fruits or flowers (Powzyk, 1997). They also feed on bark, galls, and mushrooms. Indris consume soil on a regular basis. The consumption of soil may be a means to cope with chemical deterrents often found in plants. The analysis of the microbial ecology of the gut can offer valuable perspectives and tools for investigating and monitoring primate health and improving conservation efforts. The microbial communities inhabiting primates and other taxa profoundly affect host health, nutrition, physiology, and immune systems. Microbial communities might be sensitive to alterations in the external environment, and microbial diversity seems to correlate with habitat quality with direct health consequences. The intestinal microbiota of primates is disturbed, however, when animals are held in captivity (Nishida & Ochman, 2021), necessitating the analysis of wild animals. The application of microbial analyses to conservation is currently in its infancy but holds enormous potential to improve conservation strategies and monitoring and promoting primate health (Correa *et al.*, 2021).

In many animals, lactic acid bacteria are beneficial gut commensals that contribute to gut homeostasis through maintaining a microbial balance (Walter, 2008). Research for microorganisms in wild animals are very rare due to the difficulty in culturing and the lack of access to appropriate microbiological tools at the laboratory level in field conditions (Schwab *et al.*, 2009). Therefore, the

isolation of microorganisms in the wild environment represents a major challenge. The time between sampling and analysis as well as the modalities for collection and storage of samples are important factors that could impact negatively the viability of fecal microorganisms. This study aimed at isolating lactic acid bacteria from fecal samples of *Indri indri* previously characterized via culture-independent methods (Correa *et al.*, 2021), and at analyzing isolates using a functional genomics approach.

Materials and Methods

Sample origin and collection

Individual fecal sample from 18 indris were obtained in December 2018 in Maromizaha Forest New Protected Area (NPA, latitude 18°57'S and 19°00'S, longitude 48°26'E and 48°31'E) in Madagascar. Sampled subjects belonging to five different family groups that occupy different territories within the area (Correa *et al.*, 2021). Fecal samples were collected immediately after defecation. Approximately 1 g of fecal sample was collected into screw-cap tubes with an integrated plastic shovel-like tool attached to the cap, containing 10 ml of de Man Rogosa Sharpe soft agar (0.7 %) with 0.05 % cysteine hydrochloride (mMRS). The small plastic shovel-like tool attached to the cap of the screw cap tubes was used to scoop up the fecal samples taken from the middle to avoid soil contamination. All samples were maintained on ice for about 10 d before arrival at the lab where the samples were maintained at -80 °C until the analysis.

Isolation and purification of lactic acid bacteria

Aliquots of approximately 1 g of fecal sample were serially diluted with saline. Aliquots of 1 ml from each dilution (10^{-1} to 10^{-9}) were inoculated onto mMRS agar supplemented with mupirocin (100 mg L^{-1}) (Applichem). Plates were incubated under anaerobic conditions by using the GasPak EZ

Anaerobic Pouch system (BD) at 37 °C for 48-72 h. The representative colony of each morphology was picked and individually streaked onto mMRS agar. The pure cultures of lactic acid bacteria were then stored in 20 % (v/v) glycerol at -80 °C until further analysis.

DNA extraction

The total DNA of each strain isolated from fecal samples was extracted using Wizard®Genomic DNA Purification Kit (Promega), following the suppliers' instructions with minor modifications in the cell lysis. The cell pellet from 2 mL of a 24 h culture was resuspended in 220 µL of a TE solution (10 mM Tris-HCl + 2 mM EDTA) containing Lysozyme (Sigma-Aldrich) 50 g/L and in 480 µL of 50 mM EDTA. This solution was then incubated overnight at 37 °C. At the end of the incubation the extraction procedure was completed according to the protocol. The DNA thus obtained was resuspended in DNA rehydration solution (10 mM Tris-HCl pH = 7.4; 1 mM EDTA pH = 8.0) (Promega). The quantification of the extracted DNA was carried out with the Infinite 200 Pro Spectrofluorimeter (TECAN).

LAB typing by BOX-PCR

For each isolate, BOX-PCR fingerprinting was performed to identify clonal isolates of the same strain using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') as described (Michelini *et al.*, 2016). Briefly, Box-PCR was carried out in a 20 µl amplification mixture containing 1.5 mM $MgCl_2$, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStartTaq plus DNA polymerase MasterMix kit, Qiagen), additional 0.05 mM of each dNTP, 70 ng DNA template and 2 µM primer. Amplification was carried out with an annealing temperature of 50 °C. PCR products were separated on a 2 % (w/v) agarose gel. Gels were stained with 0.5 mg L^{-1} ethidium bromide and visualized by UV illumination.

Identification of bacterial strains by sequencing of 16S rRNA genes

The partial 16S rRNA was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) as described (Dos Santos *et al.*, 2019). PCR fragments were purified using a NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) following the manufacturer's instructions. Amplicons were sequenced by service of EurofinsMWG Operon Biotech (Germany).

Whole genome sequencing and assembly

The genome of isolates were sequenced using a MiSeq platform (Illumina) at Istituto Zooprofilattico Sperimentale (Teramo, Italy). The generated data were depleted of adapter sequences, quality filtered, assembled and annotated through the PATRIC web resources using the RAST server (www.patricbrc.org). The raw reads were assembled and annotated by the comprehensive genome analysis service at PATRIC (Wattam *et al.*, 2017) with default "auto" parameters for the assembly and annotation with RAST (Aziz *et al.*, 2008). Basic information on the quality of the assembled genomes and basic genomic features are shown in Table S1. The genome accession numbers were deposited to Genbank with the bioproject number PRJNA957770 and the accession numbers JARXQJ000000000 (*Lp. plantarum* Z1), JARXQK000000000 (*Lp. plantarum* Z3) and JARXQL000000000 (*Lp. plantarum* D6).

Phylogenetic analysis of *Lactiplantibacillus plantarum* genomes

For comparison with the genomes of indri isolates of *Lp. plantarum*, genomes of ninety-six *Lp. plantarum* strains from different origins were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). The genomes of *Lp. plantarum* were rarefied by including 19 strains from cereal fermentations, 20 strains from vegetable fermentations, 13 strains from dairy fermentations, 8 strains from meat fermentations, 13 strains from insects, 18 strains from humans and

5 strains of unknown origin (Table S2). The selection of genomes prioritized strains which are available in the FUA strain collection and closed genomes (Table S2). All genomes were re-annotated by Prokka (Seemann, 2014) with default settings. Core genes were extracted using Roary (Page *et al.*, 2015). The phylogenetic tree was inferred using IQ-TREE (Minh *et al.*, 2020) based on the core gene alignment with the best fit model predicted using ModelFinder (Kalyaanamoorthy *et al.*, 2017) and 1000 bootstrap values. *Lactiplantibacillus argentoratensis* (CP032751) and *Lactiplantibacillus pentosus* (AZCU00000000) were used as outgroups and the tree was visualized with iTOL (Letunic & Bork, 2021). The pairwise average nucleotide identity (ANI) of *Lp. plantarum* strains was calculated using FastANI (Jain *et al.*, 2018).

In silico analysis of antibiotic resistance and phenolic metabolism

Amino acid sequences from all *Lp. plantarum* strains were used to build a database. Fifteen genes coding for metabolism of hydroxycinnamic acids and hydroxybenzoic acids (Gaur & Gänzle, 2023) and the Comprehensive Antibiotic Resistance Database (McArthur *et al.*, 2013) were used as query sequences. Blastp (Altschul *et al.*, 1997) was performed with default parameters and the cut-off values of 70% query cover and 40% amino acid identity. These BLAST cut-offs typically identify proteins that have same general function as the biochemically characterized query sequence although MDR transporters with this level of similarity may have a different substrate specificity. Results were curated manually to remove replicate hits of query sequences against a single gene in the genomes. The heat map was drawn by iTOL.

In silico comparison of plantaricin biosynthesis genes

Initially, genes coding for bacteriocin production were searched with BAGEL4 (Van Heel *et al.*, 2018). Further analyses used the genome of *Lp. plantarum* WCFS1 (GCA_000203855.3) as reference

strain. The *pln* genes were downloaded from NCBI and were used as query sequences for BLAST analysis following the same procedures as described above.

Characterization of sugar fermentation patters

Sugar fermentation was assayed with the API 50 CHL kit (BioMerieux, France). In brief, bacterial cells collected from overnight mMRS agar plates were suspended in modified CHL broth which was supplemented with 0.025 % cysteine hydrochloride. Then cells were incubated in an anaerobic jar at 37 °C for 5 d before the API 50 CHL test strips were evaluated.

Determination of minimum inhibitory concentrations (MICs)

The EUCAST susceptibility testing protocol ("Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution," 2003) was used for the determination of the MICs of the antibacterial agents suggested by EFSA. Exponentially growing bacterial cultures were suspended in saline solution to achieve a turbidity equivalent to 0.5 McFarland standard and then diluted to a final concentration of 1.2×10^5 CFU mL^{-1}. LSM broth (IsoSensitest broth (90%) and MRS broth (10%) adjusted to pH 6.7 were used for MIC microdilution test.

Bacterial suspensions were added to serial dilutions of the antibiotics in a 96-well Microtiter® plate. The MIC was defined as the lowest antibiotic concentration which prevented visible growth after 24 h of incubation at 37 °C. All tests were performed in triplicate.

Analysis of bacteriocin production

Prior to each experiment, strains Z1, Z3 and D6 were subcultured in 10 mL of MRS at 37 °C (pH 6.5) three consecutive times with 1% inoculum and the last culture was used as the inoculum for all experiments. The spot on the lawn bioassay (Denkova *et al.*, 2017) was used to screen for antimicrobial activity of isolates. *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739,

Enterococcus faecalis ATCC 8043, and *Pseudomonas aeruginosa* M19 were used as indicator bacteria. The indicator bacteria were cultured in tryptic soy agar supplemented with yeast extract (TSA, Merck).

One ml of each indicator organism (5×10^5 cfu/ml) was inoculated into 15 ml of semisolid TSA agar (TS broth plus 0.7% bacteriological agar) maintained at 50 °C and then poured into a petri dish. After solidification, 100 µl of neutralized cell-free supernatant from each isolate was applied as a drop and allowed to dry. Neutralized cell-free supernatant was prepared as follows: each isolate was cultured overnight in 10 ml MRS broth in anaerobic conditions. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD). Cells were removed by centrifugation, the supernatant was sterilized by filtration and 100 µl of the unadjusted aliquot of CFS was added to the plate. The remaining cell-free supernatant was adjusted to pH 6.0 with 1 N NaOH. Then, a 100 µl aliquot of the pH adjusted cell-free supernatant was filtered and added to the plate. To confirm the production of a proteinaceous compound, the neutralized cell-free supernatants were treated with 1 g/L of proteinase K (1,000 U/mL) for 1 h at 25 °C and filter sterilized and placed on the plates. The TSA plates were incubated at 37 °C or 30°C aerobically for 24 h. Screenings for bacteriocin producing LAB were repeated twice for each isolate.

Metabolism of phenolic acids

Metabolism of phenolic acids was analysed after addition of caffeic, p-coumaric and ferulic acids as substrates. Phenolic acids and their metabolites produced by isolates during fermentation were analyzed according to (Chinnici *et al.*, 2011) with modifications. Samples were diluted 1:2 with HPLC eluent A and filtered with 0.45 µm cellulose filters. The HPLC instrument (Jasco Europe Srl, Cremella, Italy) was equipped with a quaternary gradient pump Jasco PU-2089, an autosampler Jasco AS-2057 Plus Intelligent Sampler and a Jasco UV/Vis MD-910 PDA detector and a Jasco FP-2020

Plus Fluorescence detector set at λ_{ex} 260nm and λ_{em} 305 nm. Samples were eluted at 35 °C with a flow of 0.8 mL min^{-1} on a C18 Poroshell 120 (Agilent Technologies, Milano, Italy), 2.7 µm, (4.6 x 150 mm). Elution solvents were 2 % acetic acid in HPLC grade water (Eluent A) and 2 % acetic acid in HPLC grade acetonitrile (Eluent B). Gradient elution was as follow: from 2 % to 5 % B in 10 min, 5 % to 10 % B in 7 min, 10 % to 18 % B in 6 min, 18 % to 20 % B in 3 min, 20 % to 30 % B in 3 min, 30 % to 50 % B in 3 min, 50% to 100 % B in 4 min, followed by re-equilibration. Quantification of phenolic acids, 4-ethylphenol, 4-ethylguaiacol and 4-vinylphenol was performed at UV wavelengths corresponding to their maximum absorbance. These latter were obtained from triplicate injections of standard solutions of caffeic acid, p-coumaric acid, ferulic, 4-ethylphenol, 4-ethylguaiacol and 4-vinylphenol (Merck, Darmstadt, Germany).

Results

Isolation and preliminary characterization of *Lactiplantibacillus plantarum*

Fecal samples from 18 individuals were studied, one of which was sampled two times (Table S3), but isolates were obtained only from two subjects. A total of 6 isolates was obtained, three each from the two subjects Dary and Zafy, belonging to different family groups. Dary was a six-month old belonging to group 2 MZ of unknown sex because the sex can be recognized only at 2-3 years of age. Zafy was a six years old male belonging to group 8 MZ. Isolates from fecal samples of Dary and Zafy were designated as D1, D2 and D3, and as Z1, Z2, and Z3, respectively. The isolation was performed in MRS added with mupirocin, a selective medium for bifidobacteria. Bifidobacteria were not identified from the MRS added with mupirocin plates. The isolates share the same BOX-PCR profiles except for Z3 which shows an additional band (Figure S1). Isolates from the same individual therefore likely represent clonal isolates of the same strain. The 16S rRNA analysis showed that all strains shared 99.9 % sequence identity with *Lp. plantarum*. The carbohydrate fermentation pattern

was determined with the API kit (Table S4). The broad spectrum of carbohydrate fermentation conforms to the tentative identification as *Lp. plantarum.*

Phylogenetic analysis of strains of *Lactiplantibacillus plantarum.*

The identification of isolates as strains of *Lp. plantarum* was confirmed by construction of a core genome phylogenetic tree and by calculation of the pairwise ANI values with reference genomes (Figure 1). The pairwise ANI of indri isolates to all other strains of *Lp. plantarum* was higher than 95% (Figure 1). Strains Z1, Z3 and D6 are closely related to each other. Genome alignment by MAUVE (Darling *et al.*, 2004) demonstrated that the genomes of strains Z1 and Z3, Z1 and D6, and Z3 and D6 differ only in 8, 5, and 6 SNPs, respectively. Accordingly, the three strains clustered together in the phylogenetic tree of *Lp. plantarum.* Other strains with the same source or from the same geographical origin did not cluster together (Figure 1). The indri isolates shared more than 99.8% ANI with *Lp. plantarum* ZS2058, an isolated from a cabbage fermentation in China. Of the reference strains of *Lp. plantarum*, several genomes of strains with different geographic origin were also highly similar. For example, *Lp. plantarum* FUA3073, an isolate from ready-to-eat meat in Canada, shared pairwise ANI values of more than 99% with an olive isolate from Ireland, an isolate from fermented vegetables from China, and an isolate from fermented sorghum obtained in Botswana (Figure 1 and Table S1).

Genotypic and phenotypic analysis of antimicrobial resistance in *Lp. plantarum*

The *in silico* analysis of genes coding for antimicrobial resistance revealed that 17 genes were present in more than 90% of the genomes analysed and are thus part of the core genome of *Lp. plantarum* (Figure 2). Most of these genes encode for transport proteins of the Major Facilitator Superfamily or for ABC transporters, but other cell wall / cell membrane proteins and ribosomal protection proteins

were also represented (Figure 2). Eleven additional genes coding for antimicrobial resistance were part of the accessory genome of *Lp. plantarum*.

The phenotypic resistance of indri isolates of *Lp. plantarum* was assessed with a panel of 8 therapeutic antibiotics (Table 1). The inherent resistance of *Lp. plantarum* to vancomycin (Zhang *et al.*, 2018; Zheng *et al.*, 2020) has been confirmed with an MIC of more than 256 mg L^{-1}. All three strains were relatively resistant to kanamycin, streptomycin and chloramphenicol but the MIC values did not exceed the cut-off values established by the European Food Safety Authority (EFSA) for *Lp. plantarum* (Table 1) (Rychen *et al.*, 2018).

Genomic analysis and phenotypic analysis of the metabolism of hydroxycinnamic acids and hydroxybenzoic acids by indri isolates of *Lp. plantarum*

The metabolism of phenolic acids was initially evaluated by *in silico* analysis of the 9 genes that are known to contribute to conversion of phenolic acids in *Lp. plantarum* (Figure 2). Of the genes related to metabolism of hydroxycinnamic acid, the esterases Lp_0796 and Lp_2953, the reductase HcrB and the decarboxylase Pad were part of the core genome of *Lp. plantarum*. More than 50% of genomes included the gene for the vinyl phenol reductase VprA but only few genomes encoded for the esterase Lp_1092. Of the genes related to metabolism of hydroxybenzoic acids, the intracellular esterase TanB and the decarboxylase Lp_2953 (LpdC) were part of the core genome while only few genomes encoded for the extracellular tannase TanA (Figure 2). The genetic potential of the indri isolates thus matched that of the majority of strains of *Lp. plantarum*.

Metabolites produced by the three indri isolates of *Lp. plantarum* are shown in Figure 3. Ferulic acid was converted by reduction to dihydroferulic acid and by decarboxylation to vinylguajacol. Ethylguajacol was not detected, nevertheless, the concentration of vinylguajacol decreased between day 1 and day 10 of incubation. Because no new peaks emerged in the chromatograms, vinylguajacol

was presumably consumed through chemical reactions with other media components. Coumaric and caffeic acids were converted by decarboxylation only (Figure 3). 4-Ethylphenol was the major metabolite from coumaric acid; in contrast, vinylcatechol was the major metabolite from caffeic acid after 1 d of incubation but was further converted to ethylcatechol. The metabolites produced from hydroxycinnamic acids showed only minor differences among the three indri isolates of *Lp. plantarum*.

Genotypic and phenotypic analysis of bacteriocin production and antimicrobial activity.

Analysis of the genomes for the presence of putative bacteriocin operons with Bagel identified genes encoding for the two peptide bacteriocins plantaricin JK and plantaricin FE. The bacteriocin producing operons were highly similar in sequence and identical in organization when compared to the plantaricin gene loci in *Lp. plantarum* WCFS1 (Figure 4) (Diep *et al.*, 2009). Culture supernatants from the indri isolates of *Lp. plantarum* but not neutralized culture supernatants exhibited inhibitory activity against *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa* or *Enterococcus faecalis*, indicating that inhibition of these pathogens by indri isolates relates to acid production but not bacteriocin formation.

Discussion

This study describes the functional genomic analysis of three *Lp. plantarum* isolates from *Indri indri*. The sequence based analysis of intestinal samples from the same 18 individual indris documented that intestinal microbiota consist predominantly of *Proteobacteria* (40%), *Bacteroidota* (29%), *Synergistota* (17%) and *Bacillota* (11%) (Correa *et al.*, 2021). Our experimental design aimed to isolate bifidobacteria. In keeping with the low abundance of *Actinomycetota* in the intestinal microbiota (Correa *et al.*, 2021), bifidobacteria were not isolated from any of the samples but the isolation retrieved 3 strains of *Lp. plantarum*. Mupirocin is used as a selective agent for cultivation

of bifidobacteria in food and fecal samples and most lactobacilli including *Lp. plantarum* are sensitive to mupirocin (Simpson *et al.*, 2004; Miranda, Carvalho & Nero, 2014). The three strains of *Lp. plantarum* isolated in this study thus represent only a subset of lactobacilli that were present in indris' fecal samples. Because *Lp. plantarum* is not a stable representative of vertebrate intestinal microbiota (Walter, 2008; Duar *et al.*, 2017), its presence in fecal samples likely reflects its presence in the diet of indris. In humans, live dietary microbes are readily detected in fecal samples (Dal Bello *et al.*, 2003; Pasolli *et al.*, 2020). Because sequencing of 16S rRNA gene amplicons did not identify lactobacilli as dominant members of the intestinal communities (Correa *et al.*, 2021), strains of *Lp. plantarum* are only a minor component of the fecal communities of microbes of indris.

Lp. plantarum has been described as an organism with a nomadic lifestyle that is associated with plants but also temporarily persists in the intestine of vertebrates and insects (Siezen & van Hylckama Vlieg, 2011; Martino *et al.*, 2016). To assess whether indri isolates differ from other isolates of *Lp. plantarum*, the genomes of the three strains were compared to 96 genomes of *Lp. plantarum* that are available on NCBI. The number of genomes that we analysed is not greater than that used in past studies (Siezen & van Hylckama Vlieg, 2011; Martino *et al.*, 2016) but the increased availability of genome sequence data for *Lp. plantarum* allowed rarefaction of the genome dataset to obtain approximately equal representation of isolates from cereal fermentations, vegetable fermentations, dairy fermentations, meat fermentations, insects, and humans (Table S2). The indri isolates of *Lp. plantarum* were highly related (ANI > 99.8%) to an isolate obtained from a cabbage fermentation in China. The carbohydrate fermentation pattern of indri isolates did not show differences to other strains of *Lp. plantarum* (Bringel *et al.*, 2005; Alhaag *et al.*, 2019). Genome analyses also identified a bacteriocin operon that is virtually identical to *Lp. plantarum* WCFS1 and highly similar to bacteriocin loci in other strains of *Lp. plantarum* (Diep *et al.*, 2009). Plantaricin KJ and plantaricin

EF are class IIb two peptide bacteriocins with a narrow spectrum of activity (Maldonado, Ruiz-Barba & Jiménez-Díaz, 2004; Diep *et al.*, 2009). This narrow spectrum of activity conforms to the role of bacteriocins of lactic acid bacteria to support competition against close relatives that occupy the same niche rather than the wider microbial community (Kommineni *et al.*, 2015). Accordingly, the beneficial effect of *Lp. plantarum* in a mouse model of diet-induced obesity was independent of the antimicrobial activity of plantaricin EF, however, the two peptide bbacteriocin was reported to directly improve barrier properties of intestinal epithelia (Heeney *et al.*, 2019).

The "One Health" approach places the issue of antibiotic resistance in a critical global context (Caudell *et al.*, 2020; Koutsoumanis *et al.*, 2021). This approach has been well studied for livestock but needs more research for wildlife (Diallo *et al.*, 2020). Nonhuman primates live in wild areas of varying conservation status and are unlikely to be exposed to human use of antibiotics. Analysis of the *Lp. plantarum* genomes for genes coding for antimicrobial resistance identified 17 genes as part of the core genome and an additional 11 genes as part of the accessory genome. The genomes of the indri isolates included the core but not the accessory genes coding for antimicrobial resistance and are therefore linked to vertical inheritance rather than horizontal gene transfer. The presence of genes coding for antimicrobial resistance in bacterial strains that were unlikely to be in contact with human made antibiotics is not without precedent. Bacterial antimicrobial resistance is ancient and antibiotic resistance genes were identified in human populations that were not in contact with human-made antibiotics (D'Costa *et al.*, 2011; Clemente *et al.*, 2015). Antimicrobial resistance in *Lp. plantarum* has been linked to defense against antimicrobial metabolites from plants, fungi, and other bacteria (Pswarayi *et al.*, 2022). In keeping with the assumption that core genome antimicrobial resistance in *Lp. plantarum* likely targets secondary plant metabolites with antimicrobial activity, the phenotypic

antibiotic resistance of indri isolates was below the cut-off values established by EFSA (Rychen *et al.*, 2018).

Indris are folivorous nonhuman primates living exclusively on trees. They developed a very large cecum and colon due to their need to digest a large amount of vegetal material (Powzyk & Mowry, 2007). Indris feed on many plant species showing "behavioral flexibility" in the diet. Indri individuals in the Maromizaha forest utilized 138 different species of plants for food (Randrianarison *et al.*, 2022). The indri diet is dominated by leaves, especially young leaves (85%), fruits (13%), and other foods (2%), including flowers (1 %), buds (0.4%), and bark/moss (0.02%). Indris are geophagic and soil represents about 0.6% of the daily diet (Randrianarison *et al.*, 2022). All these foods may be considered a source of essential nutrients but also of beneficial microorganisms (Borruso *et al.*, 2021). Natural exposure to environmental microbes is important for maintaining microbial diversity. (Poly)phenols and tannins are present in many feed plants of indris, e.g., *Ravensara* sp. (Lauraceae), *Tina striata* (Sapindaceae), and *Xylopia lemurica* (Annonaceae) (Randrianarison *et al.*, 2022). Plant tannins form indigestible complexes with digestive enzymes and ingested proteins and thus limit nitrogen assimilation (Karl J. Siebert, Nataliia V. Troukhanova & Lynn Penelope Y, 1996; Scalbert *et al.*, 2000). The fact that indris consume soil can be considered a practice for detoxifying plants that, due to anti-nutritive or toxic compounds, might not be edible (Borruso *et al.*, 2021). Another hypothesis about geophagy is that soil consumption enhances the bioactivities of plant. For example, chimpanzees ingest plants with relevant pharmacological properties, such as antimalarial activity, which is enhanced by soil components (Klein, Fröhlich & Krief, 2008).

Some lactic acid bacteria are adapted to grow in plants where phenolic compounds are abundant (Duar *et al.*, 2017; Gaur & Gänzle, 2023). Specifically, *Lp. plantarum* is known to be more tolerant to phenolic compounds than other bacterial groups (López de Felipe, de Las Rivas & Muñoz, 2021;

Pswarayi *et al.*, 2022). Conversion of phenolic acids by decarboxylation or by reduction is a mechanisms for detoxification as the antimicrobial activity of the products is lower when compared to the substrates (Sánchez-Maldonado, Schieber & Gänzle, 2011). This study confirms and extends prior observations that key enzymes for metabolism of phenolic compounds are part of the core genome of *Lp. plantarum* (Gaur & Gänzle, 2023; Gaur, Chen & Gänzle, 2023). All *Lp. plantarum* strains converted the hydroxycinnamic acids caffeic acid, ferulic acid and *p*-coumaric acid by decarboxylation and / or reduction to the corresponding dihydrocinnamic acids. Studies with mutants of *Furfurilactobacillus milii in vitro* and *in situ* provide confidence in attributing the phenotype, conversion of hydroxycinnamic acids, to the genotype, i.e. the presence or absence of decarboxylases or reductases (Gaur *et al.*, 2022). The ability of *Lp. plantarum* to metabolize phenolic compounds supports the hypothesis that diet-derived intestinal microbes help to decrease the adverse effects of dietary phenolic compounds.

Indris live in familiar groups composed of a socially monogamous and stable reproductive pair, sometimes with their offspring and with sub-adults of up to six family members. Each group lives in a home range of about 0.2 km² (Bonadonna *et al.*, 2017). These groups live separately even if they occasionally share the same territory (Powzyk & Mowry, 2007; Bonadonna *et al.*, 2017, 2020). In the present work, 18 subjects belonging to five different groups were studied. From these subjects, we were able to isolate lactobacilli only from two individuals, Dary, six months old (strain D 6), and Zafy, six years old (strains Z1 and Z3) (Correa *et al.*, 2021), which belong to the family groups 2 MZ and 8 MZ, respectively. The home range distance between 2 MZ and 8 MZ groups is about 2.5 km. The analysis of the genomes of strains D6 (2 MZ family group) and Z1 and Z3 (8 MZ family group) showed that they are very similar, with fewer than 10 SNPs. In outbreak investigations, a cut-off of 10 - 20 SNPs is used to delineate the same strain (Pightling *et al.*, 2018). The three isolates D6, Z1

and Z3 thus share a very recent common ancestor even if their hosts belong to different family groups. Due to the considerable distance separating the two individuals, a direct exchange of intestinal microbes is less likely than acquisition of the same strain by consumption of the same plants or soil containing the strain of *Lp. plantarum.*

Microbiome research can improve conservation outcomes but few efforts have been made to integrate the biodiversity of host-associated microbiota as an essential component of wildlife management practices and thus to evaluate approaches for maintaining microbial diversity to achieve conservation objectives (Trevelline *et al.*, 2019). Symbiotic gut microorganisms in herbivorous species, such as indris, are essential not only for the digestion of recalcitrant plant fibers but also for the key role in degrading antinutritive plant secondary metabolites that are harmful to the host (Wakibara *et al.*, 2001). In this scenario, strains of *Lp. plantarum* with the ability to degrade phenolic compounds could significantly improve the host's ecological fitness.

One reason for indri's failure to grow in a controlled environment could be the insufficient dietary variation that determines the loss of essential microbial taxa from the gastrointestinal tract, altering the microbiome's functional capacity and nutritional efficiency. A functionally-limited microbiome could have severe health implications for the host, which may have lost key toxin- and plant-degrading capabilities (Clayton *et al.*, 2018). Innovative ideas and best practices are critical for developing programs and policies to prevent species extinction. For wildlife, such as primates, better knowledge of the microbial composition of wild individuals is the basis for achieving "microbiome rescue".

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Competing interests

The authors declare no conflict of interest

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Author contribution statement

N.Q., G.G., M.M., F.C., D.S., L.B., and A.C.M. conducted experimentation and prepared figures; C.P., D.V. and C.S. prepared sections of the manuscript draft; M.G.G. and P.M. designed the study and wrote the manuscript draft; all authors reviewed and approved the manuscript.

Data Availability Statement

All data that was generated or used in the manuscript are contained in the manuscript, online supplementary files, or genomes sequences submitted to GenBank.

Supporting Information

Table S1. Genome features of Z1, Z3 and D6 used in the present study.

Table S2. Genomes used for the phylogenetic analysis of *Lp. plantarum* from different origins.

Table S3. Individuals from which fecal samples were obtained and analysed.

Table S4. Carbohydrate fermentation pattern of isolates.

Figure S1. BOX PCR patterns of strains D6 (lane 2), Z1 (lane 3) and Z3 (lane 4). Lane 1 shows GenRuler^{TM} 1-kb DNA ladder (ThermoFisher Scientific). The arrow highlights the additional band in Z3 strain.

Figure legends

Figure 1. Phylogenetic tree of 97 strains of *Lactiplantibacillus plantarum* based on the maximum likelihood method. The color strip in different colors represents strain isolation sources. The genomes used for the phylogenetic tree are provided in the supplementary Table S2.

Figure 2. Heatmap showing the presence of genes coding for metabolism of phenolic compounds and for antibiotic resistance in ninety-seven strains of *Lp. plantarum* strains. Different colors and shapes represent different gene sets. The following genes were used the BLAST analysis for metabolism of phenolic compounds (left to right): Est_1092, WP_015825406, hydroxycinnamic acid esterase (Esteban-Torres *et al.*, 2015); HcrB, YP_004889276.1, hydroxycinnamic acid reductase (Santamaría *et al.*, 2018a); lp_2945 (LpdC), WP_003644796, hydroxybenzoic acid decarboxylase (Rodríguez *et al.*, 2008); Lp_0796, YP_004888771.1, hydroxycinnamic acid esterase (Esteban-Torres *et al.*, 2013); Lp_2953, WP_011101978.1, hydroxycinnamic acid esterase (Reverón *et al.*, 2017); Pad, WP_003641609, hydroxycinnamic acid decarboxylase (Rodríguez *et al.*, 2008); TanB, YP_004890536, hydroxybenzoic acid esterase (Iwamoto *et al.*, 2008); VrpA, WP_011102053, vinylphenol reductase (Santamaría *et al.*, 2018b); TanA, WP_003640628, extracellular tannase (Jiménez *et al.*, 2014).

Figure 3. Metabolites from hydroxycinnamic acids produced by the three strains of *Lp. plantarum* after 24 h or 10 days of fermentation in the presence of ferulic acid **(Panel 1)**, coumaric acid **(Panel 2)** or caffeic acid **(Panel 3)**. DHF: Dihydroferulic acid; 4-VG: 4-vinylguaiacol; 4-VP: 4-vinylphenol; 4-EP: 4-ethylphenol; 4-VC: 4-vinylcathecol; 4-EC: 4-ethylcatechol. For the same compound at the same time of sampling (24 h or 10 days), different letters indicate significant differences at $p \leq 0.05$

Figure 4. The plantaricin (*pln*) loci from three strains of *Lactiplantibacillus plantarum* Z3, Z1, and D6, with *Lp. plantarum* WCFS1 (13) as a reference. Operons are color-coded based on their predicted

functions: blue, unrelated to bacteriocin formation but signifies the upper boundary of *pln* loci; yellow, structural genes for the two-peptide bacteriocin JK; pink, structural genes for four putative proteins, of which *PlnN* appears to contain an N-terminal double-glycine leader consensus; orange, structural genes for a quorum-sensing network necessary to express all genes in the *pln* locus; red, structural genes for the two-peptide bacteriocin EF; purple, structural genes for *plnGH*, an ABC-transporter and an accessory protein, respectively, while the role other five genes in the *pln* bacteriocin biosynthesis is unknown.

Table 1. Resistance of indri isolates of *Lp. plantarum* against therapeutic antibiotics.

Strains	**Antibiotic (mg L^{-1})**							
	Ampicillin (2 mg L^{-1})*	Gentamicin (16 mg L^{-1})	Kanamycin (64 mg L^{-1})	Streptomycin (n.r.)	Erythro-mycin (1 mg L^{-1})	Clindamycin (4 mg L^{-1})	Tetracycline (32 mg L^{-1})	Chlor-amphenicol (8 mg L^{-1})
Z1 and D6	0.125	2	32	32	0.125	0.5	4	2
Z3	0.125	1	32	32	0.125	0.5	4	2

*In bracket antibiotics cut-off as defined by EFSA for *Lp. plantarum* (Rychen *et al.*, 2018); n.r., not required

Tree scale: 0.01

Isolation source

- Lemur
- Cereal
- Meat
- Dairy
- Human
- Vegetable
- Insect

ANI

95 95.5 96 96.5 97 97.5 98 98.5 99 99.5 100

AZCU000000000
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GCA 001302645.1
LP51
GCA 004683785.1
GCA 012689225.1
GCA 003611015.1
FUA3073
GCA 002407395.1
LP03
GCA 002205775.2
GCA 003352125.1
GCA 003627355.1
LP24
LP23
GCA 000410795.1
FUA3319
FUA3171
FUA3309
GCA 016415605.1
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GCA 004028315.1
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FUA3247
FUA3183
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GCA 001296095.1
Z1
D6
Z3
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GCA 001675425.1
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Tree scale: 0.01

Isolation source

- Lemur
- Cereal
- Meat
- Dairy
- Human
- Vegetable
- Insect

Phenolic genes

- Est_1092
- HcrB
- lp_2945
- lp_0796
- lp_2953
- Pad
- TanB
- VrpA
- MprB
- TanA

Antibiotic resistent genes

- MDR/MFS Transporter
- ABC-F Transporter
- ABC-F Ribosomal Protection Protein
- Cell Membrane/Wall Protein
- Dihydrofolate Reductase
- Vancomycin Resistance Gene
- Disinfectants
- Multidrug (Aminoglycoside, Macrolide, Phenicol, Diaminopyrimidine, Oxazolidinone)
- Lincosamides
- Fosfomycin
- Tetracycline
- Fluoroquinolone
- Multidrug (Streptogramin, Macrolide, Oxazolidinone, Pleuromutilin, Tetracycline, Lincosamide, Phenicol)
- Macrolide, Fluoroquinolone, Rifamycin
- Bacitracin
- Pleuromutilin
- Fusidic acid
- Defensin (Cationic Peptides)
- Diaminopyrimidines
- Vancomycin

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LP51
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GCA 012689225.1
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GCA 001296095.1
Z1
D6
Z3
GCA 001278015.1
GCA 001675425.1
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TanA
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Lp_2953
Lp_0796
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HcrB
VrpA
pad
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gb|AAW38464.1|ARO:3004572|Staphylococcus (lmrS)
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gb|KIX81495.1|ARO:3002813|lmrB
gb|ABV18113.1|ARO:3001329|mdtG
gb|APB03215.1|ARO:3003981|tetB(48)
gb|APB03214.1|ARO:3003980|tetA(48)
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gb|AYV52072.1|ARO:3002882|lmrD
gb|ABH10964.1|ARO:3002830|vgaALC
gb|ACX92986.2|ARO:3002832|vgaD
gb|WP_10467188.1|ARO:3003948|efrA
gb|CDO61516.1|ARO:3003949|efrB
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gb|ABX00624.1|ARO:3002881|lmrC
gb|AAA99504.1|ARO:3002987|bcrA
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gb|CAA36304.1|ARO:3000251|msrA
gb|AAC76093.1|ARO:3002986|bacA
gb|CAC99773.1|ARO:3003770|Listeria (mprF)
gb|EOD99669.1|ARO:3002875|dfrE
gb|ABO40886.1|ARO:3004645|dfrI
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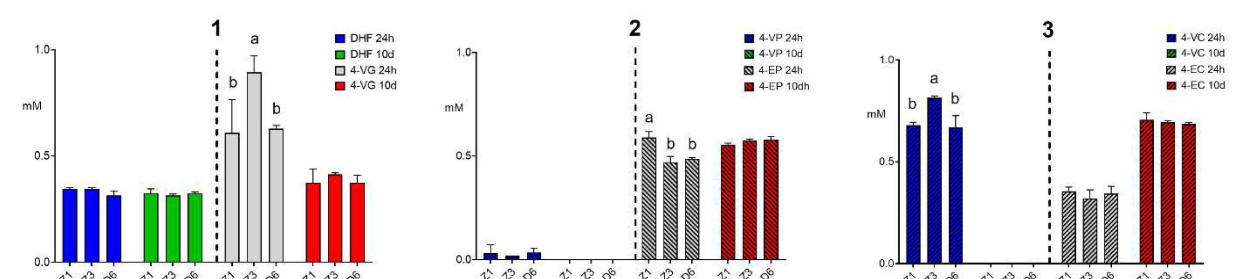
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Z1
Z3
D6
2
4-VP 24h
4-VP 10d
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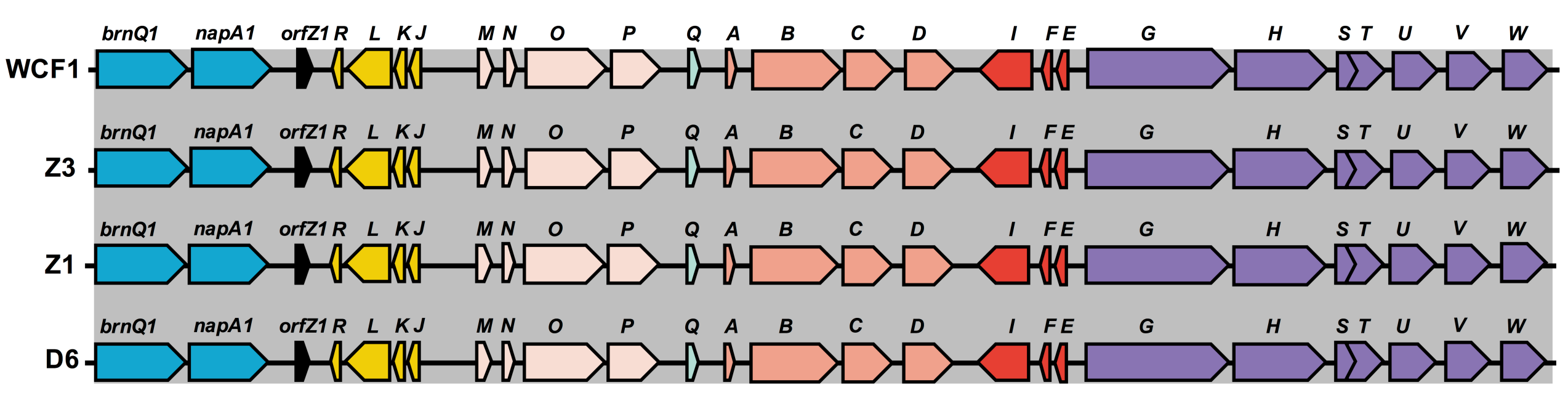
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B
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F
E
G
H
S
T
U
V
W
WCF1
Z3
Z1
D6

Online supplementary material to

Physiological and genomic characterization of *Lactiplantibacillus plantarum* isolated from *Indri indri* in Madagascar

Nanzhen Qiao, Gautam Gaur, Modesto Monica, Fabio Chinnici, Donatella Scarafile, Luigi Maria Borruso, Antonio Castro Marin, Caterina Spiezio, Daria Valente, Camillo Sandri, Michael G. Gänzle, Paola Mattarelli

Table S1. Genome features of Z1, Z3 and D6 used in the present study.

Table S2. Genomes used for the phylogenetic analysis of *Lp. plantarum* from different origins.

Table S3. Individuals from which fecal samples were obtained and analysed.

Table S4 Carbohydrate fermentation pattern of isolates

Figure S1. BOX PCR patterns of strains D6 (lane 2), Z1 (lane 3) and Z3 (lane 4). Lane 1 shows GenRuler^{TM} 1-kb DNA ladder (ThermoFisher Scientific).

Table S1. Genome features of D6, Z1 and Z3 used in the present study.

Strain	Number of assembled contigs	Genome length	Average GC percentage	Number of predicted ORFs	tRNA	rRNA	L50	N50
D6	24	3,228,147	44.55	3158	61	2	5	268711
Z1	24	3,228,236	44.56	3159	61	2	5	268711
Z3	24	3,226,195	44.55	3164	61	2	5	268711

Table S2. Genomes used for the phylogenetic analysis of *Lp. plantarum* from different origins.

Provided as separate excel file.

Table S3. Individuals from which fecal samples were obtained and analysed.

Indris's name	Sex[a] and age	Group	date of sampling
Bevolo	F; > 6 a	group 1 (1MZ)	2018-12-05
Cami	F; 1 a	group 1 (1MZ)	2018-12-05
Jery	M; > 6 a	group 1 (1MZ)	2018-12-05
Tovo A[b]	F; 2a	group 2 (2MZ)	2018-12-05
Dary[c]	U; 0.5 a	group 2 (2MZ)	2018-12-05
Max	M; >6 a	group 2 (2MZ)	2018-12-05
Soa	F; > 6 a	group 2 (2MZ)	2018-12-05
Tovo 2017 B[b]	F; 2 a	group 2 (2MZ)	2018-12-07
Anà	F; 1 a	group 3 (3MZ)	2018-12-07
Maha Gaga	M; >; 6 a	group 3 (3MZ)	2018-12-07
Mena	F; >; 6 a	group 3 (3MZ)	2018-12-07
Eva	F; > 6 a	group 4 (4MZ)	2018-12-06
Koto	M; >6 a	group 4 (4MZ)	2018-12-06
Befotsy	F; > 6 a	group 6 (6MZ)	2018-12-05
Zokibe	M; > 6 a	group 6 (6MZ)	2018-12-05
Bema Soandro	F; > 6 a	group 8 (8MZ)	2018-12-06
Emé	M; 1 a	group 8 (8MZ)	2018-12-06
Jonah	M; > 6 a	group 8 (8MZ)	2018-12-06
Zafy	M; 6 a	group 8 (8MZ)	2018-12-06

[a] F, female; M, male; U, undetermined

[b] Tovo has been sampled two times.

[c] Dary has disappeared in the first year of his life (nel 2018) so its sex remains unknown

Table S4. Carbohydrate fermentation pattern of isolates

Compounds[a)]	Strains		
	D6	Z3	D1
L-Arabinose	+	+	+
D-Ribose	+	+	+
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Rhamnose	w	w	w
D-Mannitol	+	+	+
D-Sorbitol	+	+	+
N-AcetylGlucosamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin Ferric Citrate	+	+	+
Salicin	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose	+	+	+
D-Sucrose	+	+	+
D-Trehalose	+	+	+
D-Melezitose	+	+	+
Gentiobiose	w	w	w
D-Turanose	+	+	+
Potassium Gluconate	w	w	w

[a)] Glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl-β-xylopyranoside, L-sorbose, dulcitol, inositol,Methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, D-melibiose, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate and 5-ketogluconate were not metabolized.

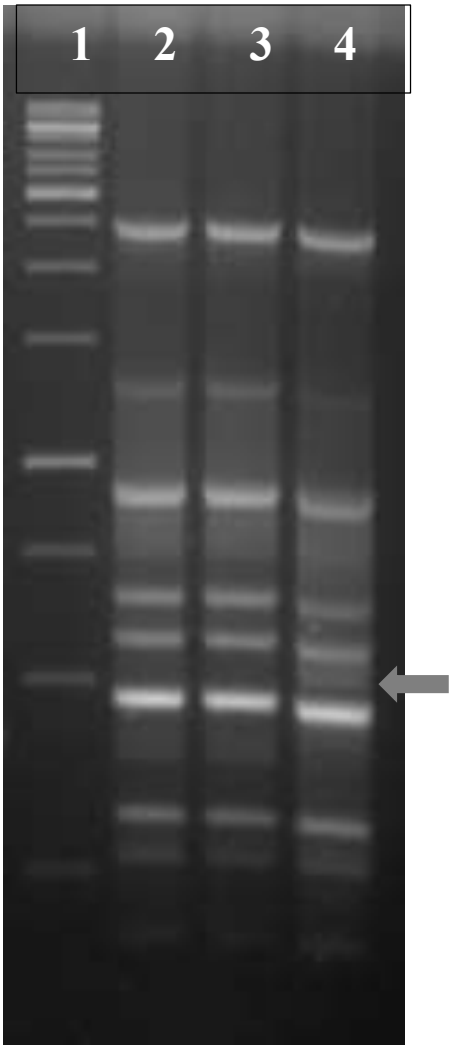

Figure S1. BOX PCR patterns of strains D6 (lane 2), Z1 (lane 3) and Z3 (lane 4). Lane 1 shows GenRuler^{TM} 1-kb DNA ladder (ThermoFisher Scientific). The arrow highlights the additional band in Z3 strain.