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Comparative genomics and single-cell transcriptomics reveal the ecological adaptation strategies of *lactobacillus* with different lifestyles

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ABSTRACT: Lactobacilli with different lifestyles show different ecological adaptabilities and host interaction abilities in the intestine. This study conducted a comparative genome analysis of 232 strains of lactobacilli, combined with carbohydrate-active enzyme and colonization factor analysis, and found that the genome of free-living lactobacilli is larger than that of host-adapted lactobacilli and the CAZymes spectrum is closely related to its lifestyle. In vivo experiments showed that host-adapted lactobacilli have significantly better colonization ability in the intestine than nomadic lactobacilli, and can still maintain a high number of live bacteria after stopping intake. Single-cell transcriptome technology was further used to explore the effects of lactobacilli intake on intestinal cells from the host perspective, and it was found that epithelial cells and immune cells (especially T cells and B cells) were most significantly affected by lactobacilli. Specifically, host-adapted lactobacilli (such as Lactobacillus reuteri) showed stronger anti-inflammatory effects in regulating Cd4+Tn cells, Cd4+Th/Treg cells, and Cd8ααT cells, and significantly downregulated the differentiation of pro-inflammatory cells such as Th1, Th2, and Th17. These findings not only clarify the relationship between Lactobacillus lifestyle and its genomic characteristics, colonization ability and immunomodulatory function, but also provide an important theoretical basis for the formulation of precise probiotic intervention strategies based on Lactobacillus.

Keywords: Lactobacillus; lifestyle; single-cell transcriptome; colonization; immune cells

1. Introduction

Lactobacillus has a long history of human use and plays an important role in the production of fermented foods (e.g., vegetables, meat, and especially fermented dairy products) and is classified as edible strain by the European Food Safety Authority [1]. *Lactobacillus* has a variety of probiotic functions, such as antimicrobial, anti-inflammatory and immunomodulatory properties[2], and is also widely used in probiotic products[3, 4]. Since 2020, the genus *Lactobacillus* has been reclassified and currently includes 25 genera, which include more than 200 species or subspecies[5]. *Lactobacillus* can be divided into three main lifestyles: free-living, nomadic and host-adapted based on differences in habitat, metabolic capacity, growth

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temperature, etc. [6]. Free-living *Lactobacillus* species are mainly found in environmental or plant-related niches, such as *Lentilactobacillus buchneri* (*L. buchneri*) and *Levilactobacillus brevis* (*L. brevis*). Nomadic *Lactobacillus* species, such as *Lactiplantibacillus plantarum* (*L. plantarum*) and *Lacticaseibacillus rhamnosus* (*L. rhamnosus*), can live freely between the host and the environment, with little evidence that they specialize in a particular habitat. They are found in invertebrate hosts, various body sites of vertebrates (e.g., intestines, mouth, vagina), and food materials such as meat, fish, vegetables, and raw or fermented dairy products [7], showing flexible metabolic and stress response capabilities. In contrast, host-adapted *Lactobacillus* species, such as *Limosilactobacillus johnsonii* (*L. johnsonii*), *Limosilactobacillus reuteri (L. reuteri),* and *Ligilactobacillus ruminis* (*L. ruminis*), exhibit reduced metabolic flexibility but are enriched in adhesion and immune interaction genes, reflecting their long-term adaptation to vertebrate or invertebrate hosts [6].

Microbial lifestyles are closely related to their colonization and physiological functions within the host. Pathogenic *E. coli* can utilize sugars that are not available to commensal bacteria, thereby gaining a colonization advantage in the mouse gut [8]. Some enteric pathogens have a dual lifestyle between animal and plant hosts, and their animal virulence factors such as the type III secretion system (T3SS) have a limited role in plants, suggesting a differential colonization strategy between hosts [9]. In the human gut, the loss of spore-forming ability often accompanies host adaptation in bacteria of the phylum Firmicutes, and despite their greater abundance in individuals, their prevalence and transmission are reduced, suggesting that they are associated with host-specific adaptation[10]. In addition, the intestinal pathogen *Enterococcus gallinarum* has diverged into different lineages through intrahost evolution, with mucosal-adapted strains evading host immunity, and a similar evolutionary pattern has been observed in host-adapted *L. reuteri* in a monoculture model[11]. It has been shown that endogenous *L. reuteri* is more likely to colonize the gut than nomadic *L. plantarum*[12] and that the two may rely on different mechanisms in alleviating diarrhoea caused by *E. coli* infection [13, 14]. However, current studies on *Lactobacillus* has focused on the physiological properties[15] and probiotic effects of specific strains [16, 17], and there is a lack of systematic investigation on the relationship between its lifestyle and colonization ability. As a microorganism with diverse lifestyles, *Lactobacillus* is also worthy of in-depth study of its colonization differences and function in different hosts, thus providing important guidance for the development of personalized probiotic therapies.

Omics technology provides a new research method for revealing the phenotypic characteristics of microorganisms and their interaction mechanisms with the host. Among these methods, comparative genomics can be used to analyze the genetic diversity of strains systematically and reveal differences in their metabolic potential, environmental adaptability and host interactions [18]. In the human intestinal microbiome, single cells are highly heterogeneous, and simple macro-omics methods cannot meet these requirements. It is necessary to introduce single-cell analysis to solve biological problems such as microbial function at the strain level [19]. Previous studies have used single-cell methods to reveal changes in immune responses in acute liver failure [20] and the enrichment mechanism of *Bacteroides* spp. mediated by the

dietary fiber inulin [21]. Therefore, combining genomic and single-cell level analysis is an effective means to reveal changes in intestinal cell populations and their heterogeneity after *Lactobacillus* administration, which will help to provide a theoretical basis for analyzing the regulatory mechanism of probiotics on intestinal microecology.

This study conducted a comprehensive comparative genomic analysis of three representative *Lactobacillus* species with different lifestyles (host-adapted, nomadic, and free-living) to explore how ecological adaptation shapes their genomic structure and functional potential. By integrating genomics, animal experiments, and single-cell transcriptomics analyses, we aimed to elucidate the relationships between lifestyle-related genomic characteristics, gut colonization capacity, and host immune regulation. This integrative approach provides a new perspective for understanding the evolutionary and functional mechanisms of ecological differentiation among *Lactobacillus* species in the host gut environment.

2. Methods

2.1 Experimental strains

The genome analysis in this study involved five *Lactobacillus* species including a total of 232 strains (*L. johnsonii*, *L. reuteri*, *L. rhamnosus*, *L. plantarum* and *L. brevis*). All genome sequence information was from the conserved strains at the Food Microbial Strain Conservation Center of Jiangnan University, and the V3 and V4 regions of the 16S rRNA gene were sequenced and sketched via the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Habitat information, genome size, and GC content of these strains were also collected. The four different strains used in the experiment were *L. plantarum* CCFM8610, *L. plantarum* QHLJZD20L2, *L. reuteri* 325, and *L. reuteri* FSH2M2. *L. reuteri* was isolated from animal fecal samples, which is in line with the host-adapted lifestyle of *L. reuteri*; *L. plantarum* included strains isolated from both food and fecal samples. strains, which is consistent with their nomadic lifestyle.

2.2 Genetic analysis of enzymes related to carbohydrate metabolism

Based on the existing reports, the differences in 3 classes of enzymes, glycoside hydrolases(GHs), glycosyltransferases(GTs), and polysaccharide lyases(PLs), were analyzed among different lifestyles and among different species of *Lactobacillus* to represent the overall characterization of their CAZymes[22, 23]. The annotation method of CAZymes was based on the past reports[24], and the screening conditions were protein sequences > 80 aa, sequence similarity > 45%, reference sequence coverage > 50%, and a threshold value of e-value < $1e^{-5}$.

2.3 Genetic analysis of key colonization factors

Intestinal colonization factors mainly include mucus binding proteins, quorum sensing genes (luxS), flagellar genes (pili), serine-rich glycoprotein adhesion, S-layer proteins and antimicrobial peptides[25, 26]. Using the English names of these key colonization factors as search keywords, reference sequences were retrieved and downloaded from the Refseq database of the NCBI and the KEGG database to construct a reference protein sequence library. The protein sequences of *Lactobacillus* were subjected to BLASTP

comparison with the above constructed reference protein sequence library, with comparison thresholds of an e-value < $1e^{-5}$, reference sequence coverage > 50% and sequence similarity > 45%[27].

2.4 Experiments on the intestinal colonization of Lactobacillus in mice with different lifestyles

2.4.1 Preparation of intragastric bacterial solution

After activating the *Lactobacillus* for 2 generations, inoculated it into fresh MRS medium, cultured it at 37℃ for 18 h, and then centrifuged it at 6000 r/min for 3 min. The supernatant was discarded, the bacterial sludge was washed twice with sterile saline, and the bacterial sludge was resuspended in saline. The bacterial sludge was counted with MRS medium until the concentration of the resuspended bacterial sludge reaches about 2.5×10^{10} CFU/mL. Each mouse was intragastrically gavaged with 0.2 mL per day, and the amount of gavage was about 5×10^9 CFU[28].

2.4.2 Experimental group design

According to the differences in the *Lactobacillus* administered by oral gavage, the mice were randomly divided into 5 groups, namely: *L. plantarum* CCFM8610 group, *L. plantarum* QHLJZD20L2 group, *L. reuter* 325 group, *L. reuteri* FSH2M2 group and blank control group. The experiment lasted for 42 days in total, and the first 7 days (day (-7) to day 0) were the adaptation period. From day 1 to day 14, the mice were treated with mixed broad-spectrum antibiotics by oral gavage. From day 16 to day 20, fecal microbiota transplantation was performed on the mice using fecal samples from normal people to construct a humanized mouse model. Faecal samples were processed according to G. Hohmann et al[29]. From day 22 to day 25, each mouse in the blank control group was gavaged with normal saline, with an oral gavage volume of 0.2 mL/day. After gavage on day 25, 5 mice were euthanized, and the complete intestine was dissected and taken to collect the contents of different intestinal segments. The mice in the other experimental groups were gavaged with *Lactobacillus* from day 22 to day 25, with a gavage dose of 5 × 109 CFU/day, and 5 mice were killed on day 25 (after gavage), day 26, day 27, and day 28, respectively, and the complete intestine was dissected and the contents of different intestinal segments were collected. The feces of the remaining 5 mice in the blank control group and 5 mice in each experimental group were continuously collected until day 35.

2.5 Method for determining the number of Lactobacillus in intestinal contents and feces

Five independent biological replicates were performed for each group. A certain amount of intestinal contents and feces samples (0.05 g) were weighed, and the total DNA in the feces and intestinal contents of the mice was extracted via the Fast DNA Spin Kit for Feces. The total DNA template of the bacterial flora (three parallel experiments were performed for each sample) was used to amplify *L. plantarum* and *L. reuteri* from the total DNA via the primers LPL-F, LPL-R, LRE-F, and LRE-R[30]. Referring to the instructions of the Supermix Kit, a 10 μL system was selected for RT-qPCR. The number of live *L. plantarum* and *L. reuteri* in different samples was quantified via the $2^{-\Delta\Delta CT}$ method.

2.6 Effects of different lifestyle Lactobacillus on healthy hosts

The mice were randomly divided into 3 groups (n = 5 per group): the *L. plantarum* CCFM8610 intervention group (*L. plantarum* group), the *L. reuteri* 325 intervention group (*L. reuteri* group) and the control group (CO group). The experiment was divided into 3 weeks. The first week was the adaptation period, and all the mice were free to eat. The second and third weeks constituted the intervention period. The control group mice were gavaged with 0.2 mL/mouse sterile saline every day; the intervention group mice were gavaged with 0.2 mL/mouse *Lactobacillus* every day. On the 22nd day, the mice were euthanized, and the colon tissues were dissected and collected in cell culture medium for single-cell transcriptome sequencing.

2.7 Colonic epithelial cell isolation

Three mice were randomly selected from each group, and the colon was cut into 1 cm long segments and rinsed twice with cold PBS. The tissue was placed in an EP tube, minced using surgical scissors and subsequently resuspended by adding PBS, and the tissue was enriched by passing through a 70 μm cell strainer. Centrifugation was performed at 300 × g for 5 min at 4℃, most of the supernatant was discarded, and the cellular precipitate at the bottom of the centrifuge tube was blown and resuspended. Trypsin (0.25% HBSS solution) was added to digest the tissue at 37℃ until single cells were obtained (mixing every 8 min during this period). After termination of digestion, added 3 mL of erythrocyte lysis buffer and incubated at room temperature for 3 min. The mixture was subsequently centrifuged at 300 × g for 5 min at 4℃ and discard the supernatant. Added an appropriate amount of pre-cooled PBS buffer (2% FBS) and gently aspirated to resuspend the cells. Cell viability > 85%, nucleation > 70%, and clumping < 15% were confirmed according to the manufacturer's recommendations.

2.8 Single-cell transcriptome sequencing library construction and sequencing

Using a droplet-based sequencing platform (10 × Genomics), the Chromium Single Cell 3' Reagent Kit (v3.1 Chemistry, 10 × Genomics, USA) was used to capture single cells and generate cDNA libraries with cell barcodes and unique molecular identification label number (UMI) information for sequencing. The cDNA library was subsequently sequenced using Illumina HiSeq 4000 (Illumina, USA). A total of 3,630,392,261 reads were obtained from the sequencing results.

2.9 Data quality control and gene expression quantification

Cell Ranger software (10 × Genomics, CA, USA) was used to split the original single-cell RNA sequencing data and process the cell barcodes, and the mouse reference genome was aligned to generate the original gene-cell expression matrix. The R software package Seurat was used to import and process the original expression matrix. The following indicators were used to perform quality control on the original data to remove low-quality data: (1) cells with a total number of genes detected of less than 200; (2) UMI number > 99th percentile; (3) cells with a mitochondrial gene expression ratio > 25%; (4) the number of cells with this gene detected was < 3. After quality control, the “DoubleFinder” package was used to predict and remove potential double cells. The expression matrix data after removing the double cells were

normalized (the normalization method was log normalization) to obtain a new expression matrix for subsequent downstream data analysis[31].

2.10 Unsupervised Dimensionality Reduction and Cluster Analysis

The "Seurat" package was used to perform dimensionality reduction and cluster analysis on the expression matrix obtained[32]. First, the "FindVariableFeatures" function was used to identify highly variable features and obtain highly variable genes. The expression matrix was then scaled and the matrix was subjected to unsupervised principal component analysis via the "RunPCA" function. In addition, the "RunHarmony" function of the "harmony" package was used to correct the single-cell RNA datasets of different samples to remove batch effects. The principal component analysis results were then visualized and the appropriate dimensions were selected to reduce the dimensionality of the dataset. Finally, the cells were unsupervisedly clustered with the selected dimensionality reduction dimension, and the cluster analysis results of the cells were visualized using UMAP (Uniform Manifold Approximation and Projection) and t-SNE (t-Distributed Stochastic Neighbor Embedding).

2.11 Identification of differentially expressed genes in cell populations and cell annotation

The differentially expressed genes (DEGs) of the cell populations were identified using the "Seurat" package[32]. The highly expressed genes of each cell population were annotated using the "FindAllMarkers" function (the test method was the Wilcoxon rank sum test), and genes with $|\log_2 \text{fold change}| > 0.25$ and FDR < 0.01 were selected as DEGs[33]. Subsequently, each cell type was identified and annotated by combining known marker genes and DEGs of this dataset. See Supplementary Table 1 for specific identification of cell types and representative marker genes.

2.12 KEGG pathway enrichment analysis of cell subgroups

The DEGs of the cell subgroups were enriched based on the KEGG database using the "clusterProfiler" package, and pathways with significant effects ($P < 0.05$) were retained.

2.13 Data statistics and analysis

The statistical and analysis software used in this study included GraphPad Prism 9 (v 9.0.0). For comparisons between two groups, the t-test was used for data that met the normal distribution and homogeneity of variance, and the Mann-Whitney test was used for data that did not meet the above conditions. Spearman correlation analysis was used for correlation analysis. Anosim analysis was used for inter-group differences in the principal component analysis (PCA) data. Unless otherwise specified, all results in this study were considered statistically significant when $P < 0.05$. R language (v 4.3.1) and Python (v 3.11) were used for statistical analysis and single-cell transcriptome analysis.

3. Results

3.1 Comparative analysis of the genomes of Lactobacillus species with different lifestyles

To explore the genomic evolutionary characteristics of *Lactobacillus* strains with different lifestyles, this study analyzed the differences in total reads, GC content, CAZymes and key colonization factor genes in the genomes of 232 *Lactobacillus* strains. The results of genome analysis revealed that there were differences in genome size and GC content between host-adapted *Lactobacillus* (*L. reuteri*, *L.johnsonii*) and nomadic *Lactobacillus* (*L. plantarum*, *L. rhamnosus*) ($P < 0.05$, Supplementary Figure 1A and 1B). Free-living lactobacilli (*L. brevis*) also tend to have larger genomes than host-adapted ones, but the scale and representativeness of the current dataset have some limitations. The three lifestyles of lactobacilli can be distinguished by the two dimensions of genome size and GC content (PERMANOVA: $P < 0.05$; Figure 1A).

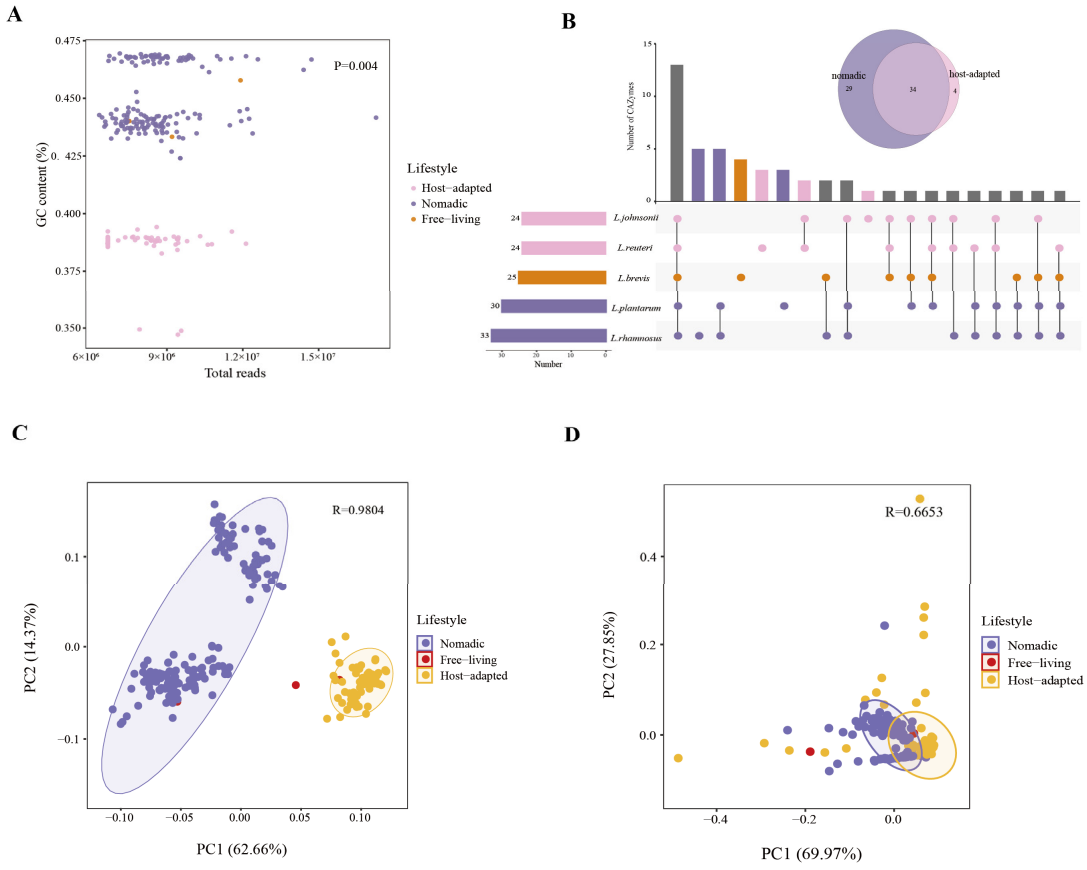

Figure. 1 Comparative genomic analysis of *Lactobacillus* strains with different lifestyles
(A) Correlation analysis between *Lactobacillus* genome size and GC content (%), PERMANOVA test ($P < 0.05$)
(B) The relationship between different lactic acid bacteria and the number of carbohydrate-active enzymes (CAZymes); the Venn diagram shows the overlap between nomadic and host-adapted lactobacilli in CAZymes. The gray column in the upset diagram represents the presence of the enzyme in all five lactic acid bacteria, pink represents the presence of the enzyme only in the host-adapted type, purple represents the presence of the enzyme in the nomadic type, and orange represents the presence of the enzyme only in the free type.
(C) PCA plots of *Lactobacillus* CAZyme genes according to lifestyle (test of variance: ANOSIM).
(D) PCA plots of *Lactobacillus* gut colonization-related genes according to lifestyle (test of variance: ANOSIM).

Lactobacillus has a strong ability to utilize carbohydrates, which plays important roles in its colonization and function in the gastrointestinal tract. This study analyzed carbohydrate-active enzymes (CAZyme) in *Lactobacillus* strains with different lifestyles, and the numbers of GHs, GTs and PLs obtained are shown in Supplementary Figure 2. Among them, the GH0, GH1, GH115, and GH13 families were the

most abundant. A comprehensive comparison of the three lifestyles of *Lactobacillus* revealed that each of them has a unique CAZyme gene family, of which 26 CAZyme gene families exist only in nomadic *Lactobacillus*, which is the largest number among the three types of *Lactobacillus*. Further comparison of nomadic and host-adapted *Lactobacillus* strains showed that there was a large overlap in gene family composition between the two strains. We found that 34 of these gene families, such as GH0, GH2, GH13_11, and GT2, exist in both nomadic and host-adapted strains (Figure 1B). Multiple carbohydrate-active enzyme families, including GH1, GH3, GH13, GH32, and GH78, were detected in *L. plantarum*, while these families were not found in *L. reuteri*. Conversely, enzyme families specific to *L. reuteri*, such as GH115, GH120, GH13_18, and GH43_26, were not found in *L. plantarum*. Except for some strains, the CAZymes profiles of different *Lactobacillus* strains were strongly correlated with their lifestyles, and PCA analysis could distinguish nomadic and host-adapted *Lactobacillus* strains to a certain extent (Figure 1C).

Some intestinal tissue anchoring/adhesion structures and some effector metabolites of bacteria play important roles in host-microbe interactions[25, 26]. Therefore, the distribution of currently known colonization-related genes in different *Lactobacillus* species was further explored and used as a certain basis to investigate the colonization ability of different lifestyle *Lactobacillus* species in the host. As shown in Supplementary Figure 1C, the distributions of different colonization factors among different *Lactobacillus* species have some differences. Except for the S-layer protein gene, which was found only in *L. brevis* (nomadic), all the other colonization factors were distributed among different *Lactobacillus* species. The numbers of genes encoding flagella (LPXTG, sortase, pilus) related genes were all significantly higher in nomadic *Lactobacillus* than in host-adapted *Lactobacillus* ($P < 0.05$, Mann-Whitney test). Based on the colonization-related genes, PCA plots showed (Supplementary Figure 1D) that different species of *Lactobacillus* were significantly segregated between species ($P < 0.05$) and lifestyles (Figure 1D).

3.2 Colonization of nomadic L. plantarum and host-adapted L. reuteri in vivo

Genomic comparisons revealed substantial differences between *L. plantarum* and *L. reuteri*, including genome size, GC content, and carbohydrate metabolic profiles, consistent with their distinct ecological origins. These genomic and functional distinctions indicate divergent colonization strategies. Therefore, *L. plantarum* (nomadic) and *L. reuteri* (host-adapted) were selected as representative species for *in vivo* validation. We then used RT-qPCR to quantify the location of *Lactobacillus* in the intestines of humanized mice and their changes over time. The RT-qPCR results of fecal samples from mice at different time points revealed (Figure 2A) that the abundance of all *Lactobacillus* strains in the intestine decreased rapidly after the cessation of gavage. Except for *L. reuteri* FSH2M2, the number of fecal *Lactobacillus* strains remained in a relatively stable after the 7th day. A comprehensive comparison of the results for *L. plantarum* (nomadic type) and *L. reuteri* (host-adapted type) revealed that on the 5th day, the number of live *L. plantarum* strains decreased significantly ($P < 0.05$, Mann–Whitney test), while the number of live *L. reuteri* strains did not change significantly, and the number of live *L. reuteri* strains was significantly higher

than that of *L. plantarum* strains($P < 0.05$, Mann–Whitney test). By day 9, the abundance of *L. reuteri* was higher than that of *L. plantarum* (20-fold, p = 0.1564, Mann–Whitney test).

As shown in Figure 2C, *Lactobacillus* in humanized mice on day 4 mainly stayed in the cecum and colon. All *Lactobacillus* strains except *L. reuteri* 325 had the lowest viable counts in the ileum, and there was no significant difference between the viable counts in the cecum and the colon (Figure 2B). Compared with *L. plantarum* (nomadic) and *L. reuteri* (host-adapted) in terms of the distribution of *Lactobacillus*, *L. plantarum* remained slightly higher than *L. reuteri* in the colon and cecum on the 4th day, but the results were not significant.

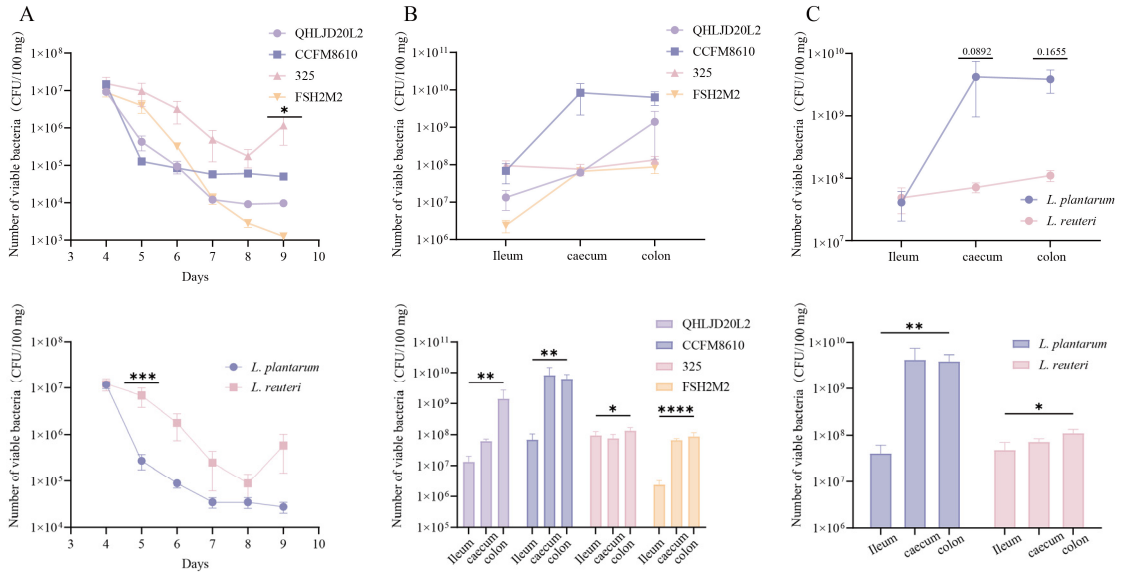

Figure. 2 Colonization sites and changes of *Lactobacillus* in mice

(A) Mean values and standard deviations of CFU counts of different strains (top) and species (bottom) of *Lactobacillus* in the feces of mice from day 4 to day 9.

(B) Mean and standard deviation of CFU counts of different strains of *Lactobacillus* in the contents of different intestinal segments of mice on day 4

(C) Mean and standard deviation of CFU counts of different species of *Lactobacillus* in the contents of different intestinal segments of mice on day 4. * is significant, $P < 0.05$

3.3 Heterogeneous response of immune cells to the administration of different lifestyles of Lactobacillus

There are significant differences in the colonization and distribution of host-adapted and nomadic *Lactobacillus* in the intestine. Currently, related studies have focused mainly on CAZymes, glycogen metabolism genes [34], and bile salt hydrolase (BSH) [35] activities to clarify the colonization relationship between *Lactobacillus* and the host. However, studies have shown that *L. plantarum* can regulate the host immune system and may affect its colonization process [36]. Based on the above findings, this study further explored the correlation between the different cell types in the mouse intestine and *Lactobacillus* colonization from the perspective of the host to further reveal the molecular mechanism of *Lactobacillus*-host interaction.

We found that after *Lactobacillus* administration (Figure 3A), the proportions of epithelial cells and pericytes increased, whereas the proportion of immune cells decreased, but the changes in the proportions of all cell types were not significant ($P > 0.05$, Mann–Whitney test). Therefore, this study further explored the changes in gene expression in mouse intestinal cells after the administration of nomadic *Lactobacillus* or

host-adapted *Lactobacillus*. The DEGs screened are shown in Figure 3B. Overall, 1186 and 1422 DEGs were identified in *L. plantarum* (*L. plantarum* group) and *L. reuteri* (*L. reuteri* group), respectively. After *Lactobacillus* administration, epithelial cells (739), immune cells (655) and Fibroblast-like cells (FLCs, 449) produced the most DEGs (Figure 3C-E). Next, this study performed functional enrichment based on the screened DEGs and found that, consistent with the overall DEG results, the differential functions were mainly concentrated in epithelial cells, immune cells and FLCs (Supplementary Figure 3A).

The modulation of host immune cell gene expression by the administration of different *Lactobacillus* strains may be one of the ways in which *Lactobacillus* affects host intestinal cells and exerts its probiotic function. Therefore, this study further compared the effects of the experimental group on the function of host immune cells compared with the control group. In immune cells (Supplementary Figure 3B and C), host-adapted *L. reuteri* caused more changes than nomadic *L. plantarum*. Both the *L. plantarum* group and the *L. reuteri* group changed 46 signaling pathways, including the MAPK signaling pathway and the interleukin 17 (IL-17) signaling pathway of host colonic immune cells (Figure 3F, $P < 0.05$). In addition, compared with the control group, there were 6 metabolic pathways that showed significant differences only in the *L. reuteri* group (Figure 3F), including the T cell receptor signaling pathway of host immune cells and natural killer cell-mediated cytotoxicity pathways, while *L. plantarum* had no significant effect on these pathways. These findings indicate that although the types of pathways affected by *Lactobacillus* in host immune cells are consistent, the regulatory abilities of *Lactobacillus* with different lifestyles are still quite different.

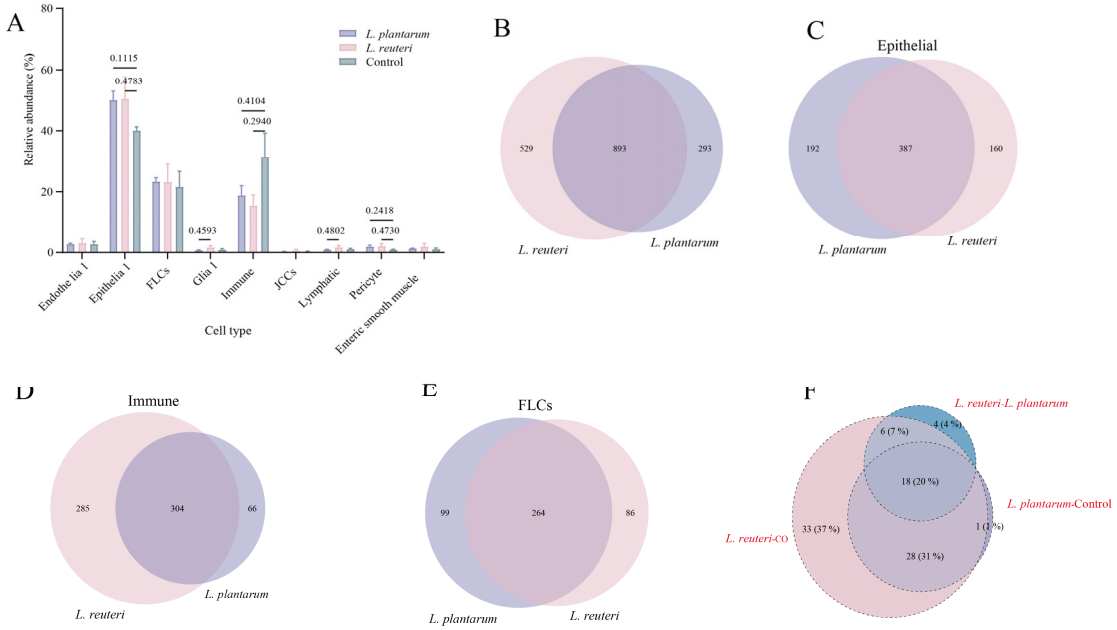

Figure. 3 Host cell changes after *Lactobacillus* administration
(A) Bar graphs demonstrating the percentages of different cell types, mean ± SEM;
(B) Wayne plots of DEGs of mouse intestinal colonocytes in different groups compared with the control group
(C-E) Compared with the control group, epithelial cells (C), immune cells (D) and fibroblasts
cells (E), number of DEGs in epithelial cells (C), immune cells (D) and fibroblast-like cells (E) Wayne plot
(F) Wayne plots demonstrating the functional number of differences between the RE, PL, and CO groups in a two-by-two comparison.

3.4 Different lifestyles of Lactobacillus lead to heterogeneity in mouse colonic T cells

Immune cell subsets may play distinct roles in *Lactobacillus* colonization, so the immune cells identified previously were regrouped, and different immune cell types were identified with reference to classic immune cell subset marker genes. The results are shown in Supplementary Figure 4A and 4B.

Functional enrichment at the immune cell level was performed to investigate the differences in the effects of different lifestyles of *Lactobacillus* intake on immune cells in the mouse colon. The results showed (Figure 4A) that among the different immune cell types, the functions of only four cell types differed between the *L. plantarum* group and the *L. reuteri* groups. Compared with those in the *L. plantarum* group, only the metabolic pathways present in B cells were up-regulated in the *L. reuteri* group, and the rest of the cells were down-regulated in the *L. reuteri* group. Among T cells, the *L. reuteri* group more significantly down-regulated host immune response functions, including the differentiation of pro-inflammatory-related cells (Th1, Th2 and Th17), and enhancement of T-cell receptor signaling pathways, compared to the *L. plantarum* group ($P < 0.05$). The *L. plantarum* group also had similar changes; therefore, *L. reuteri* (host-adapted) with more down-regulation of the relevant pathways may have a stronger probiotic function in terms of the associated regulation of inflammation and this regulatory capacity is associated with T-cells in the host's intestinal tract.

On this basis, in-depth analysis of T cell subpopulation types and DEGs to explore the heterogeneity of mouse intestinal T cells caused by different lifestyle *Lactobacillus* could facilitate the understanding of the targets of *Lactobacillus* to regulate host immune action. By UMAP analysis, we categorized T cells into 13 cell groups (Figure 4B) and based on marker genes (Supplementary Figure 4C) identified 9 cell subpopulations (Figure 4C). A comparison of the DEGs between the *L. plantarum* and *L. reuteri* groups showed that DEGs were mainly present in the $Cd4^+$ Tn cells, $Cd4^+$ Th/Treg cells and Cd8αα T cells between the *L. plantarum* and the *L. reuteri* groups (Figure 4D). These findings suggest that the differences in the regulatory capacity of *Lactobacillus* in T cells with different lifestyles may be related to these three cell subtypes.

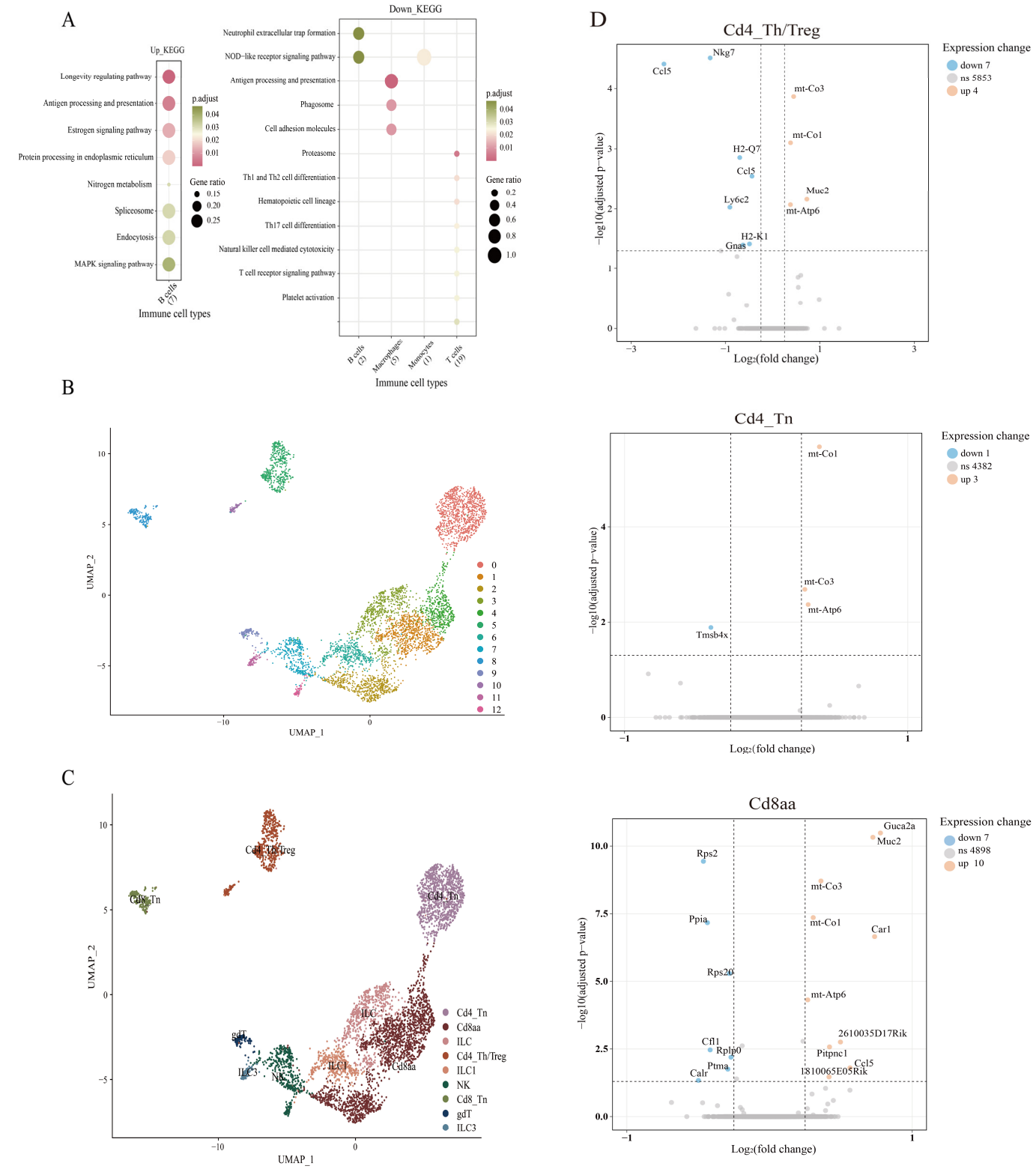

Figure. 4 Effects of different lifestyles of *Lactobacillus* on immune cells
(A) KEGG enrichment analysis of DEGs in *L. plantarum* and *L. reuteri* groups
(B) UMAP plots show T cell populations in mouse colon tissue, colors indicate T cell populations.
(C) UMAP plot demonstrating the distribution of T cell subpopulations in mouse colon tissue, colors indicate immune cell subpopulations;
(D) Volcano plot of differentially expressed genes in $Cd4^{+}$Th/Treg cells, $Cd4^{+}$Tn cells and Cd8αα T cells

4. Discussion

Genomic analyses showed significant differences between different lifestyle *Lactobacillus* species at the genome level, with host-adapted *Lactobacillus* species having smaller genomes and lower GC contents

than nomadic and free-living *Lactobacillus* species. Consistent with the findings of Walter et al [6], genome reduction was strongly associated with host adaptation in *Lactobacillus* species, with host-adapted species having significantly smaller genome sizes compared to nomadic and free-living species. Our previous study[37] also found that nomadic *Lactobacillus* populations tended to have larger genomes, while host-adapted *Lactobacillus* had relatively smaller genomes and host-adapted *Lactobacillus* species had lower GC contents than nomadic and free-living *Lactobacillus*. This may be the same as the Salmonella genome undergoes evolution during host adaptation[38], and studies have shown that relatively high levels of host adaptation may be associated with genome reduction and metabolic specialization [10], Host-adapted *Lactobacillus* undergoes the loss or decay of some of its functional genes during colonization of the host.

Differences at the genome level are reflected not only in size and GC content, but also be reflected in functional gene composition, especially metabolic genes. Therefore, we further compared the differences in carbohydrate utilizing enzymes and colonization-related genes in *Lactobacillus* from different lifestyles. The results showed that nomadic lactobacilli have more carbohydrate utilization enzyme genes, which may reflect their ability to adapt to complex metabolic environments. For example, *L. plantarum* Lp84-3 isolated from dairy samples contained 148 GTs and 118 GHs, with GH1, GH13 and GT4 being the most abundant in the GHs family[39]. Similarly, the genome of *L. plantarum* QS7T isolated from Sichuan pickled vegetables contained 108 genes from 4 CAZymes gene families, including 43 glycoside hydrolases (GH) genes, 31 glycosyltransferases (GT) genes, 22 carbohydrate esterases (CE) genes and 12 accessory activity (AA) genes[40]. Compared with other lactic acid bacteria, more two-component systems (TCSs) were found in the genome of *L. plantarum*. TCSs may not only be responsible for regulating the sugar metabolism of *L. plantarum* LP-F1, but a large number of TCSs also promote and improve the survival and adaptability of *L. plantarum* in various environments [41]. While the number of carbohydrate-active enzymes in host-adapted *L. reuteri* is mainly enriched with enzymes such as GH2, GH13, and GH36. It lacks polysaccharide lyases and accessory active family proteins, and is more inclined to utilize host-derived carbohydrates such as soluble oligosaccharides and disaccharides [42]. In addition, we found that different lactobacilli can be distinguished at the colonization factor level, regardless of whether they are classified according to species or lifestyle. This may be due to long-term adaptation to different hosts[6] or other selective pressures such as the external environment[7], which cause differences in the colonization-related genome composition of different ecotypes of Lactobacilli; at the same time, the retention or loss of these functional modules by different species during evolution also leads to their differences.

The above differences in functional gene composition suggest that *Lactobacillus* strains from different lifestyles may also have some differences in their colonization ability and adaptive strategies in the host gut. *L. reuteri*, in particular, has undergone host-specific evolutionary differentiation in different hosts through natural selection[43]. This long-term co-evolution may enhance its retention and colonization capabilities in the gut. In contrast, *L. plantarum*, as a nomadic strain, has not undergone similar host-oriented selection. Instead, it relies on its large, highly plastic genome to adapt to diverse environments[44]. Therefore, although

it possesses a wide range of metabolic capabilities, its specificity in stable intestinal colonization is relatively weak. In this study, we selected two representative strains for colonization experiments and found that after the cessation of *Lactobacillus* administration, the intestinal viable counts of nomadic *Lactobacillus* declined rapidly, whereas the viable counts of host-adapted *Lactobacillus* declined more slowly, and ultimately host-adapted *Lactobacillus* had a higher number of viable host-colonized organisms than nomadic *Lactobacillus*. Host-adapted *Lactobacillus* strains are more adaptable in the host because some unnecessary functional genes are lost during co-evolution with the host, whereas ancestral genes lost in large genome *Lactobacillus* strains such as *L. plantarum* have been offset by the emergence of many new genes through duplication and horizontal gene transfer (HGT), resulting in higher intraspecific variability in these species, which leads to higher ecological distributions of the species as a whole [45]. This is also consistent with the findings of Wang et al. [12], who reported that *L. reuteri* isolated from host feces has a stronger intestinal colonization ability and stronger competitive advantage than exogenous *L. plantarum*. In terms of colonization distribution, this study found that both host-adapted *Lactobacillus* and nomadic *Lactobacillus* were mainly distributed in the cecum and colon segments in the host intestine, but *L. reuteri* 325 (host-adapted) was also distributed in the ileum segment. Meisel et al. [46] found that daily oral administration of *L. reuteri* led to a significant increase in the relative abundance of *L. reuteri* in the small intestine, but not in the cecum and colon. This may be related to differences in the immunity of the mice[47] and strain specificity[48] .

The colonization of *Lactobacillus* in the gastrointestinal tract is closely related to the host's dietary structure, intestinal environment and immune status. Based on the previous Genome and colonization results, this study further explored the differences in the effects of different lifestyles of *Lactobacillus* on the host. Using scRNA-seq technology, 9 cell types, including immune cells, epithelial cells, fibroblast-like cells, and lymphocytes were identified in mouse colon tissue. By screening the DEGs of different cell types, it was found that epithelial cells were the most affected by *Lactobacillus* intake among the cell types in mouse colon tissue, followed by immune cells. This may be due to the lactic acid produced by *Lactobacillus* plays a key role in epithelial cell development [49]. Moreover, *Lactobacillus* has immunomodulatory effects on the host, with *L. reuteri* inducing $CD4^{+}CD8\alpha\alpha^{+}$ double-positive T cells in the epithelium to maintain the intestinal immune environment [50]; whereas *L.plantarum*-derived metabolites promote the production of IL12a by dendritic cells, which enhances the $CD8^{+}$ T cell immune response [51] . Notably, *Lactobacillus* enterica and the related metabolite retinoic acid may also suppress colitis by modulating the crosstalk between intestinal epithelial cells and immunity [52].

Our data suggest that among T cells, compared with *L.plantarum*, host-adapted *Lactobacillus*, represented by *L. reuteri*, was able to more significantly down-regulate the differentiation of host proinflammatory-related cells (Th1, Th2, and Th17), with stronger modulation of inflammatory aspects, and that the differences were mainly concentrated in $Cd4^{+}$ Tn cells, $Cd4^{+}$ Th/Treg cells, and Cd8ααT cells. This discrepancy may be due to the fact that different *Lactobacillus* strains may act through different modes of

immunomodulation [53], and different modes of immunomodulation activate or inhibit different immune pathways and effector cells. For example, M. Olivares [54] et al. investigated the ability of two different types of *Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716 to modulate immune responses and found that *Lactobacillus fermentum* CECT5716 acted as an immunostimulant while *Lactobacillus salivarius* CECT5713 acted as an anti-inflammatory agent.

This study deepens the understanding of the evolutionary and functional diversity of *Lactobacillus* from a lifestyle perspective. Host-adapted, nomadic and free-living lactobacilli, which are formed under the drive of ecological niche specialization, show significant differences in genome downsizing, metabolic function reconfiguration and immunomodulatory strategies. Our results suggest that host-adapted *Lactobacillus* not only possesses a greater intestinal colonization capacity, but also exhibits unique anti-inflammatory effects through the regulation of specific T-cell subpopulations. These findings suggests that microbial lifestyle may be a key ecological and evolutionary factor shaping host-microbe interactions and provide a theoretical basis for precise probiotic screening and intervention on basis of microbial ecotypes.

5. Conclusions

This study demonstrates that the lifestyle of *Lactobacillus* is closely associated with its colonization potential and host immune modulation. Comparative genomics of 232 *Lactobacillus* strains revealed that host-adaptive strains possess smaller genomes and lower GC content compared to free-living and nomadic strains. Furthermore, the CAZymes profiles of different *Lactobacillus* strains showed strong correlation with their lifestyles, and these genomic features were associated with enhanced colonization capacity *in vivo*. Specifically, host-adaptive *L. reuteri* survived longer in the gut and maintained higher viable cell counts than nomadic *L. plantarum*. Single-cell transcriptomic analysis further revealed that *Lactobacillus* ingestion significantly impacts host epithelial and immune cells, with host-adaptive strains exhibiting stronger immunomodulatory effects. Notably, *L. reuteri* significantly downregulated pro-inflammatory T cell subsets (Th1, Th2, and Th17) and modulated $Cd4^{+}$Tn, $Cd4^{+}$Th/Treg, and Cd8ααT cells, thereby influencing host immunity. In summary, understanding these lifestyle-dependent strategies not only advances the mechanistic basis of host-microbiota interactions but also provides a theoretical framework for the precise screening and application of probiotic strains tailored to host health needs.

Competing interests

The Authors declare no Competing Financial or Non-Financial Interests.

Data availability

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/sra/PRJNA1280272, reference number PRJNA1280272. All code used in analyses are available by request from the corresponding author.

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Ethics approval and consent to participate

The animal care and study protocols were approved by the Ethics Committee of Jiangnan University, China (JN.No20230515c0400815[202], JN.No20231030c1150205[518]) and JN.No20220315c1800630[058].

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