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Original Article

Morphine-induced dysbiosis and metabolic remodeling of the gut microbiota is transmissible via fecal microbiota transplantation in mice

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Abstract: Objective: Opioids such as morphine are widely used analgesics but are highly addictive. Emerging evidence implicates the gut microbiota in modulating opioid-associated pathophysiology. However, the global metabolic impact of morphine on gut microbial function and the causal transmissibility of morphine-induced dysbiosis remain to be fully elucidated. Methods: Adult C57BL/6 mice received repeated morphine administration for four consecutive days. Fecal metabolomic profiling was performed using integrated gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS) platforms. Gut microbial composition was analyzed by 16S rRNA gene sequencing and terminal restriction fragment length polymorphism (T-RFLP) profiling. To determine causality and transmissibility, fecal microbiota transplantation (FMT) was conducted from morphine-treated donor mice into antibiotic-depleted recipient mice. Results: Repeated morphine administration induced a profound global reprogramming of fecal metabolic profiles, with clear separation between morphine-treated and control mice. A total of 179 fecal metabolites were annotated. Morphine significantly increased diethylene glycol, N-acetyl-L-glutamic acid, and urea, while decreasing 3-aminoisobutyric acid, 2-aminobutyric acid, and pseudouridine. Pathway enrichment analysis revealed that amino acid-centered metabolic pathways, including branched-chain amino acid biosynthesis, arginine biosynthesis, and aminoacyl-tRNA biosynthesis, were the most prominently disrupted. Morphine exposure also induced a distinct dysbiotic microbial signature characterized by expansion of *Enterococcus faecalis* and *Alistipes indistinctus,* and depletion of *Prevotella melaninogenica*. Importantly, FMT from morphine-treated donors into microbiota-depleted recipients successfully transferred both the dysbiotic microbial structure and metabolic phenotype. Post-transplant recipient mice exhibited microbial profiles closely resembling morphine-treated donors, demonstrating that the morphine-induced dysbiosis is transmissible in the short term in the absence of direct drug exposure. Conclusion: Repeated acute morphine exposure alters fecal amino acid and nitrogen metabolism and drives a transmissible dysbiotic state in the short term. These findings highlight a potential association between morphine exposure, metabolic remodeling, and gut dysbiosis, suggesting the gut microbiota may be a relevant factor in opioid-associated intestinal interactions.

Keywords: Morphine, gut microbiota, metabolomics, fecal microbiota transplantation, dysbiosis

Introduction

Opioid analgesics exert their therapeutic effects primarily by binding to opioid receptors in the brain, spinal cord, and peripheral tissues and are widely prescribed for the management of moderate to severe pain [1, 2]. However, opioids are also highly addictive, and opioid misuse has escalated into a major global public health crisis, resulting in millions of individuals suffering from opioid use disorder and tens of thousands of overdose-related deaths annually [3-7]. Among opioids, morphine remains one of the most extensively used and abused com-

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pounds in both clinical and non-medical settings [8].

Morphine addiction is a chronic, relapsing disorder of the central nervous system characterized by compulsive drug-seeking and taking behavior, neurochemical remodeling, altered molecular transport in neural extracellular spaces, maladaptive behavioral plasticity, and increased mortality [9-11]. The neurobiological mechanisms underlying morphine addiction have traditionally focused on mesolimbic dopamine reward circuitry, glutamatergic transmission, synaptic plasticity, and mitochondrial metabolism [12, 13]. Chronic opioid exposure alters neuronal excitability, dopamine release, astrocytic energy metabolism, and receptor signaling [14]. However, these brain-centered mechanisms alone cannot fully explain the broad systemic physiological and metabolic consequences accompanying opioid addiction, suggesting the involvement of peripheral regulatory systems.

In recent years, the gut microbiota has emerged as a critical regulator of host metabolism, immunity, and neurophysiology through the microbiota-gut-brain axis [15-18]. Gut microbial metabolites - including short-chain fatty acids, bile acids, amino acids, and tryptophan derivatives - directly influence neuroinflammation, neurotransmitter synthesis, immune activation, and blood-brain barrier integrity [19-21]. Accumulating evidence indicates that substance abuse, including opioids, profoundly disrupts gut microbial homeostasis and contributes to metabolic and immune dysfunction [22-24]. Previous studies have demonstrated that morphine alters the *Firmicutes/Bacteroidetes* ratio [19], reduces the abundance of beneficial *Lachnospiraceae* [25], increases *Enterococcus faecalis*, disrupts intestinal barrier integrity, and perturbs bile acid metabolism [26], highlighting the growing need for precise detection and characterization of specific enteric bacteria [27]. Moreover, targeted manipulation of gut microbiota has been shown to modulate morphine withdrawal and behavioral responses via Toll-like receptor-mediated immune signaling pathways.

While previous studies have well documented that morphine induces descriptive compositional shifts in the gut microbiota and perturbs specific pathways such as bile acid metabolism, critical knowledge gaps remain. Specifically, the global metabolic reprogramming at the systems level - particularly concerning microbial amino acid and nitrogen turnover - has not been fully characterized. More importantly, previous literature largely presents associative observations during ongoing opioid exposure. It remains fundamentally unknown whether this morphine-induced metabolic and microbial configuration is merely a transient, drug-dependent fluctuation, or if it constitutes a functional phenotype capable of being causally transferred to a naïve host. To address this, our study uniquely incorporates fecal microbiota transplantation (FMT) to evaluate the direct transmissibility of the morphine-altered ecosystem.

In the present study, we investigated the effects of repeated morphine administration on gut microbial metabolism and community structure using an integrated metabolomics and 16S rRNA sequencing approach. Furthermore, we employed a microbiota-depleted mouse model combined with fecal microbiota transplantation to determine whether the morphine-induced gut microbial phenotype is stably transmissible. Our findings demonstrate that morphine induces coordinated reprogramming of gut microbial metabolism and community composition and that this dysbiotic configuration is sufficient to be transferred to naïve hosts. These results establish a causal link between morphine exposure, microbial metabolic remodeling, and stable gut dysbiosis, providing new mechanistic insight into the role of the gut microbiome in opioid-associated pathology.

Materials and methods

Animal treatments and experimental design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Chicago (No. 70917). Adult male and female C57BL/6 mice were used in this study and randomly assigned to three groups: morphine donor mice (Mdor), fecal microbiota recipient mice (Mrec), and mock-transplanted control mice (Mcon). The experimental timeline is illustrated in **Figure 1**. Specifically, the Mdor group received repeated morphine injections and served as fecal microbiota donors for transplantation. The Mrec

group consisted of microbiota-depleted mice that received FMT from Mdor mice. The Mcon group served as mock-transplanted controls and received phosphate-buffered saline (PBS) instead of donor fecal suspensions. Wide-spectrum antibiotics (ABX) were administered to Mrec mice on days 8-10 to deplete endogenous gut microbiota prior to FMT.

Morphine was obtained from the Department of Anesthesia and Critical Care, University of Chicago, dissolved in sterile saline, and administered intraperitoneally at 10 mg/kg once daily for four consecutive days. Control mice received an equivalent volume of sterile saline.

Fecal metabolomic analysis

Fecal samples were collected at designated time points and stored at -80°C until analysis. Prior to metabolomic analysis, samples were homogenized in distilled water and subjected to a two-step derivatization procedure using methoxyamine followed by BSTFA containing 1% TMCS. Metabolite profiling was performed using an Agilent 6890N gas chromatograph coupled with a time-of-flight mass spectrometer (GC/TOF-MS). Separation was achieved on an Agilent DB-5ms capillary column (30 m × 250 μm i.d.) with helium as the carrier gas at a constant flow rate of 1.0 mL/min. Mass spectra were acquired under electron impact ionization at 70 eV in full-scan mode over a mass range of m/z 30-600. In addition, GC/MS and HPLC/MS analyses were employed to determine endogenous metabolites in the biological samples.

For LC/MS analysis, the extracted solutions were directly injected without derivatization. Chromatographic separation was performed using an Agilent 1200 LC system, and mass spectrometry was conducted on an Agilent 6220 MSD TOF-MS equipped with a dual-sprayer electrospray ionization (ESI) source. TOF-MS data were acquired in both positive and negative ion modes. The annotated compounds from GC/MS and LC/MS analyses were combined and imported into SIMCA-P 15 software (Umetrics, Umeå, Sweden) for multivariate statistical analysis. To further enhance detection sensitivity of trace metabolites, a chip-based nanoLC/Q-TOF-MS microfluidic platform developed by Agilent was also employed as a complementary approach to conventional GC/MS and LC/MS analyses.

Thousands of fecal metabolites were quantified. Differential metabolites were selected based on variable importance in projection (VIP) > 1 in the OPLS model and $P < 0.05$. Corresponding fold changes were calculated and the most significantly altered metabolites were further verified. Multivariate analyses, including principal component analysis (PCA) and OPLS analysis, were performed for group discrimination. Canonical metabolic pathways were generated, and Fisher's exact test was applied to determine the statistical significance of the associations between metabolites and specific pathways.

Gut microbiota depletion and fecal microbiota transplantation

C57BL/6J mice used for microbiota depletion were maintained in a gnotobiotic facility supported by the UChicago Digestive Diseases Research Core Center (DDRCC) and Microbiome Center. To deplete endogenous gut microbiota, mice received a wide-spectrum antibiotic (ABX) cocktail consisting of 0.5 g/L vancomycin (Macklin, V871983), 1 g/L streptomycin (Macklin, S875203), 1 g/L metronidazole (Macklin, M813526), 1 g/L ampicillin (Macklin, A830931), and 1 g/L neomycin (Macklin, N6063), administered orally for three consecutive days.

Following antibiotic treatment, FMT was performed as previously described [28]. Briefly, fresh fecal samples were collected from Mdor mice on day 7. Approximately 10 mg of pooled feces was suspended in 2 mL of sterile PBS, and 0.2 mL of the supernatant was administered to each Mrec mouse by oral gavage. After the first gavage, all mice were transferred to a specific pathogen-free (SPF) environment. Fecal transplantation was conducted once daily for seven consecutive days, and sterile cages were replaced after each gavage. Control recipient mice (Mcon) received PBS only. Fecal samples from Mrec and Mcon mice were collected at designated time points for subsequent analyses.

Enteric microbiome analysis

Bacterial DNA was extracted from frozen stool samples using a DNeasy PowerSoil (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The DNA concentration and

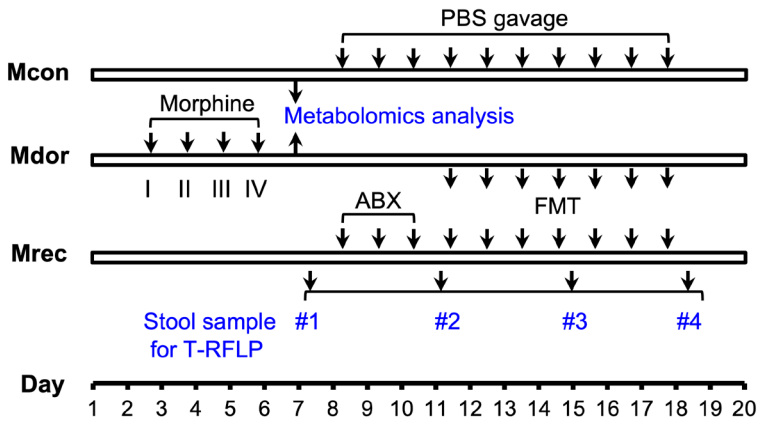


Figure 1. Experimental design and timeline of morphine administration and fecal microbiota transplantation. Schematic overview of the experimental workflow. Adult C57BL/6 mice were randomly assigned to three groups: morphine donor mice (Mdor), fecal microbiota recipient mice (Mrec), and mock-transplanted control mice (Mcon), N = 4 for each group. Mdor mice received intraperitoneal injections of morphine (10 mg/kg/day) for four consecutive days. Mrec mice underwent broad-spectrum antibiotic treatment on days 8-10 to deplete endogenous microbiota, followed by fecal microbiota transplantation (FMT) from Mdor donors for seven consecutive days. Mcon mice received PBS instead of donor feces. Fecal samples were collected at designated time points for metabolomic and microbiome analyses.

integrity were measured using NanoDrop 2000 spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. The V3-V4 region of the bacterial 16S rRNA gene was amplified using barcoded primers (338F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3') containing Illumina adapter sequences as well as a 12-bp barcode. This barcode-based primer approach allowed sequencing of multiple samples in a single 454 sequencing run without the need for physical partitioning.

Sequencing was performed using an Illumina MiSeq platform at the Argonne National Laboratory. Raw sequences were processed using the QIIME2 toolkit (NIH BIOF 089, Microbiome Bioinformatics with QIIME 2, January 2021). OTUs were clustered at 97% sequence identity using CD-HIT, and representative sequences were aligned with PyNAST. Taxonomic classification was performed using the RDP Classifier. A phylogenetic tree was constructed using FastTree, and unweighted UniFrac distances were calculated for beta diversity analysis, including principal coordinates analysis (PCoA).

Statistical analysis

All experiments were performed at least three times or repeated in three batches of independent experiments. All data are presented as mean ± standard deviations (SD). For normally distributed data with equal variance, differences between two groups were analyzed using the unpaired Student's t-test, and multiple group comparisons were performed using one-way analysis of variance (ANOVA). For the high-dimensional untargeted metabolomic and microbiome data, PCA was utilized for dimensionality reduction and visualization of global metabolic and microbial community clustering. Metabolite pathway enrichment analysis was performed using the MetaboAnalyst platform, with significance determined by hypergeometric tests. To control for false positives in high-throughput omics datasets, raw *P*-values were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure. $P < 0.05$ was considered statistically significant.

Results

Repeated morphine administration induces a global reprogramming of fecal metabolic profiles

Microbial dysbiosis is not only reflected by changes in microbial composition but is also accompanied by profound alterations in microbial metabolic function. To determine whether repeated morphine exposure reshapes gut microbial metabolism, fecal metabolomic profiling was performed in control (Mcon) and morphine-treated donor mice (Mdor) according to the experimental timeline shown in **Figure 1**. Using a combined GC/MS and LC/MS platform, a total of 179 metabolites were reliably annotated in fecal extracts.

PCA of fecal metabolites revealed a clear and robust separation between the Mcon and Mdor

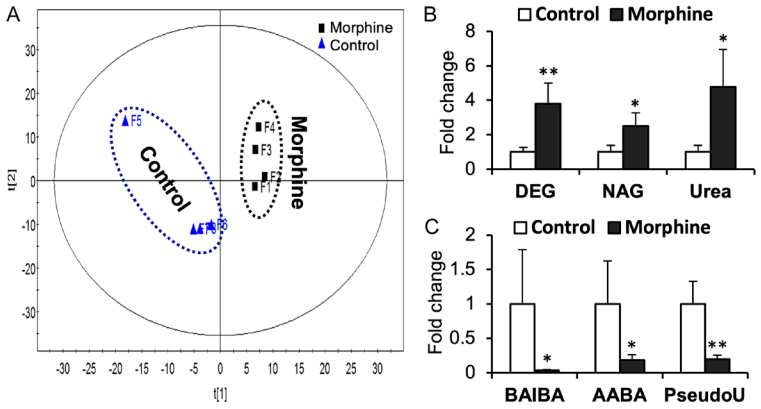


Figure 2. Global fecal metabolomic alterations induced by repeated morphine administration. A. Principal component analysis (PCA) score plot of fecal samples showing metabolic profiling in morphine group (F1-F4) and control group (F5-F8). B. Three representative upregulated metabolites: diethylene glycol (DEG), N-acetyl-L-glutamic acid (NAG), and urea. C. Three representative downregulated metabolites: 3-aminoisobutyric acid (BAIBA), 2-aminobutyric acid (AABA), and pseudo uridine (PseudoU) (N = 4). *P < 0.05, **P < 0.01 vs. control.

groups (2 components, R2X = 0.469, R2Y = 0.947, Q2 = 0.561), indicating a pronounced global metabolic shift induced by repeated morphine administration (**Figure 2A**). Among the differential metabolites, diethylene glycol (DEG), N-acetyl-L-glutamic acid (NAG), and urea were significantly increased in the Mdor group compared with the Mcon group (**Figure 2B**), whereas 3-aminoisobutyric acid (BAIBA), 2-aminobutyric acid (AABA), and pseudouridine (PseudoU) were markedly decreased (**Figure 2C**).

Functionally, these metabolites are predominantly involved in amino acid turnover, nitrogen handling, and nucleoside metabolism. The elevation of NAG and urea suggests enhanced nitrogen flux and protein catabolism, whereas the depletion of BAIBA and AABA indicates suppression of branched-chain and non-proteinogenic amino acid metabolism. In parallel, the decrease in PseudoU reflects disrupted nucleoside turnover. Collectively, these results demonstrate that repeated morphine administration induces a profound reprogramming of fecal metabolite composition, reflecting not only compositional changes but also a major functional remodeling of gut microbial metabolic activity.

Morphine exposure primarily disrupts amino acid-centered microbial metabolic pathways

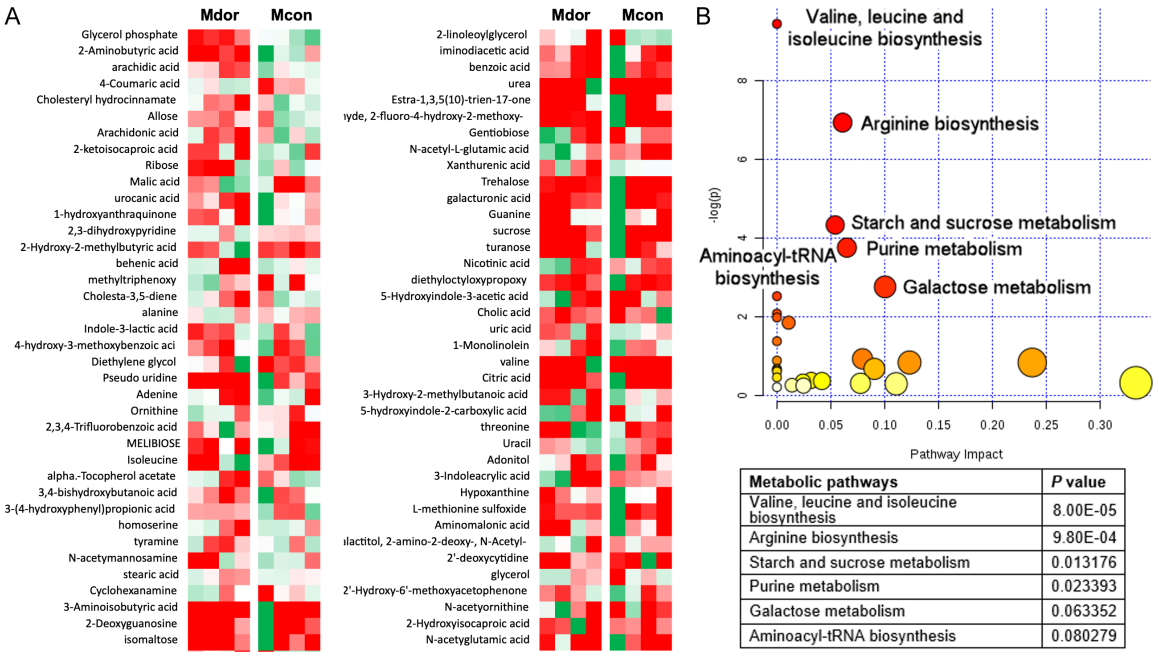
To further delineate the functional features of the morphine-induced metabolic disturbance, differential metabolites were visualized by heatmap analysis. As shown in **Figure 3A**, morphine treatment resulted in a global reshaping of the fecal metabolic landscape compared with controls, with broad shifts in multiple metabolite classes.

Subsequent pathway enrichment analysis using the MetaboAnalyst platform identified multiple metabolic pathways that were significantly affected by morphine exposure (**Figure 3B**). Notably, the most prominently enriched pathways were centered on amino acid metabolism, including valine, leucine and isoleucine biosynthesis, arginine biosynthesis, and aminoacyl-tRNA biosynthesis. In addition, pathways related to starch and sucrose metabolism, galactose metabolism, and purine metabolism were also significantly altered.

These findings indicate that amino acid biosynthesis and nitrogen-related metabolic processes constitute the primary functional hub disrupted by repeated morphine exposure, while carbohydrate and nucleotide metabolism represent secondary but coordinated metabolic adaptations. Thus, morphine does not randomly perturb gut microbial metabolism but selectively targets core biosynthetic and translational pathways essential for microbial growth, energy utilization, and host-microbe metabolic coupling.

Morphine exposure induces gut microbial dysbiosis characterized by expansion of pathogenic taxa and depletion of protective species

To determine whether the observed metabolic alterations were associated with changes in gut microbial composition, 16S rRNA gene sequencing was performed on fecal samples



Metabolic pathways	*P* value
Valine, leucine and isoleucine biosynthesis	8.00E-05
Arginine biosynthesis	9.80E-04
Starch and sucrose metabolism	0.013176
Purine metabolism	0.023393
Galactose metabolism	0.063352
Aminoacyl-tRNA biosynthesis	0.080279

Figure 3. Morphine selectively disrupts amino acid-centered microbial metabolic pathways. A. Heatmap depicting differential fecal metabolites between Mcon and Mdor mice. Each row represents a metabolite, and each column represents an individual mouse. Data were unit variance scaling and utilized for heap map plot. The red color represents the trend of decrease, and green represents a rising trend. B. Pathway enrichment analysis of significantly altered metabolites using MetaboAnalyst. Morphine predominantly affected amino acid biosynthesis and nitrogen-related pathways, including branched-chain amino acid biosynthesis, arginine biosynthesis, and aminoacyl-tRNA biosynthesis, along with secondary perturbations in carbohydrate and purine metabolism. The lower panel shows metabolic pathways with *P* values.

collected from Mcon, Mdor, and Mrec groups (**Figure 4A**). Taxonomic profiling demonstrated that morphine exposure significantly altered the structure of the enteric microbiome. Compared to the Mcon group, the Mdor group exhibited a marked shift in microbial composition. Importantly, following FMT, the microbial profile of Mrec mice closely resembled that of the Mdor group rather than the Mcon group, indicating successful and stable transfer of the morphine-induced dysbiotic microbiota (**Figure 4A**).

At the species level, morphine treatment significantly increased the relative abundance of *Enterococcus faecalis* and *Alistipes indistinctus,* while markedly suppressing the abundance of *Prevotella melaninogenica* (**Figure 4B**). These changes were consistently observed in both the Mdor and Mrec groups when compared with the Mcon group. *E. faecalis* is a well-recognized pathobiont associated with epithelial barrier disruption and inflammatory activation [29]. Interestingly, the marked expansion of *Alistipes indistinctus* mirrors dysbiotic patterns frequently observed in stress-related and inflammatory models. Conversely, the abundance of *Prevotella melaninogenica* was significantly decreased in both morphine-treated and recipient mice.

Collectively, these results demonstrate that morphine induces a specific dysbiotic microbial signature characterized by expansion of inflammation-associated taxa and depletion of potentially protective commensals. The close concordance of microbial alterations between Mdor and Mrec mice further indicates that this dysbiotic configuration is robust and transmissible.

Morphine-induced gut dysbiosis is transmissible to recipient mice via FMT

To establish whether the morphine-associated gut microbial alterations were causally transmissible, fecal samples from Mdor mice were transplanted into antibiotic-treated recipient mice. Following broad-spectrum antibiotic treatment, recipient mice displayed minimal fecal

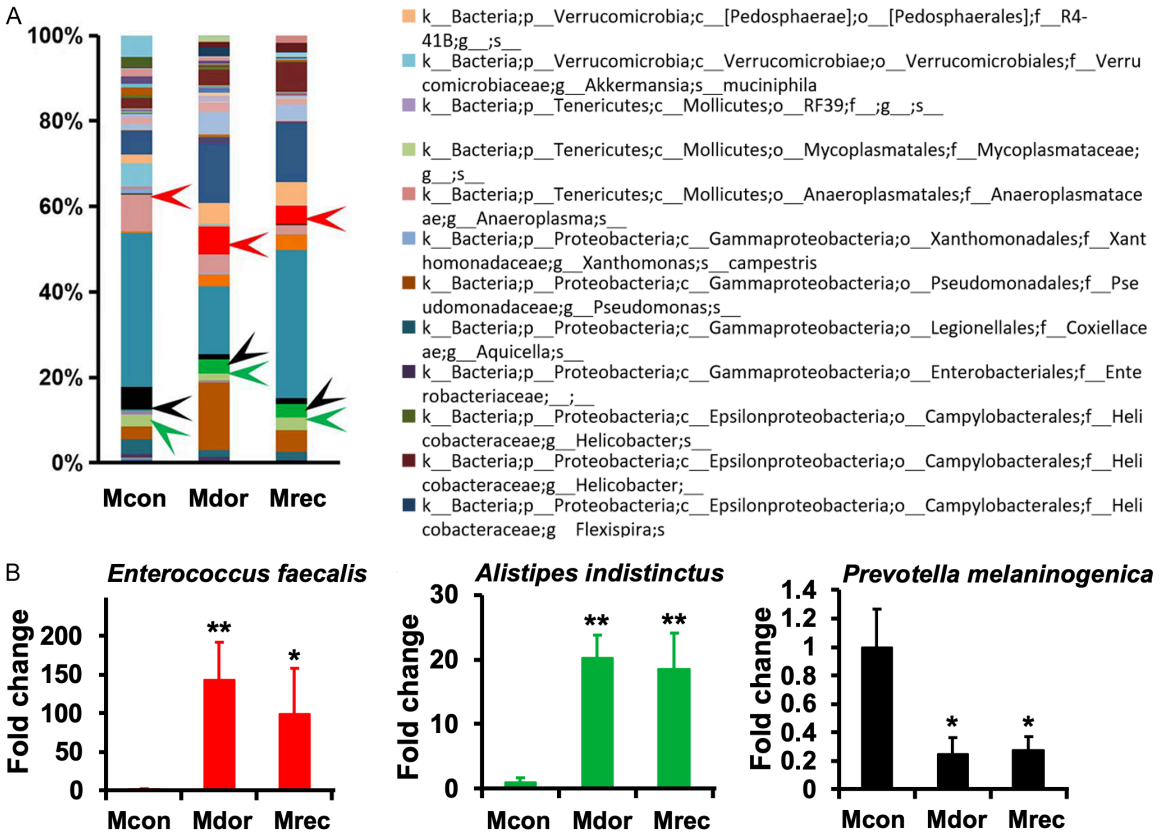


Figure 4. Morphine alters gut microbial composition at the community and species levels. Effects of morphine exposure on enteric microbiome composition changes. A. Representative taxonomic compositions of the microbiota from control mice (Mcon), donor mice after morphine injections (Mdor), and gut microbiome gavaged recipient mice (Mrec). B. Relative microbial abundance changes of the three gut microbiome species in different groups. Red, black and green arrows indicate three species: *Enterococcus faecalis*, *Alistipes indistinctus* and *Prevotella melaninogenica*, respectively. *, $P < 0.05$; **, $P < 0.01$ vs. control.

bacterial loading. After FMT, bacterial load in recipient mice increased progressively over time, indicating efficient recolonization of the gut microbiota (**Figure 5A**).

T-RFLP analysis further demonstrated that the microbial profiles of Mdor mice were clearly distinct from those of Mcon mice at all time points (**Figure 5B**). Prior to FMT, the T-RFLP profiles of recipient mice were comparable to those of control mice. However, following transplantation with morphine-derived fecal microbiota, the microbial composition of recipient mice shifted and closely resembled that of Mdor donor mice. Consistently, PCA based on T-RFLP profiles revealed that recipient mice initially clustered with control mice before transplantation but subsequently shifted to cluster with morphine-treated donor mice after FMT (**Figure 5C**).

Importantly, these results demonstrate that the morphine-induced gut microbial phenotype is not merely a secondary consequence of ongoing drug exposure but represents a dysbiotic state that is sufficient to be transferred to naïve hosts. Thus, morphine-altered gut microbiota exhibits both stability and transmissibility, providing strong causal evidence that repeated morphine exposure reshapes the gut microbial ecosystem in a manner that can be propagated independently of direct morphine administration.

Discussion

Opioids are among the most widely prescribed analgesics but are also highly addictive substances with profound systemic effects [6]. Morphine, a prototypical opioid, is the most representative drug of this class and has been

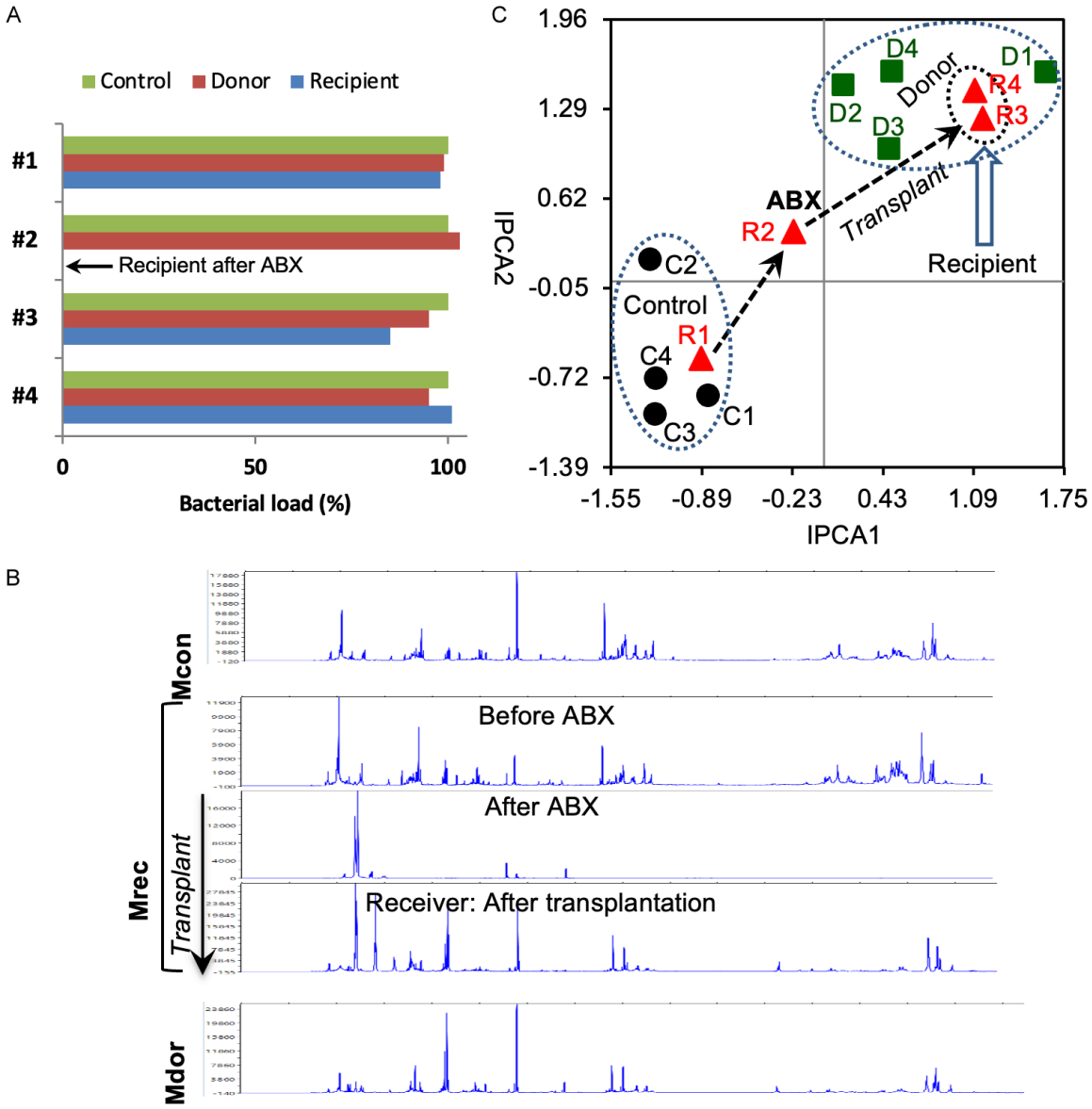


Figure 5. Morphine-induced gut dysbiosis is transmissible via fecal microbiota transplantation. A. Bacterial load quantification in recipient mice shows progressive recolonization after FMT from Mdor donors. B. Fecal terminal restriction fragment length polymorphism (T-RFLP) chromatograms representing different bacterial populations. Fecal samples were collected from each group at four time points, pooled, and homogenized prior to DNA extraction. C. Principal component analysis (PCA) based on T-RFLP profiles illustrating gut microbiome composition across groups. Black circles: Mcon; green squares: Mdor; red triangles: Mrec. Numbers indicate sampling time points: before antibiotic treatment (1), after antibiotic treatment (2), post-FMT at day 15 (3), and post-FMT at day 18 (4). Post-transplant, the microbiota of Mrec (R3, R4) closely resembled the donor (Mdor) profile, demonstrating transmissibility of morphine-induced dysbiosis.

extensively used to model opioid addiction. Addiction is a complex chronic disorder characterized by progressive neuroadaptive changes involving synaptic plasticity, intracellular signaling, gene transcription, and protein synthesis [30]. In recent years, morphine has been increasingly recognized not only as a central nervous system-acting drug but also as a critical modulator of gut microbiota composition and metabolism. With the rapid development of high-throughput sequencing and metabolomic technologies, new insights have emerged regarding the structure and function of the gut microbial ecosystem [31]. Our findings extend

correlative dysbiosis following opioid exposure. The integrated metabolomic approach reveals the profound disruption of microbial amino acid and nitrogen handling. Furthermore, the primary novelty of this study lies in demonstrating the causal transmissibility of this altered state. By utilizing an FMT model, we provide direct evidence that the morphine-reprogrammed microbial and metabolic phenotype can be robustly transferred to naïve, microbiota-depleted hosts in the absence of ongoing drug administration. This indicates that the gut microbiota is not merely a passive bystander affected by morphine, but an active, transmissible vector of opioid-associated metabolic remodeling.

Notably, morphine exposure significantly increased DEG, NAG, and PseudoU. While NAG and PseudoU are centrally involved in amino acid turnover, nitrogen handling, and nucleotide metabolism, the elevation of DEG may reflect distinct alterations in microbial fermentation pathways or xenobiotic metabolism. Together, these divergent metabolic shifts indicate that morphine directly disrupts fundamental microbial biosynthetic and translational networks [32-34]. Consistent with these changes, pathway enrichment analysis revealed that the most significantly affected pathways were amino acid-centered metabolic routes, including branched-chain amino acid biosynthesis, arginine biosynthesis, and aminoacyl-tRNA biosynthesis. Beyond serving as substrates for protein synthesis, amino acids also function as key signaling molecules linking microbial metabolism to host neurochemical regulation. Previous studies have reported that morphine alters gut tryptophan metabolism and increases systemic serotonin levels, highlighting a microbiota-brain metabolic coupling mediated by microbial metabolites such as short-chain fatty acids and branched-chain amino acids [33-35]. Glutamate metabolism is particularly relevant to opioid addiction, as glutamate acts as the precursor for γ-aminobutyric acid (GABA), a key inhibitory neurotransmitter involved in reward, reinforcement, and relapse [36, 37]. Enhanced glutamatergic signaling has been tightly linked to morphine-induced neuroplasticity, hyperalgesia, and sensory neuron sensitization in the peripheral and central nervous systems [38-40]. Therefore, the morphine-induced elevation of glutamate-related metabolites observed in our study may represent a peripheral metabolic interface that contributes to central neuroadaptive changes during opioid exposure. Collectively, these findings demonstrate that morphine does not merely induce random metabolic fluctuations in the gut but instead selectively targets amino acid, nitrogen, and translational metabolic hubs, which are fundamental to both microbial growth and host-microbiota neurochemical communication.

The metabolomic alterations observed in this study are tightly coupled to morphine-induced restructuring of the gut microbial community. Using 16S rRNA profiling and T-RFLP analysis, we demonstrate that morphine exposure results in a distinct dysbiotic microbial signature characterized by a significant expansion of *Enterococcus faecalis* and *Alistipes indistinctus*, accompanied by the depletion of *Prevotella melaninogenica*. Enterococcus faecalis is a well-established pathobiont that disrupts epithelial tight junctions, impairs barrier integrity, and promotes intestinal inflammation [29]. Furthermore, while some *Alistipes* species are commensal, their overrepresentation is frequently linked to depressive phenotypes, stress-induced gut dysbiosis, and sustained inflammatory states. The profound expansion of Alistipes indistinctus observed in our morphine-treated mice may therefore contribute to the neuroadaptive and inflammatory consequences of opioid exposure [41-43]. Conversely, the marked reduction in *Prevotella melaninogenica*, a taxon often involved in complex carbohydrate fermentation and short-chain fatty acid (SCFA) production, may reflect a critical loss of microbial metabolic diversity and buffering capacity, predisposing the host to intestinal barrier dysfunction.

Consistent with prior reports, morphine has also been shown to disrupt bile acid metabolism through a Gram-positive, TLR2-mediated mechanism, thereby impairing the hepatic-intestinal metabolic cycle [44-46]. Toll-like receptors play a central role in host-microbiota immune sensing, and opioid-induced microbial shifts may amplify innate immune signaling through TLR pathways, further reinforcing a chronic low-grade inflammatory state in the intestinal microenvironment [47, 48]. Together, these findings indicate that morphine induces a coordinated dysbiotic program characterized

by expansion of pathogenic taxa, suppression of protective commensals, and functional metabolic rewiring, thereby providing a mechanistic basis for opioid-associated intestinal inflammation and systemic immune dysregulation.

A major strength of the present study is the demonstration that morphine-induced microbial and metabolic alterations are causally transmissible via fecal microbiota transplantation. Following antibiotic-mediated microbiota depletion, recipient mice displayed minimal bacterial loads. After transplantation with feces from morphine-treated donor mice, the recipient microbiota rapidly reconstituted and adopted a microbial structure that closely mirrored the donor profile. Both T-RFLP profiling and PCA analysis confirmed that the post-transplant microbiome of recipient mice shifted from a control-like configuration to a morphine-donor-like configuration, demonstrating the stability and transferability of the morphine-induced dysbiotic ecosystem. Importantly, this transmissibility occurred in the absence of direct morphine exposure, indicating that the dysbiotic microbiota itself represents a self-sustaining pathological state, rather than a transient pharmacological consequence. This finding provides strong causal evidence supporting the concept that gut microbiota remodeling is not merely a byproduct of opioid exposure, but an active biological mediator of morphine-associated metabolic and inflammatory effects. It further suggests that microbe-derived metabolites, rather than morphine alone, may serve as persistent modulators of host physiology during and after opioid exposure.

Finally, we acknowledge certain limitations in our study. The sample size used for the metabolomic and microbiome profiling was relatively small (N = 4 per group). While this limited sample size may reduce statistical power for detecting low-abundance microbial taxa or trace metabolites, it is important to note that the mice were maintained under strictly controlled conditions within a specialized gnotobiotic facility, which minimizes inter-individual environmental and dietary variations. Furthermore, despite the small cohort, the metabolic and microbial shifts induced by morphine were robust enough to reach statistical significance and achieve clear clustering in multivariate analyses. Future studies utilizing larger cohorts and extended post-transplantation monitoring will be valuable to further validate these findings and explore the long-term dynamics of morphine-induced dysbiosis.

Taken together, our study establishes a mechanistic framework in which repeated morphine administration reprograms gut microbial amino acid and nitrogen metabolism, drives a shift toward a pro-inflammatory microbial community, and generates a stable, transmissible dysbiotic state. This dysbiosis is sufficient to be transferred to naïve hosts and may contribute to intestinal barrier dysfunction, immune activation, and systemic metabolic disturbances. These findings highlight the gut microbiota as a previously underappreciated biological amplifier of opioid-induced pathology. Importantly, they suggest that therapeutic strategies aimed at restoring microbial metabolic homeostasis - such as targeted probiotics, microbial metabolite supplementation, or microbiota-directed interventions [49] - may represent promising adjunctive approaches for mitigating the adverse effects of chronic opioid exposure and potentially reducing addiction-associated pathophysiology.

Conclusions

This study demonstrates that repeated morphine administration induces significant alterations in gut microbial composition and fecal metabolic profiles in mice. Morphine exposure reshaped key bacterial taxa, characterized by increased *Enterococcus faecalis* and *Alistipes indistinctus* and decreased *Prevotella melaninogenica*, and caused marked disturbances in amino acid-related metabolic pathways, including significant changes in NAG and PseudoU. Importantly, these dysbiotic and metabolic signatures were successfully transferred to microbiota-depleted recipient mice through fecal microbiota transplantation, indicating that morphine-induced gut microbial changes are transmissible and stable. Together, these findings reveal a close link between morphine exposure, gut microbiota dysbiosis and metabolic reprogramming, and suggest that the gut microbiome may serve as a potential therapeutic target in opioid-associated intestinal dysfunction and addiction.

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Disclosure of conflict of interest

None.

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