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Metabolite-Driven Immunosuppression in High-Risk NSCLC — with Anti-Tumor Immunity Shaped by Probiotic Strategy, Donor Origin, and Conserved Microbial Mediators

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ABSTRACT: Elevated serum cytokeratin 19 fragment (CYFRA 21-1) in non-small cell lung cancer (NSCLC) links to immunosuppression, but whether live gut bacteria or their metabolites mediate this effect remains unclear. Using stratified fecal microbiota transplantation in a Lewis lung carcinoma model, we demonstrate that cell-free supernatants, not bacterial pellets, from high CYFRA 21-1 NSCLC donors impair anti-tumor immunity by depleting systemic T cells. Intervention with *Bifidobacterium animalis* subsp. *lactis* V9 restored tumor control via two distinct pathways: direct supplementation expanded CD8⁺ T cells and promoted Th1 immunity, while preconditioned microbiota transplantation acted through Th17/regulatory T cell modulation. Donor origin further shaped outcomes: mouse-derived microbiota enabled stable engraftment and robust cytotoxic responses, whereas human-derived transplants induced greater taxonomic shifts but carried opportunistic pathogens and favored Th17 polarization. Integrated metagenomic and untargeted metabolomic profiling revealed that carboxylic acid derivatives, particularly creatine, L-serine, and L-carnitinamide, were consistently associated with therapeutic response across all interventions and donor types. These metabolites represent conserved mediators of microbiota-immune crosstalk. Our findings establish microbial metabolites as central effectors of immunosuppression in high-risk NSCLC and highlight delivery strategy and donor compatibility as critical determinants of microbiome-based therapeutic efficacy.

Keywords: *Bifidobacterium*; Cancer Immunotherapy; Fecal Bacterial Transplantation; Gut Microbiota; Metabolomics; T Cell Immunity; Tumor Microenvironment

1. Introduction

The gut microbiota has emerged as a pivotal regulator of anti-tumor immunity, with growing evidence implicating its role in shaping the tumor immune microenvironment (TIME) [1, 2]. While numerous studies have correlated microbial taxonomic profiles with responses to cancer immunotherapy, thereby identifying candidate “beneficial” or “detrimental” taxa, the functional contributions of microbiota-derived metabolites remain comparatively underexplored [3]. Through continuous metabolic activity, the gut microbiota generates a diverse array of bioactive molecules, such as short-chain fatty acids, tryptophan catabolites, and

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secondary bile acids [4]. These metabolites can translocate into systemic circulation via the portal vein and directly modulate immune cell function within the TIME [5]. Mechanistically, they influence immunity through metabolic reprogramming and epigenetic regulation [6, 7]. For instance, short-chain fatty acids enhance CD8⁺ T cell expansion and effector cytokine production via G protein-coupled receptor signaling, thereby augmenting anti-tumor responses [8]. Exercise-induced microbial formate enhances immune checkpoint inhibitor efficacy by boosting CD8⁺ T cell function via nuclear factor erythroid 2-related factor-2 [9]. In contrast, microbial endotoxins such as lipopolysaccharide can drive the accumulation of myeloid-derived suppressor cells, fostering an immunosuppressive milieu that blunts immunotherapy efficacy [10]. The microbial metabolite 4-hydroxybenzeneacetic acid promoted the infiltration of immunosuppressive polymorphonuclear myeloid-derived suppressor cells in the tumor microenvironment through activating the JAK2/STAT3 pathway and upregulating CXCL3 transcription, consequently driving colorectal cancer progression [11]. In non-small cell lung cancer (NSCLC), elevated serum cytokeratin 19 fragment (CYFRA 21-1) levels correlate with high tumor burden and systemic immunosuppression [12, 13]; however, it remains unknown whether this phenotype is primarily mediated by live microbes or their soluble metabolic products. Resolving this distinction is essential to define the mechanistic axis of microbiota-driven immunomodulation and to guide the development of metabolite-targeted interventions.

Probiotics represent a promising strategy to harness microbial-immune crosstalk for therapeutic benefit. Among them, *Bifidobacterium* species have garnered particular interest due to their well-documented immunoregulatory properties [14–16]. Notably, *Bifidobacterium* supplementation has been shown to restore responsiveness to anti-PD-1 therapy in murine melanoma models and has progressed to early-phase clinical trials [16]. Commensal *Bifidobacterium*-derived extracellular vesicles modulate the therapeutic effect of anti-PD-1 therapy in NSCLC by upregulating PD-L1 expression [17]. Yet, most existing studies have examined probiotics in isolation, without accounting for their interactions with the resident microbiota [18]. Moreover, the mode of probiotic delivery may critically influence its biological impact. Direct supplementation enables immediate colonization and sustained metabolite secretion in the gut lumen, whereas using probiotics as preconditioning agents, entailing steps such as donor gavage, fecal collection, cryopreservation, and transplantation, introduces ecological bottlenecks that may alter bacterial viability, community context, and functional output [19]. These methodological differences are hypothesized to yield divergent microbial assemblages and metabolite landscapes, thereby steering anti-tumor immunity through distinct immunological pathways. Thus, a systematic comparison of delivery strategies is warranted to inform rational design of probiotic-combination trials in oncology.

Fecal microbiota transplantation (FMT) offers a powerful means to reconfigure the gut ecosystem, with proven efficacy in recurrent *Clostridioides difficile* infection and growing exploration in cancer immunotherapy [20]. A major translational challenge, however, lies in the species-specific nature of host-microbe interactions: microbial consortia identified as optimal in murine models often fail to colonize or function predictably in human recipients [21]. These discrepancies stem from fundamental differences in

host genetics, diet, immune physiology, and gut anatomy between mice and humans [22]. Consequently, the extent to which findings from mouse-to-mouse FMT studies generalize to human-derived transplants remains uncertain. A direct, parallel comparison of mouse- versus human-derived responder microbiota, assessing their engraftment dynamics, immunomodulatory capacity, and metabolic outputs in a controlled murine host, represents a critical but underexplored approach to evaluate preclinical fidelity and refine donor selection criteria.

Building on this framework, the present study was designed to test three interrelated hypotheses: (1) that microbial metabolites, rather than live bacteria, are the primary mediators of immunosuppression in NSCLC patients with high CYFRA 21-1 levels; (2) that direct supplementation with *Bifidobacterium animalis* subsp. *lactis* V9 (*B. lactis* V9; a probiotic species with known anti-inflammatory effect [23, 24] and *B. lactis* V9-preconditioned microbiota transplantation differentially reprogram the TIME through distinct immune and metabolic mechanisms; and (3) that the donor species, mouse versus human, imposes a fundamental constraint on the ecological stability, immunological impact, and metabolic reprogramming capacity of transplanted microbiota.

Using stratified FMT from NSCLC patients and Lewis lung carcinoma (LLC) tumor-bearing mice, combined with multi-omics profiling and functional immune assays, this work delineates the causal roles of microbial form (live vs. metabolite), delivery strategy (direct vs. preconditioned), and donor origin (syngeneic vs. xenogeneic) in shaping anti-tumor immunity. In doing so, it identifies a core set of conserved metabolites linked to therapeutic response, offering a foundation for the development of precision microbiome-based interventions with enhanced clinical translatability.

2. Materials and methods

2.1 Ethics statement

We strictly adhered to the ethical standards of the Declaration of Helsinki in conducting all procedures involving human participants. The investigator-initiated clinical study was formally reviewed and approved by the Ethics Committee of the People's Hospital of Inner Mongolia Autonomous Region (Approval No.: 2021007). All mouse procedures were conducted in strict accordance with the Guidelines for Ethical Review of Laboratory Animal Welfare and were approved by the Inner Mongolia Agricultural University Laboratory Animal Welfare and Ethics Committee (Approval No. NND20223076).

2.2 Collection and processing of donor fecal samples

Fresh fecal samples were collected from human and mouse donors for use in subsequent FMT experiments: (i) three immunotherapy-naïve NSCLC patients with elevated serum CYFRA 21-1 levels; (ii) three NSCLC patients who achieved a partial response following treatment with *B. lactis* V9 (2 g/day) in combination with immunochemotherapy (21-day cycle, four cycles total); and (iii) LLC-bearing mice treated for 12 days with *B. lactis* V9 along with anti-PD-1, pemetrexed, and cisplatin. We defined the feces collected in (ii) and (iii) as the human- and mouse-derived responder microbiota. The baseline

characteristics of human donors were shown in Table S1. The human donors included in the study were all patients with advanced NSCLC, all of whom were diagnosed by pathology and had a disease stage of III-IVB. They had not received immunotherapy, radiotherapy or chemotherapy. Gender, age, BMI, dietary habits and the history of antibiotic use were standardized and matched to minimize the influence of individual confounding factors on the analysis of results. The serum CYFRA 21-1 levels of three NSCLC patients with elevated serum CYFRA 21-1 levels were all above 5.0 ng/mL (clinical detection threshold).

Written informed consent was obtained from all human participants prior to sample collection. Fresh stool samples were transported to the laboratory within 1 h under anaerobic conditions to preserve microbial viability. A standardized protocol was employed to process collected samples: fresh feces were homogenized in sterile phosphate-buffered saline (PBS, pH 7.4) at a 1:5 (w/v) ratio by vortexing. Particulate matter was removed by filtration through sterile 200- μ m filter bags. The resulting filtrate was transferred into 50-mL nuclease-free centrifuge tubes and centrifuged at $50 \times g$ for 5 min at 4 °C. The supernatant was supplemented with 10% (v/v) glycerol as a cryoprotectant and snap-frozen in liquid nitrogen. To minimize degradation of microbial and metabolite components, the total time from sample collection to freezing was strictly limited to 4 h.

For murine donors, fresh fecal pellets were collected on the day of euthanasia and immediately homogenized in sterile PBS at a 1:1 (w/v) ratio using a low-speed tissue homogenizer. The homogenate was filtered through a sterile 100- μ m mesh to remove undigested debris, mixed with 10% (v/v) glycerol, and stored in liquid nitrogen until use.

Prior to FMT, recipient mice received a broad-spectrum antibiotic cocktail via oral gavage once daily for five consecutive days to deplete the endogenous gut microbiota and create a permissive niche for donor engraftment. The cocktail consisted of vancomycin hydrochloride (100 mg/kg), neomycin sulfate (200 mg/kg), metronidazole (200 mg/kg), and ampicillin sodium (200 mg/kg) [25] (all from Solarbio Science & Technology Co., Ltd., Beijing, China). On the day of transplantation, frozen fecal samples were thawed and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was filtered through a sterile 0.22- μ m membrane to generate fecal supernatant (for fecal supernatant transfer, FST). The bacterial pellet was resuspended in PBS, and the concentration was adjusted to 1×10^9 – 1×10^{10} CFU/mL to prepare the bacterial fraction (for fecal bacterial transplantation, FBT). Each recipient mouse received 0.2 mL of the respective preparation via oral gavage daily for five consecutive days. A two-day colonization period followed transplantation to allow stable engraftment of the donor microbiota.

2.3 *Bifidobacterium animalis subsp. lactis* V9 (*B. lactis* V9)

B. lactis V9 (Kex02), originally isolated in 2005 from the gut of a healthy Mongolian child, was provided by the Key Laboratory of Dairy Biotechnology and Engineering at Inner Mongolia Agricultural University and is deposited in the China General Microbiological Culture Collection Center (CGMCC No. 5470).

2.4 Mouse model construction

Six-week-old female C57BL/6J mice were sourced from Beijing Huafukang Bioscience Co. (Beijing, China) and housed under specific pathogen-free conditions at the Key Laboratory of Dairy Biotechnology and Engineering, Inner Mongolia Agricultural University. Mice were maintained at $22 \pm 2^\circ\text{C}$ with $50 \pm 10\%$ humidity on a 12-h light/dark cycle and provided autoclaved food and water *ad libitum*.

Following a one-week acclimatization period, mice underwent antibiotic pretreatment and FMT as described above. The dorsal right flank of each mouse was shaved and disinfected with 70% ethanol, and 0.1 mL of LLC cell suspension (5×10^5 cells/mouse; Wuhan Procell Life Science & Technology Co., Ltd., Wuhan, China) was injected subcutaneously. Seven days after tumor cell inoculation, once palpable tumors had formed, mice received intraperitoneal injections of anti-PD-1 (Bio X Cell, Lebanon, NH, USA) at 10 mg/kg every three days for a total of four doses. A single dose each of pemetrexed (100 mg/kg; Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) and cisplatin (6 mg/kg; TargetMol, Boston, MA, USA) was administered concomitantly. For direct *B. lactis* V9 supplementation, lyophilized bacterial powder was resuspended in sterile saline to a final concentration of 1×10^9 CFU/0.2 mL and administered daily by oral gavage for 12 consecutive days. To generate *B. lactis* V9-preconditioned microbiota, donor mice or NSCLC patients received daily *B. lactis* V9 for 12 days or four treatment cycles (21 days per cycle), respectively. Fresh fecal samples were collected immediately after the final gavage subsequent transplantation.

At the end of the intervention period, fecal samples were collected for metagenomic and metabolomic analyses. Mice were then euthanized, and tumors were excised, weighed, and processed for further analysis. Spleens were harvested for flow cytometry.

2.5 Flow cytometry of splenocytes

Fresh spleens were harvested from mice and immediately placed in pre-chilled PBS. Tissues were mechanically dissociated using the plunger of a sterile syringe and passed through a 70- μm cell strainer to generate single-cell suspensions. Lymphocytes were isolated by density gradient centrifugation using mouse spleen lymphocyte separation medium (Solarbio Science & Technology Co., Ltd., Beijing, China). The isolated cells were resuspended in Gibco RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 μL of Leukocyte Activation Cocktail containing BD GolgiPlug™ (BD Biosciences, Franklin Lakes, NJ, USA), then incubated for 5 h at 37°C in a 5% CO_2 humidified atmosphere.

Following incubation, cells were centrifuged, and the supernatant was discarded. The pellet was resuspended in 100 μL of cell staining buffer. Dead cells were labeled using Fixable Viability Stain 700 (BD Biosciences, Franklin Lakes, NJ, USA), and nonspecific Fc receptor binding was blocked by incubation with purified rat anti-mouse CD16/CD32 (clone 2.4G2; BD Biosciences, Franklin Lakes, New Jersey, USA).

Surface staining was performed by adding fluorochrome-conjugated antibodies (all from BD Biosciences unless otherwise noted) and incubating the cells at 4°C in the dark for 30 min. The antibody panel included: FITC-conjugated rat anti-mouse CD11b (clone M1/70), BV510-conjugated rat anti-mouse Ly-6G (clone 1A8), PE-conjugated rat anti-mouse F4/80 (clone T45-2342), APC-Cy7-conjugated hamster anti-mouse CD3 ϵ (clone 145-2C11), BB700-conjugated mouse anti-mouse NK1.1 (clone PK136),

BV421-conjugated rat anti-mouse I-A/I-E (clone M5/114.15.2), PE-Cy7-conjugated hamster anti-mouse CD11c (clone HL3), BV421-conjugated rat anti-mouse CD4 (clone GK1.5), BV510-conjugated rat anti-mouse CD8a (clone 53-6.7), and BB700-conjugated rat anti-mouse CD25 (clone PC61).

After surface staining, cells were washed twice with and resuspended in 100 μ L of cell staining buffer, before being fixed and permeabilized using the Transcription Factor Buffer Set according to the manufacturer's protocol (BD Biosciences, Franklin Lakes, New Jersey, USA). Intracellular cytokine and transcription factor staining was performed by adding the following antibodies and incubating at 4 °C in the dark for 45 min: FITC-conjugated rat anti-mouse IFN- γ (clone XMG1.2), PE-Cy7-conjugated rat anti-mouse IL-4 (clone 11B11), PE-conjugated rat anti-mouse IL-17A (clone TC11-18H10), and APC-conjugated rat anti-mouse FOXP3 (clone FJK-16s; Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometry data were acquired on a MoFlo Astrios™ EQ cell sorter (Beckman Coulter, Brea, CA, USA), and analysis was performed using FlowJo software (v10.8; BD Biosciences).

2.6 Fecal metagenomics analysis

Total genomic DNA was extracted from mouse fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. DNA purity was assessed by measuring the A260/A280 ratio (target range: 1.8-2.0) using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA integrity was confirmed by visual inspection following electrophoresis on a 1% agarose gel, and DNA concentration was quantified with a Qubit 4.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of high-quality DNA per sample was used to construct sequencing libraries with the NEBNext Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), targeting an insert size of approximately 350 bp. Paired-end sequencing (PE150) was performed on an Illumina NovaSeq 6000 platform, with a minimum sequencing depth of 3 Gb per sample.

Raw sequencing data were processed using a standardized bioinformatics pipeline. Initial quality assessment was conducted with FastQC (v0.11.9). Low-quality reads (Phred quality score, $Q < 20$) and adapter sequences were removed using Trimmomatic [26], yielding high-quality clean reads for downstream analysis. To exclude host-derived sequences, clean reads were aligned to the mouse reference genome (GRCm39) using Bowtie2 (v2.4.5); only unmapped reads were retained as putative microbial sequences.

Taxonomic profiling was performed using MetaPhlAn 4.0, which leverages clade-specific marker genes to assign microbial taxa from phylum to species level and estimate their relative abundances [27]. Alpha diversity was evaluated using the Shannon index, while beta diversity was assessed via principal coordinate analysis (PCoA) based on Bray-Curtis distance. Statistical significance of intergroup differences in microbial community structure was determined using permutational multivariate analysis of variance (PERMANOVA; *adonis* test). Differentially enriched taxa between experimental groups were identified through linear discriminant analysis (LDA) effect size with an LDA score > 4 . Additionally, a microbial

co-occurrence network was constructed in Cytoscape (v3.9.1), retaining only significant correlations (Spearman's $|\rho| > 0.8$ and $P < 0.05$).

2.7 Fecal untargeted metabolomics analysis

Untargeted metabolomic profiling of mouse fecal samples was performed using ultra-high-performance liquid chromatography coupled with mass spectrometry. An appropriate amount of each fecal sample was weighed and extracted with 600 μL of methanol spiked with 4 ppm 2-amino-3-(2-chlorophenyl) propionic acid as an internal standard (Aladdin, Shanghai, China). Samples were vortexed for 30 s, homogenized using a tissue homogenizer at 50 Hz for 120 s, and sonicated at room temperature for 10 min. Following centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was filtered through a 0.22- μm membrane, and the resulting filtrate was collected for analysis.

Chromatographic separation was performed on a Thermo Vanquish UHPLC system equipped with an ACQUITY UPLC® HSS T3 column (2.1 $\times$ 100 mm, 1.8 μm ; Waters, Milford, MA, USA). The column temperature was maintained at 40 °C, with a flow rate of 0.3 mL/min and an injection volume of 2 μL . Gradient elution was employed with two mobile phase systems: in positive ionization mode, solvent A consisted of water with 0.1% formic acid and solvent B of acetonitrile with 0.1% formic acid; in negative ionization mode, solvent A was water containing 5 mM ammonium formate and solvent B was acetonitrile [28].

Mass spectrometry detection was performed on a Thermo Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization source. Data were acquired in both positive and negative ion modes with a resolving power of 60,000 over a scan range of m/z 100–1000. Tandem mass spectra were generated using higher-energy collisional dissociation for structural characterization.

Raw data files were converted to mzXML format using ProteoWizard [29]. Peak detection, retention time alignment, and intensity calibration were performed using the XCMS package in R [30]. Metabolite identification was achieved by matching accurate mass and MS/MS fragmentation patterns against the Human Metabolome Database (HMDB) [31] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [32]. Functional annotation and pathway enrichment were conducted using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>). Microbiota-metabolite interaction networks were constructed and visualized using Cytoscape (v3.9.1).

2.8 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (v21.0; IBM Technology Co., Armonk, NY, USA). All data are presented as mean $\pm$ standard error of the mean. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated with Levene's test. Appropriate parametric (one-way ANOVA with LSD post-hoc test) or non-parametric (Kruskal-Wallis test with Dunn's post-hoc analysis) tests were selected based on these assessments, with specific sample sizes (n) and statistical methods detailed in each figure legend. Data visualization was achieved using R (v4.0.2) and Origin 2017

(OriginLab Corporation, Northampton, MA, USA), and final figure assembly and refinement were performed in Adobe Illustrator 2020 (Adobe Inc., San Jose, CA, USA). Statistical significance was defined as $P < 0.05$.

2.9 Data declaration

The raw metagenomic sequencing data of mouse-derived fecal sample were generated in our previous research, which is currently under review for publication. That work focuses on addressing the structural characteristics of the mouse gut microbiota post-transplantation, where linear discriminant analysis (LDA) was employed to screen and identify the dominant microbial taxa among different groups. For the current research, we reused the aforementioned mouse-derived metagenomic raw data, but conducted a novel extended analysis: we compared these data with the newly generated human-derived fecal metagenomic data to clarify the similarities and differences in microbial functional gene composition, metabolic pathway distribution, and interspecific homology between mouse and human fecal microbiota.

It should be emphasized that the analysis content, research focus, and scientific conclusions of this study are completely different from those of the aforementioned under-review work. The mouse-derived metagenomic data herein only serve as a reference for cross-species comparison, and no original figures, tables, or descriptive content from the under-review manuscript are duplicated. The raw data of mouse fecal metagenomics on day 31 have been deposited in CNGB Sequence Archive of China National GeneBank DataBase (CNGBdb; <https://db.cngb.org/cnsa/>) with the accession number CNP0007653 for public access upon publication of the under-review work.

We also declare that raw flow cytometry data generated from the same sample queue were used here. Previous studies have mainly focused on evaluating the percentage variables of T cell subsets (Th1, Th2, Th17, Treg cells) during NSCLC Immunochemotherapy. In this study, we derived Th1/Th2 and Th17/Treg ratios, focusing on immune balance and evaluating the overall polarization status of the immune system in recipient mice after fecal microbiota transplantation.

3. Results

3.1 Soluble metabolites from high CYFRA 21-1 NSCLC donors drive T cell depletion and attenuate anti-tumor immunity

Elevated serum CYFRA 21-1 levels and tumor-associated gut microbiota dysbiosis are hallmarks of advanced NSCLC and are closely linked to impaired anti-tumor immunity and profound resistance to therapy [3, 33]. However, it remains unclear whether live gut bacteria or their soluble metabolites are the principal mediators of immune dysfunction in this high-risk subgroup. To dissect their individual contributions, fecal bacterial pellets (for FBT) and cell-free supernatants (for FST) were prepared from three immunotherapy-naïve NSCLC patients with elevated CYFRA 21-1 levels and administered to antibiotic-pretreated recipient mice prior to subcutaneous engraftment of LLC cells. The effects of FBT and FST on tumor progression and systemic immunity were evaluated during subsequent immunochemotherapy

consisting of pemetrexed, cisplatin, and anti-PD-1. Mice receiving PBS alone served as vehicle controls (Figure 1A).

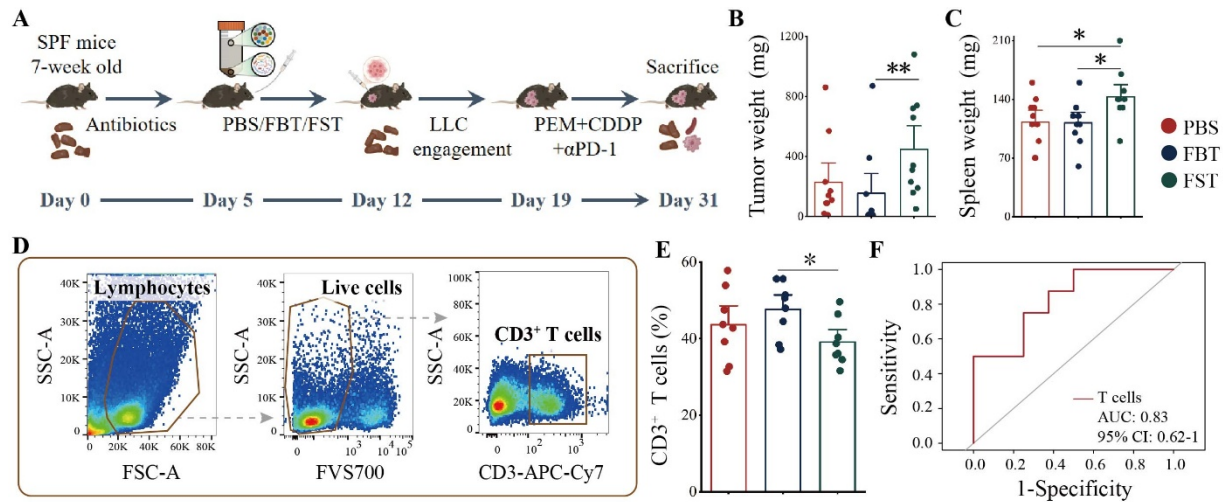


Figure 1. Soluble metabolites from high CYFRA 21-1 NSCLC donors suppress anti-tumor immunity by depleting T cells. (A) Experimental timeline: Specific-pathogen-free (SPF) mice received a 5-day oral antibiotic cocktail, followed by oral gavage of phosphate-buffered saline (PBS), fecal bacteria transplantation (FBT) or fecal supernatant transfer (FST) prepared from NSCLC donors with elevated CYFRA 21-1 levels. Seven days later, mice were subcutaneously engrafted with Lewis lung carcinoma (LLC) cells. Starting on day 12, they received immunochemotherapy (a single dose of cisplatin [CDDP] and pemetrexed [PEM] plus four doses of anti-PD-1). Fresh fecal samples were collected at three time points: baseline (day 0), on the day of LLC engraftment (day 12), and after chemoimmunotherapy completion (day 31) for metagenomic sequencing. Mice were euthanized on day 31, and spleen and tumor tissues were harvested for subsequent analyses. (B) Final tumor weight ($n = 10$ per group). (C) Spleen weight ($n = 10$ per group). (D) Gating strategy for splenic CD3⁺ T cell populations. (E) Frequencies of total T cells ($n = 8$ per group). (F) Receiver operating characteristic curves for CD3⁺ T cells. diagonal line indicates no discrimination; AUC = area under the curve. Error bars represent SEM. Data distribution and variance homogeneity were assessed using the Shapiro-Wilk and Levene's tests, respectively. Significant significance was determined by Kruskal-Wallis test with Dunn's post-hoc analysis (B) or one-way ANOVA with LSD post-hoc test (C, E). * $P < 0.05$, ** $P < 0.01$.

FST-administered mice developed significantly larger tumors than FBT recipients ($P < 0.01$; Figure 1B), indicating that soluble microbial components compromise treatment efficacy. Moreover, spleen weight was significantly higher in the FST group compared to both FBT and PBS controls ($P < 0.05$; Figure 1C), suggestive of systemic immune dysregulation or compensatory splenomegaly. Flow cytometric analysis of splenic immune populations revealed that the FST group exhibited a marked reduction in total CD3⁺ T cell proportions relative to FBT group (Figure 1D, E; $P < 0.05$). We further performed receiver operating characteristic analysis and found that CD3⁺ T cells yielded an area under the curve (AUC) of 0.83 (Figure 1F), suggesting it may be effectively characterized the immune status of recipient mice after fecal microbiota transplantation.

These results demonstrate that, in the context of high CYFRA 21-1 NSCLC, gut-derived soluble metabolites, not intact bacteria, are the dominant drivers of immune suppression during chemoimmunotherapy, primarily through systemic depletion of T lymphocytes.

3.2 Gut bacteria and their metabolites differentially modulate microbial community structure in recipient mice

Given the stronger tumor-promoting effect of gut microbial metabolites, we next characterized how FBT and FST differentially reshape the gut microbiota of recipient mice. Fresh fecal samples were collected from PBS, FBT, and FST groups at three time points: baseline (day 0), on the day of LLC engraftment (day 12), and after chemoimmunotherapy completion (day 31) for metagenomic sequencing.

At baseline (day 0), α -diversity (Shannon index) was comparable across groups ($P > 0.05$; Figure 2A), confirming equivalent starting microbiota. Antibiotic pretreatment markedly reduced microbial diversity at day 12 and day 31 in all groups ($P < 0.05$ vs. Day 0, Table S2), indicating that the endogenous microbiota has been effectively depleted. Nevertheless, both FBT and FST partially restored α -diversity, with Shannon indices significantly higher than in PBS controls ($P < 0.001$). Notably, by day 31, the FBT group exhibited a significantly higher Shannon index than the FST group ($P < 0.05$; Figure 2A), indicating that live bacterial transfer supports more robust and sustained recovery of microbial diversity than metabolite exposure alone.

At the phylum level, all groups were dominated by Firmicutes and Bacteroidetes, followed by Proteobacteria, Desulfobacterota, and Verrucomicrobiota (Figure 2B). The Firmicutes-to-Bacteroidetes (F/B) ratio, a commonly used, albeit imperfect, indicator of gut ecosystem stability, showed a progressive decline from FBT to FST to PBS at day 31 ($P < 0.05$; Figure 2C). This gradient suggests that donor-derived components from high CYFRA 21-1 NSCLC patients induce a differential dysbiosis effect, with whole bacteria exerting a more disruptive effect than metabolites.

At day 31, 232 bacterial species were differentially abundant in the FBT group (102 increased, 130 decreased) and 209 in the FST group (93 increased, 116 decreased) relative to PBS (Figure 2D). Of these, 196 species overlapped between FBT and FST (Figure 2E, Table S3). The FBT-unique alterations included depletion of *Prevotella copri*, *Bacteroides uniformis*, *Paraprevotella clara*, *Phascolarctobacterium faecium*, alongside enrichment of *Eubacterium_J plexicaudatum* and *Duncaniella muris*, both more abundant in FBT than FST ($P < 0.01$; Figure 2F). These taxa may contribute to the resilience of the FBT-associated microbiota and help mitigate dysbiosis despite an elevated F/B ratio.

In contrast, FST induced 13 unique species-levels changes relative to PBS (Figure 2G), notably the depletion of recognized beneficial species such as *Muribaculum intestinale*, *Lactobacillus taiwanensis*, and *Limosilactobacillus reuteri*.

Collectively, these results indicate that while FBT drives more extensive structural remodeling of the gut microbiota, it is the functional output, particularly the metabolite profile, of the donor microbiota that predominantly mediates immunosuppression. Thus, microbial metabolites, rather than community composition *per se*, serve as the primary effector mechanism impairing anti-tumor immunity through disruption of host immune surveillance.

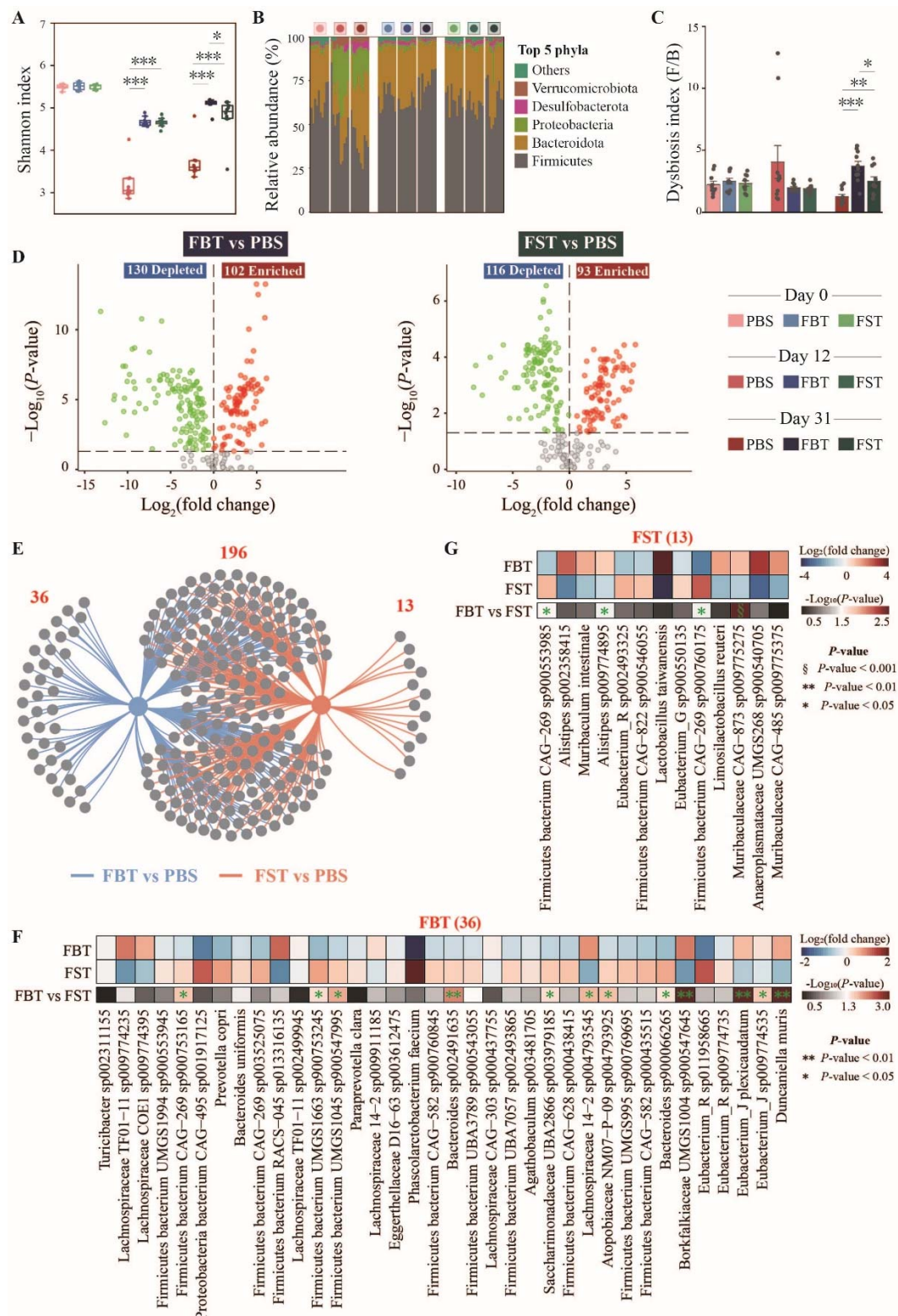


Figure 2. Live bacteria and metabolites from high CYFRA 21-1 NSCLC donors differentially reshape gut microbiota structure. (A) Shannon index of fecal microbiota ($n = 10$ per group). Boxplots show median (center line), interquartile range (box), and $1.5 \times$ interquartile range (whiskers), with individual data points overlaid as dots. (B) Relative abundance of the top five bacterial phyla across all groups ($n = 10$ per group). (C) Firmicutes-to-Bacteroidetes (F/B) ratio as a dysbiosis index. Error bars represent SEM. (D) Volcano plots showing differentially abundant species ($P < 0.05$) in FBT vs. PBS (left) and FST vs. PBS (right). Each dot represents one species (red: enriched; green: depleted). (E) Venn network plot of overlapping and unique differentially abundant species between FBT and FST (vs. PBS). (F-G) Heatmaps of (F) FBT-specific and (G) FST-specific differential species. Upper panels: $\log_2(\text{fold change})$; lower panels: statistical significance ($-\log_{10}[P\text{-value}]$).

Data distribution and variance homogeneity were assessed using the Shapiro-Wilk and Levene's tests, respectively. Significant differences were evaluated using the Kruskal-Wallis test with Dunn's post-hoc analysis (A, C) or one-way ANOVA with LSD post-hoc analysis (D, F-G). * $P < 0.05$, ** $P < 0.01$, § $P < 0.001$.

3.3 Direct *B. lactis* V9 supplementation and preconditioned microbiota transplantation elicit distinct anti-tumor immune responses

Provided that fecal supernatant from high CYFRA 21-1 NSCLC patients impairs anti-tumor immunity through systemic T cell depletion, and our previous study found that *B. lactis* V9 could block metabolite-induced tumorigenic effects and transfer of *B. lactis* V9-remodeled microbiota and metabolites recapitulated antitumor effect (unpublished data), we compared the effects of two intervention strategies on the immune status in recipient mice: (1) direct daily oral supplementation with live *B. lactis* V9 (FBT+V9 and FST+V9 groups) and (2) transplantation of fecal microbiota from donors previously preconditioned with *B. lactis* V9 (FBT-R and FST-R groups; Figure S1).

They differentially shaped host immunity. Based on our prior observation that FST compromises chemoimmunotherapy by depleting T cells, we focused on splenic T cell subsets using flow cytometry (Figure 3A). Direct *B. lactis* V9 supplementation more effectively restored systemic T cell populations: the proportions of CD3⁺ T cells were significantly higher in the FBT+V9 and FST+V9 groups than in their preconditioned counterparts (FBT-R and FST-R; $P < 0.05$; Figure 3B). Consistent with the intrinsic T cell-depleting effect of FST, the FST+V9 group also exhibited a lower CD3⁺ T cell frequency than the FBT+V9 group ($P < 0.05$; Figure 3B).

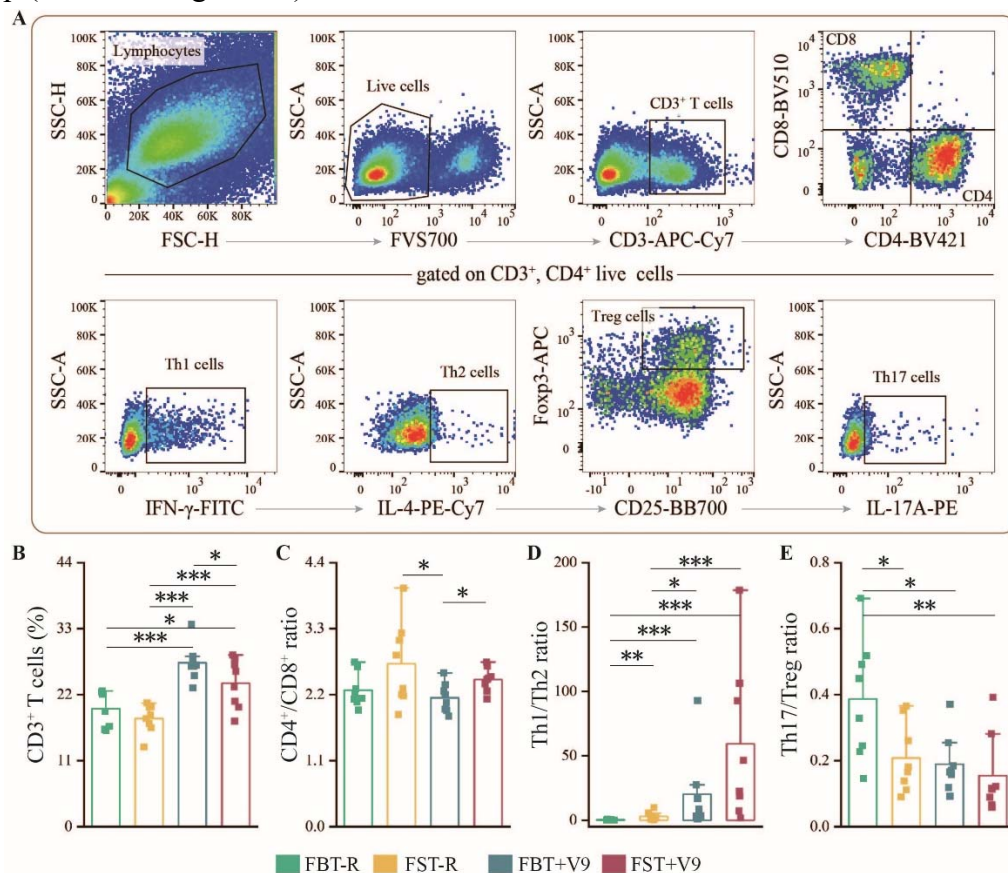


Figure 3. Direct *B. lactis* V9 supplementation and preconditioned microbiota transplantation enhance anti-tumor immunity via distinct immune pathways. (A) Gating strategy for splenic T cell subsets ($n = 8$ per group). (B) Frequency of CD3⁺ T cells. (C-E) Ratios of (C) CD4⁺/CD8⁺, (D) Th1/Th2, and (E) Th17/Treg. Error bars represent SEM. Data distribution and variance homogeneity were assessed using the Shapiro-Wilk and Levene's tests, respectively. Significant differences were evaluated using the Kruskal-Wallis test with Dunn's post-hoc analysis (C-E) and one-way ANOVA with LSD post-hoc analysis (B). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

We further examined T cell polarization patterns. A reduced CD4⁺/CD8⁺ ratio typically reflects expansion of CD8⁺ cytotoxic T lymphocytes (CTLs), which are critical for tumor cell killing. The FBT+V9 group displayed a significantly lower CD4⁺/CD8⁺ ratio than both the FST-R and FST+V9 groups (Figure 3C; $P < 0.05$), indicating that combining intact donor bacteria with direct *B. lactis* V9 supplementation fosters a CTL-enriched immune milieu.

Next, we assessed T helper (Th) cell balance. Th1 cells produce IFN- γ and TNF- α to drive anti-tumor cellular immunity, whereas Th2 cells secrete IL-4 and IL-10, which can promote immunosuppression when overactivated. The Th1/Th2 ratio was significantly elevated in both direct supplementation groups (FBT+V9 and FST+V9) compared to FBT-R, and FST-R groups (Figure 3D; $P < 0.05$), demonstrating that live *B. lactis* V9 administration robustly skews immunity toward a Th1-polarized, anti-tumor state. In contrast, the FBT-R group exhibited a significantly lower Th1/Th2 ratio than even the FST-R group ($P < 0.01$), suggesting that *B. lactis* V9-preconditioned microbiota may suppress tumors through Th2-biased or Th1-independent mechanisms.

Finally, we evaluated the Th17/regulatory T cell (Treg) axis, as a reduced ratio is often linked to poor tumor prognosis. Strikingly, the FBT-R group showed a significantly higher Th17/Treg ratio than all other intervention groups ($P < 0.05$; Figure 3E), implying that microbiota preconditioning may enhance anti-tumor immunity via Th17 pathway activation or Treg modulation.

Collectively, these findings reveal that while both strategies inhibit tumor growth, they engage distinct immunological programs: direct *B. lactis* V9 supplementation drives a Th1/CTL-dominated response with broad T cell restoration, whereas *B. lactis* V9-preconditioned microbiota transplantation appears to act through Th17-oriented or CD4⁺ T cell-modulatory pathways, without fully reversing FST-induced T cell loss.

3.4 Direct *B. lactis* V9 supplementation and preconditioned microbiota transplantation drive distinct gut microbiota ecosystems

Although both direct *B. lactis* V9 supplementation and preconditioned microbiota transplantation modulate anti-tumor immunity, the gut microbiota serves as a central mediator of these divergent immunological outcomes. To dissect how each strategy shapes microbial ecology, fresh fecal samples were collected from six experimental groups (Figure S1), at baseline (day 0), on the day of LLC cell inoculation (day 12), and after chemoimmunotherapy completion (day 31), and subjected to shotgun metagenomic sequencing.

At day 0, α -diversity (Shannon index) differed no significantly among experimental groups ($P > 0.05$; Figures S2A, B), confirming comparable initial microbiota composition. By day 12 and day 31, however, mice receiving direct *B. lactis* V9 supplementation (FBT+V9 and FST+V9) exhibited significantly higher Shannon indices than those in the PBS group ($P < 0.001$), while no significant difference was observed among the FBT-R, FST-R and PBS groups ($P > 0.05$; Figure S2A), indicating that live probiotic administration potently enhances microbial α -diversity. Consistent with this, principal coordinate analysis

based on Bray-Curtis distance revealed distinct clustering of microbial communities by intervention type at both day 12 and day 31 compared to their respective PBS groups (Figures S2C-F), demonstrating that each strategy drives the assembly of a unique gut ecosystem.

Differential abundance analysis identified species-level shifts associated with each treatment (Figures 4A-D). At day 12, compared to their respective PBS, the FBT-R, FST-R, FBT+V9, and FST+V9 groups harbored 131 (63 enriched, 68 depleted), 132 (63 enriched, 69 depleted), 113 (58 enriched, 55 depleted), and 114 (49 enriched, 65 depleted) significant differentially abundant species, respectively. By day 31, these numbers shifted to 59 (43 enriched, 16 depleted), 63 (46 enriched, 17 depleted), 237 (106 enriched, 131 depleted), and 207 (96 enriched, 111 depleted), respectively, revealing a marked expansion of microbial perturbations in the direct supplementation groups over time.

Focusing on bacterial species uniquely altered in only one experimental group ($P < 0.05$), we observed a striking divergence between intervention strategies that intensified over time. At day 12, the FBT-R, FST-R, FBT+V9, and FST+V9 groups harbored eight, five, 16, and 19 unique differential species, respectively (Figure 4C). By day 31, this disparity widened dramatically: FBT-R and FST-R exhibited only two and two unique species, whereas FBT+V9 and FST+V9 contained 25 and eight, respectively (Figure 4D).

Among these group-specific taxa, direct *B. lactis* V9 supplementation (FBT+V9 and FST+V9) was associated, relative to PBS controls, with the depletion of tumor-enriched species, including *Bacteroides caecimuris* and *Helicobacter_C japonicus*, and the enrichment of *Anaerostipes caccae*, *Akkermansia muciniphila*, and *Barnesiella intestinihominis*. In contrast, preconditioned microbiota transplantation (FBT-R and FST-R) induced minimal unique microbial shifts, particularly at day 31, underscoring the more dynamic and extensive ecological remodeling driven by direct probiotic supplementation over time. Notably, we did not detect colonization of *B. lactis* V9 in the recipient gut, suggesting that the effects of direct supplementation may be mediated by transient metabolic activity during the administration period and/or by modulation of the resident microbiota.

To assess ecological interactions, we constructed microbial co-occurrence networks using strong correlations ($|\text{rho}| > 0.60$; Figure 4E), excluding links also present in the PBS group. At day 12, the FBT+V9 and FST+V9 groups exhibited 2071 and 128 significant correlations, respectively, compared to only 25 and 20 in the FBT-R and FST-R groups. By day 31, while FBT+V9 and FST+V9 groups showed reduced network complexity, the direct supplementation groups retained substantially denser and more interconnected networks (103 and 93 vs. 24 and 34 correlations, respectively). The majority of these associations were positive, suggesting enhanced cooperative interactions among taxa.

Collectively, these results demonstrate that direct *B. lactis* V9 supplementation induces broader, more dynamic, and ecologically cohesive microbial remodeling than preconditioned microbiota transplantation. This enhanced inter-microbial connectivity and diversity may underlie its superior capacity to restore immune homeostasis and support anti-tumor responses.

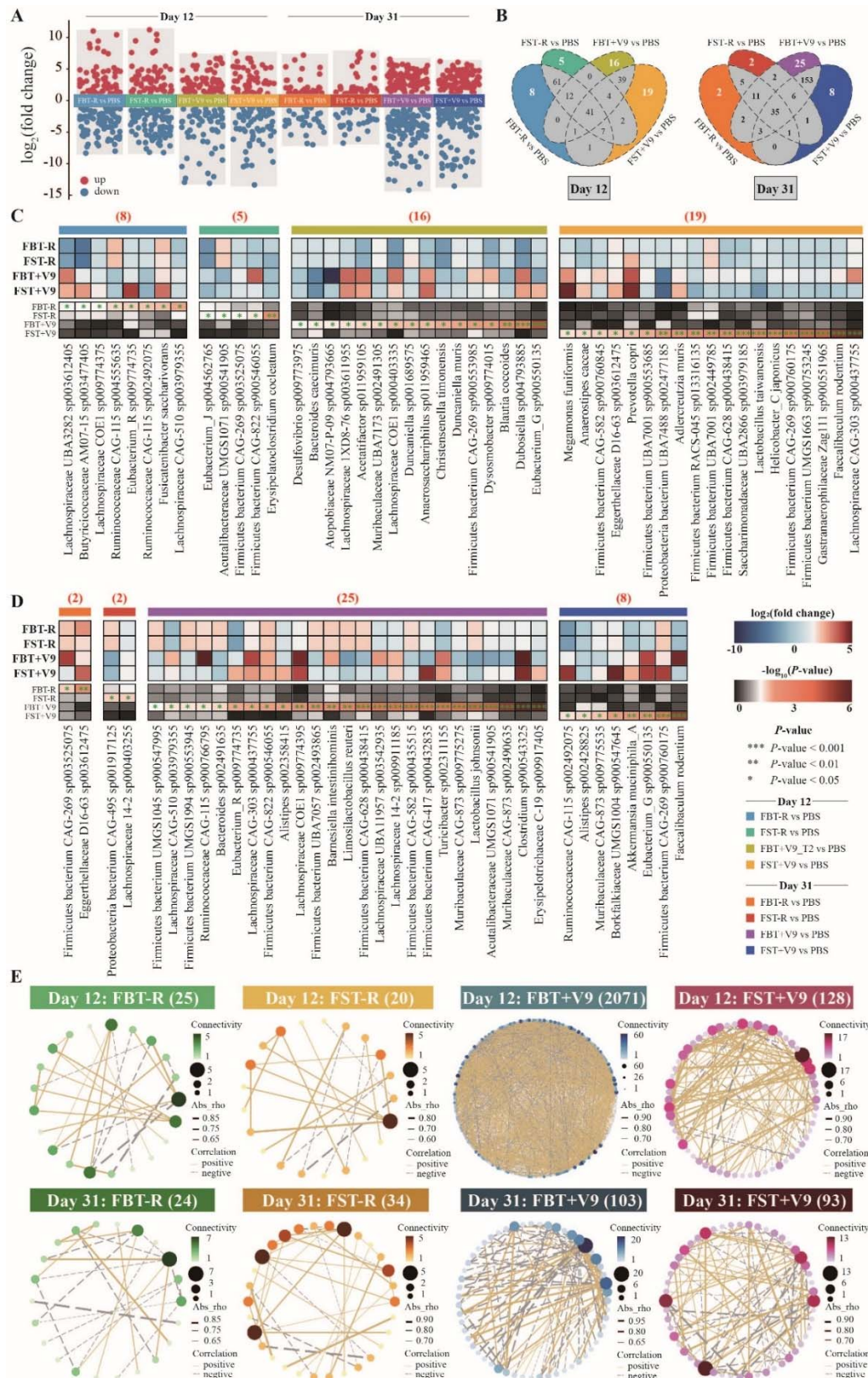


Figure 4. Direct *B. lactis* V9 supplementation induces more extensive and stable microbial remodeling than preconditioned transplantation. (A) Volcano plots of differentially abundant species (vs. PBS) at day 12 and day 31 for FBT-R, FST-R, FBT+V9, and FST+V9. Red (up): increased ($FC > 2$, $P < 0.05$); blue (down): decreased ($FC < 0.5$, $P < 0.05$). (B) Venn diagram of unique and shared differential species across intervention groups (vs. PBS). (C-D) Heatmaps of group-specific differential species at (C) day 12 and (D) day 31, with species count shown at the top. Upper: log₂(fold change); lower: -log₁₀(P-value). (E) Microbial co-occurrence networks ($|\rho| > 0.60$), excluding correlations also present in PBS. Node size and color reflect connectivity degree; beige solid lines = positive correlations; gray dashed lines = negative correlations ($n = 10$ per group). Significant differences were evaluated using Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Differentially abundant metabolites were classified into seven major chemical categories. Carboxylic acids and their derivatives were the most prevalent class in both comparisons. (D-E) Proportions of subclasses of differential carboxylic acid derivatives of the two compared pairs. (F-G) Heatmaps of differentially abundant metabolites in (F) amino acids, peptides, and analogues and (G) alcohols and polyols. Color intensity reflects relative metabolite abundance (purple = low, yellow = high). (H-I) Microbe-metabolite correlation networks for (H) amino acids/peptides and (I) alcohols/polyols. Node color: bacterial species (green), hub taxa (yellow), metabolites (purple/orange), hub metabolites (red/pink). Node size reflects number of connections; edges represent connections.

To explore functional links between microbial taxa and metabolic shifts, we constructed microbe-metabolite correlation networks based on Spearman's rank correlations. Among amino acids, peptides, and analogues, the bacterial species most frequently correlated with metabolite variation included *Dysosmobacter* sp000403435, *Lawsonibacter* sp000492175, *Lachnospiraceae* UBA9502 sp004554205, and *Lachnospiraceae* UMGS1370 sp009774615. Within this subclass, 4-acetamido-2-aminobutanoic acid, *N*²-succinyl-L-arginine, creatine, and L-serine exhibited the broadest associations with microbial taxa (Figure 5H). In the alcohols and polyols category, 2,3-dinor-8-iso prostaglandin F1 α and L-carnitinamide showed the most extensive microbial connectivity. Notably, *Lawsonibacter* sp000492175 display strong correlations across both amino acid- and alcohol-related metabolites (Figure 5I), thereby implicating this taxon as a potential integrator of cross-pathway metabolic regulation. Among them, creatine, L-serine, and L-carnitine are key small molecule metabolites that regulate tumor metabolic reprogramming. Their function and abundance determine the malignant phenotype and systemic immune status of tumors.

Together, these findings demonstrate that both direct *B. lactis* V9 supplementation and preconditioned microbiota transplantation reshape the gut metabolome in distinct yet functionally relevant ways. These metabolite-driven shifts likely contribute to the observed differences in anti-tumor immunity by establishing unique metabolic microenvironments that either support or constrain immune cell function.

3.6 Mouse- and human-derived responder microbiota drive distinct anti-tumor immune and gut microbiota phenotypes

Having established that *B. lactis* V9, enhanced microbiota, whether delivered via direct supplementation or preconditioned transplantation, boosts anti-tumor immunity using human NSCLC donors, we next sought to evaluate how donor species origin influences these effects. To bridge the gap between preclinical models and clinical relevance, we compared responder-derived donor materials generated in a dedicated preliminary study: fecal samples were collected from *B. lactis* V9-treated, immunochemotherapy-responding LLC tumor-bearing mice (mouse-derived; “-M”) and from *B. lactis* V9-treated, immunochemotherapy-responding NSCLC patients (human-derived; “-R”). Fecal bacterial pellets (FBT-M, FBT-R) and cell-free supernatants (FST-M, FST-R) prepared from these donor samples were administered to antibiotic-pretreated recipient mice prior to subcutaneous LLC engraftment (Figure S3A).

In the previous dedicated preliminary study, it showed that both mouse- and human-derived responder microbiota significantly reduced tumor volume and weight relative to PBS controls, confirming anti-tumor efficacy regardless of donor origin. Therefore, we analyzed their effects of different interventions on the host immunity and found that the underlying immune mechanisms diverged markedly. Recipients of mouse-derived microbiota (FBT-M and FST-M) exhibited significantly higher proportions of splenic CD3⁺ T cells than those receiving human-derived material (FBT-R and FST-R; $P < 0.01$; Figure 6A). Moreover, the FST-M group displayed a significantly lower CD4⁺/CD8⁺ ratio than FST-R ($P < 0.01$; Figure 6B), indicative of a more robust CTL response.

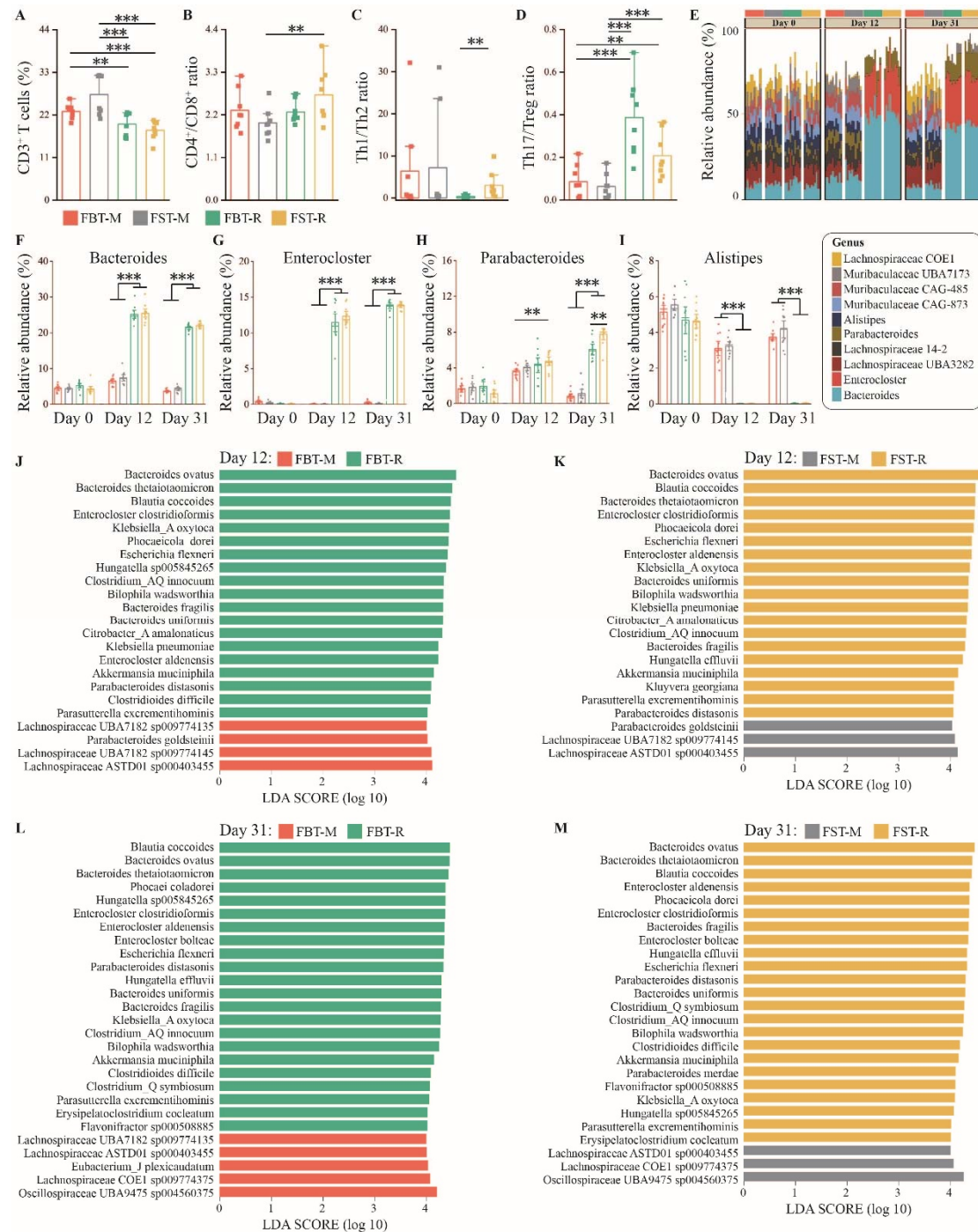


Figure 6. Mouse- and human-derived responder microbiota drive divergent immune and microbial phenotypes. (A) CD3⁺ T cell frequency ($n = 8$ per group). (B-D) Ratios of (B) CD4⁺/CD8⁺, (C) Th1/Th2, and (D) Th17/Treg ($n = 8$ per group). (E) Relative abundance of top 10 genera. (F-I) Relative abundance of four significant differentially abundant bacterial genera, including *Bacteroides*, *Enterocloster*, *Parabacteroides*, and *Alistipes*. (J-M) Linear discriminant analysis effect size (LDA score > 4.0 , $P < 0.05$) of taxa enriched between FBT-M and FBT-R groups at (J) day 12 and (L) day 31, between FST-M and FST-R groups at (K) day 12 and (M) day 31 ($n = 10$ per group). Error bars represent SEM. Data distribution and variance homogeneity were assessed using the Shapiro-Wilk and Levene's tests, respectively. Significant differences were evaluated using the Kruskal-Wallis test with Dunn's post-hoc analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Although no overall difference in Th1/Th2 balance was observed between donor types, the FBT-R group showed a Th2-skewed profile relative to FST-R (Figure 6C), suggesting context-dependent modulation within human-derived cohorts. In contrast, human-derived transplants consistently induced a significantly higher Th17/Treg ratio than mouse-derived ones ($P < 0.01$; Figure 6D), pointing to a

preferential engagement of Th17-mediated immunity. Th17 cells have dual roles in tumor immunity, being both pro-tumorigenic and anti-tumorigenic. In our study, we found that tumor suppression was accompanied by an increase in the Th17/Treg ratio, indicating that Th17 cells are more inclined to support the anti-tumor immune phenotype. However, whether this reflects expansion of anti-tumorigenic Th17 cells or primarily Treg suppression requires further functional characterization. Collectively, these findings indicate that while both donor types suppress tumor growth, they do so through immunologically distinct pathways: mouse-derived microbiota favor systemic T cell expansion and CTL activation, whereas human-derived microbiota skew responses toward Th17/Treg modulation.

To assess how donor origin shapes gut microbial ecology, fecal samples were collected from all groups at baseline (day 0), pre-treatment (day 12), and post-therapy (day 31) for metagenomic sequencing. No differences in α -diversity were detected at day 0 (Figures S3B), confirming equivalent starting conditions. By day 12 and day 31, compared to the no differences in α -diversity between human-derived groups and PBS controls, mouse-derived groups exhibited significantly higher Shannon indices than PBS controls ($P < 0.001$; Figure S3B), reflecting more resilient microbial recovery. Similar to the human-derived groups, principal coordinate analysis revealed clear clustering by mouse-derived species at both time points (Figure S3C).

At the genus level, four of the top 10 taxa showed differential abundance at day 31: *Bacteroides*, *Enterocloster*, and *Parabacteroides* were significantly enriched in human-derived recipients, whereas *Alistipes* predominated in mouse-derived groups (Figures 6F-I). Linear discriminant analysis effect size further revealed that human-derived transplants introduced a broader spectrum of differentially enriched species (Figures 6J-M). At day 12 (Figure 6J, K), FBT-R and FST-R harbored 19 and 19 enriched species, respectively, including immunomodulatory taxa such as *Hungatella effluvii* and *Akkermansia muciniphila*, compared to only four and three species in FBT-M and FST-M groups (including the beneficial *Parabacteroides goldsteinii*). By day 31 (Figure 6L, M), FBT-R alone retained 22 enriched species, including the anti-tumor-associated *Bacteroides thetaiotaomicron* and *Akkermansia muciniphila*. Notably, human-derived microbiota also carried potential opportunistic pathogens such as *Escherichia flexneri* and *Klebsiella_A oxytoca*.

Microbial co-occurrence network analysis demonstrated that mouse-derived communities formed denser, more highly interconnected networks than human-derived ones (Figures 4E, S3D), suggesting superior colonization stability and ecological synergy in syngeneic transplants. In contrast, human-derived microbiota induced more extensive compositional turnover, likely due to host-microbe incompatibilities and adaptation barriers.

These results highlight a critical limitation of conventional murine models: while human responder microbiota can modulate immunity and suppress tumors, they do so through non-overlapping pathways and with reduced ecological integration compared to mouse-derived donors. This underscores the urgent need

for advanced humanized mouse models that better recapitulate the human gut environment to improve the translational fidelity of microbiome-based therapeutic development.

3.7 Mouse- and human-derived responder microbiota reprogram the gut metabolome through donor-specific pathways

Given that mouse- and human-derived responder microbiota drive distinct anti-tumor immune responses and establish divergent microbial ecosystems, we investigated whether these differences are mirrored in systemic metabolic reprogramming. Untargeted metabolomic profiling of fecal samples from recipient mice revealed that donor origin is a dominant factor shaping the gut metabolome. Partial least squares discriminant analysis showed clear separation between mouse- and human-derived recipient groups, confirming that microbiota source fundamentally dictates metabolic output (Figure 7A).

Comparative analysis identified 351 differentially abundant metabolites between FBT-R and FBT-M groups (165 increased, 186 decreased; Figure 7B) and 405 between FST-R and FST-M groups (186 increased, 219 decreased; Figure 7C). Functional classification using MetaboAnalyst 6.0 revealed that carboxylic acids and derivatives was the most prevalent chemical class in both comparisons (Figures 7D, E). Within this category, amino acids, peptides, and analogues accounted for 59.7% (FBT-R vs. FBT-M) and 54.7% (FST-R vs. FST-M) of differential metabolites, while alcohols and polyols formed the second largest group (22.4% and 26.6%, respectively; Figure 7F). Subclass-level analysis further highlighted divergent metabolic signatures: human-derived groups exhibited enrichment across a broader array of amino acid-related compounds (Figure 7G), whereas mouse-derived groups showed more pronounced shifts in alcohols and polyols (Figure 7H).

To identify hub microbial drivers of these metabolic differences, we constructed microbe-metabolite correlation networks. In the amino acid, peptide, and analogue network, central bacterial taxa included *Clostridioides difficile*, *Blautia coccoides*, *Parasutterella excrementihominis*, *Hungatella effluvii*, and *Eubacterium_J plexicaudatum*, which strongly correlated with metabolites such as creatine, L-serine, L-aspartic acid, linatine, and saccharopine (Figure 7I). The alcohol and polyol network was centered on seven molecules: 13(S)-HOTrE, 25-hydroxy-24-epi-brassinolide, L-carnitinamide, L-arabinonate, L-arabinono-1,4-lactone, (10S)-juvenile hormone III acid diol, and hyocholate (Figure 7J). Notably, *Blautia coccoides* and *Hungatella effluvii* emerged as hub species in both networks, suggesting their role as cross-pathway metabolic regulators.

Critically, three metabolites, creatine, L-serine, and L-carnitinamide, were consistently altered across all comparisons, regardless of donor species or administration mode (bacterial vs. supernatant). Their recurrence across experimental contexts implicates them as conserved mediators of microbiota-immune crosstalk, potentially functioning as universal metabolic signals in anti-tumor immunity.

In summary, mouse- and human-derived responder microbiota rewire host metabolism through distinct, donor-specific pathways, which likely contribute to their divergent immunological effects. The identification of conserved core metabolites offers promising targets for microbiome-informed immunotherapies that may

operate independently of species-specific microbial composition, thereby enhancing translational applicability across preclinical and clinical settings.

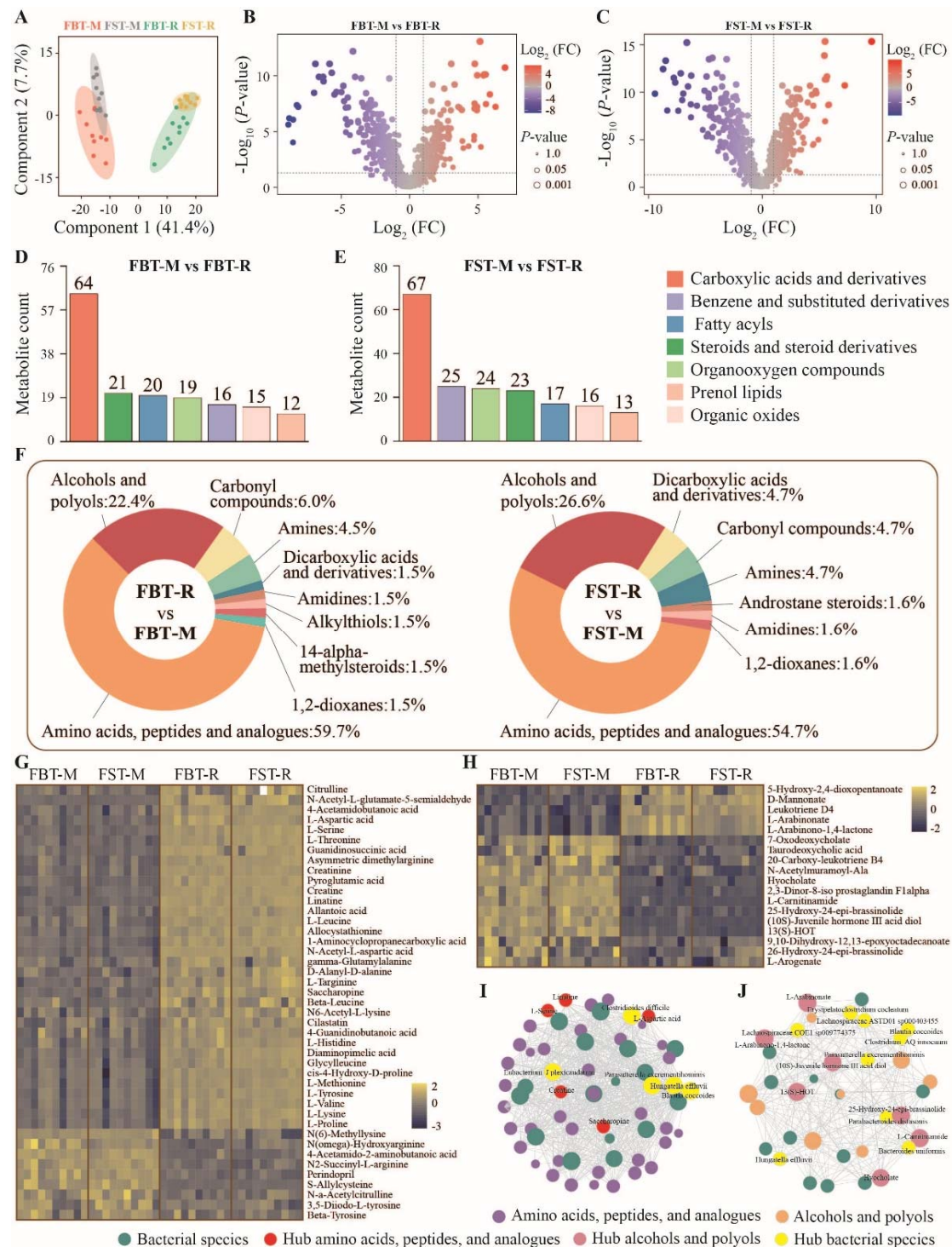


Figure 7. Donor origin dictates metabolomic reprogramming in recipient mice. (A) Partial least squares discrimination analysis of fecal metabolomes ($n = 10$ per group). (B-C) Volcano plots of significant differentially abundant metabolites in (B) FBT-M vs. FBT-R and (C) FST-M vs. FST-R. (D-E) Numbers of significant differentially abundant metabolites between (D) FBT-M and FBT-R and (E) FST-M and FST-R. Differentially abundant metabolites were classified into seven major chemical categories. Carboxylic acids and their derivatives were the most prevalent class in both comparisons. (F) Proportions of subclasses of differential carboxylic acid derivatives of the two compared pairs. (G-H) Heatmaps of differentially abundant metabolites in (G) amino acids, peptides and analogues and (H) alcohols and polyols. Color intensity reflects relative metabolite abundance (purple = low, yellow = high). (I-J) Microbe-metabolite correlation networks for (I) amino acids/peptides and (J) alcohols/polyols. Node color: bacterial species (green), hub taxa (yellow), metabolites (purple/orange), hub metabolites (red/pink). Node size reflects number of connections; edges represent connections.

4. Discussion

This study was designed to address three interrelated gaps in microbiome-oncology research: (1) whether microbial metabolites or live bacteria drive immunosuppression in high-risk NSCLC; (2) how the mode of probiotic delivery, direct supplementation versus preconditioned microbiota transplantation, affects anti-tumor immunity; and (3) whether donor species origin, mouse versus human, fundamentally shapes the ecological and functional outcomes of fecal microbiota transplantation. By integrating stratified FMT, multi-omics profiling, and immune phenotyping in a syngeneic LLC model, our findings provide mechanistic clarity and conceptual advances with direct implications for clinical translation.

We first demonstrate that in NSCLC patients with elevated serum CYFRA 21-1, a biomarker that defines a clinically relevant, high-risk subgroup characterized by immunosuppression and poor response to immunochemotherapy [12, 13], gut microbial metabolites, not intact bacteria, are the primary mediators of impaired anti-tumor immunity. Fecal supernatant transfer from these donors recapitulated systemic T cell depletion and accelerated tumor progression during chemoimmunotherapy, whereas FBT did not. This finding directly links microbiota-derived metabolic dysfunction to a defined patient population with unmet clinical need. Notably, the FBT group exhibited an elevated F/B ratio, a metric frequently associated with metabolic disease and oncogenesis [34], yet did not promote tumor growth. This paradox underscores a critical principle: functional metabolite output, rather than conventional compositional indices, more accurately reflects the immunoregulatory capacity of the gut microbiota. These results align with emerging evidence that microbial metabolic products, not taxonomic presence alone, dictate systemic immune states and shape the TIME [35-37].

Second, we reveal that even a single probiotic strain, *B. lactis* V9, can steer the host immune system toward divergent anti-tumor trajectories depending on delivery strategy. Direct supplementation most effectively restored the peripheral T cell compartment, promoted expansion of CD8⁺ cytotoxic T lymphocytes, polarized immunity toward a Th1-dominant phenotype, and was accompanied by enhanced gut microbial α -diversity and stronger species co-occurrence networks, hallmarks of a resilient, anti-tumor-supportive ecosystem. In addition, no colonization of *B. lactis* V9 was detected in the gut of recipient mice receiving direct *B. lactis* V9 supplementation, indicating that it may induce sustained immune reprogramming by inducing cumulative ecological and metabolic changes in the resident microbiota through daily repeated gavage. In contrast, *B. lactis* V9-preconditioned microbiota transplantation suppressed tumors with comparable efficacy but operated primarily through Th17-associated pathways and modulation of the Th17/Treg balance, without fully reversing T cell loss. These results extend prior work showing *Bifidobacterium* enhances checkpoint blockade efficacy [16, 38, 39] and support the model proposed by Spencer et al. (2021) [40], wherein probiotic effects are mediated through functional interactions with the resident gut community. Critically, this distinction carries direct clinical relevance: for CYFRA 21-1-high NSCLC patients, direct *B. lactis* V9 supplementation may represent a more effective combinatorial strategy

to restore anti-tumor immunity, while preconditioned microbiota transplantation could offer a viable alternative for individuals intolerant to live probiotics.

Third, FMT holds considerable promise for treating microbiota-related diseases [41, 42], yet its application in oncology remains largely exploratory. A major obstacle to clinical translation stems from fundamental physiological, immunological, and microbial differences between preclinical mouse models and human patients [43-45]. Addressing this gap, we present a systematic comparison of responder-derived microbiota from syngeneic mice versus NSCLC patients and demonstrate that donor origin profoundly shapes colonization dynamics, immune polarization, and metabolic reprogramming. Owing to host-microbe compatibility, mouse-derived microbiota colonized more rapidly and stably in recipient mice, formed synergistic inter-species networks, and elicited a stronger CTL response. In contrast, human-derived microbiota, shaped by divergent host genetics, diet, and lifestyle, induced more extensive restructuring of community architecture, carried potential opportunistic pathogens such as *Escherichia flexneri* and *Klebsiella_A oxytoca*, and preferentially skewed immunity toward a Th17/Treg balance. The differential immune outcomes between mouse- and human-derived microbiota raise the question of whether these differences reflect intrinsic functional disparities between the microbial consortia or represent a xenogeneic response of the murine host to human-origin microbes. Several lines of evidence suggest that microbial functional differences likely contribute substantially to the observed immune polarization. First, the distinct taxonomic and metabolomic profiles between the two donor types, particularly the enrichment of Th17-associated taxa (e.g., *Bacteroides*, *Parabacteroides*) in human-derived microbiota, align with their Th17-skewed immune outcome. Second, the conserved metabolites identified across both donor types (creatine, L-serine, L-carnitinamide) were consistently associated with anti-tumor responses, suggesting that metabolite function may transcend donor origin. Nevertheless, we cannot exclude the possibility that host recognition of xenogeneic microbial antigens contributes to the Th17/Treg bias observed in human-derived microbial recipients. Dissecting the relative contributions of microbial function versus host xenoreactivity will require future studies employing germ-free mice colonized with defined human- or mouse-derived microbiota under matched metabolic conditions, as well as assessment of host antibody responses to donor microbes. These findings establish donor species as a decisive determinant of FMT efficacy and provide a mechanistic explanation for the frequent disconnect between murine studies and human trials. Based on the practical application scenarios of FMT in clinical NSCLC patients, this result suggests that strict screening and elimination of opportunistic pathogens in donor feces should be carried out before implementing human microbiota transplantation in clinical practice. At the same time, attention should be paid to the regulation of Th17/Treg balance to avoid excessive Th17 polarization causing inflammatory reactions that affect treatment efficacy. The colonization advantage of mouse-derived microbiota provides phenotype reference for clinical screening of core microbiota with high colonization and CTL induction activity. Consequently, future efforts should prioritize the development of humanized mouse models that better recapitulate human

gut physiology and immunity to improve the predictive validity of microbiota-based interventions in immuno-oncology.

A central discovery across all intervention strategies and donor sources was the consistent identification of three metabolites, creatine, L-serine, and L-carnitinamide, as conserved core mediators of microbiota-driven anti-tumor immunity. These molecules emerged as hub nodes in microbe-metabolite correlation networks and are biologically plausible regulators of immunometabolism. Creatine enhances T cell anti-tumor activity by improving mitochondrial function [46–48]; in melanoma patients, intratumoral creatine levels correlate positively with immunotherapy response [49]. L-serine, a central player in one-carbon metabolism, governs T cell proliferation and differentiation [50–52]; its deficiency induces CD8⁺ T cell exhaustion [53], whereas supplementation can reinvigorate cytotoxic function [54]. L-carnitinamide is closely linked to fatty acid oxidation, a critical energy pathway for memory T cells and Treg cells [55], and its analog has been shown to suppress M2-like polarization of tumor-associated macrophages in colorectal cancer models [56]. In our study, L-carnitinamide drives the CTL/Th1 dominant immune response in the direct *B. lactis* V9 supplementation group, while creatine and L-serine jointly mediate key metabolites regulating Th17/Treg balance in *B. lactis* V9-preconditioned and human-derived microbiota transplantation groups. This multi metabolite synergistic immune regulatory pattern constitutes a conserved mechanism for microbiota-immunity interactions in high-risk NSCLC. Their recurrence across diverse experimental contexts highlights their potential as universal, species-agnostic targets for microbiome-informed immunomodulation. While these correlative findings do not establish causality, the consistent association of creatine, L-serine, and L-carnitinamide across diverse experimental contexts positions them as high-priority candidates for future mechanistic investigation.

Strengths of this work include its hypothesis-driven, multi-layered experimental design that isolates the effects of microbial form (live vs. metabolite), intervention strategy (direct vs. preconditioned), and donor origin (mouse vs. human). The integration of metagenomic sequencing, untargeted metabolomics (analyzed via MetaboAnalyst 6.0), and comprehensive flow cytometry enabled causal tracing from microbial inputs to metabolic intermediates to immune outputs.

Nevertheless, several limitations warrant consideration. First, the human donor cohort was limited to three NSCLC patients per group, which may constrain the generalizability of microbial and metabolomic associations. Second, the specific chemical identities and mechanisms of the immunosuppressive metabolites present in fecal supernatants remain uncharacterized. Third, although FBT and FST were comparable in terms of component sources, the contribution of dose effects to phenotype differences cannot be completely ruled out. Future research can further analyze the respective contributions of live bacteria and metabolites more accurately through quantitative normalization of metabolites or dose-response curve studies. Fourth, all human-derived FMT experiments were conducted in immunocompetent C57BL/6 mice, which lack human immune receptors, mucosal interfaces, and dietary context, potentially underestimating

functional fidelity. Finally, metabolite concentrations in systemic circulation or the tumor microenvironment, more direct mediators of immune modulation, were not measured.

Together, these findings redefine the mechanistic hierarchy of microbiota-immunity interactions in high-risk NSCLC: microbial metabolites, not live bacteria, are the dominant effectors of immunosuppression, and their modulation by probiotic intervention is critically dependent on delivery strategy and donor compatibility. By identifying conserved metabolite mediators and exposing the translational constraints of cross-species FMT, this work provides a functional framework for designing safer, more effective microbiome-based therapies. Future efforts should focus on metabolite-targeted adjuvants, personalized delivery regimens, and human-relevant preclinical models to realize the full potential of microbiota modulation in immuno-oncology.

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Competing interests

The authors declare no competing interests.

Data availability statement

Raw metagenomic sequencing data have been deposited in the CNGB Sequence Archive of the China National GeneBank DataBase (CNGBdb; <https://db.cngb.org/cnsa/>) under accession numbers CNP0008945. These data will be publicly accessible upon publication.

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