

Probiotic prioritization manuscript draft

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Shared Metabolomic Signatures Across Alcohol and Caffeine-Related Exposures and Probiotic Prioritization via Genome-Scale Modeling

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Interpretation

The primary model ranks predicted gut–brain-relevant microbial functions. A separate experimental-priority model ranked *L. plantarum* ATCC 14917 first among probiotic-deployable candidates. The mouse experiment used HMPM2111, a different strain. It does not validate the primary ranking or the exact top-ranked secondary-model strain. Direct testing of the primary leaders remains future work.

Open reporting items

Figure 3's Venn counts total 1,916 alcohol features and 404 caffeine annotations, whereas the text reports 1,919 and 405. The distinction between feature counts and matched or deduplicated annotations needs reconciliation. No figure counts have been changed in this copy.

Complete endpoint-specific animal counts and statistical outputs are still needed. Figures 5–6 should be read as preliminary; the immunofluorescence images remain descriptive pending blinded animal-level quantification. The draft also notes incomplete pathway-analysis session history and reliance on cached host-transcriptomic contrasts.

Project page and current materials

<https://www.jalencai.com/research/gut-brain-axis/>

Reader note added September 21, 2026. The source document follows on the next page.

Shared Metabolomic Signatures Across Alcohol and Caffeine-Related Exposures and Probiotic Prioritization via Genome-Scale Modeling

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Abstract. **Background:** Alcohol exposure and caffeine-induced sleep restriction may share fecal metabolomic changes that can inform probiotic prioritization. **Methods:** Public metabolomics data from alcohol-exposed rats and caffeine-induced sleep-restricted mice were compared for directionally concordant metabolite annotations and pathways. AGORA2 genome-scale metabolic modeling and supervised pairwise ranking prioritized strains using gut-brain-relevant mediator proxies, while a separate fixed-weight experimental-priority score integrated the primary ranking with public host-transcriptomic evidence, curated literature, feasibility, safety, and uncertainty. An in vivo mouse study compared control, caffeine, and caffeine plus *Lactiplantibacillus plantarum* HMPM2111 (LP) groups using behavioral and prefrontal-cortex molecular endpoints. **Results:** Nine metabolite annotations changed in the same direction across datasets. The primary ranking favored Akkermansia lineages overall and *Bifidobacterium animalis* among probiotic-deployable candidates. In the experimental-priority model, *L. plantarum* ATCC 14917 ranked first among 114 probiotic-deployable candidates, although sensitivity analyses showed dependence on curated and LP-specific evidence. HMPM2111 was distinct from the highest-ranked LP strain, and preliminary behavioral and transcript patterns shifted toward control values after LP treatment. **Conclusion:** This framework generates testable probiotic hypotheses from heterogeneous exposure datasets, and the LP experiment provides preliminary species-level proof-of-concept evidence for probiotic intervention in the caffeine-related model.

Keywords: alcohol; caffeine; gut microbiota; microbiota-gut-brain axis; metabolomics; probiotics; genome-scale metabolic modeling

1. Introduction

Substance use disorders involve persistent neuroadaptations in reward, stress, and executive-control circuits [1,2]. Alcohol can produce tolerance, withdrawal, craving, and systemic injury [3], while caffeine is a widely consumed psychoactive compound associated with reinforcement, withdrawal, anxiety, and sleep disruption [4,5]. Despite distinct pharmacology, both exposures can influence stress and neurobehavioral regulation.

The gut microbiota communicates with the central nervous system through neural, immune, endocrine, barrier, and metabolite-mediated pathways collectively described as the microbiota–gut–brain axis [6]. Short-chain fatty acids, tryptophan derivatives, bile-acid metabolites, and GABA-

related compounds can influence inflammatory tone and neural signaling, motivating interest in probiotic and psychobiotic interventions [7,8]. Alcohol-related gut dysbiosis and permeability correlate with behavioral severity [10], and caffeine-induced sleep restriction alters the mouse gut microbiome and fecal metabolome [11].

Whether these distinct exposures share a directionally consistent fecal metabolomic signature and whether that signature can guide strain-level probiotic selection remain unclear. We therefore integrated public alcohol- and caffeine-related rodent metabolomics, identified concordant metabolite and pathway changes, used AGORA2-based machine-learning ranking to prioritize candidate strains [13], and evaluated *Lactiplantibacillus plantarum* (LP; formerly *Lactobacillus plantarum* [9]) as an experimentally feasible proof-of-concept benchmark.

2. Materials and Methods

2.1. Public metabolomics datasets

The alcohol dataset was derived from the rat alcohol-use-disorder study reported by Wang et al. [3] and comprised the positive-ion fecal untargeted liquid chromatography-mass spectrometry feature table from 16 rats (8 control and 8 alcohol-exposed; 5,490 features) subjected to chronic intermittent ethanol voluntary drinking. Positive-ion data defined the primary principal component analysis (PCA), differential analysis, and cross-substance overlap, whereas the 5,945-feature negative-ion table was analyzed as a complementary sensitivity analysis. Source data are available through the National Microbiology Data Center under accession NMDCX0000138.

The caffeine dataset was derived from the caffeine-induced sleep restriction mouse study reported by Song et al. [11]. Because sample-level fecal intensities were unavailable for reanalysis, the published differential-metabolite table and original study statistics were used. The analysis used the 405 annotated metabolites reported in Supplementary Table S4 that met the source-study screening criteria of $p < 0.05$, fold change > 1.2 , and variable importance in projection (VIP) > 1 relative to controls.

2.2. Alcohol metabolomics preprocessing and differential analysis

Alcohol feature intensities were matched to sample metadata, transformed as $\log_{10}(x + 1 \times 10^{-8})$, and filtered to remove zero-variance features. Principal component analysis was performed on mean-centered, unit-variance-scaled data. Group separation was evaluated by permutational multivariate analysis of variance (PERMANOVA) with 999 permutations in the full scaled feature space and, separately, in the first seven principal components, which explained 80.8% of cumulative variance; analysis of similarities (ANOSIM) was retained as a secondary diagnostic.

Alcohol-exposed and control groups were compared feature-wise using two-sided Welch t-tests on transformed intensities. P values were adjusted by the Benjamini-Hochberg procedure, and FDR $q < 0.05$ without a minimum fold-change threshold defined the primary differential set. Positive- and negative-ion modes were analyzed separately; positive mode defined the main overlap signature, and Table 1 reports positive-mode effect sizes converted to $\log_2$ fold change.

2.3. Pathway enrichment and cross-substance overlap

Upregulated and downregulated lists were analyzed separately using MetaboAnalyst 6.0 [14]. A pathway was considered shared only when it appeared in the corresponding direction-specific analyses for both exposures. Metabolite names were harmonized for formatting and obvious synonyms, then intersected separately for concordant up- and downregulation. Because standardized

identifiers and caffeine sample-level intensities were unavailable, the comparison was direction- and name-based rather than a pooled effect-size meta-analysis.

2.4. Primary genome-scale metabolic and machine-learning model

Strain-level metabolic capabilities were derived from AGORA2 genome-scale reconstructions and simulated in COBRApy under the AGORA Western-diet medium [13,15]. Models with maximal biomass production $\leq 1 \times 10^{-6}$ were excluded; for feasible models, secretion and uptake potentials were estimated while maintaining at least 10% of strain-specific maximal biomass. Model features captured short-chain-fatty-acid production, indole and GABA potential, bile-acid modulation, and penalties for excess succinate and ethanol production.

Because most of the nine shared annotations were host-derived lipids, steroid-related signals, or xenobiotics not consistently represented as microbial exchange reactions, the primary ranker used microbiome-plausible mediator features rather than claiming direct production, consumption, or restoration of every shared metabolite. Standardized barrier-support, neurocalming, pro-inflammatory, and overall postbiotic composites were used for ranking.

2.5. Empirical labels and pairwise ranking

Empirical strain-metabolite responses were assembled from Metabolomics Workbench study ST001688 and Ricci et al. [16] (internal source label PMC9486989_FA), which reports acetate and lactate concentrations for 14 bifidobacterial or *Collinsella* culture supernatants. The target panel included acetate, propionate, butyrate, lactate, succinate, indole, indole-3-acetate, indole-3-propionate, and GABA. Responses were averaged by strain, directionally oriented, standardized within study, and combined with the mechanistic score to form the final training label (65% empirical evidence and 35% mechanistic evidence). Strains were mapped to AGORA2 models by exact identifier where possible and otherwise by audited genus-species matching. Pairwise feature differences were used to train a class-balanced logistic-regression ranker, which was applied to all feasible models and to a prespecified probiotic-deployable subset.

2.6. Primary-model validation and uncertainty

Primary-model performance was assessed by stratified five-fold cross-validation repeated across 60 data splits, a 2,000-permutation label test, and 400 bootstrap resamples for rank stability. The executed training table reports only one study identifier, so these analyses constitute internal validation; independent leave-one-study-out or external-study validation was not possible. Area under the receiver operating characteristic curve (AUC) intervals and rank-stability estimates were interpreted as model-uncertainty measures rather than confidence intervals for causal treatment effects.

2.7. Secondary experimental-priority model

The primary and secondary models answered distinct, complementary questions. The primary model ranked strains using predicted gut–brain-relevant mediator functions and remained the principal mechanistic ranking; it did not directly model restoration of all nine shared annotations. Because the strongest predicted restorative strains were not necessarily available, sufficiently characterized, or practical for immediate animal testing, a separate fixed-weight experimental-priority model was used only to select a near-term proof-of-concept benchmark. It combined four normalized positive components—primary restoration (0.40), public host-response evidence from Gene Expression Omnibus (GEO) (0.25), gut–brain plausibility evidence (0.20), and feasibility/safety (0.15)—and

subtracted a bounded uncertainty penalty with coefficient 0.15. Thus, the secondary model did not replace or retrain the primary model; it translated the primary ranking into an experimentally deployable candidate list. Local LP behavioral and molecular outcomes were excluded from all ranking inputs.

Host-response evidence used accession-derived contrasts from GEO series GSE74988 and GSE87368. Prespecified gene sets summarized epithelial barrier, mucosal repair, inflammatory, oxidative-stress, eicosanoid, and neuroimmune responses. Evidence-transfer coefficients were 1.00 for exact-strain evidence and 0.35 for a different strain of the same species; related-species transfer was evaluated only in sensitivity analysis.

Literature, feasibility, and safety components were bounded to limit sparse-evidence amplification. Predeclared sensitivity analyses removed or reduced curated literature, feasibility/safety, species transfer, or LP-specific evidence. The publication pipeline passed all 16 safeguards/tests, and 43 inputs/outputs were checksum-frozen.

2.8. Animal study, treatment allocation, and ethics

All animal procedures were approved by the Institutional Animal Care and Use Committee of Anhui University under protocol IACUC(AHU)-2025-029. Reporting was structured in accordance with ARRIVE 2.0 [19]. The study design specified 30 male specific-pathogen-free C57BL/6J mice, 8–10 weeks of age, assigned to control, caffeine, and caffeine + LP groups ($n = 10$ per group). Mice were obtained from Hefei Qingyuan Biotechnology Co., Ltd., acclimated for 7 days, housed under a 12-h light/12-h dark cycle, and randomly assigned to groups.

Control mice received double-distilled water and daily PBS gavage. Caffeine groups received 50 mg/kg caffeine intraperitoneally at 08:00 and 20:00 for 7 days with PBS or *L. plantarum* HMPM2111. LP was gavaged once daily for 14 days (2×10^8 CFU/mouse in 200 μ L PBS) after a 4-h fast, with caffeine administered on days 8–14. HMPM2111 (GDMCC No. 64520) was cultured in MRS medium and viability verified by serial-dilution plating. Behavioral testing occurred 30 min after the final caffeine injection.

2.9. Behavioral assays

The open-field test was conducted for 10 min in a $500 \times 500 \times 400$ mm arena; VisuTrack-AI quantified total distance, center distance/time, and center entries. The elevated plus maze contained two open and two closed 35×5 cm arms, with 15-cm closed-arm walls, elevated 50 cm; mice were tested for 10 min, and total entries, open-arm entry percentage, and open-arm time were quantified. Apparatuses were cleaned with 75% ethanol between animals.

2.10. Tissue collection, immunofluorescence, and quantitative PCR

Within 24 h of behavioral testing, mice were anesthetized with sodium pentobarbital and euthanized by cervical dislocation. Prefrontal-cortex tissue for qPCR was saline-perfused, snap-frozen, and stored at -80°C ; tissue for immunofluorescence was perfused/fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT, and stored at -80°C . qPCR and immunofluorescence each used $n = 3$ animals/group.

Immunofluorescence targeted Iba1 and GFAP with DAPI nuclear counterstaining. Total RNA was extracted with Sparkzol, reverse-transcribed from 1,200 ng RNA, and analyzed on a QuantStudio 3 using SYBR Green chemistry. Relative expression was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method from technical triplicates and normalized to β -actin.

2.11. Statistical analysis

All metabolomics tests were two-sided. Benjamini-Hochberg correction was applied to alcohol feature-level tests and GEO differential-expression analyses.

Behavioral and qPCR outcomes were analyzed in GraphPad Prism 8.0 using one-way analysis of variance (ANOVA) followed by Tukey multiple-comparison tests for the three treatment groups. The individual animal was treated as the statistical unit. Data are presented as mean $\pm$ standard deviation (SD), and $p < 0.05$ was considered statistically significant. Immunofluorescence images are presented descriptively because blinded animal-level quantification is not yet available.

2.12. Data and code availability

- Alcohol metabolomics are available under NMDCX0000138; caffeine data are from Song et al. [11]; host transcriptomics are from GSE74988 and GSE87368; empirical training data are from Metabolomics Workbench ST001688. Analysis code and derived outputs are available at <https://github.com/JalenCai/probiotic-prioritization-alcohol-caffeine>.

3. Results

3.1. Alcohol exposure induced broad fecal metabolomic remodeling

Positive-ion fecal metabolomics from 16 rats (8 alcohol-exposed, 8 controls) showed partial PCA separation (PC1 42.24%, PC2 11.26%; Figure 1A). PERMANOVA confirmed group-level structure in the full scaled feature space ($F = 5.506$, $p = 0.001$; 999 permutations), with concordant ANOSIM support ($R = 0.5965$, $p = 0.001$).

Of 5,490 positive-ion features, 1,919 met $FDR\ q < 0.05$ (1,646 increased; 273 decreased; Figure 1B–C). A complementary negative-ion analysis identified 48 FDR -significant features but was not used to define the primary overlap signature.

Alcohol-associated pathway enrichment included purine, histidine, butyrate, lipid, bile-acid, and sphingolipid-related processes (Figure 1D–E), supporting downstream cross-substance comparison.

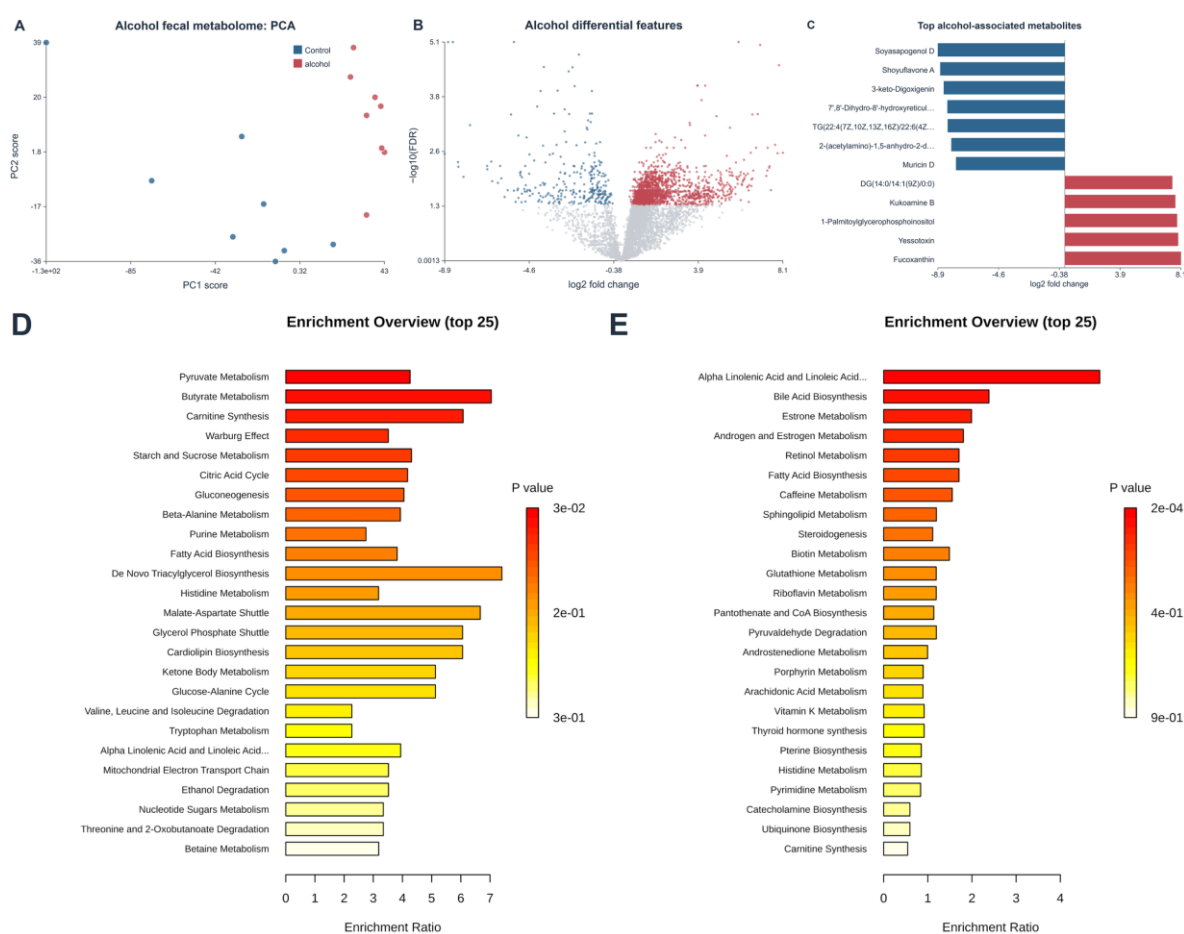


Figure 1. Alcohol-associated fecal metabolomic changes: (A) PCA; (B) differential-feature volcano plot; (C) top fold-change metabolites; (D–E) pathway enrichment for down- and upregulated metabolites.

3.2. Caffeine-related exposure was associated with fecal metabolite and pathway disruption

A public mouse caffeine-induced sleep restriction dataset was used to evaluate caffeine-related fecal metabolite changes [11]. The reported differential list contained 405 metabolites meeting the source-study criteria ($p < 0.05$, fold change > 1.2), with prominent lipid- and carnitine-related signals.

Pathway enrichment showed overlap with the alcohol dataset. Downregulated caffeine-associated metabolites were enriched in purine and histidine metabolism, phospholipid biosynthesis, and mitochondrial fatty-acid beta-oxidation (Figure 2A). Upregulated metabolites were enriched in caffeine metabolism, branched-chain amino-acid degradation, de novo triacylglycerol biosynthesis, steroidogenesis, and the malate-aspartate shuttle (Figure 2B).

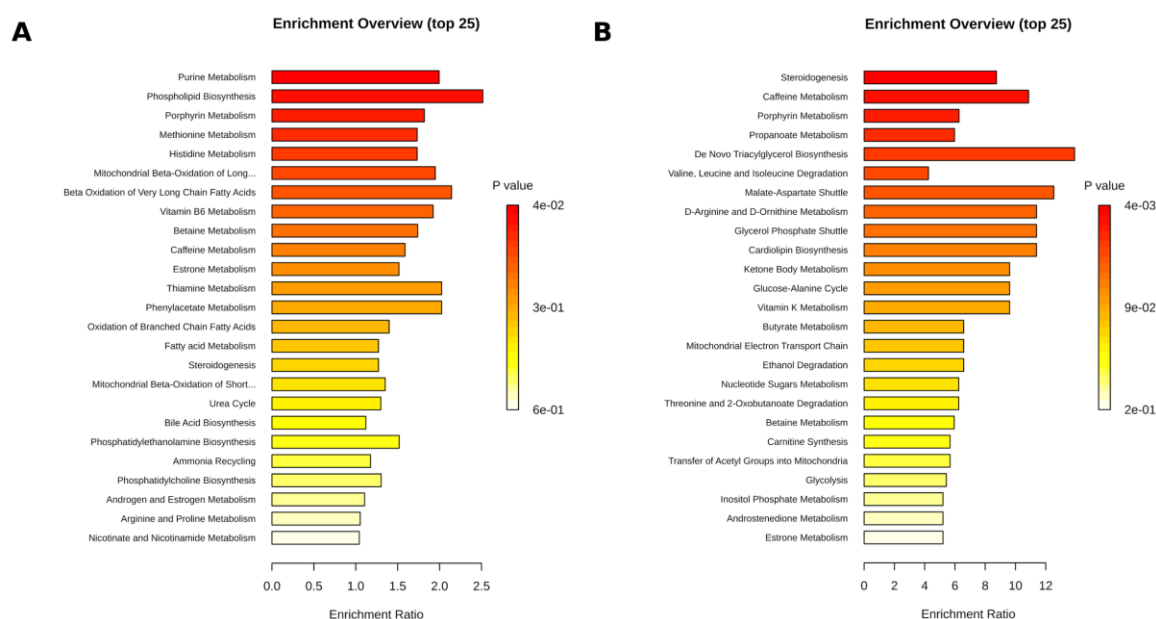


Figure 2. Caffeine-associated pathway enrichment. (A) Enrichment overview for downregulated caffeine-associated metabolites. (B) Enrichment overview for upregulated caffeine-associated metabolites.

3.3. Alcohol and caffeine shared nine directionally concordant metabolites

Direction-based overlap identified nine concordant metabolites. Seven increased in both datasets—ricinoleic acid, cortisone, corchorusoside B, postin, CE(5:0), 19-hydroxy-PGE2, and LysoPE(0:0/22:4(7Z,10Z,13Z,16Z))—and two decreased: 3-dehydroxycarnitine and O-ethyl S,S-diphenyl phosphorodithioate (Figure 3A–B; Table 1).

The nine-metabolite heatmap showed coordinated alcohol-associated shifts (Figure 3C). Because the source studies differed in species and experimental context, the overlap is interpreted as directional convergence rather than a pooled effect-size meta-analysis.

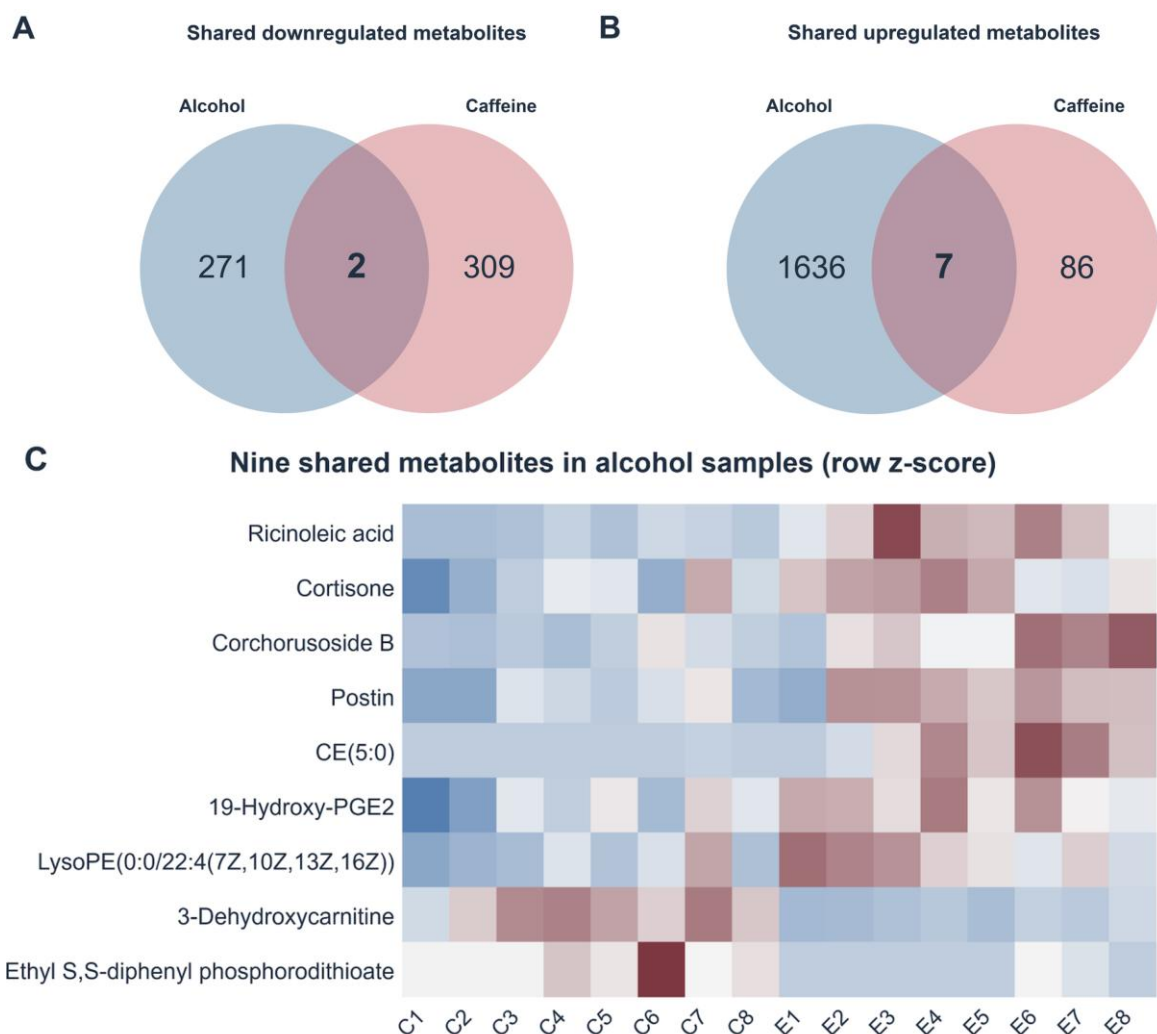


Figure 3. Cross-substance overlap: (A) shared downregulated metabolites; (B) shared upregulated metabolites; (C) heatmap of the nine shared metabolites in alcohol samples.

Table 1. Shared metabolites with concordant direction across alcohol and caffeine datasets.

Metabolite	Direction	Alcohol log2FC	Alcohol p	Alcohol FDR q	Caffeine FC	Caffeine p
Ricinoleic acid	Up	4.514	0.00389	0.0247	2.77	0.0107
Cortisone	Up	0.902	0.0165	0.0481	1.81	0.000656
Corchorusoside B	Up	2.075	0.00406	0.0251	1.62	0.0395
Postin	Up	0.749	0.00534	0.0282	62.9	2.81e-06
CE(5:0)	Up	6.016	0.000279	0.00853	1.34	0.0335
19-Hydroxy-PGE2	Up	0.677	0.0143	0.0446	7.48	0.000332
LysoPE(0:0/22:4(7Z,10Z,13Z,16Z))	Up	0.929	0.00473	0.0269	1.78	0.0249
3-Dehydroxycarnitine	Down	-2.783	1.37e-05	0.00168	0.492	0.0073
O-Ethyl S,S-diphenyl phosphorodithioate	Down	-4.647	0.000374	0.00927	0.416	0.000364

3.4. AI-guided genome-scale modeling prioritized candidate strains for metabolome restoration

Because several shared annotations were host-derived or absent as AGORA2 exchange reactions, the primary model ranked strains using microbiome-plausible mediator features—short-chain fatty acids, indole/GABA potential, bile-acid modulation, barrier support, and penalties for excess

succinate/ethanol—under a Western-diet medium [13]. Thus, rankings represent indirect gut–brain-relevant restoration potential rather than direct control of all nine metabolites.

The primary ranker showed moderate internal discrimination (mean AUC 0.749; 95% interval 0.728–0.770; accuracy 0.690), while permutation testing was not definitive ($p = 0.137$). No external-study validation was possible, so the model is interpreted as a hypothesis-generating prioritization tool.

Akkermansia muciniphila YL44 ranked highest overall, whereas *Bifidobacterium animalis* strains were most consistently prioritized in the probiotic-deployable subset (Figure 4).

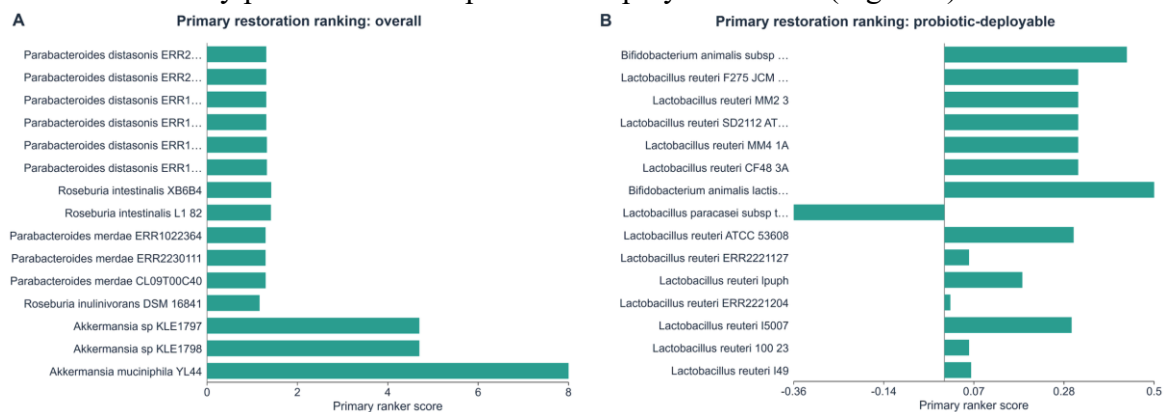


Figure 4. Primary strain prioritization: (A) top overall candidates; (B) probiotic-deployable candidates.

3.5. A secondary experimental-priority model selected LP as a near-term validation benchmark

A separate experimental-priority model addressed near-term testability rather than replacing the primary mechanistic ranking. The fixed score combined primary restoration (0.40), public GEO evidence (0.25), gut–brain plausibility (0.20), feasibility/safety (0.15), and an uncertainty penalty (0.15); wet-lab outcomes were excluded from model inputs.

The rebuilt v3 workflow preserved the distinction between metabolome restoration and experimental priority. In the primary probiotic-filtered ranking, *L. plantarum* WCFS1 ranked 46th. In the full secondary model, *L. plantarum* ATCC 14917 ranked first among 114 probiotic-deployable candidates and sixth overall; WCFS1 ranked 11th. HMPM2111 (GDMCC No. 64520), the administered strain, is distinct from both ATCC 14917 and WCFS1; therefore, the wet-lab study does not validate the exact top-ranked LP strain.

Sensitivity analyses showed that LP prioritization was conditional: the best LP rank remained first when feasibility/safety was removed or species-level transfer was disabled, but fell to 36 without curated literature, 17 without LP-specific evidence, and 51 under primary restoration alone. Wet-lab outcomes were excluded from ranking inputs, and strict taxonomy parsing excluded *L. paraplantarum* from exact LP classification.

3.6. Preliminary behavioral patterns after caffeine and LP treatment

In the caffeine model, caffeine-exposed mice showed lower open-field locomotor/center exploration and reduced elevated-plus-maze open-arm exploration, while LP + caffeine values shifted toward controls (Figure 5). Because locomotor activity also changed, anxiety-like measures should be interpreted alongside total movement.

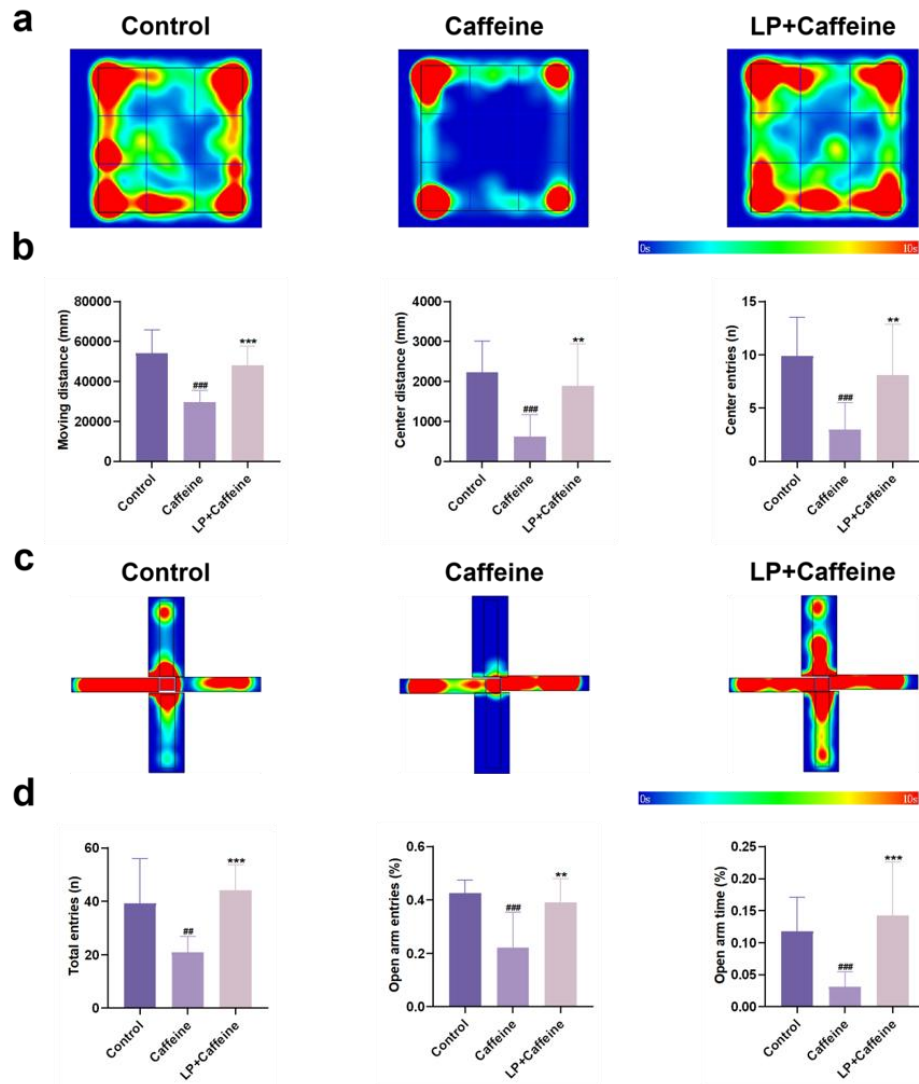


Figure 5. Preliminary behavioral readouts after caffeine and LP treatment. Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus caffeine; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus control.

3.7. Preliminary inflammatory and barrier-associated molecular patterns

Prefrontal-cortex qPCR showed higher pro-inflammatory transcripts (IL-6, IL-1 β , TNF- α) and lower IL-10, ZO-1/TJP1, and occludin/OCLN after caffeine, with directional shifts toward controls after LP treatment (Figure 6B–G). Representative Iba1/GFAP images are descriptive only because blinded animal-level quantification is not yet available (Figure 6A).

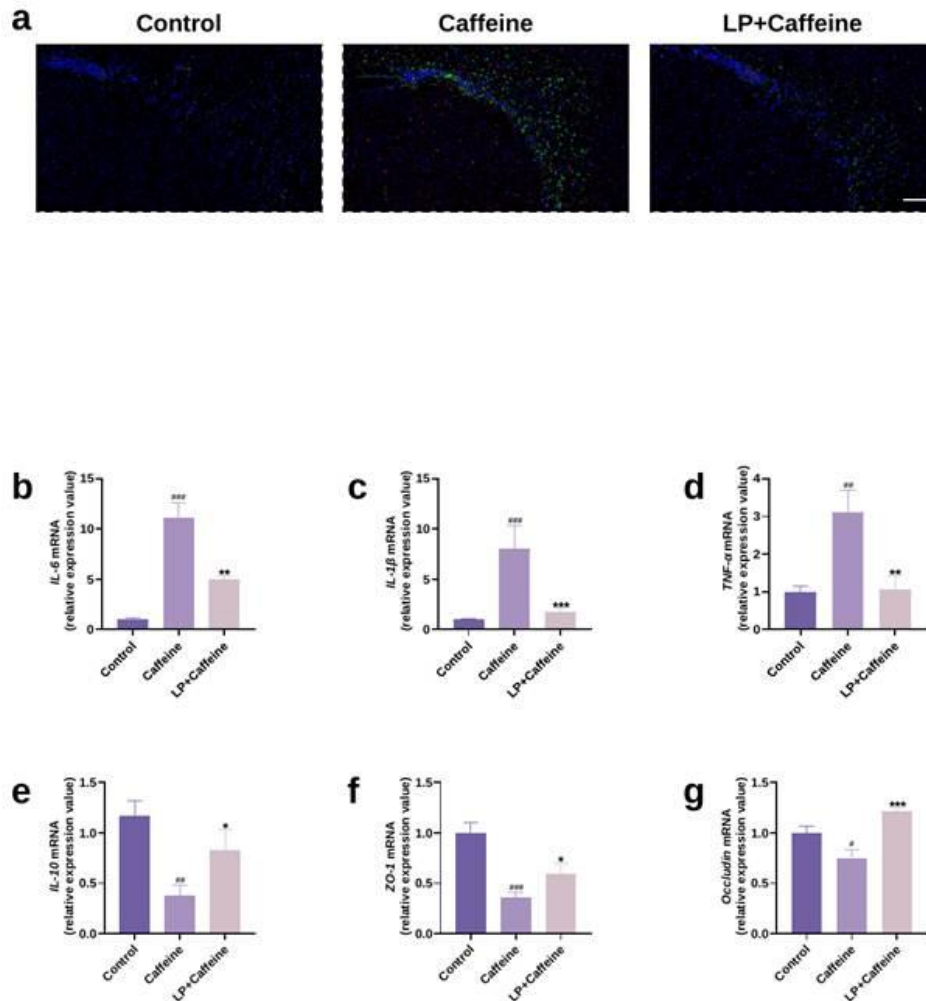


Figure 6. Preliminary prefrontal-cortex readouts after caffeine and LP treatment. (A) Representative Iba1/GFAP/DAPI panel (reported scale bar, 200 μ m; descriptive pending blinded quantification). (B–G) Relative mRNA expression by $2^{-\Delta\Delta C_t}$ normalized to β -actin.

4. Discussion

Across two heterogeneous rodent fecal-metabolomics datasets, we identified a nine-metabolite directionally concordant panel and broader pathway convergence. The result should not be interpreted as a universal “addiction metabolome”; rather, it provides an auditable cross-study hypothesis set despite differences in species, exposure paradigm, and available raw data [3,11].

The more interpretable shared signals are consistent with stress-endocrine and lipid-remodeling biology. Cortisone supports stress-axis involvement [20,21], while LysoPE, CE(5:0), and 19-hydroxy-PGE2 are compatible with membrane, sterol, and eicosanoid remodeling reported in the source studies [3,11]. Other annotations—including corchoroside B, postin, and O-ethyl S,S-diphenyl phosphorodithioate—remain hypothesis-generating and require targeted LC-MS/MS confirmation before mechanistic interpretation.

The primary AGORA2 model prioritized *A. muciniphila* overall and *B. animalis* among probiotic-deployable candidates, but its moderate internal discrimination and non-definitive permutation test support prioritization rather than efficacy claims. The secondary model served a different purpose: selecting a feasible proof-of-concept benchmark. *L. plantarum* ATCC 14917 ranked highest among

LP candidates in the full secondary model, but HMPM2111 was the tested strain and LP ranking weakened when curated or LP-specific evidence was removed. Accordingly, the wet-lab study does not validate the exact computational ranking.

The LP experiment nevertheless provides preliminary biological support: behavioral and prefrontal-cortex transcript patterns shifted toward controls after treatment. Major limitations remain, including caffeine/sleep-restriction confounding, name-based metabolite matching, unavailable caffeine animal-level intensities, incomplete MetaboAnalyst session history, cached GEO contrasts, incomplete exact wet-lab statistics, and unquantified Iba1/GFAP imaging. Future work should confirm metabolite identities by targeted MS/MS, collect harmonized microbiome/metabolite/barrier/neuroimmune measures in a single cohort, and directly compare top-ranked *Akkermansia* and *B. animalis* candidates with LP.

5. Conclusions

This study presents an auditable workflow linking cross-substance metabolomic convergence to strain-level probiotic prioritization. Genome-scale modeling reduced the candidate space, a separate experimental-priority model justified an accessible LP benchmark, and preliminary wet-lab findings provided species-level proof of concept. Targeted metabolite confirmation, complete animal-level statistics, and direct testing of top-ranked strains are required before therapeutic or ranking-superiority claims can be made.

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