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Chronic arsenic–fluoride co-exposure impairs spermatogenesis with gut microbiota–associated bile acid and lipid metabolic remodeling

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ABSTRACT

Arsenic and fluoride frequently co-occur in groundwater, but the gut-associated metabolic changes accompanying male reproductive toxicity from their co-exposure remain unclear. We established a chronic developmental drinking water co-exposure model in Sprague–Dawley rats using 100 mg/L NaF and 75 mg/L NaAsO₂. Reproductive outcomes were evaluated at postnatal day (PND) 60 and PND120, and reciprocal fecal transfer was performed between control and arsenic–fluoride (AsF) backgrounds. Developmental AsF exposure impaired sperm production and quality, disrupted reproductive hormone homeostasis, altered spermatogenic-stage markers, and induced testicular and epididymal injury. Repeated administration of AsF-donor fecal material to control recipients was accompanied by selected adverse reproductive changes, whereas control-donor material was accompanied by partial changes in the opposite direction in AsF-exposed recipients. Endpoint 16S rRNA sequencing demonstrated exposure-associated microbial community differences, and donor-material administration was accompanied by changes in selected host phenotypes. Untargeted serum metabolomics revealed prominent remodeling of bile acid- and lipid-related signals, including reciprocal Fecal microbiota transplantation-associated patterns and exploratory relationships between bile-acid-related features and reproductive hormones. These metabolic findings were accompanied by differences in colonic junction- and inflammation-related markers, intestinal–hepatic FXR–FGF15-related proteins, hepatic bile acid and lipid endpoints, and testicular autophagy-related markers. Collectively, the findings characterize reproductive hazards at the tested concentrations and support a nonexclusive gut–liver–testis framework in which donor-material-associated microbial and metabolic changes accompany partial modification of recipient phenotypes.

1. Introduction

Inorganic arsenic (As) and fluoride (F) are widespread groundwater contaminants whose occurrence and co-enrichment are governed by aquifer mineralogy, pH, redox conditions, and water–rock interactions. Global models indicate that large populations may be exposed to elevated concentrations of either contaminant, while hydrogeochemical investigations have documented As–F co-occurrence in endemic regions and groundwater basins in northern China (He et al., 2020; Kumar et al., 2020; Podgorski and Berg, 2020, 2022; Z. Wang et al., 2024; Cao et al., 2024). Mining, coal combustion, industrial discharge, phosphate-related activities, and other human activities may further release or redistribute these elements in soil–water systems (Patel et al., 2023; Shaji et al.,

2024). The World Health Organization guideline values for drinking water are 0.01 mg/L for As and 1.5 mg/L for F (World Health Organization, 2022). Their environmental co-occurrence creates a multiple-contaminant context in which biological responses cannot be inferred solely from single-contaminant studies (Mondal and Chattopadhyay, 2020). Therefore, elucidating the health effects and biological pathways associated with chronic As–F co-exposure is important for environmental hazard assessment and evidence-based risk management in co-contaminated regions.

Male reproductive function is sensitive to environmental and metabolic stress because it depends on coordinated germ-cell differentiation, blood–testis barrier integrity, steroidogenesis, and energy metabolism (Rato et al., 2012; Eisenberg et al., 2023; Kimmins et al., 2024).

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Environmental pollutants have been implicated in altered semen quality and reproductive endocrine function, although the strength of evidence varies across contaminants and study designs (Skakkebaek et al., 2022; Xu et al., 2022). Experimental studies indicate that arsenic can disturb reproductive hormones, damage testicular structure and the blood–testis barrier, and impair spermatogenesis and sperm maturation (Machado-Neves, 2022; Mahboob et al., 2025; F.-W. Niu et al., 2025; Song et al., 2026). Fluoride exposure has likewise been associated in animal models with oxidative injury, altered steroidogenesis, germ-cell damage, and reduced sperm quality (Radovanović et al., 2022; Li et al., 2023; Ommati et al., 2025; Talebi et al., 2025). Evidence concerning combined As–F reproductive toxicity is more limited (Mondal and Chattopadhyay, 2020). Our previous study showed that developmental As–F co-exposure was accompanied by testicular injury, changes in autophagy-related markers, and disruption of intestinal microbial balance (Liu et al., 2021). However, whether exposure-associated intestinal and metabolic changes contribute to the reproductive phenotype, and how joint effects vary among endpoints, remain insufficiently defined.

The gut microbiota may modify host responses to environmental chemicals by influencing xenobiotic biotransformation, intestinal barrier function, immune and endocrine signaling, and systemic metabolism (Claus et al., 2016; Licht and Bahl, 2019; Fan and Pedersen, 2021). Arsenic illustrates this bidirectional relationship: gut microorganisms participate in redox and methylation reactions that can modify arsenic metabolism and excretion, whereas arsenic exposure can alter microbial composition and metabolic output (Lu et al., 2014; Coryell et al., 2019; Yang et al., 2021; L. Chen et al., 2023). Fluoride exposure has also been associated with dose-dependent changes in microbial community structure and predicted function, although findings vary according to dose, duration, and host species (Mo et al., 2023; Yasin et al., 2025). These interactions may be especially relevant when exposure spans preconception, gestation, lactation, puberty, and adulthood because early-life microbial assembly and the reproductive endocrine system both undergo substantial developmental remodeling (Qi et al., 2021; Santos-Marcos et al., 2023; S. Wang et al., 2024).

Growing evidence suggests that the gut microbial ecosystem may influence male reproductive function through metabolic, immune, and endocrine pathways relevant to spermatogenesis (Ding et al., 2020; H. Cai et al., 2022; M. Wang et al., 2024; W. Chen et al., 2024). Experimental studies have further linked microbial disturbances to coordinated intestinal and testicular responses, while fecal transfer has been shown to modify reproductive phenotypes and systemic and testicular metabolic profiles (Hao et al., 2022; S. Niu et al., 2026). These observations support the gut microbiota as a potential contributor to environmentally induced reproductive injury.

Reciprocal fecal transfer between exposed and control animals provides an experimental approach for testing whether donor-derived fecal material can modify recipient phenotypes and therefore offers stronger evidence than cross-sectional microbial associations alone. Nevertheless, fecal transfer does not by itself establish stable microbial engraftment or microbiota-specific causation, because recipient conditioning, microbial viability, transferred microbial products, soluble metabolites, and chemical carryover may influence the observed effects (Gheorghe et al., 2021). Integrating reciprocal transfer with microbial, intestinal, metabolic, and reproductive assessments may therefore provide a broader basis for investigating gut-associated contributions to toxicant-induced male reproductive injury.

Bile acids provide a biologically plausible interface among intestinal microorganisms, hepatic metabolism, and distal-organ physiology. Primary bile acids synthesized in the liver are deconjugated and further transformed by intestinal microorganisms, generating a bile acid pool shaped jointly by host and microbial processes (Winston and Theriot, 2020; Guzik and Quinn, 2021; Collins et al., 2023). Beyond facilitating lipid absorption, bile acids signal through receptors including the farnesoid X receptor (FXR) and G-protein-coupled bile acid receptor 1. Ileal FXR induces fibroblast growth factor 15 (FGF15), which signals to the

liver to regulate bile acid synthesis and broader metabolic processes (Inagaki et al., 2005; Chiang and Ferrell, 2020; Collins et al., 2023). Lipid uptake, synthesis, and remodeling are integral to germ-cell development and steroid hormone production (Miller and Auchus, 2011; Rato et al., 2012), and experimental studies indicate that bile acid receptors can influence testicular physiology and male reproductive function (Baptissart et al., 2014; Martinot et al., 2017). Microbiota-associated bile acid and lipid remodeling could therefore accompany coordinated intestinal, hepatic, and testicular responses.

Autophagy is a highly conserved cellular quality-control process that contributes to organelle turnover, steroidogenesis, germ-cell development, and testicular homeostasis (Gao et al., 2018; Samare-Najaf et al., 2023). Previous studies of combined As–F exposure and fluoride exposure alone have documented changes in testicular autophagy-related proteins and autophagosome-like structures (Liu et al., 2021; Li et al., 2023; Talebi et al., 2025). Because static protein and ultrastructural measurements cannot determine autophagic-flux direction or functional consequence, such findings are appropriately interpreted as accompanying autophagy-related alterations rather than evidence of pathway activation or autophagy-mediated injury (Klionsky et al., 2021).

In this study, we used a relatively high-dose chronic drinking-water exposure model to characterize the reproductive hazards of combined As–F exposure. Sprague–Dawley rats were exposed to sodium arsenite and/or sodium fluoride from parental pre-mating through gestation and lactation, with male offspring continuing their assigned exposures until postnatal day (PND) 60 or PND120. The direct-exposure experiment assessed duration-associated reproductive changes, whereas reciprocal fecal transfer between control and combined-exposure backgrounds was used to evaluate FMT-associated differences in recipient phenotypes under their original drinking-water regimens. Reproductive outcomes were evaluated alongside intestinal, microbial, serum metabolomic, hepatic, and testicular endpoints. Untargeted metabolomic data were further interrogated to identify putatively annotated bile acid signals, while static autophagy-related endpoints were used to characterize accompanying testicular alterations. By integrating these assessments, this study provides a multilevel characterization of reproductive toxicity at the tested concentrations and the associated intestinal, microbial, metabolic, hepatic, and testicular responses.

2. Materials and methods

2.1. Chemicals

Sodium arsenite (NaAsO_2 ; Sigma–Aldrich, St. Louis, MO, USA) and sodium fluoride (NaF; Shanghai Fengxian Fengcheng Reagent Factory, Shanghai, China) were dissolved in distilled water to prepare the exposure solutions. The nominal concentrations were 75 mg/L NaAsO_2 and 100 mg/L NaF, equivalent to approximately 43.25 mg/L elemental As and 45.25 mg/L F, respectively.

2.2. Animals, exposure, and experimental allocation

Specific-pathogen-free Sprague–Dawley rats were housed under controlled environmental conditions with food and water provided *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethics Committee of Shanxi Medical University.

Twenty-four female and 12 male rats were allocated to four drinking-water groups: control water (Con), 75 mg/L NaAsO_2 (As), 100 mg/L NaF (F), or the combination of 75 mg/L NaAsO_2 and 100 mg/L NaF (AsF). Parental exposure began 10 days before mating. Females and males were paired at a ratio of 2:1, and exposure continued throughout gestation and lactation. After weaning, male offspring remained on the drinking-water regimens assigned to their parental groups until terminal sampling at PND60 or PND120 ($n=8$ per group).

The exposure concentrations were selected based on our previous experimental model and related reproductive-toxicity literature (Liu

et al., 2021; Mahboob et al., 2025; Ommati et al., 2025). Group-level water and food consumption was recorded weekly.

2.3. Reciprocal fecal microbiota transplantation (FMT)

Rats from the PND120 Con and AsF groups served as the respective donor sources. Sixteen male offspring maintained on control water were randomized to receive sterile saline (Con-Sal, $n = 8$) or fecal suspension derived from AsF donors (Con-AsF, $n = 8$). Sixteen male offspring maintained under combined AsF exposure were randomized to receive sterile saline (AsF-Sal, $n = 8$) or fecal suspension derived from control donors (AsF-Con, $n = 8$). All 32 recipients completed the experimental protocol and remained on their original drinking-water regimens throughout the fecal-transfer period.

On each administration day, freshly voided donor feces were collected and homogenized in approximately five volumes of sterile physiological saline. The resulting suspension was filtered to remove particulate material and centrifuged at 1,200 rpm for 3 min. After removal of the supernatant, the pellet was resuspended in sterile physiological saline to the original suspension volume. The centrifugation–resuspension washing procedure was repeated three times, after which the final pellet was resuspended to the original volume.

Freshly prepared fecal suspension or sterile physiological saline was administered by enema at 1 mL/100 g body weight twice weekly for 7 weeks, for a total of 15 administrations per recipient. Administration ended at PND110, followed by a 10-day post-administration interval before terminal sampling at PND120.

2.4. Exposure biomarker

Urinary As concentrations were measured by atomic fluorescence spectrometry according to the Chinese health industry standard WS/T 474-2015, Determination of arsenic in urine—Hydride generation atomic fluorescence spectrometry, after mixed-acid digestion and pre-reduction with thiourea and ascorbic acid. Bone F concentrations were measured using a fluoride ion-selective electrode, with the procedure adapted from WS/T 89-2015, Determination of fluoride in urine—Ion-selective electrode method. Bone samples were dried, ashed, acid-extracted, pH-adjusted, and mixed with total ionic-strength adjustment buffer before analysis. Procedural blanks and calibration standards were included, and four animals per group were analyzed.

2.5. Determination of sperm density, motility, and morphology

Prior to analysis, Dulbecco's modified Eagle medium (DMEM) and all instruments were pre-warmed to 37 °C. After euthanasia, the right cauda epididymis was rapidly excised, weighed, and minced in pre-warmed DMEM to obtain a sperm suspension.

Sperm density: A 10 μ L aliquot of sperm suspension was loaded onto a hemocytometer, and spermatozoa in 20 squares were counted under a light microscope. Sperm density was calculated as: Sperm density ($\times 10^6$ /mL) = (total sperm count in 20 squares / 2) $\times 10^6$

Sperm motility: A 10 μ L aliquot was analyzed on a hemocytometer at 37 °C, and sperm movement within 20 squares was recorded for 15 s. Motile sperm were defined as those exhibiting progressive or non-progressive movement. Sperm motility (%) = (number of motile sperm / total sperm counted) $\times 100\%$.

Sperm morphology: A 20 μ L aliquot was smeared on a glass slide, air-dried, fixed in methanol, and stained with 2% eosin. At least 500 spermatozoa per sample were examined under a light microscope. Abnormalities were classified as head or tail defects, and the abnormality rate was calculated as: Abnormality rate (%) = (number of abnormal sperm / total sperm counted) $\times 100\%$.

2.6. Measurement of serum reproductive hormones

Serum levels of reproductive hormones, including testosterone, estradiol, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (AndyGene, Beijing, China) according to the manufacturer's instructions.

2.7. Morphological examination

Testicular tissues were fixed in Bouin's solution for 48 h and subsequently transferred to 75% ethanol. Epididymal tissues and liver tissues were fixed in 4% paraformaldehyde for at least 24 h and trimmed to an appropriate size. All tissues were dehydrated through a graded ethanol series using an automated tissue processor, cleared, embedded in paraffin, and sectioned at 4 μ m. The sections were baked at 58 °C for 30 min, deparaffinized in xylene, rehydrated through graded ethanol, and stained with hematoxylin and eosin (H&E). After dehydration and clearing, the stained sections were mounted with neutral resin. Histopathological features of the testes, epididymides, and liver were examined by light microscopy.

2.8. Testicular immunohistochemistry

Paraffin sections were deparaffinized in three 15-min xylene baths, rehydrated through absolute, 85%, and 75% ethanol, and rinsed in distilled water. Citrate-buffer microwave antigen retrieval was performed for 20 min. Sections were cooled, washed three times in phosphate-buffered saline (PBS), incubated with 3% H₂O₂ for 25 min in the dark, and blocked with 3% bovine serum albumin (BSA) for 20 min. Primary antibodies against ACRV1 (HUABIO HA722543, 1:300) or SYCP3 (Proteintech 23024-1-AP, 1:1000) were applied overnight at 4 °C. After PBS washing, HRP-conjugated goat anti-rabbit secondary antibody (Servicebio GB23303, 1:100) was applied for 30 min at 37 °C. DAB development was monitored microscopically, followed by hematoxylin counterstaining, differentiation, bluing, graded dehydration, clearing, and neutral-resin mounting. Image acquisition was performed using a 3D digital section scanner (model P250 FLASH; Jinan Danjier Electronics Co., Ltd., China). HALO software (Indica Labs, USA) quantified DAB-positive tissue area. The three fields were averaged to one animal-level value; $n=4$ animals per group.

2.9. Colonic immunofluorescence

Paraffin-embedded colonic tissues were sectioned at 3 μ m. Sections were baked at 60 °C for 3 h, deparaffinized, rehydrated, treated with hydrogen peroxide solution, and subjected to heat-induced antigen retrieval in sodium citrate buffer. After blocking with 5% BSA for 15 min, separate sections were incubated overnight at 4 °C with primary antibodies against ZO-1 or Occludin (1:200; Proteintech Group, Wuhan, China), or TNF- α , IL-1 β , or IL-10 (1:200; Santa Cruz Biotechnology, USA). After washing with PBS, the sections were incubated with the appropriate fluorescence-conjugated secondary antibodies (1:100) for 2 h at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and the sections were mounted using an antifade mounting medium.

Images were acquired using a fluorescence microscope under identical acquisition settings for each marker. Representative images were obtained at 200 $\times$ and 400 $\times$ magnification, with scale bars of 50 and 20 μ m, respectively. Three animals were randomly selected from each group, and six non-overlapping fields per animal were analyzed. The measurements from the six fields were averaged to obtain one value for each animal; individual fields were not treated as independent biological replicates.

2.10. Transmission electron microscope (TEM)

Testicular tissues were rapidly excised and cut into approximately 1 mm³ pieces, followed by fixation in 2.5% glutaraldehyde at 4 °C for 2 h. After rinsing in PBS, samples were post-fixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in epoxy resin.

Ultrathin sections (approximately 70 nm) were prepared using an ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan).

2.11. Western blot analysis

Tissue samples were homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (100:1:1, v/v/v). After centrifugation at 12,000 rpm at 4 °C for 10 min, the supernatant was collected as total protein. Protein concentration was determined using a BCA Protein Assay Kit (Boster, China).

Equal amounts of protein (30 µg) were separated by SDS-PAGE (10% resolving gel, 5% stacking gel) and transferred onto nitrocellulose membranes. Membranes were blocked and then incubated overnight at 4 °C with primary antibodies against FXR, SHP, CYP7A1, CYP8B1, FGF15, and BSEP (Abclonal Biotechnology Co., Ltd., Wuhan, China); ATG5, ATG7, SYCP3, and PGK2 (Santa Cruz Biotechnology, USA); PPARα, PPARγ, and PGC-1α (Proteintech Group, Inc., Wuhan, China); DAZL (Abclonal Technology, Wuhan, China); Beclin1 and β-actin (Abcam, Cambridge, UK); and SQSTM1/p62 (p62) and LC3B (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturers' recommended dilutions.

After washing, membranes were incubated with HRP-conjugated secondary antibodies (1:4000, Boster, China) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Boster, China) and imaged using a ChemiDoc™ MP Imaging System (Bio-Rad, USA). Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.12. 16S rRNA gene sequencing and microbial statistics

Fecal DNA was extracted, and the V3–V4 region was amplified with primers 341F/805R and sequenced on an Illumina NovaSeq 6000. QIIME 2 and DADA2 were used for filtering, denoising, chimera removal, and amplicon sequence variant (ASV) inference, followed by taxonomic assignment against SILVA release 138 (Bolyen et al., 2019; Callahan et al., 2016; Quast et al., 2013). Analyses used six animals per group and a common ASV feature space.

2.13. Untargeted metabolomics and bile-acid-focused interrogation

Serum metabolites were extracted by cold methanol precipitation and analyzed on an UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) coupled to a Q-Exactive high-resolution mass spectrometer in positive and negative ion modes. A pooled quality-control sample was injected periodically. XCMS, CAMERA, and metaX were used for feature detection, alignment, and quality-control (QC)-based signal-drift correction. Features were annotated by accurate mass and, when available, MS/MS matching against HMDB, KEGG, and an in-house library. Reporting followed metabolomics minimum standards and confidence principles (Sumner et al., 2007; Schymanski et al., 2014).

The bile acid analysis was a post hoc, biologically informed interrogation of the untargeted dataset, not a targeted quantitative assay. Table S4 reports all bile-acid-related features and their statistical evidence; Table S5 lists 12 detected bile acid species and confidence categories. Intensities are relative signals and species names are putative

annotations. PLS-DA performance was assessed in mixOmics using threefold stratified cross-validation repeated 100 times with centroid distance (Rohart et al., 2017). Genus–bile-acid correlations used animal-level paired data, whereas bile-acid–hormone relationships used group-level summaries; both were treated as exploratory Spearman analyses. Network visualization and statistical analyses were conducted using R (version 4.5.2).

2.14. Exploratory database-derived analysis

Disease-related genes were retrieved from the GeneCards database (<https://www.genecards.org/>) using the following keywords: azoospermia, hypogonadism, male infertility, spermatogenesis, spermatogenic failure, teratozoospermia, and testicular diseases (Safran et al., 2010). Redundant entries were removed to generate a non-duplicated disease gene set. Arsenic- and fluoride-associated genes were obtained from the Comparative Toxicogenomics Database (<http://ctdbase.org/>; Davis et al., 2023). Only human-derived interactions (Homo sapiens) were retained. Gene-level integration was performed by aggregating interaction evidence, including the number of supporting publications and interaction types, to construct chemical–gene association datasets. The intersection of disease-, arsenic-, and fluoride-related genes was identified using R software (version 4.3.0) and visualized using Venn diagrams to define common candidate targets.

To explore protein interactions, overlapping genes were mapped to the STRING database (version 12.0; <https://string-db.org/>) with a minimum interaction score of 0.4 (Szklarczyk et al., 2023). The protein–protein interaction network was constructed and visualized using Cytoscape (version 3.10.1; Shannon et al., 2003). Functional modules were identified using MCODE (Bader and Hogue, 2003), and hub genes were ranked with the Degree algorithm in cytoHubba (Chin et al., 2014).

Functional enrichment analysis was performed using the clusterProfiler package in R (Wu et al., 2021). Gene Ontology (GO) analysis included biological process, cellular component, and molecular function categories, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was also performed. Pathways with adjusted P values < 0.05 were considered statistically significant.

2.15. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were primarily performed using R software (version 4.5.2). Comparisons between two independent groups were conducted using Student's *t*-test or the Wilcoxon rank-sum test, depending on the distributional characteristics of the data. Multiple-group comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Prespecified reciprocal FMT comparisons were conducted within each recipient background, with multiplicity adjustment applied where indicated.

For the PND120 Con, As, F, and AsF groups, each endpoint with verified individual-animal data was additionally analyzed using a 2 × 2 factorial model, with arsenic and fluoride exposure entered as binary factors. Type III tests with sum-to-zero contrasts were used to estimate the main effects of arsenic and fluoride and their interaction. The arsenic × fluoride interaction was evaluated before interpreting the main effects. When evidence of interaction was detected, interpretation focused on model-estimated simple effects and contrasts rather than the marginal main effects. The direction of interaction was determined from these estimated contrasts rather than by visual inspection of group means. Heteroskedasticity-consistent type 3 (HC3) standard errors were used in sensitivity analyses (Long and Ervin, 2000).

Western blot band intensities were quantified using ImageJ software (2015 version; National Institutes of Health, Bethesda, MD, USA), and statistical graphs were generated using GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA). Exact sample sizes, statistical tests, post hoc procedures, and multiplicity adjustments for individual assays

are reported in the corresponding figure legends and supplementary tables. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic arsenic–fluoride exposure impairs spermatogenesis, with reproductive phenotypes differing following FMT

The experimental design and exposure timeline are shown in Fig. 1a. Weekly food- and water-intake trajectories were summarized descriptively in Fig. S1 and Table S1. At PND120, mean water consumption was 40.88, 19.64, 41.20, and 22.69 mL/rat/day in the Con, As, F, and AsF groups, respectively. Based on nominal elemental concentrations and group-level water-consumption summaries, the estimated daily arsenic administration was 0.849 and 0.981 mg/rat/day in the As and AsF groups, respectively, whereas the estimated fluoride administration was 1.864 and 1.027 mg/rat/day in the F and AsF groups, respectively.

Internal-burden measurements confirmed the expected exposure pattern. Urinary arsenic concentrations were 22.795 ± 3.418 and 21.616 ± 1.811 mg/L in the As and AsF groups, respectively, compared with 0.152 ± 0.076 and 0.254 ± 0.125 mg/L in the Con and F groups. Bone fluoride concentrations were 1411.8 ± 161.4 and 1329.5 ± 189.4 $\mu\text{g/g}$ in the F and AsF groups, respectively, compared with 469.4 ± 63.8 and 448.8 ± 25.8 $\mu\text{g/g}$ in the Con and As groups. All corresponding exposure-related comparisons remained significant after Tukey adjustment (adjusted $P < 0.001$; Fig. S1). At the FMT endpoint, neither urinary arsenic nor bone fluoride differed significantly between Con-Sal and Con-AsF or between AsF-Sal and AsF-Con, indicating comparable terminal internal burdens within each recipient background.

Direct As and/or F exposure produced marked but endpoint-specific alterations in male reproductive function at PND60 and PND120 (Fig. 1). Sperm density was reduced in the exposed groups at both time points, with the lowest values generally observed following combined exposure. Sperm motility was markedly suppressed in the AsF group at PND60 and remained reduced at PND120, whereas sperm abnormality varied according to exposure and age without showing the same pattern. Serum testosterone was reduced in several exposure groups, while serum estradiol was elevated following combined exposure, particularly at PND120. Serum FSH and LH were generally lower at PND120 than at PND60, and intratesticular testosterone was reduced in the AsF group at PND120. Reproductive organ coefficients showed comparatively modest and endpoint-dependent changes (Fig. S2).

The PND120 Con, As, F, and AsF groups were further evaluated using 2×2 arsenic-by-fluoride factorial models (Fig. S4). Significant As $\times$ F interactions were detected for serum testosterone ($P = 0.008$), estradiol ($P = 0.009$), LH ($P = 0.010$), sperm motility ($P < 0.001$), testis coefficient ($P = 0.005$), and epididymis coefficient ($P < 0.001$). Sperm abnormality showed an interaction in the primary Type III analysis ($P = 0.030$), but this evidence was attenuated in the HC3 sensitivity analysis ($P = 0.064$) and was therefore not considered robust. No significant interaction was detected for FSH ($P = 0.083$), intratesticular testosterone ($P = 0.137$), or sperm density ($P = 0.968$). Sperm density instead showed significant main effects of both As ($P < 0.001$) and F ($P = 0.003$), while intratesticular testosterone showed a significant As main effect. These results demonstrate endpoint-specific departures from additivity, with several outcomes exhibiting joint effects that were smaller than those expected from the sum of the individual As and F effects.

In the adjusted pairwise comparisons, AsF at PND120 differed from the corresponding control in serum testosterone, estradiol, intratesticular testosterone, and sperm density, whereas the sperm-motility comparison did not reach the adjusted significance threshold. Histological examination supported the functional findings. Control testes displayed regularly organized seminiferous tubules with an intact germinal epithelium, whereas exposed animals showed varying degrees of epithelial disorganization, widened intercellular spaces, germ-cell

loss or detachment, and reduced luminal sperm content. Exposed epididymides showed epithelial swelling and vacuolization, widened luminal spaces, focal epithelial cell loss, reduced sperm content, and occasional aggregation of abnormal sperm, with more evident abnormalities at PND120 (Fig. 1; Fig. S3).

Reciprocal FMT was associated with selective changes in reproductive outcomes within the two recipient backgrounds (Fig. 1). Compared with Con-Sal recipients, Con-AsF recipients showed lower sperm density and serum testosterone following administration of AsF-donor fecal material, together with modest changes in sperm motility and abnormality. Compared with AsF-Sal recipients, AsF-Con recipients showed higher sperm motility, serum testosterone, and estradiol following administration of control-donor fecal material. FSH and LH increased numerically, whereas sperm density, sperm abnormality, and intratesticular testosterone were not consistently improved. Histologically, Con-AsF recipients showed reduced luminal sperm content, focal germ-cell detachment, and partial seminiferous-tubule atrophy relative to Con-Sal recipients. The pronounced epididymal epithelial injury and reduced luminal sperm content observed in AsF-Sal recipients were less evident in AsF-Con recipients, although normal testicular and epididymal architecture was not completely restored.

Stage-specific testicular immunohistochemistry was performed at PND120 using SYCP3 as a marker of meiotic germ cells and ACRV1 as a marker associated with postmeiotic and acrosome-forming spermatids. In the FMT groups, ACRV1-positive areas were $17.28 \pm 3.23\%$, $5.80 \pm 1.61\%$, $5.41 \pm 0.49\%$, and $24.11 \pm 5.45\%$ in the Con-Sal, Con-AsF, AsF-Sal, and AsF-Con groups, respectively. The corresponding SYCP3-positive areas were $29.75 \pm 5.97\%$, $2.62 \pm 2.11\%$, $2.16 \pm 1.73\%$, and $15.08 \pm 4.04\%$ (Fig. 2a–d). Thus, both markers decreased following administration of AsF-donor fecal material to control recipients and increased following administration of control-donor material to AsF-exposed recipients.

Consistent alterations were observed in the direct-exposure groups. ACRV1-positive areas were $23.67 \pm 6.28\%$, $5.37 \pm 2.16\%$, $12.61 \pm 4.06\%$, and $12.38 \pm 4.61\%$ in the Con, As, F, and AsF groups, respectively, while the corresponding SYCP3-positive areas were $17.21 \pm 2.71\%$, $2.44 \pm 0.97\%$, $7.91 \pm 2.93\%$, and $9.22 \pm 4.41\%$ (Fig. S5). These findings support the involvement of meiotic and postmeiotic germ-cell-associated processes, although ACRV1 and SYCP3 did not show identical exposure-response patterns.

Western blotting further showed lower DAZL, PGK2, and SYCP3 expression in Con-AsF than in Con-Sal recipients. Expression was numerically higher in AsF-Con than in AsF-Sal recipients, although these comparisons did not reach statistical significance (Fig. 2e–f). Collectively, chronic As and F exposure produced distinct internal burdens and impaired sperm quality, reproductive endocrine balance, and testicular and epididymal integrity through endpoint-specific joint effects. Repeated donor-material administration was accompanied by changes in reproductive, histological, and spermatogenic-marker outcomes, including opposite-direction changes in selected endpoints across the two recipient backgrounds.

3.2. Direct exposure alters gut microbial community structure, whereas endpoint donor-associated FMT metrics show limited convergence

Bray–Curtis ordinations were analyzed separately for the direct-exposure and FMT recipient models (Fig. 3a–b). In the direct-exposure model, microbial community composition differed significantly among Con60, AsF60, Con120, and AsF120 groups (PERMANOVA $R^2 = 0.336$, $P < 0.001$), without evidence of unequal multivariate dispersion (PERMDISP $P = 0.270$). The prespecified Con60 versus AsF60 and Con120 versus AsF120 comparisons were both significant after multiple-testing correction (adjusted $P = 0.0046$ and 0.0138 , respectively).

Alpha-diversity analyses included the Shannon, Simpson, Chao1, observed ASVs, Good's coverage, and Pielou's evenness indices (Fig. 3c). Genus-level composition also varied among groups. *Lactobacillus*

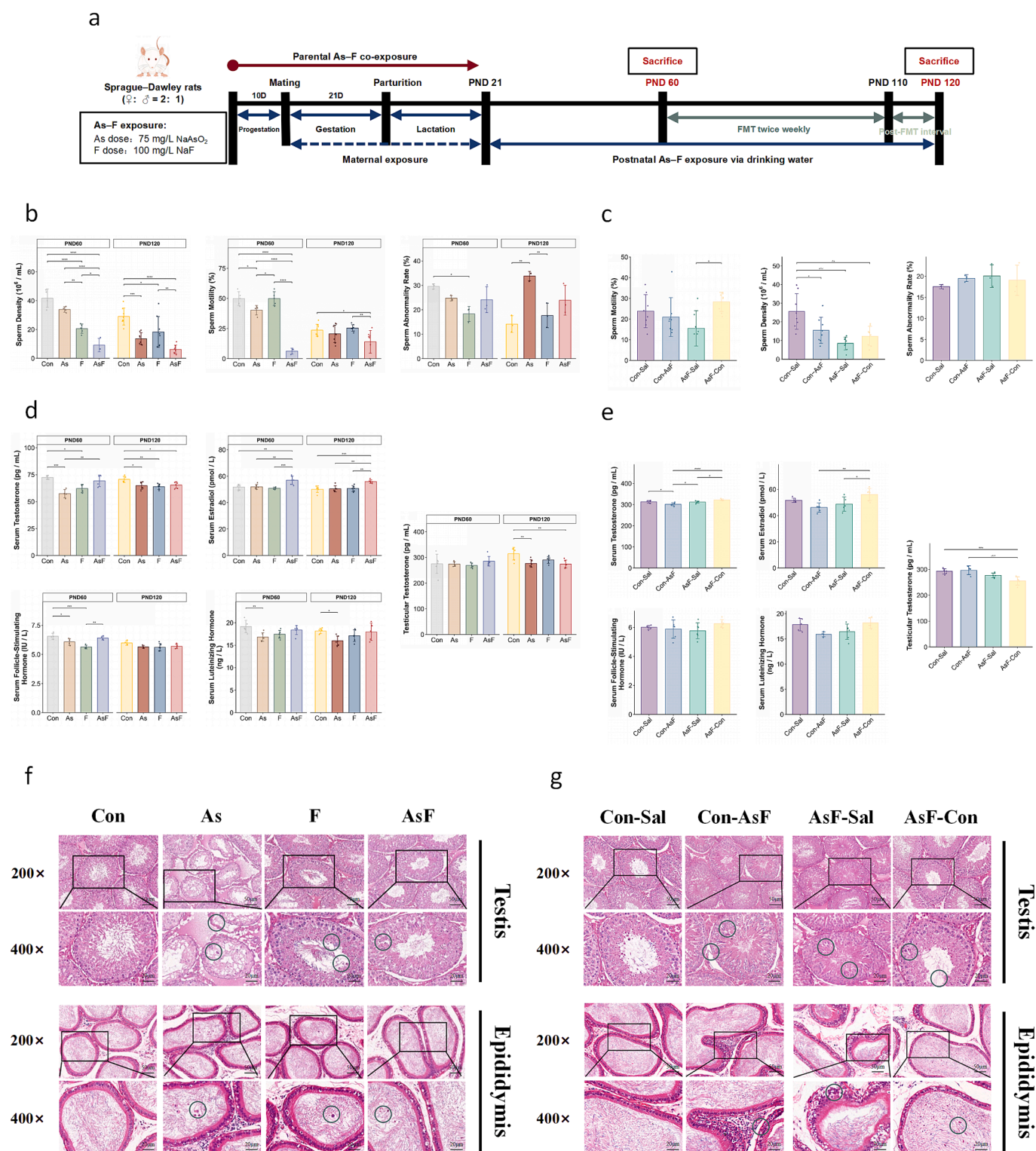
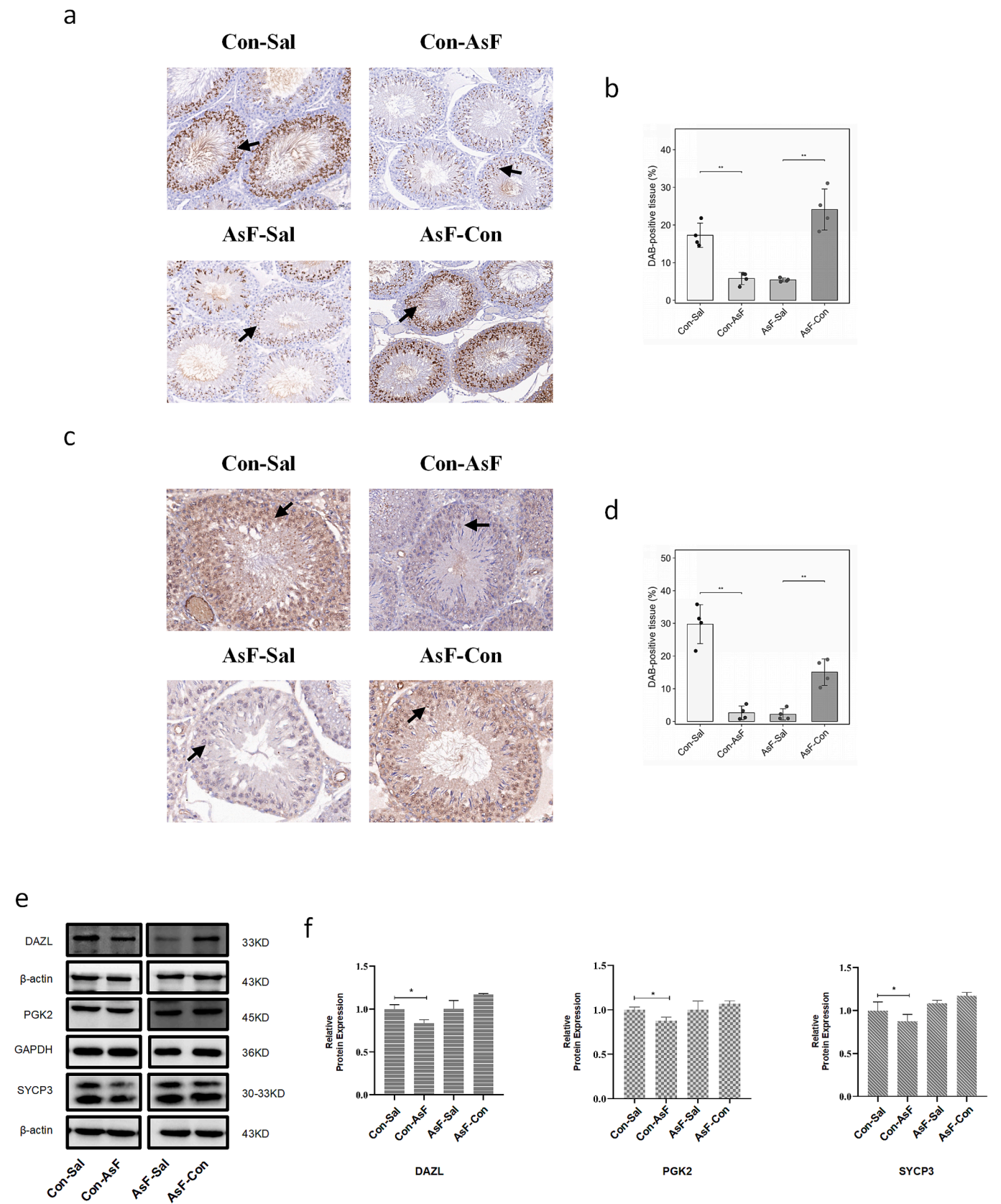


Fig. 1. Chronic developmental arsenic–fluoride exposure impairs male reproductive outcomes and is partially modified by reciprocal fecal microbiota transplantation. (a) Parental and offspring drinking-water exposure, FMT schedule, and terminal sampling. (b) Sperm density, motility, and abnormality at PND60 and PND120. (c) Sperm endpoints in FMT recipients. (d) Reproductive hormones after direct exposure. (e) Reproductive hormones after FMT. (f) Representative testis and epididymis H&E images after direct exposure. (g) Corresponding testis and epididymis H&E images after FMT. Green circles indicate representative regions of histopathological lesions for visual orientation and were not treated as independent quantitative observations. For H&E, scale bars are 50 μ m at 200 $\times$ and 20 μ m at 400 $\times$. For animal-level endpoints, individual points represent animals, and the number of plotted points indicates the biological sample size. Data are presented as mean $\pm$ SD. Factorial interaction results are detailed in Fig. S4. FMT, fecal microbiota transplantation; PND, postnatal day; H&E, hematoxylin and eosin; SD, standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



(caption on next page)

Fig. 2. Meiotic and postmeiotic spermatogenic markers and spermatogenesis-related proteins after reciprocal fecal microbiota transplantation. All panels use testicular samples from the four FMT recipient groups. (a) Representative ACRV1 IHC images at 200 × magnification (scale bar, 50 μm). (b) Animal-level quantification of ACRV1 DAB-positive tissue area. (c) Representative SYCP3 IHC images at 400 × magnification (scale bar, 20 μm). (d) Animal-level quantification of SYCP3 DAB-positive tissue area. Brown DAB denotes ACRV1 cytoplasmic/acrosome-associated or SYCP3 nuclear immunoreactivity; nuclei are blue. Black arrows indicate representative characteristic immunoreactive structures for visual orientation and were not used as independent quantitative observations. Three fields were averaged within each animal. Individual points represent animals, and the number of plotted points indicates the biological sample size. IHC data are presented as mean ± SD. (e) Representative immunoblots of DAZL, PGK2, and SYCP3. (f) Densitometric quantification of DAZL, PGK2, and SYCP3. Individual points represent animals, and the number of plotted points indicates the biological sample size. Western blot data are presented as mean ± SEM. Descriptive animal-level IHC values are reported in Results. FMT, fecal microbiota transplantation; ACRV1, acrosomal vesicle protein 1; IHC, immunohistochemistry; DAB, 3,3'-diaminobenzidine; SYCP3, synaptonemal complex protein 3; DAZL, deleted in azoospermia-like; PGK2, phosphoglycerate kinase 2; SD, standard deviation; SEM, standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed a relatively higher abundance in AsF-Con recipients, whereas *Dubosiaella*, *Bifidobacterium*, and *Escherichia-Shigella* were relatively more abundant in Con-AsF recipients (Fig. 3h–i). Supporting sequencing-depth, ASV-overlap, taxonomic-composition, linear discriminant analysis effect size (LEfSe), and donor-associated-ASV analyses are shown in Figs. S6–S8.

When the donor-reference and FMT groups were evaluated together, the overall six-group Bray–Curtis model was significant (PERMANOVA $R^2=0.307$, $P<0.001$), although multivariate dispersion also differed among groups (PERMDISP $P=0.025$). Within recipient backgrounds, the prespecified Con-Sal versus Con-AsF and AsF-Sal versus AsF-Con comparisons were not significant after adjustment (adjusted $P = 0.106$ for both comparisons, Table S2).

Recipient communities also remained distinguishable from their reciprocal donor-reference groups. Con-AsF differed from the AsF120 donor reference, and AsF-Con differed from the Con120 donor reference (adjusted $P = 0.0046$ for both comparisons). Consistently, recipient-to-donor centroid distance, donor-proximity indices, reciprocal donor-associated ASV relative abundance, and donor-associated ASV detection counts did not differ significantly within either recipient background (all adjusted $P = 0.256–0.873$; Fig. 3d–g; Table S3).

3.3. Colonic junction- and inflammation-related markers

PND120 intestinal immunofluorescence showed reciprocal marker changes across the two recipient backgrounds (Fig. 4). Administration of AsF-donor fecal material to control recipients was accompanied by reduced ZO-1 and Occludin staining and increased TNF- α expression. Administration of control-donor material to AsF-exposed recipients was accompanied by changes in the opposite direction. Both prespecified comparisons remained significant after Holm correction for ZO-1, Occludin, and TNF- α (adjusted $P = 0.002$, 0.008 , and 0.013 , respectively).

IL-1 β also increased in Con-AsF relative to Con-Sal and decreased in AsF-Con relative to AsF-Sal (adjusted $P=0.010$ for both comparisons). IL-10 showed a similar reciprocal pattern, increasing after administration of AsF-donor fecal material and decreasing after administration of control-donor fecal material (adjusted $P=0.002$ for both comparisons). The complete FMT marker panel is presented in Fig. S10, and the corresponding direct-exposure immunofluorescence profiles are shown in Fig. S9.

3.4. Untargeted serum metabolomics identifies exposure- and FMT-associated metabolic remodeling

Untargeted serum metabolomics showed broad metabolic differences following arsenic–fluoride exposure and reciprocal FMT. The two prespecified PLS-DA score plots separated the Con120, AsF120, Con-Sal, and Con-AsF groups in Model A and the Con120, AsF120, AsF-Sal, and AsF-Con groups in Model B (Fig. 5a–b).

Repeated stratified three-fold cross-validation identified three components as optimal for both models. Model A showed moderate internal classification performance, with a mean balanced error rate of approximately 0.37, whereas Model B showed stronger internal discrimination,

with a mean balanced error rate of approximately 0.14 (Fig. S11f–g).

Chemical-class annotation showed that lipid-related metabolites constituted a major component of the differential features. Glycerophospholipids represented the largest annotated class, followed by fatty acyls, glycerolipids, sphingolipids, steroids and derivatives, and bile-acid-related features (Fig. 5c). The two FMT comparisons showed different directional patterns: decreases predominated in Con-AsF relative to Con-Sal, whereas increases predominated in AsF-Con relative to AsF-Sal (Fig. 5d–f).

Pathway enrichment analysis mapped the differential features to glycerophospholipid metabolism, fatty acid biosynthesis, glycerolipid and ether-lipid metabolism, ketone-body metabolism, lipid digestion and absorption, cAMP signaling, and autophagy-related pathways (Fig. 5g–h). Additional PCA, heatmap, PLS-DA, VIP-ranked feature, and pathway results are presented in Figs. S11–S14.

3.5. Bile-acid-related features show reciprocal FMT-associated patterns and structured endocrine relationships

A bile-acid-focused analysis of the untargeted metabolomics dataset identified relative bile-acid-related features that differed across the AsF120 versus Con120, Con-AsF versus Con-Sal, and AsF-Con versus AsF-Sal comparisons (Fig. 6a–c). Among the predefined 12-feature panel, TUDCA-, glycodeoxycholic acid-, 7-ketodeoxycholic acid-, GCDCA-3G-, and 7-SCA-related signals showed significant overall group differences after multiple-testing correction (Fig. 6d).

TUDCA-related signal intensity was higher after direct AsF exposure, whereas the 7-ketodeoxycholic acid-related signal was lower. GCDCA-3G showed the clearest reciprocal FMT-associated pattern, increasing in Con-AsF relative to Con-Sal and decreasing in AsF-Con relative to AsF-Sal. Changes in the 7-SCA-related signal were more evident in the FMT comparisons than in the direct Con120 versus AsF120 comparison.

In the exploratory display based on group-level summaries, TUDCA showed an inverse relationship with FSH and testosterone, GCDCA-3G with LH, and 7-SCA with LH and estradiol, whereas GUDCA showed a positive relationship with testosterone (Fig. 6e). Animal-level genus–bile-acid analysis identified 50 nominally significant correlations involving 22 genera and 11 bile-acid-related features (Fig. 6f). Representative associations included positive correlations of Family_XIII_AD3011_group with TUDCA, GCDCA-3G, and 7-SCA, as well as correlations involving *Roseburia*, *Negativibacillus*, *NK4A214_group*, *Allobaculum*, *Ligilactobacillus*, *Ralstonia*, *Blautia*, and *Akkermansia*. These exploratory results indicate that a subset of bile-acid-related signals co-varied with specific genera but do not establish directionality or mediation.

The integrated three-layer network retained 15 representative genera, four bile-acid-related features—TUDCA, GUDCA, GCDCA-3G, and 7-SCA—and four reproductive hormones. The final network contained 22 genus–bile acid edges and six bile acid–hormone edges (Fig. 6g–h).

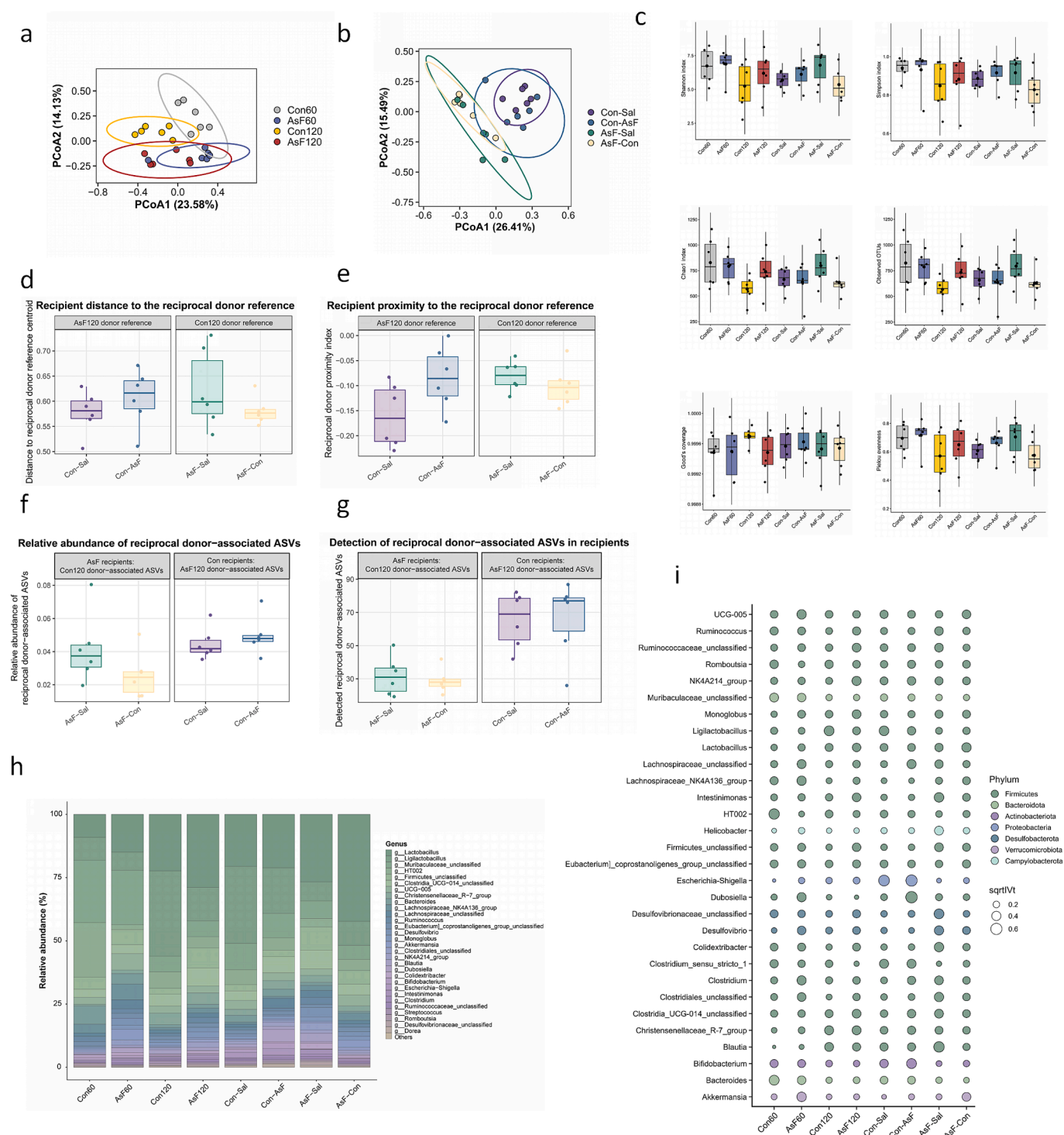


Fig. 3. Arsenic-fluoride exposure and reciprocal fecal microbiota transplantation are associated with remodeling of gut microbial communities. (a) Bray-Curtis PCoA for the direct-exposure groups. (b) Separate Bray-Curtis PCoA for the four FMT-recipient groups; donor references were not included. (c) Shannon, Simpson, Chao1, observed-ASV, Good's-coverage, and Pielou-evenness indices across the eight groups. (d) Recipient distance to the reciprocal donor reference. (e) Reciprocal donor proximity. (f) Relative abundance of reciprocal donor-associated ASVs. (g) Detected count of reciprocal donor-associated ASVs. (h) Phylum-level microbial composition. (i) Dominant-genus bubble plot. $n = 6$ animals/group. Adjusted alpha-diversity, PERMANOVA, and PERMDISP results are provided in Table S2, and definitions and statistical results for the donor-associated restructuring metrics are provided in Table S3. FMT, fecal microbiota transplantation; PCoA, principal coordinates analysis; ASV, amplicon sequence variant; PERMANOVA, permutational multivariate analysis of variance; PERMDISP, permutational analysis of multivariate dispersions.

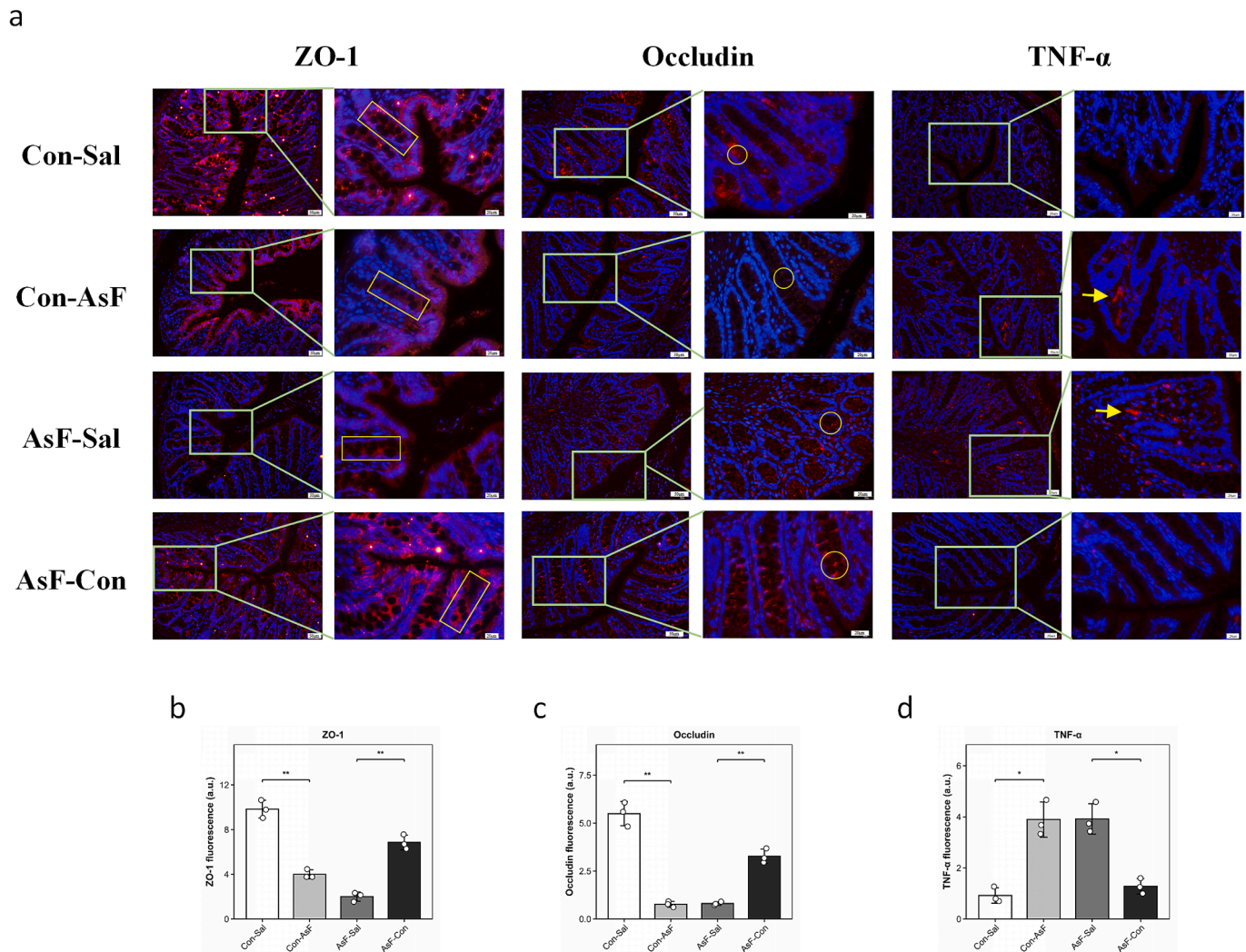


Fig. 4. Reciprocal fecal microbiota transplantation is associated with colonic tight-junction and inflammatory-marker changes. Representative $200\times$ and $400\times$ immunofluorescence images and corresponding animal-level quantification of (a) ZO-1, (b) Occludin, and (c) TNF- α . Scale bars are $50\mu\text{m}$ at $200\times$ and $20\mu\text{m}$ at $400\times$. Six fields were averaged within each animal. Individual points represent animals, and the number of plotted points indicates the biological sample size. Yellow boxes highlight representative continuous ZO-1 staining, circles provide goblet-cell orientation, and arrows identify representative TNF- α immunoreactivity. Holm-adjusted P values for the prespecified reciprocal contrasts were 0.002, 0.008, and 0.013, respectively. Full marker panels are presented in Figs. S9 and S10. FMT, fecal microbiota transplantation; ZO-1, zonula occludens-1; TNF- α , tumor necrosis factor alpha. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Reciprocal FMT is associated with enterohepatic signaling and hepatic metabolic responses at PND120

PND120 liver histology and biochemical measurements differed across the FMT groups (Fig. 7a). Con-Sal recipients showed relatively preserved hepatic architecture, whereas Con-AsF recipients displayed hepatocellular disorganization, lipid vacuolation, and localized inflammatory-cell infiltration. These alterations were less pronounced in AsF-Sal than in AsF-Con recipients.

Serum total bile acids and hepatic injury and lipid-related indicators showed corresponding FMT-associated patterns. Total bile acids, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hepatic total cholesterol, triglycerides, and LDL tended to increase after administration of AsF-donor fecal material, whereas several of these measures shifted in the opposite direction after administration of control-donor fecal material. Serum total bile acids were significantly lower in AsF-Con than in AsF-Sal. Direct-exposure liver histology and biochemical results are shown in Fig. S15.

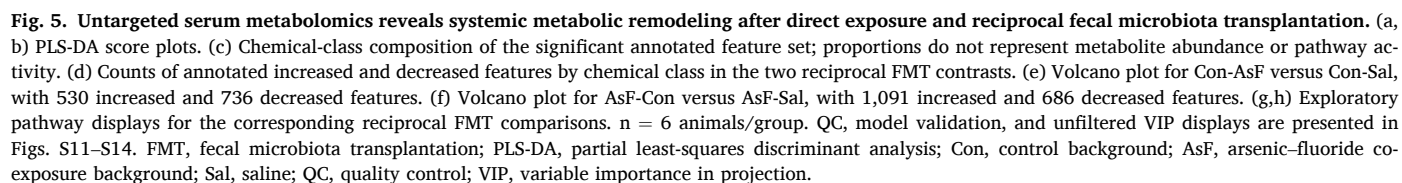
Intestinal FXR and FGF15 expression tended to decrease in Con-AsF relative to Con-Sal and to increase in AsF-Con relative to AsF-Sal

(Fig. 7c-d). In the liver, FXR, FGF15, FGFR4, and SHP were lower following administration of AsF-donor fecal material. Administration of control-donor fecal material increased hepatic FXR, FGF15, and SHP expression, whereas FGFR4 showed little change (Fig. 7e-f).

Proteins involved in hepatic bile acid synthesis and transport also showed reciprocal or directionally opposing patterns. CYP8B1 was higher in Con-AsF than in Con-Sal, accompanied by an upward trend in CYP7A1 and a downward trend in BSEP. CYP7A1 and CYP8B1 were lower and BSEP was higher in AsF-Con than in AsF-Sal, although not all comparisons reached statistical significance (Fig. 7g-h). PPAR α was reduced after administration of AsF-donor fecal material and increased after administration of control-donor fecal material, while PGC-1 α and PPAR γ showed similar directional changes (Fig. 7i-j).

3.7. Reciprocal FMT is associated with testicular autophagy-related ultrastructure and protein expression

PND120 testicular TEM showed group-dependent ultrastructural changes in Leydig cells, Sertoli cells, spermatogonia, and spermatocytes (Fig. 8a). Con-Sal recipients showed relatively preserved cellular



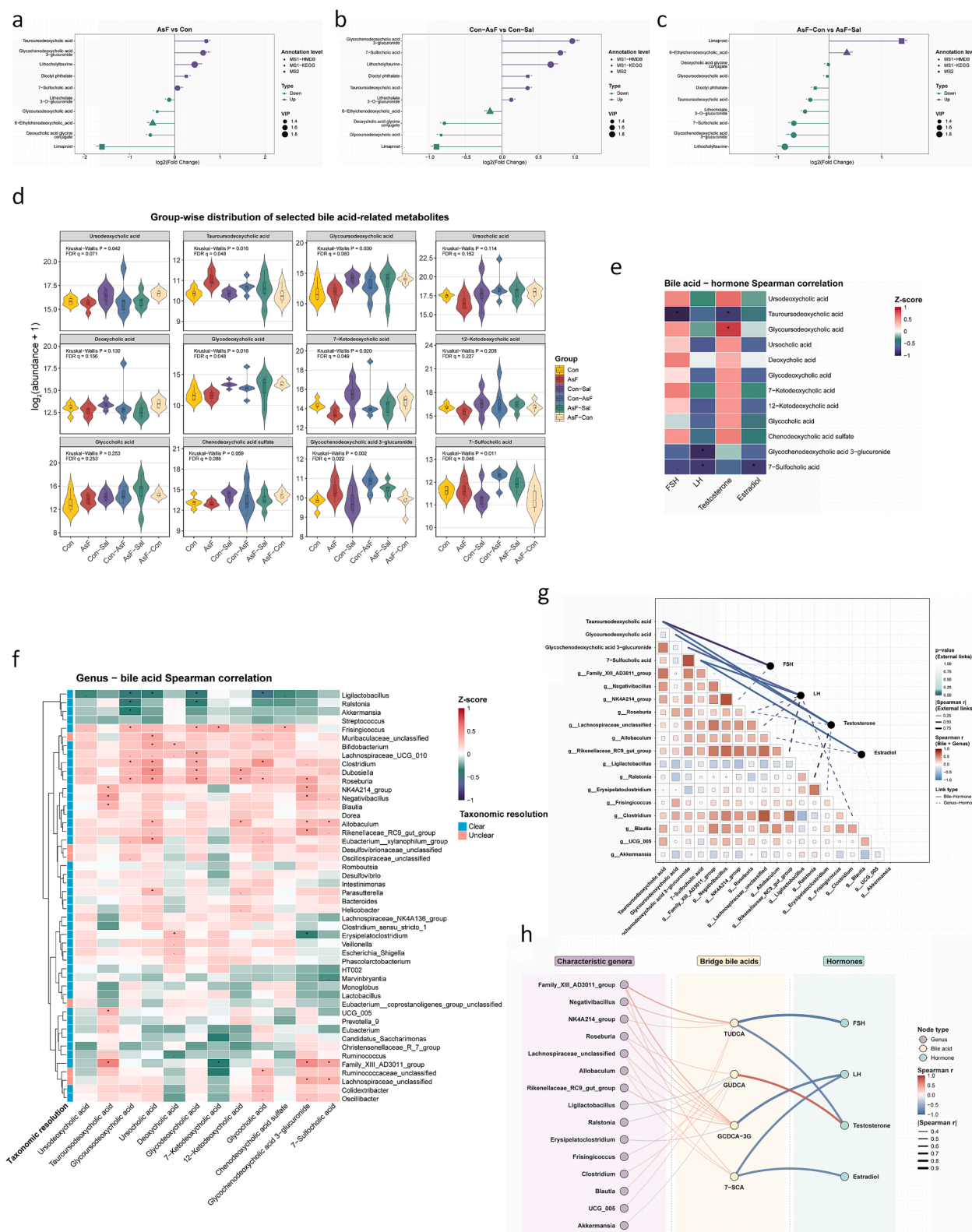
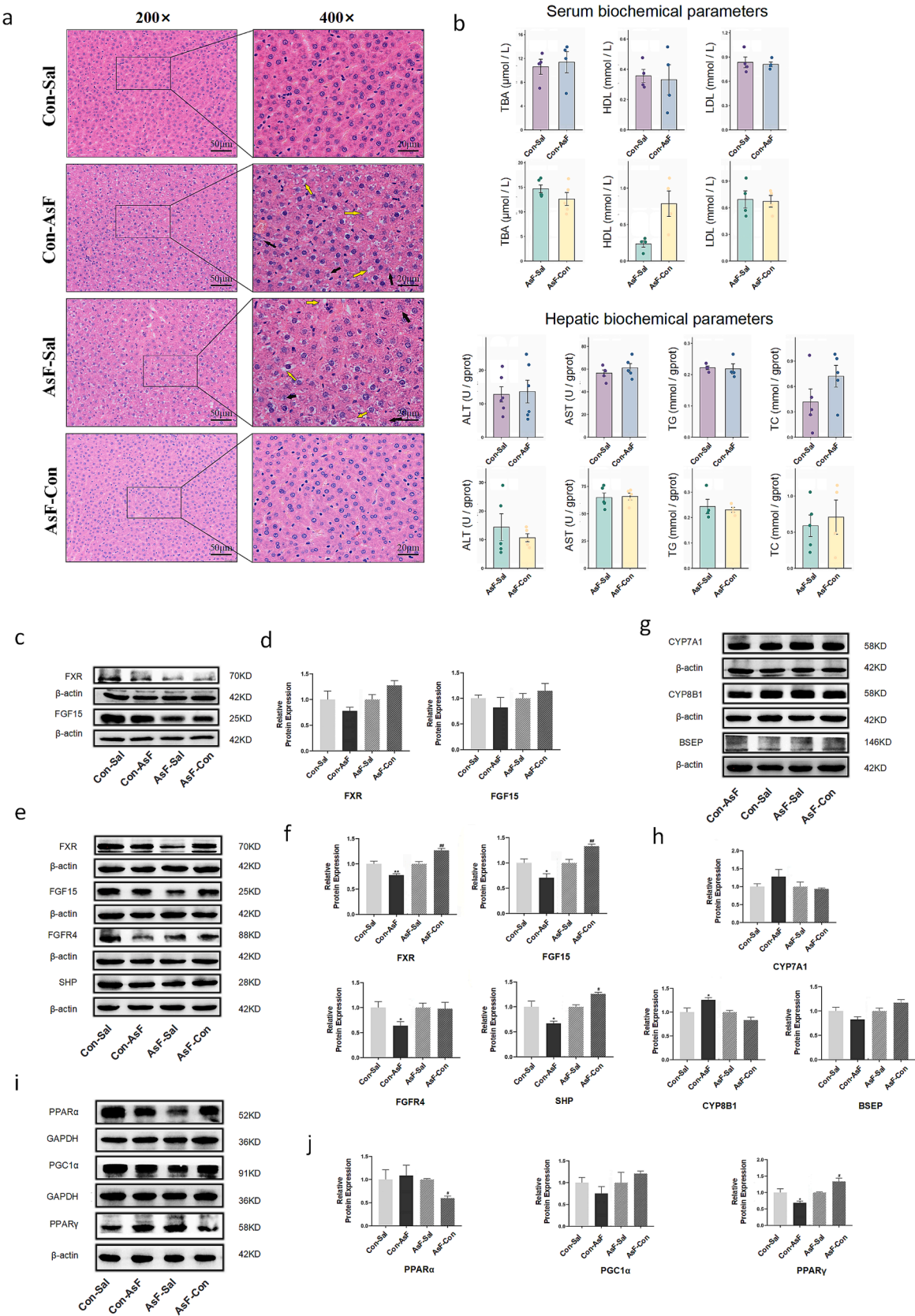


Fig. 6. Bile-acid-related metabolic remodeling is associated with gut microbial and reproductive endocrine changes. (a–c) VIP lollipop plots for the direct exposure and reciprocal FMT contrasts. (d) Relative signal distributions for 12 putatively annotated bile acid species. (e) Exploratory bile-acid–hormone Spearman correlation heatmap based on group-level summaries. (f) Animal-level genus–bile-acid Spearman correlations. (g) Focused combined correlation map linking representative genera, bile-acid-related signals, and reproductive hormones. (h) Three-layer genus–bile-acid–hormone network. $n = 6$ animals/group for metabolomics and genus–bile-acid analyses. The bile-acid-related signals were derived from untargeted LC–MS/MS and were not confirmed or absolutely quantified using authentic standards; annotation information and statistical evidence are provided in Tables S4 and S5. VIP, variable importance in projection; FMT, fecal microbiota transplantation; LC–MS/MS, liquid chromatography–tandem mass spectrometry.



(caption on next page)

Fig. 7. Reciprocal fecal microbiota transplantation is associated with coordinated enterohepatic and hepatic metabolic responses. All panels use FMT-recipient samples. (a) Representative liver H&E images; scale bars are 50 μ m at 200 $\times$ and 20 μ m at 400 $\times$. Yellow arrows indicate lipid vacuoles, and black arrows indicate inflammatory cell infiltration. (b) Serum and hepatic biochemical indices. (c,d) Representative intestinal FXR and FGF15 immunoblots and corresponding densitometric quantification. (e,f) Representative hepatic FXR, FGFR4, FGF15, and SHP immunoblots and quantification. (g,h) Representative hepatic CYP7A1, CYP8B1, and BSEP immunoblots and quantification. (i,j) Representative hepatic PPAR α , PGC-1 α , and PPAR γ immunoblots and quantification. For animal-level biochemical and Western blot endpoints, individual points represent animals, and the number of plotted points indicates the biological sample size. Biochemical data are presented as mean $\pm$ SD, whereas Western blot densitometric data are presented as mean $\pm$ SEM. These data support coordinated enterohepatic and hepatic responses but do not establish liver-mediated causal transmission to the testis. FMT, fecal microbiota transplantation; H&E, hematoxylin and eosin; FXR, farnesoid X receptor; FGF15, fibroblast growth factor 15; FGFR4, fibroblast growth factor receptor 4; SHP, small heterodimer partner; CYP7A1, cholesterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; BSEP, bile salt export pump; PPAR α , peroxisome proliferator-activated receptor alpha; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR γ , peroxisome proliferator-activated receptor gamma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

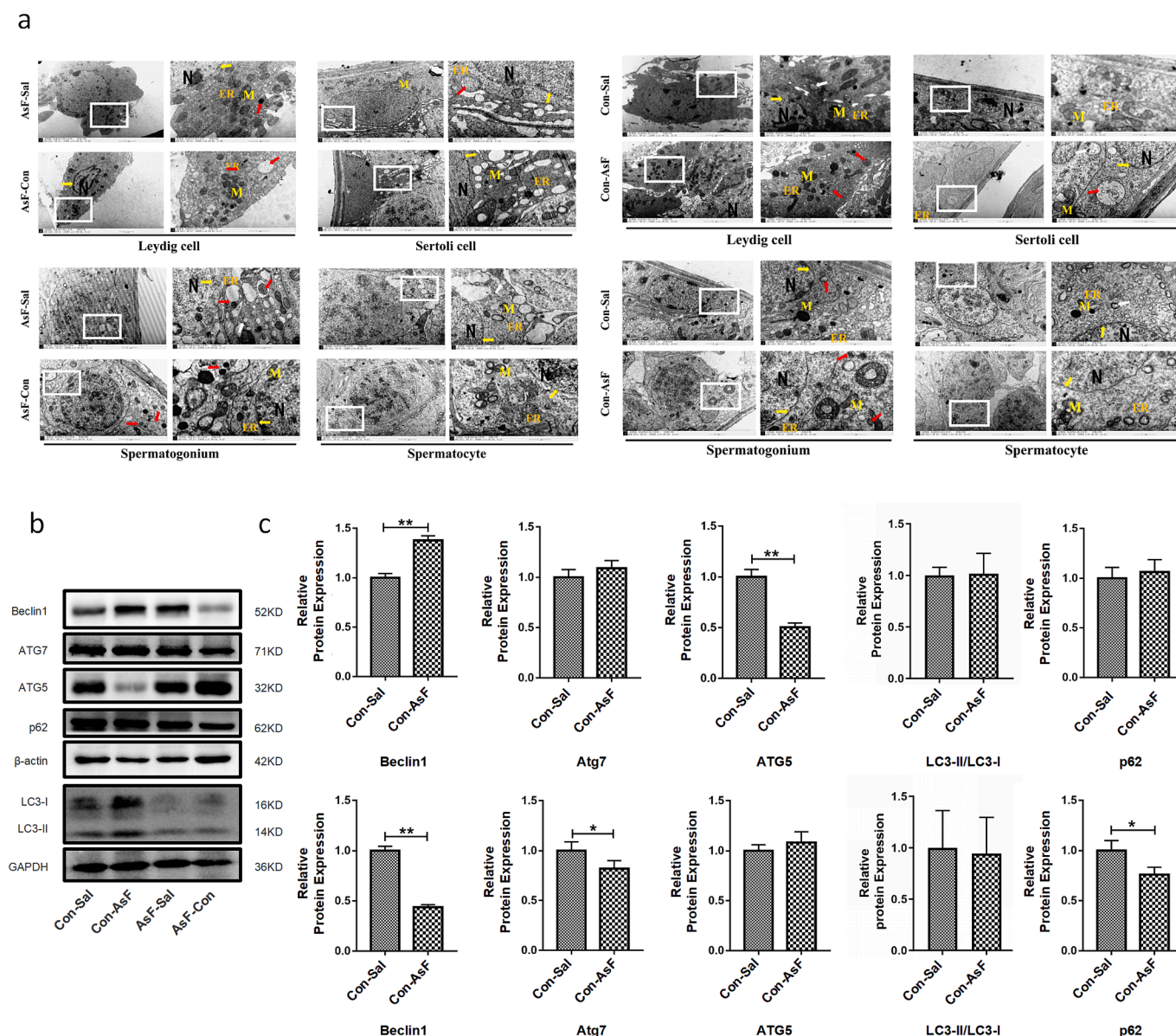


Fig. 8. Microbiota transfer is associated with testicular ultrastructural and autophagy-related responses. (a) Representative transmission electron micrographs of Sertoli cells, Leydig cells, spermatogonia, and spermatocytes. The left panels were acquired at approximately 10,000 $\times$ magnification (scale bar, 2 μ m), and the right panels show enlarged views of the white-boxed regions in the corresponding left panels at approximately 30,000 $\times$ magnification (scale bar, 1 μ m). N, nucleus; M, mitochondrion; ER, endoplasmic reticulum. Yellow arrows indicate the nuclear membrane, white arrows indicate putative autophagic precursor structures, and red arrows indicate autophagosome-like structures. (b) Representative immunoblots of Beclin1, ATG5, ATG7, LC3-I/LC3-II, and p62. (c) Densitometric quantification. Individual points represent animals, and the number of plotted points indicates the biological sample size. Western blot densitometric data are presented as mean $\pm$ SEM. The static ultrastructural and protein measurements do not establish the direction of autophagic flux. TEM, transmission electron microscopy; ATG5, autophagy-related protein 5; ATG7, autophagy-related protein 7; LC3, microtubule-associated protein 1 light chain 3; p62, sequestosome 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphology without prominent autophagosome-like structures. In Con-AsF recipients, autophagosome-like structures were observed in Leydig and Sertoli cells. In AsF-Sal recipients, these structures were most apparent in Leydig cells and were also observed in Sertoli cells and spermatogonia. Their distribution and abundance differed after administration of control-donor fecal material to AsF-exposed recipients. Direct-exposure ultrastructural images at PND60 and PND120 are presented in Fig. S16.

Autophagy-related protein expression also differed across the FMT groups (Fig. 8b–c). Compared with Con-Sal, Con-AsF recipients showed higher Beclin1 and lower ATG5 expression, whereas LC3-II/LC3-I, p62, and ATG7 showed smaller increases. Compared with AsF-Sal, AsF-Con recipients showed lower Beclin1, p62, and ATG7 expression, accompanied by a modest increase in ATG5 and a modest reduction in LC3-II/LC3-I. These findings demonstrate group-specific alterations in testicular autophagy-related ultrastructure and protein-expression profiles following reciprocal FMT.

3.8. Exploratory toxicogenomic analysis identifies overlapping cellular-stress pathways

The original database-derived analysis was retained in Fig. S17. UpSet analysis identified 51 genes shared among seven male reproductive disease-related categories: azoospermia, hypogonadism, male infertility, spermatogenesis, spermatogenic failure, teratozoospermia, and testicular diseases. Intersection with arsenic- and fluoride-associated genes from the Comparative Toxicogenomics Database identified 64 genes shared across the disease, arsenic, and fluoride domains.

GO and KEGG enrichment analyses identified biological processes and pathways involving oxidative stress, reactive-oxygen-species responses, inflammation, apoptosis regulation, lipid and atherosclerosis, AGE–RAGE signaling, TNF signaling, p53 signaling, cellular senescence, NF- κ B signaling, FoxO signaling, mTOR signaling, and autophagy-related pathways.

Protein–protein interaction, MCODE module, and cytoHubba analyses identified highly connected candidates including IL1B, CASP3, TP53, AKT1, PTEN, BCL2, NFE2L2, RELA, MAPK3, CASP8, CASP9, CDKN1A, CTNNB1, CCND1, and ANXA5 (Fig. S17).

4. Discussion

The present study characterized reproductive and multi-organ responses to chronic, relatively high-dose As–F co-exposure across developmental and postnatal periods. At the tested concentrations, exposure was associated with impaired sperm quality, altered reproductive hormones and organ coefficients, testicular and epididymal lesions, changes in spermatogenic-stage markers, intestinal and microbial alterations, systemic metabolic remodeling, hepatic responses, and testicular autophagy-related changes. Factorial analyses further showed that the joint effects of arsenic and fluoride differed among endpoints and were frequently less than additive on the analyzed scale rather than uniformly synergistic. Repeated administration of donor-derived fecal material was accompanied by partially reciprocal differences in selected reproductive, colonic, microbial, metabolic, hepatic, and testicular endpoints. Collectively, these findings support coordinated responses across the gut, liver, and testis, but do not establish a single serial pathway or exclude direct toxicant effects and complementary gut–testis, endocrine, inflammatory, or liver–testis routes.

The reproductive findings are consistent with previous experimental evidence that arsenic and fluoride can interfere with spermatogenesis, steroidogenesis, sperm maturation, redox homeostasis, and testicular structure (Machado-Neves, 2022; Li et al., 2023; F.-W. Niu et al., 2025; Talebi et al., 2025; Song et al., 2026). The exposure design extended from parental premating through gestation and lactation and then continued in male offspring to PND60 or PND120, covering several

sensitive periods of reproductive development. The observed changes in sperm density, motility, abnormality, reproductive hormones, and testicular and epididymal morphology therefore reflect the combined influence of developmental exposure history and continued postnatal exposure. Comparisons between PND60 and PND120 broaden the temporal characterization of the phenotype, although these terminal cohorts do not independently separate chronological age from cumulative exposure duration.

The ACRV1 and SYCP3 findings add stage-associated information to the conventional sperm and histopathological endpoints. ACRV1, also known as SP-10, is an acrosomal protein predominantly expressed during the round-spermatid stage and is therefore a marker of post-meiotic spermatid differentiation (Reddi, 2023). In contrast, SYCP3 is a core component of the chromosome axial and lateral elements of the synaptonemal complex and is associated with spermatocytes undergoing meiotic prophase (Guo et al., 2025; Yang and Wang, 2026). The lower ACRV1- and SYCP3-positive areas observed in exposed animals are therefore consistent with disturbances involving postmeiotic spermatids and meiotic spermatocytes, respectively. Partially reciprocal descriptive patterns were also observed among FMT recipients. Nevertheless, positive-area measurements cannot distinguish altered protein abundance from changes in the number or relative representation of marker-positive germ cells, do not constitute stereological quantification of germ-cell populations, and do not evaluate functional acrosome integrity or sperm DNA fragmentation. Because mating success, pregnancy rate, and litter outcomes were not assessed, the reproductive conclusions should remain restricted to impaired spermatogenesis, sperm quality, and testicular injury rather than infertility.

The endocrine findings similarly require context-dependent interpretation. Altered serum and intratesticular testosterone, estradiol, FSH, and LH indicate disruption of reproductive endocrine homeostasis, which may involve Leydig-cell steroidogenesis, substrate availability, hepatic steroid handling, and hypothalamic–pituitary–gonadal feedback (Miller and Auchus, 2011; Rato et al., 2012; Santos-Marcos et al., 2023). However, estradiol did not respond in the same direction in the direct-exposure and reciprocal FMT experiments. This divergence argues against treating estradiol as a linear marker of microbiota-associated reproductive injury or a simple indicator of damage and rescue. Exposure history, aromatization, enterohepatic recycling, microbial deconjugation, hepatic clearance, and endocrine feedback could all contribute, but aromatase activity, estrogen conjugates, microbial β -glucuronidase activity, hepatic steroid-metabolizing enzymes, and longitudinal hormone profiles were not measured (Ridlon and Bajaj, 2015; Santos-Marcos et al., 2023). The hormone data therefore indicate context-dependent endocrine modulation without identifying the responsible regulatory level.

The microbial findings provide evidence of exposure- and FMT-associated community restructuring. The significant overall PERMANOVA results, together with nonsignificant PERMDISP tests, indicate group-associated differences in community composition that were not explained solely by unequal multivariate dispersion. In contrast, none of the alpha-diversity indices remained significant after adjustment, suggesting that compositional restructuring was more apparent than changes in within-sample richness or evenness. These results are biologically plausible because the gut microbiota can influence xenobiotic biotransformation, intestinal homeostasis, immune signaling, bile acid transformation, and host metabolism (Fan and Pedersen, 2021; Collins et al., 2023). The reciprocal changes in colonic ZO-1, Occludin, and TNF- α staining further show that donor-material administration was accompanied by differences in the colonic junction- and inflammation-related milieu. However, these tissue markers are not direct measurements of intestinal permeability and do not demonstrate systemic or testicular inflammation.

The reciprocal FMT design provides useful but qualified evidence concerning an intestinal contribution to the phenotype. Within each recipient background, animals remained on the same assigned drinking-

water regimen, allowing saline- and donor-material-treated groups to be compared without changing the underlying exposure assignment. Directionally reciprocal differences across selected spermatogenic, endocrine, colonic, metabolic, and hepatic endpoints strengthen the internal coherence of the experiment. However, within-background microbial comparisons were not significant after adjustment, recipient communities remained distinguishable from the reciprocal donor references, and recipient-to-donor distance, donor proximity, reciprocal donor-associated ASV abundance, and detected-ASV metrics did not demonstrate stable donor-community convergence. The observed changes are therefore best interpreted as FMT-associated modification of selected recipient phenotypes rather than proof of stable engraftment or microbiota-specific causation.

Nonmicrobial transfer also remains a relevant alternative explanation. The original inocula could not be retrospectively quantified for arsenic, fluoride, or soluble metabolites, and transient transfer cannot be excluded, particularly in the Con-AsF comparison. The absence of terminal differences in urinary arsenic or bone fluoride within the corresponding recipient backgrounds reduces concern about persistent separation in terminal internal burden but does not exclude transient exposure earlier during the seven-week administration period. In addition, recipients were sampled 10 days after the final administration, so the onset, stability, and longer-term persistence of the observed changes cannot be determined. The findings are compatible with partial involvement of the intestinal microbial ecosystem or other transferred fecal components, but they do not establish complete phenotypic rescue or reversibility of the original exposure effects (Gheorghe et al., 2021).

Untargeted metabolomics showed that reciprocal donor-material administration was accompanied by extensive systemic metabolic remodeling, with lipid-related features prominent among the annotated differential signals. However, the metabolite-class plots describe the composition of retained features rather than the total abundance or activity of an entire lipid class. Cross-validated discrimination was also stronger in the AsF-background model than in the control-background model, indicating that the robustness of metabolomic separation differed between comparisons. The PLS-DA, VIP, and pathway-enrichment results should therefore be used to prioritize candidate metabolic processes rather than as independent evidence of pathway activation.

Bile acids provide a biologically plausible interface between microbial and host metabolism because intestinal microorganisms mediate bile acid deconjugation, oxidation, epimerization, and secondary transformation, while bile acids reciprocally regulate microbial ecology and host signaling (Guzior and Quinn, 2021; Collins et al., 2023; Wise and Cummings, 2023). TUDCA and GCDCA-3G were among the bile-acid-related features showing reciprocal FMT-associated patterns, whereas 7-SCA showed recipient-group differences without a marked Con120-AsF120 difference. This distinction indicates that the direct-exposure and FMT contrasts are not interchangeable and that recipient background may shape the detected metabolic response. TUDCA, GUDCA, GCDCA-3G, and 7-SCA also appeared as bridge features in the exploratory genus–bile-acid–hormone network.

These bile acid results require several analytical qualifications. They were obtained through a post hoc, bile-acid-focused interrogation of untargeted LC–MS/MS data, were expressed as relative signal intensities, and were not confirmed using authentic standards or a targeted quantitative assay. The displayed names therefore represent putative annotations rather than definitive molecular identifications. Moreover, genus–bile-acid associations were correlation-based, and bile-acid–hormone relationships used group-level summaries rather than animal-level paired observations. These analyses identify coordinated variation and candidate relationships but cannot establish directionality or mediation.

The intestinal–hepatic FXR–FGF15 pathway offers a plausible physiological context for the coordinated bile acid and lipid findings. Intestinal FXR-induced FGF15 is an established endocrine signal that

regulates hepatic CYP7A1-dependent bile acid synthesis, while hepatic FXR-, FGFR4-, SHP-, CYP8B1-, BSEP-, PPAR-, and PGC-1 α -related processes participate in bile acid transport and lipid homeostasis (Inagaki et al., 2005; Chiang and Ferrell, 2020). The observed differences in intestinal and hepatic proteins, liver histology, serum total bile acids, and hepatic lipid indices are therefore compatible with altered enterohepatic and hepatic metabolic regulation. Experimental studies also indicate that bile acid receptors can influence testicular steroidogenesis, spermatogenesis, and male fertility, and other FMT or pollutant models have reported coordinated hepatic, circulating, and testicular metabolic changes (Baptissart et al., 2014; Martinot et al., 2017; Hao et al., 2022; Yang et al., 2026).

The present data, however, do not demonstrate that hepatic changes mediated the testicular phenotype. Targeted bile acids and related metabolites were not measured simultaneously in portal blood, systemic circulation, liver, and testis, and no isotope tracing, bile acid supplementation, or tissue-specific FXR/TGR5 intervention was performed. The observed multi-organ coordination is therefore consistent with several nonexclusive routes: gut-derived metabolites or inflammatory signals may act directly on the testis; hepatic processing may modify circulating metabolic signals; endocrine changes may influence both liver and testis; and arsenic and fluoride may act directly on either organ. The gut–liver–testis concept should consequently be presented as an integrative framework supported by coordinated compartmental changes, rather than as a demonstrated unidirectional gut-to-liver-to-testis mechanism.

Lipid remodeling may be relevant to reproductive injury because cholesterol availability, mitochondrial lipid handling, and fatty acid oxidation contribute to Leydig-cell steroidogenesis, Sertoli-cell support, germ-cell development, spermiogenesis, and sperm membrane maturation (Miller and Auchus, 2011; Rato et al., 2012; Gao et al., 2018). Phosphatidylethanolamine, which contributed to the autophagy-related pathway enrichment, also participates in LC3 lipidation during autophagosome formation (Tanida et al., 2004). Nevertheless, pathway enrichment driven by an annotated circulating lipid cannot demonstrate LC3 lipidation or autophagic activity in testicular cells. It provides biological plausibility for a relationship between lipid remodeling and autophagy-related processes but not a mechanistic bridge.

The testicular TEM and Western blot findings should therefore be interpreted as accompanying autophagy-related alterations. Static measurements of LC3-II/LC3-I, p62, Beclin1, ATG5, and ATG7, together with autophagosome-like structures, cannot distinguish increased autophagosome formation from impaired lysosomal degradation or determine whether the response is protective, injury-promoting, or secondary to metabolic stress (Klionsky et al., 2021). Canonical apoptotic markers and testicular inflammatory cytokines were not measured, so apoptotic and inflammatory injury remain possible parallel or interacting mechanisms. The colonic cytokine measurements do not provide evidence of testicular inflammation. Likewise, the database-derived human gene analysis identifies convergence on oxidative-stress, inflammatory, apoptotic, lipid, and autophagy-related pathways but remains exploratory public-data context rather than validation in the experimental rats or in human biological samples.

The study has several strengths, including an exposure design spanning developmental and postnatal periods, factorial analysis of verified PND120 endpoints, reciprocal within-background donor-material comparisons, donor-reference analyses in a common ASV space, animal-level treatment of histological measurements, and integration of reproductive, intestinal, microbial, metabolic, hepatic, and testicular observations. Its main environmental limitation is the exposure intensity. The nominal 75 mg/L NaAsO₂ and 100 mg/L NaF exposure concentrations were above the corresponding WHO drinking-water guideline values; accordingly, this model is best interpreted as an experimental hazard model rather than a direct simulation of environmentally representative human exposure. Group-level water-consumption data and terminal urinary arsenic and bone fluoride confirm

exposure separation but cannot provide individual body-weight-normalized, cumulative, or absorbed doses. The results do not estimate human risk magnitude, establish safe exposure limits, or demonstrate effects at environmentally representative concentrations.

Taken together, the strongest inference is that chronic As-F co-exposure impaired spermatogenesis at the tested concentrations and produced endpoint-specific joint effects, while reciprocal donor-material administration was accompanied by partial modification of selected recipient phenotypes. Coordinated changes in microbial composition, the colonic microenvironment, bile-acid- and lipid-related signals, enterohepatic and hepatic markers, reproductive hormones, and testicular endpoints support a nonexclusive multi-organ framework for further testing. Lower-dose and dose–response experiments, chemically characterized and strain-resolved FMT, longitudinal donor-restructuring analyses, targeted bile acid quantification, tissue-specific metabolomics and receptor intervention, fertility testing, and dynamic autophagic-flux assays will be required to determine which components are causal and whether the findings extend to environmentally relevant exposures.

CRedit authorship contribution statement

Jinyao Chen: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Yufen Cui:** Validation, Investigation, Data curation. **Yingda Wang:** Validation, Investigation, Data curation. **Ruipeng Kong:** Validation, Investigation, Data curation. **Penghui Liu:** Methodology, Investigation, Conceptualization. **Qiang Su:** Methodology, Investigation, Data curation. **Yidi Li:** Writing – review & editing, Formal analysis. **Hengyi Guo:** Validation, Methodology. **Yu Fu:** Writing – review & editing, Validation. **Qian Zhao:** Supervision, Resources. **Ben Li:** Supervision, Resources. **Xiaolin Tian:** Methodology, Funding acquisition. **Xiaoyan Yan:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiaoyan Yan reports financial support was provided by National Natural Science Foundation of China. Xiaoyan Yan reports financial support was provided by Department of Science and Technology of Shanxi Province. Xiaolin Tian reports financial support was provided by Department of Science and Technology of Shanxi Province. Xiaoyan Yan reports financial support was provided by Shanxi Provincial Department of Education. Jinyao Chen, Qiang Su, Yidi Li, Zhichao Jia, Shaoping Pan, Xiaolin Tian, Xiaoyan Yan has patent #CN202610156223.X pending to Shanxi Medical University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary figures and tables providing additional data on exposure characterization, reproductive outcomes, gut microbiota, metabolomic alterations, and hepatic and testicular findings. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2026.110520>.

Data availability

The data supporting the findings are available from the corresponding author on reasonable request. Supplementary Tables S1–S5 provide the statistical and annotation records described in the text.

References

- Bader, G.D., Hogue, C.W.V., 2003. An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* 4, 2. <https://doi.org/10.1186/1471-2105-4-2>.
- Baptissart, M., Vega, A., Martinot, E., et al., 2014. Bile acids alter male fertility through G-protein-coupled bile acid receptor 1 signaling pathways in mice. *Hepatology* 60, 1054–1065. <https://doi.org/10.1002/hep.27204>.
- Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
- Cai, H., Cao, X., Qin, D., Liu, Y., Liu, Y., Hua, J., Peng, S., 2022. Gut microbiota supports male reproduction via nutrition, immunity, and signaling. *Front. Microbiol.* 13, 977574. <https://doi.org/10.3389/fmicb.2022.977574>.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Cao, W., Zhang, Z., Fu, Y., Zhao, L., Ren, Y., Nan, T., Guo, H., 2024. Prediction of arsenic and fluoride in groundwater of the North China Plain using enhanced stacking ensemble learning. *Water Research* 259, 121848. <https://doi.org/10.1016/j.watres.2024.121848>.
- Chen, L., Li, C., Zhong, X., Lai, C., Zhang, B., Luo, Y., Guo, H., Liang, K., Fang, J., Zhu, X., Zhang, J., Guo, L., 2023. The gut microbiome promotes arsenic metabolism and alleviates the metabolic disorder for their mammal host under arsenic exposure. *Environment International* 171, 107660. <https://doi.org/10.1016/j.envint.2022.107660>.
- Chen, W., Zou, H., Xu, H., Cao, R., Zhang, H., Zhang, Y., Zhao, J., 2024. The potential influence and intervention measures of gut microbiota on sperm: It is time to focus on testis-gut microbiota axis. *Front. Microbiol.* 15, 1478082. <https://doi.org/10.3389/fmicb.2024.1478082>.
- Chiang, J.Y.L., Ferrell, J.M., 2020. Bile acid receptors FXR and TGR5 signaling in fatty liver diseases and therapy. *American Journal of Physiology-Gastrointestinal and Liver*. *Physiology* 318, G554–G573. <https://doi.org/10.1152/ajpgi.00223.2019>.
- Chin, C.-H., Chen, S.-H., Wu, H.-H., Ho, C.-W., Ko, M.-T., Lin, C.-Y., 2014. cytoHubba: Identifying hub objects and sub-networks from complex interactome. *BMC Systems Biology* 8 (Suppl 4), S11. <https://doi.org/10.1186/1752-0509-8-S4-S11>.
- Claus, S.P., Guillou, H., Ellero-Simatos, S., 2016. The gut microbiota: A major player in the toxicity of environmental pollutants? *npj Biofilms Microbiomes* 2, 16003. <https://doi.org/10.1038/npjbiofilms.2016.3>.
- Collins, S.L., Stine, J.G., Bisanz, J.E., Okafor, C.D., Patterson, A.D., 2023. Bile acids and the gut microbiota: Metabolic interactions and impacts on disease. *Nat Rev Microbiol* 21, 236–247. <https://doi.org/10.1038/s41579-022-00805-x>.
- Coryell, M., Roggenbeck, B.A., Walk, S.T., 2019. The human gut microbiome's influence on arsenic toxicity. *Curr Pharmacol Rep* 5, 491–504. <https://doi.org/10.1007/s40495-019-00206-4>.
- Davis, A.P., Wieggers, T.C., Johnson, R.J., Sciaky, D., Wieggers, J., Mattingly, C.J., 2023. Comparative toxicogenomics database (CTD): Update 2023. *Nucleic Acids Research* 51, D1257–D1262. <https://doi.org/10.1093/nar/gkac833>.
- Ding, N., Zhang, X., Zhang, X.D., Jing, J., Liu, S.S., Mu, Y.P., Peng, L.L., Yan, Y.J., Xia, G.M., Bi, X.Y., Chen, H., Li, F.H., Yao, B., Zhao, A.Z., 2020. Impairment of spermatogenesis and sperm motility by the high-fat diet-induced dysbiosis of gut microbes. *Gut* 69, 1608–1619. <https://doi.org/10.1136/gutjnl-2019-319127>.
- Eisenberg, M.L., Esteves, S.C., Lamb, D.J., Hotelling, J.M., Giwerzman, A., Hwang, K., Cheng, Y.-S., 2023. Male infertility. *Nat Rev Dis Primers* 9, 49. <https://doi.org/10.1038/s41572-023-00459-w>.
- Fan, Y., Pedersen, O., 2021. Gut microbiota in human metabolic health and disease. *Nat Rev Microbiol* 19, 55–71. <https://doi.org/10.1038/s41579-020-0433-9>.
- Gao, F., Li, G., Liu, C., Gao, H., Wang, H., Liu, W., Chen, M., Shang, Y., Wang, L., Shi, J., Xia, W., Jiao, J., Gao, F., Li, J., Chen, L., Li, W., 2018. Autophagy regulates testosterone synthesis by facilitating cholesterol uptake in Leydig cells. *Journal of Cell Biology* 217, 2103–2119. <https://doi.org/10.1083/jcb.201710078>.
- Gheorghe, C.E., Ritz, N.L., Martin, J.A., Wardill, H.R., Cryan, J.F., Clarke, G., 2021. Investigating causality with fecal microbiota transplantation in rodents: Applications, recommendations and pitfalls. *Gut Microbes* 13, 1941711. <https://doi.org/10.1080/19490976.2021.1941711>.

- Guo, S., Zhang, Y., Fei, C., Liu, X., Xia, W., Luo, M., Wei, G., Qin, W., Xiong, C., Li, H., Yin, Y., He, X., Zhou, L.Q., 2025. Deciphering meiotic chromatin organization by SYCP3. *Nucleic Acids Research* 53, gkaf460. <https://doi.org/10.1093/nar/gkaf460>.
- Guzior, D.V., Quinn, R.A., 2021. Review: Microbial transformations of human bile acids. *Microbiome* 9, 140. <https://doi.org/10.1186/s40168-021-01101-1>.
- Hao, Y., Feng, Y., Yan, X., et al., 2022. Gut microbiota–testis axis: FMT mitigates high-fat diet-diminished male fertility via improving systemic and testicular metabolome. *Microbiology Spectrum* 10, e00028–e122. <https://doi.org/10.1128/spectrum.00028-22>.
- He, X., Li, P., Ji, Y., Wang, Y., Su, Z., Elumalai, V., 2020. Groundwater arsenic and fluoride and associated arsenicosis and fluorosis in China: Occurrence, distribution and management. *Expo Health* 12, 355–368. <https://doi.org/10.1007/s12403-020-00347-8>.
- Inagaki, T., Choi, M., Moschetta, A., et al., 2005. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metabolism* 2, 217–225. <https://doi.org/10.1016/j.cmet.2005.09.001>.
- Kimmins, S., Anderson, R.A., Barratt, C.L.R., Behre, H.M., Catford, S.R., De Jonge, C.J., Delbes, G., Eisenberg, M.L., Garrido, N., Houston, B.J., Jørgensen, N., Krausz, C., Lismar, A., McLachlan, R.I., Minhas, S., Moss, T., Pacey, A., Priskorn, L., Schlatt, S., Trasler, J., Trasande, L., Tüttelmann, F., Vazquez-Levin, M.H., Veltman, J.A., Zhang, F., O'Bryan, M.K., 2024. Frequency, morbidity and equity — the case for increased research on male fertility. *Nat Rev Urol* 21, 102–124. <https://doi.org/10.1038/s41585-023-00820-4>.
- Klionsky, D.J., Abdel-Aziz, A.K., Abdelfattah, S., et al., 2021. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). *Autophagy* 17, 1–382. <https://doi.org/10.1080/15548627.2020.1797280>.
- Kumar, M., Goswami, R., Patel, A.K., Srivastava, M., Das, N., 2020. Scenario, perspectives and mechanism of arsenic and fluoride co-occurrence in the groundwater: A review. *Chemosphere* 249, 126126. <https://doi.org/10.1016/j.chemosphere.2020.126126>.
- Li, Y., Zhang, J., Sun, L., Zhao, H., Jia, X., Zhang, Y., Li, Y., 2023. Fluoride-induced sperm damage and HuR-mediated excessive apoptosis and autophagy in spermatocytes. *Biol Trace Elem Res* 201, 295–305. <https://doi.org/10.1007/s12011-022-03138-1>.
- Licht, T.R., Bahl, M.I., 2019. Impact of the gut microbiota on chemical risk assessment. *Current Opinion in Toxicology* 15, 109–113. <https://doi.org/10.1016/j.cotox.2018.09.004>.
- Liu, P., Li, R., Tian, X., Zhao, Y., Li, M., Wang, M., Ying, X., Yuan, J., Xie, J., Yan, X., Lyu, Y., Wei, C., Qiu, Y., Tian, F., Zhao, Q., Yan, X., 2021. Co-exposure to fluoride and arsenic disrupts intestinal flora balance and induces testicular autophagy in offspring rats. *Ecotoxicology and Environmental Safety* 222, 112506. <https://doi.org/10.1016/j.ecoenv.2021.112506>.
- Long, J.S., Ervin, L.H., 2000. Using heteroscedasticity-consistent standard errors in the linear regression model. *The American Statistician* 54, 217–224. <https://doi.org/10.1080/00031305.2000.10474549>.
- Lu, K., Abo, R.P., Schlieper, K.A., Graffam, M.E., Levine, S., Wishnok, J.S., Swenberg, J. A., Tannenbaum, S.R., Fox, J.G., 2014. Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: An integrated metagenomics and metabolomics analysis. *Environ Health Perspect* 122, 284–291. <https://doi.org/10.1289/ehp.1307429>.
- Machado-Neves, M., 2022. Arsenic exposure and its implications in male fertility. *Anim. Reprod.* 19, e20220119. <https://doi.org/10.1590/1984-3143-ar2022-0119>.
- Mahboob, S.Z., Jahan, S., Badshah, H., Virk, R., David, M., Bano, R., Alzahrani, J., Ayub, M., Azizullah, A., Nazish, M., Kamal, A., Ullah, K., Naseem, M.T., 2025. Reproductive toxicity in male rats induced by chronic arsenic exposure involves hormonal and structural changes. *Sci Rep* 15, 29037. <https://doi.org/10.1038/s41598-025-14929-6>.
- Martino, E., Baptissart, M., Vega, A., et al., 2017. Bile acid homeostasis controls CAR signaling pathways in mouse testis through FXR α . *Scientific Reports* 7, 42182. <https://doi.org/10.1038/srep42182>.
- Miller, W.L., Auchus, R.J., 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews* 32, 81–151. <https://doi.org/10.1210/er.2010-0013>.
- Mo, Z., Wang, J., Meng, X., Li, A., Li, Z., Que, W., Wang, T., Tarnue, K.F., Ma, X., Liu, Y., Yan, S., Wu, L., Zhang, R., Pei, J., Wang, X., 2023. The dose–response effect of fluoride exposure on the gut microbiome and its functional pathways in rats. *Metabolites* 13, 1159. <https://doi.org/10.3390/metabo13111159>.
- Mondal, P., Chattopadhyay, A., 2020. Environmental exposure of arsenic and fluoride and their combined toxicity: A recent update. *J of Applied Toxicology* 40, 552–566. <https://doi.org/10.1002/jat.3931>.
- Niu, F.-W., Liu, M.-D., Yao, K., Yang, R., Gao, L., Zhai, J.-X., Wang, C., Zhang, S.-H., Xu, D.-X., Zhang, Z.-H., 2025. Mitochondrial ROS-associated integrated stress response is involved in arsenic-induced blood-testis barrier disruption and protective effect of melatonin. *Environment International* 197, 109346. <https://doi.org/10.1016/j.envint.2025.109346>.
- Niu, S., Du, Y., Liu, B., Feng, Z., Wang, G., Li, C., Wang, Y., Sun, Q., Yu, F., Zhou, G., Ba, Y., 2026. Fluoride-induced male reproductive toxicity: Dual-tissue pyroptosis in the gut–testis axis mediated by inflammatory cytokines. *Journal of Hazardous Materials* 514, 142537. <https://doi.org/10.1016/j.jhazmat.2026.142537>.
- Ommati, M.M., Jin, Y., Zamiri, M.J., Retana-Marquez, S., Nategh Ahmadi, H., Sabouri, S., Song, S.Z., Heidari, R., Wang, H.-W., 2025. Sex-specific mechanisms of fluoride-induced gonadal injury: A multi-omics investigation into reproductive toxicity and gut microbiota disruption. *J Agric Food Chem* 73, 2527–2550. <https://doi.org/10.1021/acs.jafc.4c10190>.
- Patel, K.S., Pandey, P.K., Martín-Ramos, P., Corns, W.T., Varol, S., Bhattacharya, P., Zhu, Y., 2023. A review on arsenic in the environment: Contamination, mobility, sources, and exposure. *RSC Adv.* 13, 8803–8821. <https://doi.org/10.1039/D3RA00789H>.
- Podgorski, J., Berg, M., 2020. Global threat of arsenic in groundwater. *Science* 368, 845–850. <https://doi.org/10.1126/science.aba1510>.
- Podgorski, J., Berg, M., 2022. Global analysis and prediction of fluoride in groundwater. *Nat Commun* 13, 4232. <https://doi.org/10.1038/s41467-022-31940-x>.
- Qi, X., Yun, C., Pang, Y., Qiao, J., 2021. The impact of the gut microbiota on the reproductive and metabolic endocrine system. *Gut Microbes* 13, 1894070. <https://doi.org/10.1080/19490976.2021.1894070>.
- Quast, C., Pruesse, E., Yilmaz, P., et al., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>.
- Radovanović, J., Antonijević, B., Čurčić, M., Baralić, K., Kolarević, S., Bulat, Z., Đukić-Čosić, D., Buha Djordjević, A., Vuković-Gaćić, B., Javorac, D., Antonijević Miljković, E., Carević, M., Mandinić, Z., 2022. Fluoride subacute testicular toxicity in wistar rats: Benchmark dose analysis for the redox parameters, essential elements and DNA damage. *Environmental Pollution* 314, 120321. <https://doi.org/10.1016/j.envpol.2022.120321>.
- Rato, L., Alves, M.G., Socorro, S., Duarte, A.I., Cavaco, J.E., Oliveira, P.F., 2012. Metabolic regulation is important for spermatogenesis. *Nat Rev Urol* 9, 330–338. <https://doi.org/10.1038/nrurol.2012.77>.
- Reddi, P.P., 2023. Transcriptional regulation of spatiotemporal gene expression within the seminiferous epithelium: Mouse Acrv1 gene as a model. *Andrology* 11, 904–910. <https://doi.org/10.1111/andr.13410>.
- Ridlon, J.M., Bajaj, J.S., 2015. The human gut sterolbiome: Bile acid-microbiome endocrine aspects and therapeutics. *Acta Pharmaceutica Sinica B* 5, 99–105. <https://doi.org/10.1016/j.apsb.2015.01.006>.
- Rohart, F., Gautier, B., Singh, A., Lê Cao, K.-A., 2017. mixOmics: An R package for omics feature selection and multiple data integration. *PLoS Computational Biology* 13, e1005752. <https://doi.org/10.1371/journal.pcbi.1005752>.
- Safran, M., Dalah, I., Alexander, J., Rosen, N., Iny Stein, T., Shmoish, M., Nativ, N., Bahir, I., Doniger, T., Krug, H., Sirota-Madi, A., Olender, T., Golan, Y., Stelzer, G., Harel, A., Lancet, D., 2010. GeneCards version 3: The human gene integrator. *Database* 2010, baq020–baq020. <https://doi.org/10.1093/database/baq020>.
- Samareh-Najaf, M., Neisy, A., Samareh, A., Moghadam, D., Jamali, N., Zarei, R., Zal, F., 2023. The constructive and destructive impact of autophagy on both genders' reproducibility, a comprehensive review. *Autophagy* 19, 3033–3061. <https://doi.org/10.1080/15548627.2023.2238577>.
- Santos-Marcos, J.A., Mora-Ortiz, M., Tena-Sempere, M., Lopez-Miranda, J., Camargo, A., 2023. Interaction between gut microbiota and sex hormones and their relation to sexual dimorphism in metabolic diseases. *Biol Sex Differ* 14, 4. <https://doi.org/10.1186/s13293-023-00490-2>.
- Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science & Technology* 48, 2097–2098. <https://doi.org/10.1021/es5002105>.
- Shaji, E., Sarath, K.V., Santosh, M., Krishnaprasad, P.K., Arya, B.K., Babu, M.S., 2024. Fluoride contamination in groundwater: A global review of the status, processes, challenges, and remedial measures. *Geoscience Frontiers* 15, 101734. <https://doi.org/10.1016/j.gsf.2023.101734>.
- Shannon, P., Markiel, A., Ozier, O., et al., 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research* 13, 2498–2504. <https://doi.org/10.1101/gr.139303>.
- Skakkebaek, N.E., Lindahl-Jacobsen, R., Levine, H., Andersson, A.-M., Jørgensen, N., Main, K.M., Lidegaard, Ø., Priskorn, L., Holmboe, S.A., Bräuner, E.V., Almstrup, K., Franca, L.R., Znaor, A., Kortenkamp, A., Hart, R.J., Juul, A., 2022. Environmental factors in declining human fertility. *Nat Rev Endocrinol* 18, 139–157. <https://doi.org/10.1038/s41574-021-00598-8>.
- Song, X., Yang, X., Fu, W., Xing, Y., Cheng, C., Tian, X., Yang, W., Zhao, L., Lu, Y., Li, X., Cheng, A., Liu, S., Liang, C., Bai, J., Zhang, J., 2026. Prenatal and postnatal exposure to environmental level arsenic declines male reproductive function by affecting spermatogenesis and maturation in offspring. *Environmental Research* 288, 123276. <https://doi.org/10.1016/j.envres.2025.123276>.
- Sumner, L.W., Amberg, A., Barrett, D., et al., 2007. Proposed minimum reporting standards for chemical analysis. *Metabolomics* 3, 211–221. <https://doi.org/10.1007/s11306-007-0082-2>.
- Szklarczyk, D., Kirsch, R., Koutrouli, M., et al., 2023. The STRING database in 2023: Protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Research* 51, D638–D646. <https://doi.org/10.1093/nar/gkac1000>.
- Talebi, S.F., Seify, M., Bhandari, R.K., Shoorai, H., Oskuei, S.D., 2025. Fluoride-induced testicular and ovarian toxicity: Evidence from animal studies. *Biol Res* 58, 6. <https://doi.org/10.1186/s40659-025-00586-6>.
- Tanida, I., Ueno, T., Kominami, E., 2004. LC3 conjugation system in mammalian autophagy. *The International Journal of Biochemistry & Cell Biology* 36, 2503–2518. <https://doi.org/10.1016/j.biocel.2004.05.009>.
- Wang, M., Zheng, L.-W., Ma, S., Zhao, D.-H., Xu, Y., 2024a. The gut microbiota: Emerging biomarkers and potential treatments for infertility-related diseases. *Front. Cell. Infect. Microbiol.* 14, 1450310. <https://doi.org/10.3389/fcimb.2024.1450310>.
- Wang, S., Cui, J., Jiang, S., Zheng, C., Zhao, J., Zhang, H., Zhai, Q., 2024b. Early life gut microbiota: Consequences for health and opportunities for prevention. *Crit Rev Food Sci Nutr* 64, 5793–5817. <https://doi.org/10.1080/10408398.2022.2158451>.
- Wang, Z., Guo, H., Adimalla, N., Pei, J., Zhang, Z., Liu, H., 2024c. Co-occurrence of arsenic and fluoride in groundwater of Guide Basin in China: Genesis, mobility and enrichment mechanism. *Environmental Research* 244, 117920. <https://doi.org/10.1016/j.envres.2023.117920>.

- Winston, J.A., Theriot, C.M., 2020. Diversification of host bile acids by members of the gut microbiota. *Gut Microbes* 11, 158–171. <https://doi.org/10.1080/19490976.2019.1674124>.
- Wise, J.L., Cummings, B.P., 2023. The 7- α -dehydroxylation pathway: An integral component of gut bacterial bile acid metabolism and potential therapeutic target. *Front. Microbiol.* 13, 1093420. <https://doi.org/10.3389/fmicb.2022.1093420>.
- Organization, W.H. (Ed.), 2022. Guidelines for drinking-water quality, Fourth edition incorporating the first and second, addenda. ed. World Health Organization, Geneva.
- Wu, T., Hu, E., Xu, S., et al., 2021. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The. Innovation* 2, 100141. <https://doi.org/10.1016/j.xinn.2021.100141>.
- Xu, H., Jia, Y., Sun, Z., Su, J., Liu, Q.S., Zhou, Q., Jiang, G., 2022. Environmental pollution, a hidden culprit for health issues. *Eco-Environment & Health* 1, 31–45. <https://doi.org/10.1016/j.eehl.2022.04.003>.
- Yang, Y., Chi, L., Lai, Y., Hsiao, Y.-C., Ru, H., Lu, K., 2021. The gut microbiome and arsenic-induced disease—iAs metabolism in mice. *Curr Envir Health Rpt* 8, 89–97. <https://doi.org/10.1007/s40572-021-00305-9>.
- Yang, F., Wang, P.J., 2026. The synaptonemal complex: Structure, function, and clinical implications. *Biology of Reproduction*, ioag092. <https://doi.org/10.1093/biolre/ioag092>.
- Yang, W., Zou, P., He, S., Cui, H., Yang, Z., An, H., et al., 2026. Perfluorooctane sulfonic acid impairs spermatogenesis via the liver–gut microbiota–testis axis: A central role of chenodeoxycholic acid metabolism. *Journal of Advanced Research*. <https://doi.org/10.1016/j.jare.2025.06.037>.
- Yasin, M., Zohoori, F.V., Kumah, E.A., Subramanian, M., Dean, P., Orr, C.H., 2025. Effect of fluoride on gut microbiota: A systematic review. *Nutrition Reviews* 83, e1853–e1880. <https://doi.org/10.1093/nutrit/nuae202>.