

# Gut microbial arginine biosynthesis mediates the anti-obesity effect of a pasteurized *Akkermansia muciniphila* 11

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# Abstract

## Background

*Akkermansia muciniphila* is regarded as a promising next-generation probiotic for metabolic disorders, yet the postbiotic modes of action through which it exerts benefits remain poorly defined. This study explored how pasteurized *A. muciniphila* (pAkk11) might influence host metabolism by remodeling the gut microbial ecosystem.

## Results

In a 12-week intervention involving 61 overweight or obese individuals, pAkk11 intake was accompanied by an 8.0% reduction in body weight and a concurrent rise in serum L-arginine levels; arginine biosynthesis pathways were among those most closely linked to metabolic improvements. In diet-induced obese mice, multi-omics profiling showed that pAkk11 did not increase the abundance of *A. muciniphila* itself but was instead associated with a marked enrichment of *Faecalibaculum rodentium*, a species harboring upregulated arginine synthesis genes (*argC*, *argD*, *argH*) and correspondingly elevated L-arginine levels. The higher L-arginine availability coincided with evidence of nitric oxide production and AMPK signaling activation in both liver and adipose tissues. These molecular events paralleled a pattern of reduced lipogenesis, reflected by ACC phosphorylation, and enhanced lipolysis, reflected by HSL upregulation, together pointing to a rebalancing of lipid metabolism.

## Conclusions

These observations raise the possibility that pAkk11 may alleviate obesity not through direct colonization, but by ecologically reshaping the gut microbiota to augment microbial arginine synthesis, which in turn engages the host NO–AMPK axis. Such microbial metabolic reprogramming could represent a core postbiotic mechanism and may offer a new conceptual direction for microbiome-targeted strategies in metabolic disease.

## 1 Introduction

Obesity and its critical hepatic complication, non-alcoholic fatty liver disease (NAFLD), constitute a global health crisis with a persistent lack of durable therapeutic options [1]. In this context, targeting the gut microbiota—a "super organ" of metabolism profoundly shaped by diet and lifestyle—has emerged as a highly promising strategy for metabolic disease intervention [2–4]. Among potential targets, the mucin-degrading bacterium *Akkermansia muciniphila* (*A. muciniphila*, Akk) has garnered significant attention as an archetypal "next-generation probiotic" candidate due to its consistent association with host metabolic health [5, 6]. Notably, its pasteurized (postbiotic) formulation, which offers enhanced stability and safety, has demonstrated reproducible benefits in improving body weight, insulin sensitivity, and lipid profiles in

both preclinical models and human pilot studies [6, 7]. This compelling evidence firmly positions it as a viable translational agent against metabolic syndrome.

Research over the past decade has established *A. muciniphila* as a key regulator in metabolic homeostasis, with its depletion often associated with metabolic disorders [8, 9]. Its beneficial effects are multifaceted and context-dependent, whether as live or pasteurized bacteria, functioning within the complex gut microbial ecological network and influenced by the pre-existing microbiota and dietary factors [10–13]. This evolving understanding underscores Akk's role not merely as a simple effector, but as a dynamic component within the gut ecosystem.

However, despite these encouraging clinical and mechanistic outcomes, a fundamental mechanistic question remains unresolved: how does a non-viable bacterial preparation orchestrate systemic metabolic improvement? Prevailing mechanistic research over the past decade has successfully established a 'single-component, single-target' paradigm [13]. This paradigm focuses on isolated elements such as the outer membrane protein Amuc\_1100 [14], the secreted protein P9 [15], or specific phospholipids [16], which engage host receptors (e.g., Toll-like receptor 2) to mediate local effects on gut barrier function or hormone secretion [17]. While invaluable, this reductionist framework may be insufficient to capture the holistic biological activity of a postbiotic within the complex intestinal ecosystem. Emerging evidence suggests that Akk's benefits may extend beyond its own constituents; for instance, its administration can enrich co-occurring microbial clusters and modulate the production of microbial metabolites like short-chain fatty acids, hinting at a broader ecological impact [18, 19]. Crucially, groundbreaking research indicates that the efficacy of Akk supplementation itself may be contingent upon the pre-existing gut microbial context exemplified by the "deficient-supplement" principle, where metabolic benefits are most pronounced in individuals with low baseline Akk abundance [20]. This profound context-dependency underscores that Akk operates not in isolation but through intricate interactions within the microbial community. We therefore posit that a key, overlooked aspect of its mode of action is its potential role as an ecological engineer, functionally reshaping the metabolic output and functional network of the resident microbial community, rather than acting solely through its own constituent molecules.

This perspective is strongly supported by two converging frontiers of knowledge. First, the gut microbiome is now recognized as a core "endocrine organ" that governs systemic physiology through its vast repertoire of metabolites [20, 21]. The microbiome-derived metabolome significantly contributes to the circulating pool of small molecules and is established as a central regulator of host glucose and lipid metabolism [22, 23]. Among the myriads of microbial metabolites, amino acid derivatives, particularly those related to L-arginine (Arg) metabolism, have emerged as critical signaling nodes linking gut microbial activity to host metabolic status. L-arginine, a conditionally essential amino acid, serves as a pivotal precursor for nitric oxide (NO) synthesis and sits at the crossroads of multiple metabolic pathways, including the urea cycle and polyamine biosynthesis [24]. Recent landmark studies have revealed sophisticated, non-canonical sensing mechanisms for arginine (e.g., the BAG2-SAMD4B axis) that regulate cellular stress responses and survival beyond the classical mTORC1 pathway [25].

Furthermore, perturbations in hepatic arginine metabolism, specifically through enzymes like arginase 2 (ARG2), have been causally linked to disrupted tricarboxylic acid (TCA) cycle flux, mitochondrial dysfunction, and the development of fibroinflammatory liver disease in the context of obesity [26]. This positions arginine metabolism as a sensitive integrator of metabolic and inflammatory signals. However, the role of the gut microbiota as a source of systemic arginine, and how this microbial-derived arginine pool influences host energy metabolism in obesity, remains strikingly underexplored. While dietary supplementation with L-arginine has shown inconsistent effects on body weight in humans [27], the specific contribution and regulation of a microbiota-derived arginine flux to activate host metabolic pathways (such as the NO-AMPK axis) constitute a significant knowledge gap. Second, cutting-edge research is unveiling the sophisticated molecular dialogue between host and microbiota. For example, host-derived apolipoprotein L9 (APOL9) can specifically recognize symbionts to modulate immune responses, highlighting a level of selective, bi-directional regulation far more complex than simple receptor-ligand interactions [28]. These advances collectively compel a paradigm shift: from viewing Akk merely as a direct effector to understanding it as a modulator of the gut's metabolic signaling network.

Based on this synthesis, we propose a hypothesis that the systemic metabolic benefits of pasteurized *A. muciniphila* Akk11 (pAkk11) may involve such "ecological engineering," with a specific focus on the microbial arginine metabolic axis. We hypothesize that pAkk11 may act as a keystone modulator, potentially fostering a cooperative gut microbial metabolic program centered on the enhanced biosynthesis of L-arginine. This targeted microbial metabolic reprogramming is postulated to elevate the systemic flux of arginine and its downstream signaling molecules (e.g., nitric oxide), which may in turn be associated with activation of conserved energy-sensing and homeostatic pathways, such as AMPK, in peripheral metabolic tissues (e.g., liver, adipose) [29, 30]. This hypothesis directly addresses the aforementioned gap by proposing that the efficacy of pAkk11 may hinge on its ability to augment a specific microbial metabolic function (arginine synthesis), that is deficient in the obese state, rather than merely supplementing a generic bacterial component.

To explore this "ecological engineering" hypothesis and bridge the clinical-mechanistic divide, we designed a complete, reverse-translational evidence chain. We first conducted a 12-week, randomized, double-blind, placebo-controlled trial in overweight/obese individuals to confirm the efficacy of pAkk11 and identify conserved host metabolic signatures, with a particular focus on the arginine metabolome. Guided by these human findings, we then employed a diet-induced obese mouse model integrated with multi-omics (shotgun metagenomics, untargeted metabolomics and proteomics) and functional analyses to explore the potential cascade of events from the pAkk11-induced ecological shift and the specific enrichment of arginine-synthesizing commensals (e.g., *Faecalibaculum rodentium*), through the resulting alteration in the host arginine-NO metabolic axis, to the final activation of AMPK signaling and phenotypic improvement. This study aims to move beyond the traditional supplement model and define a new framework for postbiotic action based on the targeted rewiring of gut microbial metabolic networks.

## 2 Materials and methods

## 2.1 Material

Pasteurized *A. muciniphila* Akk11 (pAkk11) powder capsules (10 billion TFU/capsule) were provided by Wecare Probiotics Co., Ltd.

## 2.2 Clinical trial protocol design

This study was a randomized, double-blind, placebo-controlled clinical trial conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology (Ethics Approval No. K-2025-0565). Written informed consent was obtained before participant enrollment.

A total of 72 male and female volunteers were recruited at the First Affiliated Hospital of Henan University of Science and Technology in Luoyang under the following inclusion criteria: aged 18–65 years and BMI  $\geq 24$  kg/m<sup>2</sup>. Key exclusion criteria included previous treatment for severe gastrointestinal, hepatic or metabolic diseases; females who were pregnant, lactating or planning pregnancy; a diagnosis of irritable bowel syndrome, ulcerative colitis, liver cirrhosis or diabetes mellitus; use of antibiotics within the past three months; and a known hypersensitivity to probiotic products or any of the study formulation ingredients.

This study was conducted using a randomized, double-blind, placebo-controlled trial design. Subjects in the pAkk11 group received an intervention of pAkk11 at a dose of 10 billion TFU per day, while those in the placebo group were given maltodextrin, with the intervention duration being 12 weeks for both groups. Eligible subjects were randomly assigned to the pAkk11 group or the placebo group by a table of random number. Double-blinding was maintained throughout the trial, with the active pAkk11 intervention and placebo being identical in appearance, packaging, odor and taste. Subjects, investigators, outcome assessors and data analysts remained unaware of the group assignments, and the randomization code was sealed and held by an independent third party until unblinding was performed upon completion of the primary analysis. In addition, a comprehensive adverse reaction assessment system comprising subject self-reports, regular follow-ups and adverse reaction record forms was adopted to collect relevant information, which allowed for full monitoring of potential safety issues arising during the pAkk11 intervention and ensured the health of subjects as well as the reliability of study data.

The primary outcomes were to evaluate the ameliorative effects of pAkk11 intervention on anthropometric and body composition measures in overweight/obese adults, including body weight, BMI, body fat percentage, waist circumference (WC), wrist circumference, waist-to-hip ratio (WHR), and visceral fat index (VFI); and to assess its impacts on the serum concentrations of lipid profile parameters, namely total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG).

The intervention lasted 12 weeks, with study visits at baseline (week 0) and weeks 4, 8, and 12. Samples and data were collected at each visit. During the 12-week intervention period, 11 subjects were lost to follow-up, including 5 who discontinued due to loss of contact and 6 who withdrew for personal reasons such as work relocation. Ultimately, 61 participants were included in the per-protocol safety analysis, with overall compliance > 95%. No serious adverse events were observed during the study.

## 2.3 Sample size calculation

Sample size calculation was performed using the power analysis package (pwr package) in R software (R version 4.2.0). After comprehensive consideration of multiple factors, the study was designed to enroll 30 study participants in each group. Taking into account the potential 10%-20% subject dropout rate during the study, it was finally determined that 36 study participants would be randomly assigned to each group, i.e., this study planned to screen 72 eligible study participants for enrollment in the clinical trial. If the finally obtained samples exhibited quality deviations, additional eligible study participants would need to be recruited and enrolled to supplement the study cohort.

## 2.4 Animal model establishment and experimental design

Six-week-old male C57BL/6J mice (SPF grade) were purchased from Henan Skebes Biotechnology Co., Ltd. (Henan, China). Mice were housed and maintained at  $24 \pm 2$  °C with a humidity of  $50 \pm 10\%$  under a 12 h light/dark cycle, with free access to water and food. All animal experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology (approval No. 202403046, dated October 12, 2024) and were carried out in strict accordance with institutional guidelines and the ARRIVE guidelines. The animal use license number was SCXK (Yu) 2020-0005. After a 1-week acclimation period, mice were randomly assigned to a normal control group (Control) fed a standard chow diet or a high-fat diet (HFD) group fed a diet containing 60% fat for 12 consecutive weeks. Obesity was considered successfully induced when body weight exceeded that of age-matched control mice by > 20%. Successfully modeled mice were then randomly allocated into three groups (n = 8 per group): the HFD model group (HFD), the pAkk11 treatment group (pAkk11), and a positive control group receiving orlistat (Orlistat). During the intervention period, the Control and HFD groups received 0.9% NaCl by oral gavage once daily; the pAkk11 group received pAkk11 by oral gavage at  $1 \times 10^7$  TFU per [g or kg] body weight (bw) once daily; and the Orlistat group received orlistat (30 mg/kg) by oral gavage once daily. All groups were maintained on their respective diets throughout the intervention. The intervention lasted 8 weeks, and gavage was performed daily at 10:00 AM. Oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed in the final week of the intervention [15].

Wild-type N2 *Caenorhabditis elegans* (hermaphrodite) was preserved in the Laboratory of Probiotic Science and Technology, College of Food and Bioengineering, Henan University of Science and Technology. *Escherichia coli* OP50 was continuously subcultured and activated in LB medium, then spread on NGM plates as nematode food. Nematodes were cultured at a constant temperature of 20 °C, and subcultured using the chunk method; before the experiment, eggs were obtained and washed using

the lysis method (M9 buffer and lysis solution ratio 2:1), and inoculated onto plates to obtain synchronized nematodes. The experimental groups were designed as follows: control group (ordinary NGM), high-glucose model group (NGM containing 10 mmol/L glucose, glucose added after high-temperature sterilization), and low, medium, and high-dose arginine (Arg) intervention groups (adding 1, 5, and 10 mM Arg to the high-glucose NGM, respectively). All groups were fed OP50 normally and cultured under the same conditions.

## 2.5 Sample collection

At the end of the experimental period, mice were fasted for 12 h with free access to water before sample collection. Following anesthesia, blood samples were collected via orbital sinus puncture (retro-orbital bleeding), and mice were euthanized by cervical dislocation under anesthesia in accordance with institutional guidelines for animal experimentation. Blood samples were centrifuged at  $1,000 \times g$  for 10 min at 4 °C to obtain serum. The liver and white adipose tissues were excised and weighed. All samples were stored at – 80 °C until further analyses.

## 2.6 Histopathological analysis

Liver, epididymal white adipose tissue (WAT), and colon tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5- $\mu$ m sections. Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Immunofluorescence staining was performed with antigen retrieval, permeabilization, blocking, and antibody incubation. Briefly, sections were subjected to antigen retrieval in citrate buffer at 95 °C for 20 min and cooled to room temperature. Sections were permeabilized with 0.3% Triton X-100 in PBS for 10 min and blocked with 5% serum at room temperature for 1 h. Primary antibodies were incubated overnight at 4 °C. After PBS washing, fluorophore-conjugated secondary antibodies were incubated at room temperature for 1 h. Sections were then mounted with an antifade mounting medium containing DAPI. Images were acquired using a fluorescence microscope/slide scanner. Three sections per group were analyzed, and fluorescence intensity was quantified using ImageJ.

## 2.7 Determination of serum biochemical-related indicators

Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), and catalase (CAT) were measured according to the manufacturers' instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum levels of glucagon-like peptide-1 (GLP-1), leptin (LEP), peptide YY (PYY), adiponectin (ADPN), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), lipopolysaccharide (LPS), interleukin-25 (IL-25), lipoprotein lipase (LPL), apolipoprotein A (ApoA), apolipoprotein B (ApoB), ghrelin (GHRL),  $\gamma$ -aminobutyric acid (GABA), melatonin (MT), and insulin (INS) were determined using ELISA kits following the manufacturer's protocols (Hepeng (Shanghai) Biotech Co., Ltd., Shanghai, China). Blood glucose in mice was measured using a glucometer (Sinocare Inc., Changsha, China).

## 2.8 Western blot analysis

Total protein was extracted from adipose tissue using RIPA lysis buffer (CBW001150, Kebi Biotechnology, China) containing 1× protease inhibitor cocktail (CBW0018, Kebi Biotechnology), phosphatase inhibitor (CBW0019, Kebi Biotechnology) and PMSF (CBW0017, Kebi Biotechnology). Tissue samples were cut into small pieces and homogenized in a high-speed tissue grinder (HLK-s-4, Jingxin, China) with 3–4 magnetic beads for up to 100 s with intermittent cooling on ice. The homogenates were incubated on ice for 30 min with vortexing every 5 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were determined using a BCA protein assay kit (CBW0020, Kebi Biotechnology) according to the manufacturer's instructions. A total of 30 µg protein was mixed with 5× protein loading buffer (CBW0029, Kebi Biotechnology) at a 4:1 ratio and denatured by boiling for 15 min. The proteins were separated by SDS-PAGE on 8–10% gels and transferred to PVDF membranes (0.45 µm, IPVH00010, Millipore, USA) at 300 mA constant current for 30–40 min depending on the molecular weight of the target protein. After blocking in 3% BSA (V900933, Merck, USA) for 1 h at room temperature, the membranes were incubated with primary antibodies for 16 h at 4 °C, and then incubated with HRP-conjugated secondary antibodies (074-1506, Kirkegaard & Perry Laboratories, USA) for 30 min at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (P0018M, Beyotime, China) and imaged using the Clink ChemiScope 6100 system. Band intensities were quantified using AlphaEaseFC software. The antibodies used were as follows: rabbit polyclonal anti-AMPKα1 antibody (Proteintech, #10929-2-AP, China); rabbit polyclonal anti-phospho-AMPKα1 antibody (Affinity, #AF3423, China); rabbit polyclonal anti-ACCα antibody (Proteintech, #21923-1-AP, China); rabbit polyclonal anti-phospho-ACCα antibody (Affinity, #AF3421, China); rabbit polyclonal anti-SREBP1 antibody (Proteintech, #14088-1-AP, China); rabbit polyclonal anti-FAS antibody (Abcam, #ab82419, UK); rabbit polyclonal anti-PPARα antibody (Affinity, #AF5301, China); rabbit polyclonal anti-CPT1 antibody (Proteintech, #15184-1-AP, China); and rabbit polyclonal anti-β-actin antibody (Proteintech, #81115-1-RR, China).

## 2.9 RT-qPCR

Total RNA was extracted from liver and colon tissues using TRIzol reagent and further purified with the SteadyPure RNA Extraction Kit (Accurate Biotechnology (Hunan) Co., Ltd., Changsha, China). cDNA was synthesized using the Evo Super M-MLV Reverse Transcription Kit II (Accurate Biotechnology). Quantitative real-time PCR (qPCR) was performed using the SYBR Green Pro Taq HS Premixed qPCR Kit according to the manufacturer's instructions, with β-actin as the reference gene. Relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method [31].

## 2.10 Analysis of the gut microbiota

Total DNA was extracted from mouse feces. For 16S rRNA gene sequencing, PCR amplification was performed using specific primers targeting the V3-V4 regions of the bacterial 16S rRNA gene. After amplification, library construction was carried out with the NEBNext® Ultra DNA Library Prep Kit. For shotgun metagenomic sequencing, the extracted DNA was subjected to fragmentation, end repair, adapter ligation, and library construction directly using the same kit without PCR amplification. Following

qualification of both libraries by quality control, sequencing was performed on the Illumina NovaSeq platform (for metagenomics) and MiSeq platform (for 16S rRNA) respectively.

For 16S rRNA gene sequence data: Raw sequences were subjected to quality control, trimming, denoising, assembly, and chimeric sequence removal using the QIIME2 DADA2 plugin, resulting in high-precision Amplicon Sequence Variant (ASV) tables and their representative sequences. Subsequently, microbial diversity metrics were generated via the QIIME2 core-metrics-phylogenetic pipeline. Alpha diversity was evaluated using the Chao1 index and Shannon index, while Beta diversity was visualized via Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS) plots. Additionally, Spearman's rank correlation coefficient was computed through co-occurrence network analysis to explore the ecological associations among species.

For metagenomic sequence data, the preprocessing steps were similar to those of 16S rRNA analysis, with the difference being the subsequent taxonomic and functional annotation. Specifically, taxonomic annotation and functional annotation were performed by aligning against functional databases including KEGG, COG, and CAZy, to clarify the gene functions and metabolic potential carried by the microbial community. Quantitative metagenomic analysis was conducted by WekemoTech Group Co., Ltd. (Shenzhen, China).

## 2.11 Untargeted metabolomics analysis

Untargeted metabolomics analysis was performed on serum and fecal samples using UHPLC-MS/MS technology. A C18 column was selected as the analytical column, and the mobile phase consisted of eluent A (0.1% formic acid (FA) in water) and eluent B (methanol). Data were acquired in positive and negative electrospray ionization (ESI+, ESI-) modes within the mass range of  $m/z$  100–1500, using data-dependent acquisition (DDA, Data-Dependent Acquisition) as the scan mode. Raw data were processed with XCMS software for peak picking, peak alignment, and quantitation. Structural identification and functional annotation of metabolites were achieved based on high-quality secondary spectrum databases, as well as the KEGG, HMDB and LIPID MAPS databases.

## 2.12 *C. elegans* Morphology and Metabolic Indicator Detection

Nematode Physiological Indicators and Biochemical Component Measurement: Nematodes cultured for 96 hours were selected for relevant indicator detection. Referring to previous methods, nematode body length and width were measured using microscopy and Image J software ( $n \geq 30$  per group), and the relative fat content in the body was analyzed by Oil Red O staining ( $n \geq 25$  per group).

## 2.13 Statistical analysis

Mouse data are presented as the mean  $\pm$  standard deviation (SD). One-way analysis of variance was used to analyze the data with GraphPad Prism version 9.0 software (Graph Pad Software Inc.). The error

bars represent the SD based on the mean of at least three replicates. Statistical significance was considered at  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , and  $****p < 0.0001$ .

Clinical data were analyzed using SPSS 27.0. Continuous variables are presented as mean  $\pm$  SD (or median [IQR] for non-normally distributed data). Normality was assessed by the Shapiro-Wilk test and homogeneity of variances by Levene's test. For within-group comparisons, a paired t-test was used for normally distributed data; otherwise, the Wilcoxon signed-rank test was applied. For between-group comparisons, an independent-samples t-test was used for normally distributed data; otherwise, the Mann–Whitney U test was applied. To control for multiple comparisons, p-values were adjusted using the Benjamini-Hochberg procedure. Statistical significance was defined as adjusted  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

## 3 Results

### 3.1 Efficacy of pAkk11 in improving body weight and composition in overweight/obese individuals

#### 3.1.1 Participant demographics and baseline characteristics

During the 12-week intervention, 11 participants withdrew from the study early or were lost to follow-up due to personal reasons such as work relocation and scheduling conflicts, with a total of 61 participants completing the full 12-week intervention. The mean age was  $29.97 \pm 9.73$  years in the placebo group (16 males, 14 females) and  $32.52 \pm 12.15$  years in the pAkk11 group (15 males, 16 females). Baseline characteristics, including age, sex, body weight, BMI, fat mass, waist circumference, hip circumference, waist-to-hip ratio, and serum lipid parameters, were comparable between groups (all  $p > 0.05$ ) (Tab.S1). No gastrointestinal adverse events were reported during the intervention.

#### 3.1.2 pAkk11 intervention promotes weight loss and improves body composition

After 12 weeks of intervention, supplementation with pAkk11 elicited significantly superior reductions across all key obesity-related metrics compared to the placebo group. Specific improvements included an 8.01% decrease in body weight (Fig. 1A-a), a 12.06% reduction in fat mass (Fig. 1A-b), a 12.02% reduction in BFP (Fig. 1A-c), an 8.06% reduction in BMI (Fig. 1A-d), a 10.01% decline in VFI (Fig. 1A-e), a 17.8% reduction in Body Roundness Index (BRI,  $p < 0.001$ ) (Fig. 1A-f), a 4.02% reduction in wrist circumference (Fig. 1A-g), a 6.98% decrease in waist circumference (Fig. 1A-h), a 4.5% reduction in hip circumference (Fig. 1A-i) and a 17.71% decline in waist-to-hip ratio (Fig. 1A-j). In contrast, changes in the placebo group for primary indicators such as body weight, waist circumference, and body fat were not statistically significant. The magnitude of reduction in the pAkk11 group, predominantly within the 6–12% range, was markedly greater than that observed with placebo.

At weeks 4, 8, and 12, body weight reductions from baseline in the pAkk11 group were 3.83%, 5.06%, and 8.00%, respectively (all  $p < 0.001$  vs. baseline), with significantly greater reductions compared with the Placebo group (Fig. 1B, a-b). Weight distribution analysis revealed a clear downward shift in the pAkk11 group, along with a markedly reduced data range relative to the Placebo group (Fig. 1B-c). Notably, the proportions of participants achieving weight-loss targets of  $\geq 5\%$  and  $\geq 10\%$  were significantly higher in the pAkk11 group than in the Placebo group (Fig. 1B-d). Furthermore, pAkk11 facilitated favorable shifts in BMI categories, moving more individuals from an “obese” status toward “overweight” or “normal” status (Fig. 1B-e).

Analysis of body composition revealed that muscle mass remained unchanged in both groups ( $p > 0.05$ , Fig. 1B f), while lean body mass (LBM) was significantly reduced in the pAkk11 group ( $p < 0.001$ , Fig. 1B g) with no change in the placebo group. This LBM loss was attributable to non-muscular components (e.g., water, collagen) associated with adipose tissue reduction, rather than skeletal muscle loss, suggesting pAkk11-mediated weight loss preserves muscle mass and represents high-quality fat loss.

### **3.1.3 pAkk11 intervention improves metabolic phenotypes by elevating circulating L arginine**

Serum untargeted metabolomic profiling showed distinct metabolic separation between pAkk11 and placebo groups post-intervention (Fig.S1A), with 33 differential metabolites identified (12 upregulated, 21 downregulated; Fig.S1B). Pathway enrichment analysis highlighted significant enrichment of the arginine metabolism pathway (Fig.S1E), and pAkk11 significantly elevated serum levels of L-glutamine, L-arginine and L-ornithine (Fig. 1C) — key metabolites in arginine biosynthesis. The Mantel test confirmed significant correlations between arginine-related metabolite changes and improvements in body weight, BMI and fat mass (Fig. 1D).

Linear regression analysis further showed that elevated serum L-arginine levels were correlated with the anti-obesity effects associated with pAkk11, explaining 21–38% of the variance in improvements in body weight, fat mass, BMI, VFI and circumferential measures (all  $p < 0.05$ , Fig. 2). Collectively, these findings suggest an association between pAkk11-associated metabolic improvements in obese individuals and changes in arginine metabolism.

## **3.2 Systemic effects of pAkk11 in overweight/obese individuals**

### **3.2.1 pAkk11 intervention improves lipid metabolism**

After 12 weeks of intervention, lipid metabolism improved significantly in the pAkk11 group. Compared to baseline, serum LPL (Fig. 3A, a) level was significantly elevated, while levels of HDL-C, TC and TG (Fig. 3A, b, d-e) were significantly reduced. Furthermore, ApoA levels increased and ApoB levels decreased, resulting in a significant increase in the ApoA/ApoB ratio (Fig. 3A, f-h). In the placebo group,

apart from a slight, non-significant decrease in ApoA, none of the other lipid parameters changed significantly. Collectively, these results suggest that pAkk11 may have more favorable associations with lipid profiles compared with placebo, and favorably alter indices associated with cardiovascular risk.

### **3.2.2 pAkk11 intervention modulates appetite regulatory hormones and reduces inflammation**

After 12 weeks of intervention, pAkk11 significantly altered key hormones involved in appetite control and energy metabolism. Compared with baseline, the pAkk11 group exhibited elevated levels of hormones that promote satiety and energy expenditure. Specifically, satiety-promoting hormones (GLP-1, PYY, adiponectin/ADPN) were upregulated, while appetite- and fat storage-related hormones (insulin, ghrelin) were downregulated, with leptin showing a non-significant decreasing trend (Fig. 3B). The placebo group exhibited no significant changes in any of these hormones. Furthermore, pAkk11 alleviated systemic low-grade inflammation by significantly reducing pro-inflammatory cytokines (TNF- $\alpha$ , IL-25; Fig. 3C), with no changes in the placebo group.

### **3.2.3 pAkk11 improves sleep quality and mood and is associated with changes in neuromodulators and SCFAs**

pAkk11 significantly improved sleep quality and emotional state, as assessed by the Pittsburgh Sleep Quality Index (PSQI) and Self-Rating Anxiety Scale (SAS) (Fig. 3E), with no significant improvements in the placebo group. This was accompanied by significant elevations in sleep- and mood-related neuromodulators ( $\gamma$ -aminobutyric acid/GABA, melatonin; Fig. 3D) and gut-derived short-chain fatty acids (SCFAs): propionate and butyrate levels were significantly higher in the pAkk11 group than in the placebo group (Fig. 3F).

## **3.3 pAkk11 improves body weight and glucose–lipid metabolic phenotypes in HFD-fed obese mice**

To mechanistically dissect the metabolic benefits and underlying molecular pathways of pAkk11 observed in our human RCT, we conducted complementary in vivo experiments using a high-fat diet-induced obese mouse model. pAkk11 reversed HFD-induced body weight gain, liver hypertrophy and white adipose tissue (WAT) accumulation, with efficacy comparable to the anti-obesity drug Orlistat (Fig. 4A). pAkk11 significantly reduced final body weight and cumulative weight gain in HFD-fed mice (Fig. 4B), normalized fasting blood glucose (FBG) and homeostatic model assessment for insulin resistance (HOMA-IR) (Fig. 4C), and improved glucose tolerance and insulin sensitivity (reduced AUC in OGTT and ITT; Fig. 4D). Additionally, pAkk11 intervention was associated with improved lipid profiles, with serum lipids approaching normal levels (Fig. 4E). These data indicate that pAkk11 is associated with mitigation of HFD-induced obesity and associated glucose-lipid metabolic dysfunction in mice.

### 3.4 pAkk11 ameliorates liver and adipose tissue injury and alleviates metabolic inflammation

A long-term high-fat diet typically imposes an exacerbated burden on the liver, leading to hepatic injury, metabolic imbalance, and the induction of inflammatory responses, which causes systemic damage and disrupts the intestinal barrier integrity [32]. pAkk11 intervention normalized hepatic lipid levels in HFD-fed mice (Fig. 5A), reduced hepatic injury markers (ALT, AST;  $p < 0.05$ ) and enhanced antioxidant capacity (Fig. 5B). Furthermore, pAkk11 decreased hepatic pro-inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-6, MCP-1;  $p < 0.05$ , Fig. 5C) and reshaped gut hormone levels: anorexigenic hormones (GLP-1, PYY) and ADPN were upregulated, while leptin (LEP) was downregulated (Fig. 5D).

Histopathological analysis showed pAkk11 improved HFD-induced hepatic steatosis, reduced adipocyte hypertrophy and enhanced intestinal barrier integrity (Fig. 5E), with immunofluorescence and qPCR confirming restored intestinal barrier protein expression (Fig. 5F). Collectively, pAkk11 alleviates HFD-induced liver and adipose tissue injury, metabolic inflammation and intestinal barrier dysfunction in mice.

### 3.5 pAkk11 reshapes the serum and fecal metabolomes of HFD-fed mice

We assessed pAkk11's effects on murine serum and fecal metabolites by high-throughput untargeted profiling; serum OPLS-DA revealed distinct metabolic profiles between HFD and pAkk11 groups (Fig. 6A-a). We performed targeted quantitative profiling of serum metabolites, with a clustered heatmap revealing distinct metabolic signatures between HFD and pAkk11 groups (Fig. 6A-b). Notably, metabolites associated with lipid anabolic processes were enriched in the HFD group.

Hexadecasphinganine (a sphingolipid precursor critical for lipid droplet biogenesis) was markedly upregulated, aligning with enhanced de novo lipogenesis under high-fat feeding. In contrast, the pAkk11 intervention group exhibited a distinct signature oriented toward lipid catabolism and metabolic homeostasis. Key metabolites included LPC 18:1 (a lysophosphatidylcholine known to promote fatty acid mobilization and  $\beta$ -oxidation) [33] and L-Arginine (a precursor for nitric oxide synthesis, which enhances adipose tissue lipolysis) [34]. Furthermore, the elevation of Oxoadipic acid (a metabolic intermediate that interfaces with the TCA cycle, reflecting active fuel oxidation) [35] supports a shift toward increased lipid utilization. KEGG enrichment analysis identified significant enrichment of arginine biosynthesis and arginine/proline metabolism pathways in pAkk11-treated mice (Fig. 6A d), and network correlation analysis highlighted L-arginine as a central metabolite linked to other key metabolic markers (Fig. 6A-c).

Fecal metabolite analyses yielded consistent group-specific differences (Fig. 6B-a-b): the pAkk11 group had elevated L-arginine, NMNH (a nicotinamide derivative boosting microbial/host metabolic resilience) [36]; cysteine, an antioxidant amino acid, mitigates gut oxidative stress.[37] These metabolites reflect improved amino acid metabolism and overall metabolic health in pAkk11 group. In contrast, increased

abundances of malotetraose and palmitoyl glucuronide in HFD group indicate impaired gut carbohydrate fermentation and reduced lipid clearance, further underscoring the metabolic dysregulation induced by high-fat feeding [38] (Fig. 6B-b). Similarly, network correlation analysis of fecal metabolites identified 15 most strongly interconnected metabolites-including L-arginine (Fig. 6B-c). Venn diagram analysis identified 5 overlapping differential metabolites in serum and feces, including L-arginine and its upstream precursors (4-hydroxyproline, L-proline; Fig. 6B d), with pAkk11 significantly elevating L-arginine levels in both serum and feces (Fig. 6B e). Correlation analysis showed L-arginine was negatively correlated with body weight, inflammatory factors (MCP-1, TNF- $\alpha$ , IFN- $\gamma$ ), LEP and LPS, and positively correlated with PYY and ADPN (Fig. 6B f), linking elevated L-arginine to improved metabolic phenotypes in pAkk11-treated mice.

### 3.6 pAkk11 enriches *Faecalibaculum rodentium* and links to microbial arginine biosynthesis

Fecal microbiota analysis showed pAkk11 modified microbial alpha diversity (Fig.S2A) and induced distinct microbial community clustering (PCoA/NMDS; Fig.S2B), with the pAkk11 group clustering near the normal control. At the phylum level, pAkk11 increased Bacteroidetes and decreased Firmicutes, significantly lowering the Firmicutes/Bacteroidetes ratio (Fig. 7A, S2C). LEfSe analysis identified *Faecalibaculum* as the key discriminative genus in the pAkk11 group (Fig. 7B), and correlation analysis linked *Faecalibaculum* to improved metabolic parameters (positive with HDL-C, GLP-1, ADPN; negative with body weight, TC, TNF- $\alpha$ ; Fig. 7C).

We performed shotgun metagenomic sequencing to identify microbial genes and key taxa mediating gut arginine biosynthesis, linking microbial function, arginine metabolism, and host metabolic phenotypes. At the species level, the abundances of *Parabacteroides distasonis*, *Faecalibaculum rodentium*, *Phocaeicola vulgatus*, *Adlercreutzia equolifaciens*, *Bacteroides xylanisolvens*, *Butyricimonas virosa*, and *Bacteroides uniformis* were increased, while those of *Dysosmobacter welbionis*, *Curtobacterium flaccumfaciens*, and *Ruminococcus gnavus* were decreased (Fig.S2E). Linear discriminant analysis effect size (LEfSe) analysis further confirmed that *Faecalibaculum rodentium* was the most discriminative core differential functional bacterial species between the two groups (Fig. 7D).

Shotgun metagenomic sequencing confirmed *Faecalibaculum rodentium* as the core differential functional species in the pAkk11 group (Fig. 7D). Functional pathway enrichment analysis showed the arginine biosynthesis pathway was significantly enriched in pAkk11-treated mice (Fig.S2F), with *F. rodentium* contributing the most to this pathway (far higher than other bacterial species; Fig. 7E). Gene-level LEfSe analysis identified enrichment of key arginine synthesis genes (*argC*, *argD*, *argH/ASL*, *ALDH*) in the pAkk11 group (Fig. 7E-F), with these genes primarily derived from *F. rodentium*. Integrative correlation analysis linked *F. rodentium* abundance to elevated L-arginine levels and improved metabolic phenotypes (positive with L-arginine, HDL-C, PYY; negative with body weight, inflammatory markers, LPS) (Fig. 7G, Fig. 8), suggesting *F. rodentium* as the core microbial mediator of pAkk11-induced arginine biosynthesis activation.

### 3.7 pAkk11 activates the AMPK pathway via arginine anabolism to reduce fat accumulation

Arginine is mainly derived from exogenous intake and endogenous synthesis. However, mammals have limited capacity for endogenous arginine synthesis [39]. As key symbiotic partners in the host metabolic network, intestinal microbiota play a central regulatory role in arginine synthesis, catabolism, and metabolite conversion [40]. This microbiota-arginine interaction not only directly affects the bioavailability of arginine in the host intestine, but also regulates host energy metabolism and metabolic homeostasis through signal transduction of downstream metabolites (e.g., nitric oxide, NO). It thus serves as a vital link connecting intestinal microecology and host metabolic health. Previous studies have confirmed that NO can act as a signaling molecule to activate the AMPK signaling pathway, thereby regulating lipid metabolism [41]. Based on this, the present study focused on the arginine-NO-AMPK pathway to investigate the mechanism underlying pAkk11-mediated improvement in obesity.

First, *C. elegans* experiments showed that arginine supplementation was associated with dose-dependent reduction of HFD-induced body hypertrophy and lipid accumulation (Oil Red O staining; Fig. 9A), by inhibiting lipogenesis-related genes (*fasn*, *sbp*) and upregulating fatty acid  $\beta$ -oxidation and AMPK homolog genes (*cpt-1*, *cpt-2*, *aak-2*; Fig. 9B), supporting a conserved role of arginine in regulating lipid metabolism potentially via AMPK signaling. To validate this regulatory axis at the mammalian protein level, we performed Western blotting on adipose tissue. Compared with the CON group, HFD-fed mice exhibited significantly lower total AMPK $\alpha$ 1 protein expression and decreased ACC $\alpha$  phosphorylation (p-ACC), indicating impaired AMPK signaling. Importantly, pAkk11 treatment significantly restored both AMPK $\alpha$ 1 expression and p-ACC levels ( $p < 0.01$ ), confirming AMPK pathway activation. Consequently, pAkk11 suppressed lipogenesis, as evidenced by downregulated protein expression of SREBP-1, FAS, and ACC $\alpha$ , while concurrently enhancing fatty acid  $\beta$ -oxidation via upregulation of PPAR- $\alpha$  and CPT1 in adipose tissue (Fig. 9C). In HFD-fed mice, pAkk11 upregulated key host arginine synthesis genes (*Ass1*, *Asl*; Fig. 9D), suggesting enhanced arginine anabolism, and significantly elevated serum nitric oxide (NO) levels (a downstream metabolite of arginine; Fig. 9D). NO levels and AMPK pathway activation (indicated by *prkaa1* upregulation) were observed in pAkk11-treated mice in liver tissue (Fig. 9D). AMPK activation further reshaped lipid metabolism homeostasis: pAkk11 downregulated lipogenic genes and upregulated lipolytic genes in liver tissue (Fig. 9E), and immunohistochemical analysis of adipose tissue showed enhanced eNOS staining, elevated p-ACC (inactive acetyl-CoA carboxylase, a marker of lipogenesis inhibition) and increased HSL (hormone-sensitive lipase, a key lipolytic enzyme) expression in pAkk11-treated mice (Fig. 9G), consistent with improved serum lipid profiles (Fig. 9F). Collectively, these findings suggest that pAkk11 is associated with alleviation of obesity and activation of the arginine-NO-AMPK axis, which inhibits lipogenesis and promotes lipolysis in metabolic tissues.

## 4 Discussion

In contrast to conventional probiotic strategies that rely on live bacterial colonization and continuous intake, “next-generation probiotics” represented by *A. muciniphila* and their postbiotic preparations have attracted increasing attention in the field of metabolic health [8, 42]. Prior mechanistic research has focused on a single-component, single-target paradigm, identifying molecules like Amuc\_1100 and P9 as key effectors of Akk’s metabolic benefits [43, 44]. Nevertheless, important mechanistic gaps remain. On the one hand, most existing studies primarily report improvements in body weight and glucose-lipid metabolic phenotypes accompanied by shifts in microbial composition, while providing limited insight into which specific microbial functional units transmit these effects and which metabolic networks serve as key intermediaries [45, 46]. On the other hand, direct correspondence between mechanistic readouts in animal models and measurable indicators in human studies is often lacking, leading to variability in reproducibility and interpretability across studies [47].

This study systematically evaluated the effects of pasteurized *A. muciniphila* Akk11 on obesity intervention from both mechanistic and translational perspectives. Our clinical data show that pAkk11 induces a mean 8.0% body weight reduction in overweight and obese individuals, exceeding the efficacy of previously reported pasteurized Akk interventions [48], and this improvement is tightly linked to elevated circulating L-arginine levels. The clinical ramifications of this framework are substantive. Firstly, the robust serum arginine response serves as a direct readout of the intended microbial action, positioning it as a strong candidate pharmacodynamic biomarker for use in clinical development and eventual companion diagnostics [49]. Secondly, the unique mechanism, distinct from current hormonal or neural appetite modulators, creates a clear rationale for exploring synergistic combinations. Co-administration with GLP-1 receptor agonists, for instance, could simultaneously target central satiety and peripheral energy metabolism, potentially improving weight loss efficacy and metabolic health [50]. Most directly, the pronounced attenuation of hepatic lipid accumulation in our models provides a strong rationale for prioritizing evaluation in NAFLD/MASLD cohorts, where modulating the gut-liver axis via microbial metabolites is a paramount therapeutic strategy [51, 52].

This clinical finding was further corroborated in a high-fat diet-induced obesity mouse model. The gut microbiota is a major contributor to host amino acid homeostasis [53], yet its specific role in arginine metabolism during pAkk11 intervention has been underexplored. Our untargeted metabolomics in mice revealed that pAkk11 intervention elevated L-arginine levels by ~ 1.8-fold in serum and ~ 2.2-fold in feces. This was directly corroborated by metagenomic functional enrichment of the arginine biosynthesis pathway (KEGG map00220). Furthermore, functional attribution analyses linked the enhancement of these pathways to the contributions of specific microbial functional units, thereby strengthening a closed evidence chain connecting microbial units, functional genes, metabolic pathways, and host phenotypic outcomes. Collectively, these cross-omics, directionally consistent results suggest that the arginine biosynthesis–related metabolic axis may represent a key intermediary pathway through which pAkk11 mediates metabolic improvement. While a recent study implicated arginine as a mediator in synbiotic action against obesity [41], it did not identify the specific microbial source or fully delineate the downstream signaling cascade.

Building on these cross-omics insights, our subsequent mechanistic investigations further delineated the downstream signaling cascade through which the enhanced arginine biosynthesis axis exerts metabolic benefits, uncovering a critical role of the arginine-NO-AMPK pathway. As a conditionally essential amino acid, arginine's bioavailability is tightly regulated by both host endogenous synthesis and microbial metabolism [54]; notably, mammals have inherent limitations in de novo arginine synthesis, making the gut microbiota-derived arginine a vital complementary source. This connection not previously established in the context of Akk based interventions. Our results showed that in the HFD-induced obese mouse model, the expression levels of *Ass1* and *Asl* (two key genes involved in host arginine biosynthesis) were significantly downregulated. In contrast, pAkk11 intervention markedly upregulated the expression of these two genes. This upregulation directly accelerated the conversion of citrulline to arginine, thereby increasing arginine levels in the body and laying a material foundation for downstream NO production. NO, a key metabolite of arginine catalyzed by nitric oxide synthase (NOS), serves as a pivotal signaling molecule bridging arginine metabolism and host energy homeostasis. Accumulating evidence has established that NO can activate the AMPK pathway, a central regulator of lipid and glucose metabolism [55]; our results are consistent with this regulatory link in the context of pAkk11 intervention. Specifically, the expression of *prkaa1*, the gene encoding the AMPK $\alpha$ 1 subunit, was significantly upregulated in the liver tissue of pAkk11-treated mice, indicating robust activation of the AMPK pathway. Activation of the AMPK pathway subsequently reshaped lipid metabolism homeostasis through dual regulatory effects, inhibition of lipogenesis and promotion of lipolysis. Immunohistochemical results of adipose tissue demonstrated that pAkk11 treatment increased the phosphorylation level of acetyl-CoA carboxylase (p-ACC signal intensity increased ~ 70%), a hallmark of ACC inactivation and thus lipogenesis inhibition, and enhanced the expression of hormone-sensitive lipase (HSL), a key enzyme driving triglyceride breakdown. These molecular changes in adipose tissue were consistent with the alterations in serum lipid metabolism-related factors, forming a coherent evidence chain from microbial functional regulation to host molecular signaling and ultimately to phenotypic improvement (Fig. 10). These protein-level changes provide correlational evidence linking microbial genetic potential to host physiological outcomes. Many prior studies linking the gut microbiome to obesity have identified associations between microbial taxa or metabolites and host phenotypes [23, 56], but have often lacked this depth of mechanistic validation across molecular, cellular, and organismal scales [47].

Prevailing mechanistic models for *A. muciniphila* have largely centered on its direct interactions with the host. These include the role of its outer membrane protein Amuc\_1100 in strengthening tight junctions and modulating immune responses [18, 57], and the secreted protein P9 in stimulating glucagon-like peptide-1 (GLP-1) secretion and adipose tissue browning [15]. These studies firmly established *A. muciniphila* as a "single-agent" therapeutic entity. Our findings necessitate a conceptual expansion. Shotgun metagenomic analysis revealed that pAkk11 intervention did not significantly increase its own relative abundance but instead induced a profound functional remodeling of the gut microbiome, most prominently characterized by the enrichment of *Faecalibaculum rodentium*. This bacterium was identified as the principal contributor to the enhanced microbial arginine biosynthesis pathway, with its functional

genetic contribution for key enzymes (*argC*, *argD*, *argH*) increasing 2.5 to 3-fold. pAkk11 could foster a symbiotic partnership with a resident bacterium possessing a critical, complementary metabolic capability.

From a biological plausibility perspective, the arginine metabolic axis is well positioned to serve as a bridge between microbial signals and host metabolic homeostasis. Arginine and its related metabolic intermediates play central roles not only in nitrogen metabolism and the urea cycle, but also in nitric oxide production, redox regulation, immune cell function, and tissue repair processes [58, 59]. In parallel, ornithine and downstream polyamine pathways have been implicated in epithelial renewal, mucosal repair, and the maintenance of barrier integrity [60]. Accordingly, enhancement of arginine biosynthetic programs induced by pAkk11 could theoretically alleviate obesity-associated pathophysiology through multiple, interconnected mechanisms. On the one hand, improved arginine availability may contribute to reductions in chronic low-grade inflammation, enhanced insulin sensitivity, and normalization of lipid metabolic signaling, thereby mitigating lipid accumulation and metabolic stress in hepatic and adipose tissues. On the other hand, support of epithelial homeostasis and barrier repair may reduce endotoxin-associated burden, fostering a positive feedback loop linking inflammation, metabolism, and barrier function. In the present study, improvements in hepatic lipid deposition, reductions in inflammatory markers, and restoration of barrier-related indices following pAkk11 intervention were directionally consistent with these mechanistic expectations. While these observations support the biological relevance of the arginine axis as a potential mediator, the evidence remains largely associative. Future studies incorporating more direct host-level validation, such as protein or transcriptional assessment of key energy metabolism pathways (e.g., AMPK signaling, fatty acid oxidation, and lipolysis–lipogenesis balance), pathway-specific perturbation or rescue approaches, isotope tracing, or functional microbial reconstitution, will be instrumental in further strengthening the mechanistic link between arginine-centered microbial metabolism and host metabolic improvement.

Metabolic phenotype of host may be compromised not by the absence of a specific taxon, but by a deficit in a key biochemical function, such as arginine anabolism, that is collectively encoded. This concept provides a powerful explanatory framework for the inter-individual variability observed in microbiome interventions [61]. Our proposed “ecological-functional” model addresses a different, complementary question, how does a pasteurized *A. muciniphila* preparation produce sustained, multi-organ metabolic benefits? The answer lies in pAkk11 appears to function as an ecological recruiter, identifying and potentiating the most suitable endogenous functional module (e.g., *F. rodentium*) to restore this niche, which capacity to reprogram microbial community metabolism level. It signifies an evolution from viewing *A. muciniphila* as a delivered drug to recognizing it as a trigger for restorative host-microbe symbiosis. It argues compellingly for a future shift from taxonomy-guided probiotics to function-targeted microbial therapeutics, where interventions are designed to augment specific, deficient metabolic pathways, thereby enhancing predictability and personalization [4]. This functional perspective directly addresses the core challenge of variable clinical responsiveness. It advocates for a next-generation approach, function-centric intervention, where the therapeutic goal is restoring specific

biochemical pathways, thereby achieving more consistent and personalized outcomes than species-based probiotics [62].

Building on the concordant responses observed along the proposed mechanistic axis and the accompanying improvements in clinical phenotypes, the present study has several notable strengths and innovative aspects. First, we adopted an integrated evidence pathway that bridges mechanistic interrogation in animal models with validation in a randomized controlled human trial, thereby addressing a common limitation in microbiome-based interventions whereby mechanistic insights and clinical outcomes are often disconnected. Specifically, the animal component was used to identify candidate functional networks and key metabolic pathways, while the human intervention assessed their relevance to core clinical endpoints and evaluated whether the associated metabolic networks exhibited directionally consistent responses. This design substantially enhanced the interpretability and translational relevance of our findings. Second, the analytical focus was advanced from microbial compositional changes toward functional attribution and metabolic network remodeling. Through cross-validation of metagenomic and metabolomic data, we linked key pathways, functional genes, and metabolite networks into a relatively closed evidence chain, thereby reducing uncertainty inherent to single-omics correlation-based inference. Third, outcome assessment encompassed not only core anthropometric measures such as body weight and adiposity, but also metabolic, inflammatory, and barrier-related signals, allowing the observed intervention effects to be interpreted within a broader biological context. Collectively, these design features and evidence-integration strategies provide a more systematic framework for understanding the mode of action of postbiotic *Akkermansia* and offer a solid foundation for subsequent evaluation of its scalability and application potential.

Despite these strengths, several limitations warrant careful consideration. First, with respect to causality, the current evidence primarily supports the biological plausibility and cross-layer consistency of the arginine biosynthesis-related network as a key intermediary pathway, but remains largely associative in nature. More direct validation strategies, such as pathway-specific perturbation or rescue approaches, reconstitution of key functional microbial units, stable-isotope tracing of microbial arginine flux, or fecal microbiota transplantation from responders will be required to establish the necessity and sufficiency of this pathway in mediating the observed effects. Second, the sample size and follow-up duration of the clinical trial may limit assessment of long-term weight maintenance, inter-individual heterogeneity in response. In addition, variability in lifestyle factors such as diet and physical activity may have influenced effect magnitude, underscoring the need for larger, longer-term studies incorporating stratified or sensitivity analyses. Finally, dose-response relationships and optimal intervention regimens across different formulation formats remain to be systematically explored. Overall, future research guided by three parallel priorities, causal validation, long-term translational assessment, and intervention optimization, will be critical for further consolidating the mechanistic evidence base and clinical applicability of pAkk11.

In conclusion, this work provides evidence for the anti-obesity effects associated with pAkk11 and suggests a potential remodeling of the gut microbiome to enhance arginine biosynthesis. By showing

that its efficacy is associated with ecological remodeling of the gut microbiome to enhance arginine biosynthesis, and by mapping the subsequent signaling cascade through the NO-AMPK axis, we propose a novel mechanistic framework. These insights elevate our understanding of postbiotic actions, underscore the importance of microbial metabolic networking in host health, and pave the way for novel therapeutic strategies targeting the "gut microbiota-arginine metabolism" interface in metabolic syndrome.

## **Declarations**

### **Acknowledgments**

Graphical abstract and Schematic mechanism in the manuscript were drawn in Figdraw.

### **Competing interests**

YD, JGZ and SGF are affiliated with Wecare Probiotics R&D Centers (WPC) of Wecare Probiotics Co., Ltd. This affiliation is for institutional research collaboration purposes, and there are no additional financial agreements, grants, patents (planned, issued, or pending), consulting fees, honoraria, stock holdings, or other financial or non-financial interests related to the content of this manuscript. The remaining authors declare no competing interests.

### **Patient and public involvement**

Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

### **Informed Consent Statement**

All participants provided written informed consent prior to study enrollment.

### **Data availability statement**

The mouse gut microbiota sequencing data are publicly available in the NCBI BioProject database under the accession number PRJNA1434063 (<http://www.ncbi.nlm.nih.gov/bioproject/1434063>). The untargeted metabolomics raw data have been deposited in the MetaboLights repository under the study identifiers MTBLS14996 (human serum) and MTBLS14995 (mouse serum and fecal). To facilitate peer review, the controlled-access reviewer links for the metabolomics datasets are available at <https://www.ebi.ac.uk/metabolights/reviewerd8b537d3-90d1-42aa-97dd-c707f7d5c66c> (Access Token : mtbls\_31e07527-ef31-4c8c-8ad6-9b44886eddf7) and <https://www.ebi.ac.uk/metabolights/reviewerd4ffd35f-24d0-4464-b1ad-607dc488a2b3>. (Access Token : mtbls\_b0cc3d3c-8c39-4a76-a54a-dbc33e617f6b) We confirm that these metabolomics datasets will be made fully publicly accessible via the MetaboLights repository immediately upon the formal acceptance of the manuscript.

## Ethics approval

All animal experimental procedures in this study were approved by the Experimental Animal Welfare Ethics Committee of Henan University of Science and Technology (Approval No.: HAUST-024-M0820079, approval date: 22 August 2024) and performed in accordance with institutional guidelines and the ARRIVE guidelines. The experimental animal license number is SCXK (YU) 2020-0005. The human trial was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants before enrollment. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology (Approval code: K-2025-0565, approval date: 4 April 2025). The clinical trial registration number is NCT06964919 (accessed on 11 May 2025).

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# Figures

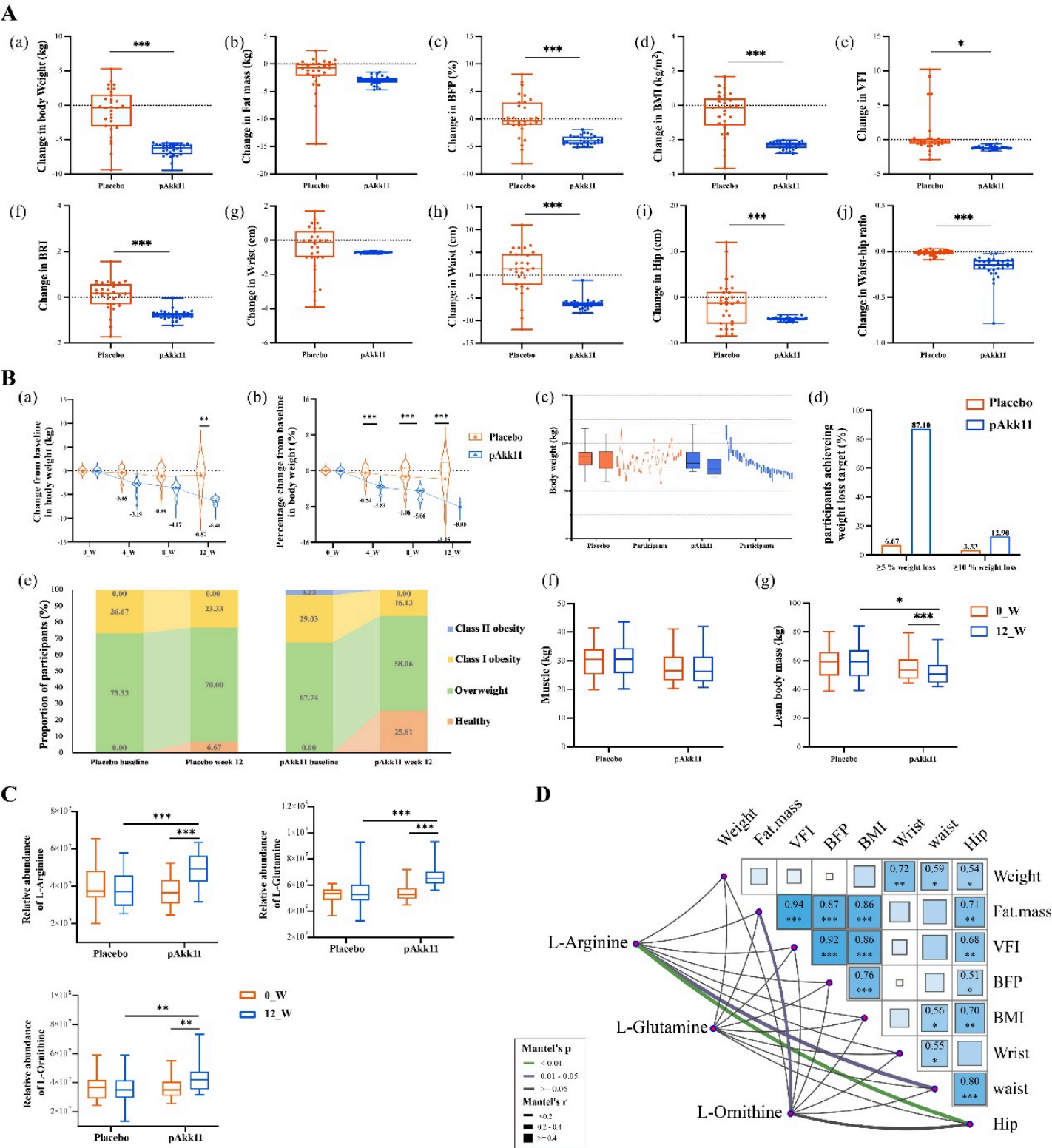


Figure 1

**Impact of pAkk11 intervention on metabolic and phenotypic parameters in overweight/obese individuals.** (A) Changes in body weight, fat mass, fat percentage (BFP), BMI, VFI, BRI, Wrist, Waist, Hip and Waist-hip ratio; (B-a) Absolute weight change (kg); (B-b) Percentage change from baseline; (B-c)

Body weight distribution at week 12; (B-d) Percentage achieving  $\geq 5\%$  and  $\geq 10\%$  weight loss, highlighting effectiveness of pAkk11 intervention; (B-e) Shifts in obesity classification from baseline to week 12; (B-f) Muscle mass; (B-g) Lean body mass (kg); (C) Relative abundance changes of key metabolites (L-Glutamine, L-Arginine, L-Ornithine); (D) Mantel test analysis of associations between key metabolites and anthropometric phenotypes.

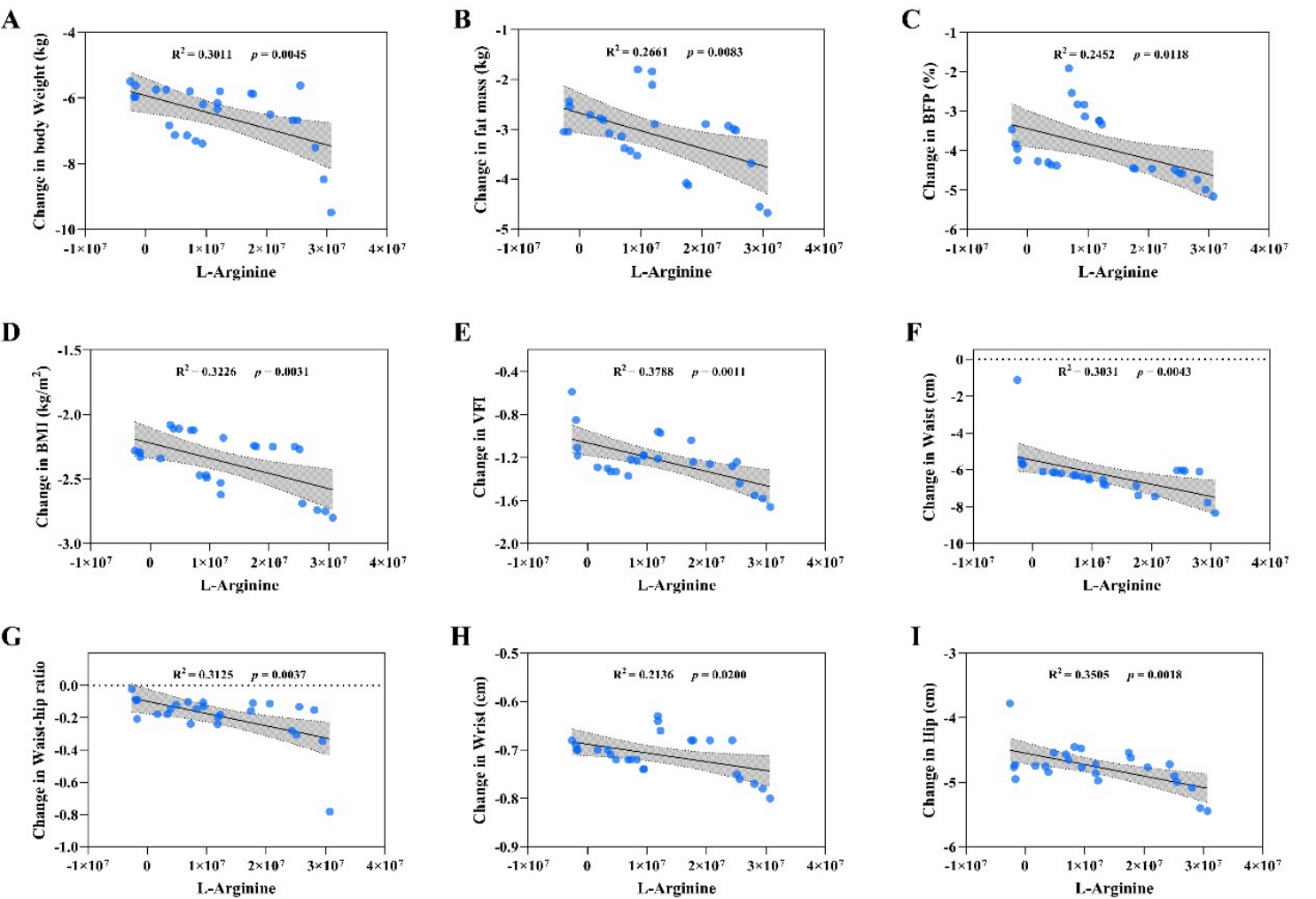


Figure 2

**Linear regression analysis of the association between changes in L-arginine levels and improvements in obesity-related phenotypes.** (A) Body weight; (B) Fat mass; (C) Body fat percentage (BFP); (D) Body mass index (BMI); (E) Visceral fat index (VFI); (F) Waist circumference; (G) Waist-to-hip ratio; (H) Wrist circumference; and (I) Hip circumference. The gray shaded areas represent the 95% confidence intervals of the regression curves.

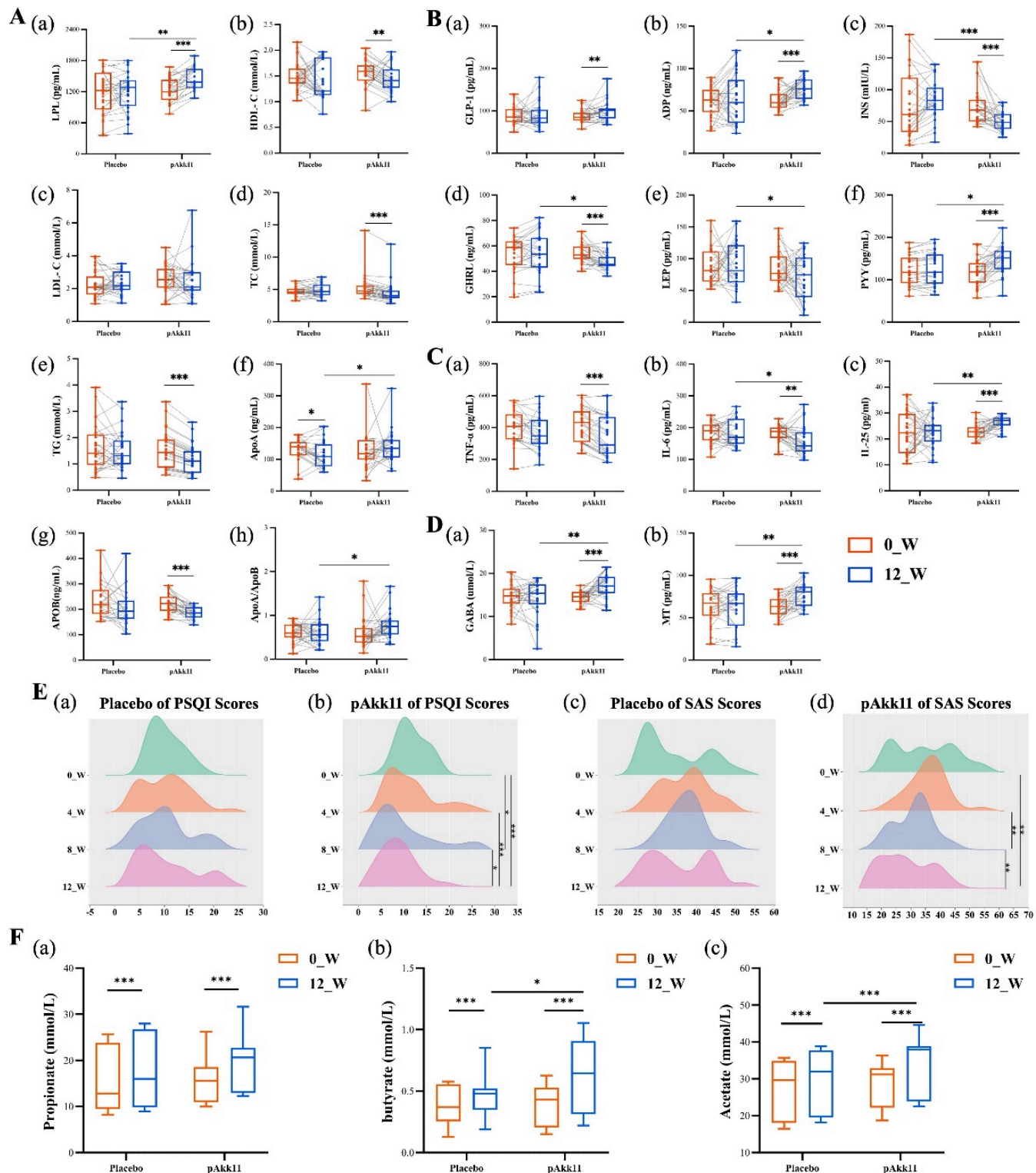


**Figure 3**

**Comparative analysis of various biochemical markers and psychological scores between placebo and pAkk11 groups at baseline (0\_W) and after 12 weeks (12\_W).** (A) Serum lipid profiles levels (LPL, HDL-C, LDL-C, TC, TG, apoA, apoB, apoA/apoB); (B) Glucose-related markers (GLP-1, ADPN, INS, GHRE, LEP, PYY); (C) Serum Inflammatory Cytokines levels (TNF- $\alpha$ , IL-6, IL-25); (D) Changes in neurotransmitter levels (GABA and MT); (E) Density plots illustrating the distribution of PSQI and SAS scores in (a)

placebo and (b) pAkk11 groups for PSQI scores, and (c) placebo and (d) pAkk11 groups for SAS scores; (F) SCFAs levels between groups at baseline and after the intervention (propionate, butyrate and acetate).

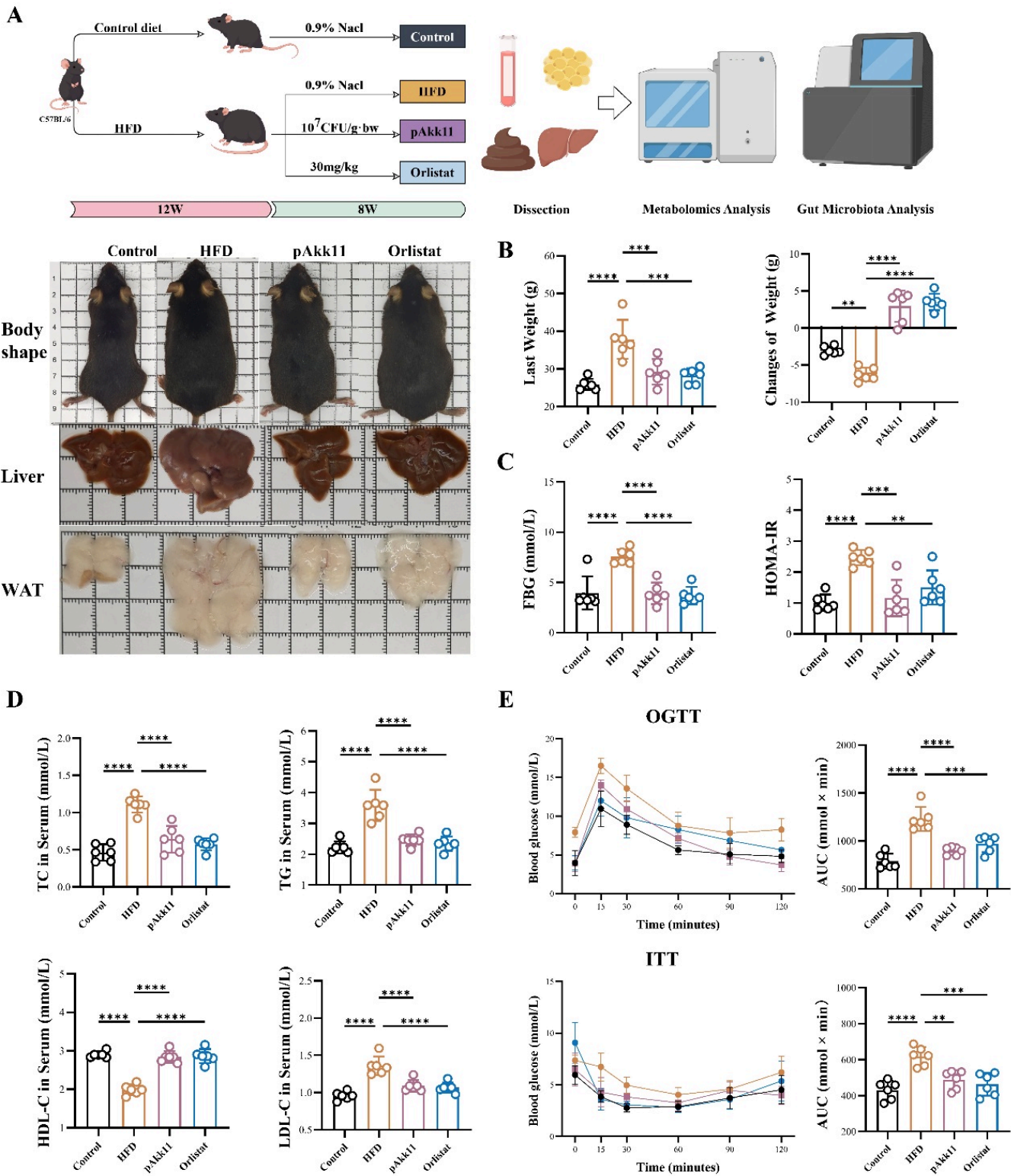


Figure 4

**pAkk11 Attenuates HFD-Induced Obesity and Metabolic Dysfunctions in C57BL/6 Mice.** (A) Schematic illustration of the animal experimental design and representative phenotypes of body shape, liver, and WAT in each group; (B) final body weight and body weight change during the intervention; (C) fasting blood glucose (FBG) and homeostasis model assessment of insulin resistance (HOMA-IR); (D) OGTT and ITT curves and corresponding AUC analyses; (E) Serum lipid levels (TC, TG, HDL-C, LDL-C).

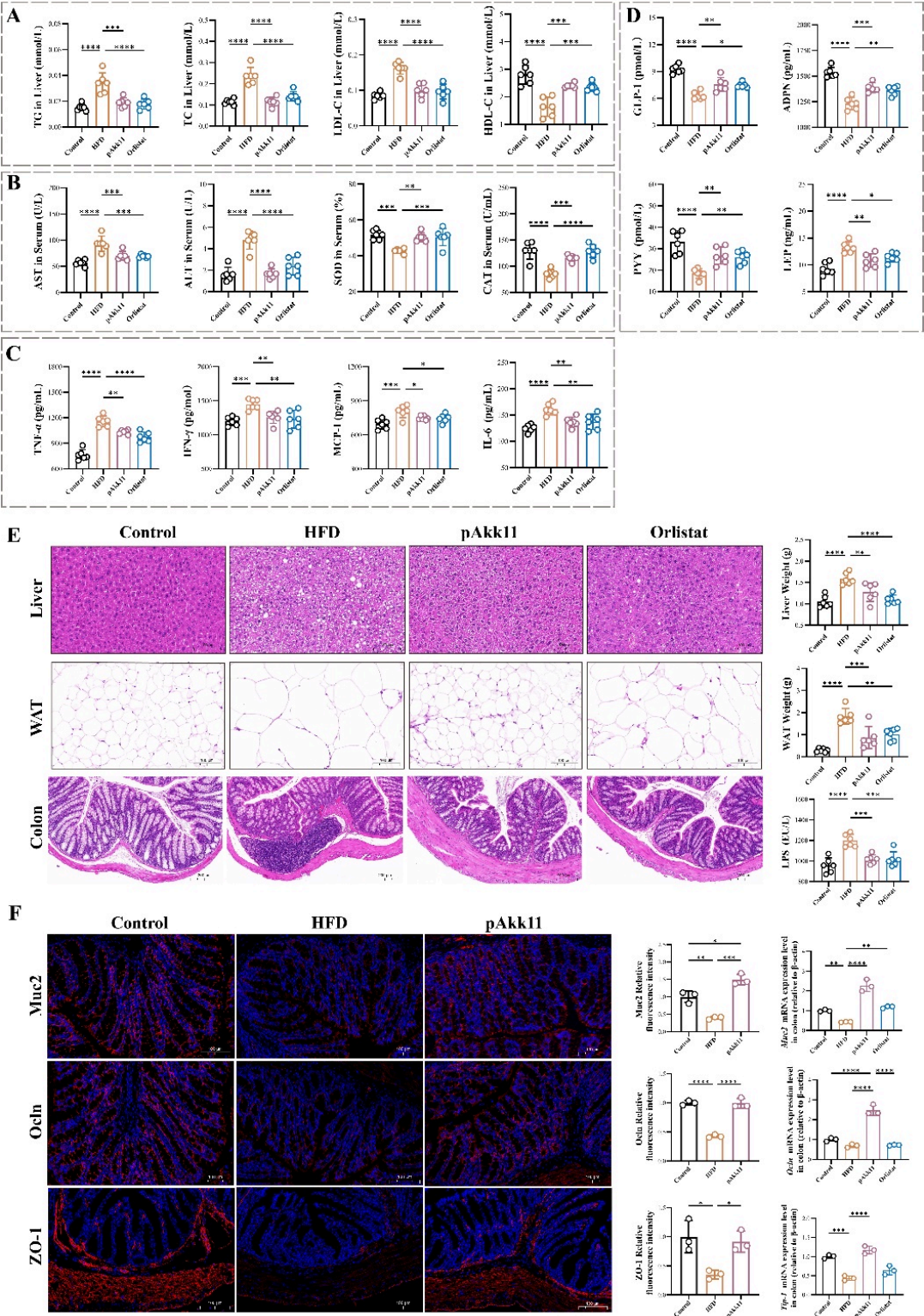


**Figure 5**

**pAkk11 ameliorates liver and adipose tissue injury and alleviates metabolic inflammation.** (A) Liver lipid levels (TC, TG, HDL-C, LDL-C); (B) Liver Function Related Indices (AST, ALT, SOD, CAT); (C) Serum Inflammatory Factors (IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, IL-6); (D) Fat-related hormone indicators (GLP-1, ADPN, PYY, LEP); (E) H&E staining of liver, WAT and colon tissue; (F) Immunofluorescence and mRNA expression analysis of Muc2, Ocln and ZO-1. Immunofluorescence staining of Muc2, Ocln and ZO-1 in colon tissues: Nuclei were counterstained with DAPI (blue); target proteins are shown in red; Quantitative analysis of relative fluorescence intensity of target proteins, and relative mRNA expression levels of *Muc2*, *Ocln* and *tjp1* (normalized to Control group, with  $\beta$ -actin as internal reference).



and serum; (B-e) Changes in the abundance of key differential metabolites in feces and serum; (B-f) correlations between L-arginine levels (serum and feces) and phenotypic indices.

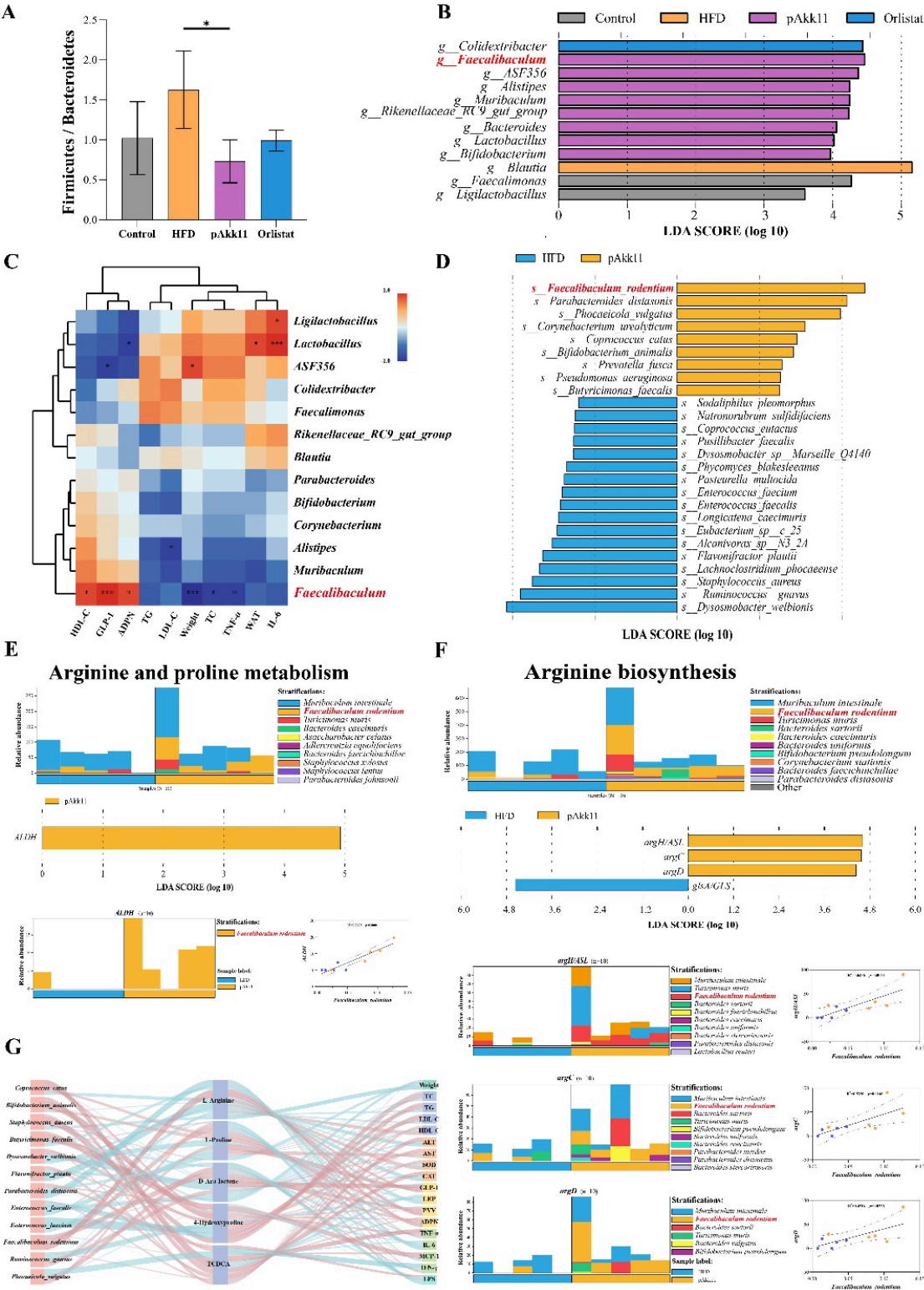


**Figure 7**

**Efficacy of pAkk11 on gut microbiota diversities and Metagenomic Analysis.** (A) The ratio of the Firmicutes to Bacteroidetes; (B) LEfSe analysis of differential gut microbial taxa at the genus level; (C)

Heatmap of correlations between gut microbiota at the genus level and obesity-related indices; (D) LEfSe analysis at Species level; (E) Hierarchical column chart of species sources related to arginine and proline metabolism and LEfSe analysis of key functional genes and hierarchical column chart of species sources; (F) Stratified bar plot of species sources contributing to arginine biosynthesis, and LEfSe analysis of key functional genes, and Species-source stratified bar charts of key functional genes (*argH*/*ASL*, *argC*, *argD* and *ALDH*); (G) Correlations between key bacterial strains, metabolites, and phenotypic indices.

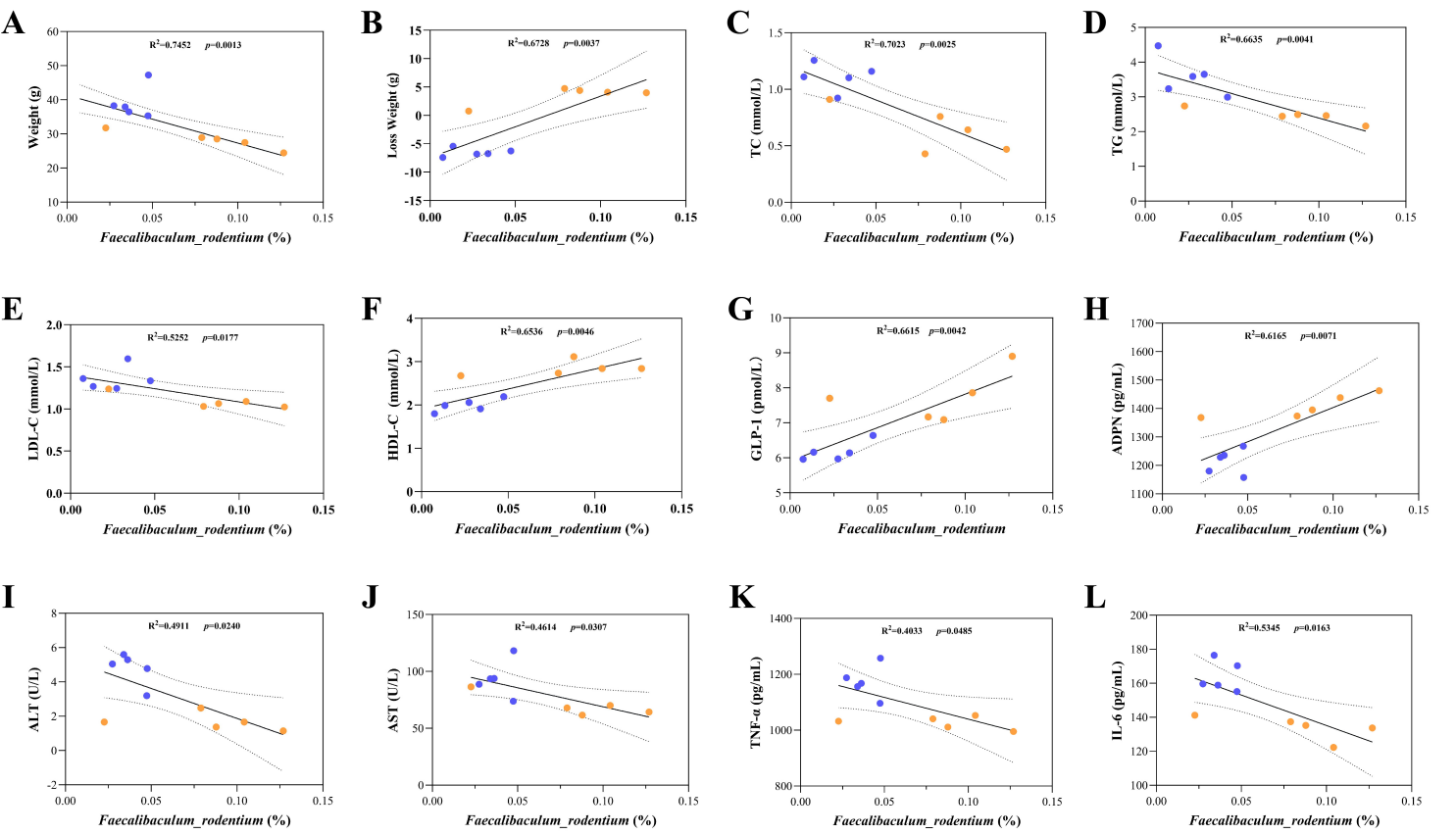


Figure 8

**Linear regression analysis of the association between the relative abundance of *Faecalibaculum rodentium* and alterations in metabolic and biochemical indicators.** (A) Weight; (B) Last weight; (C) TC; (D) TG; (E) LDL-C; (F) HDL-C; (G) GLP-1; (H) ADPN; (I) ALT; (J) AST; (K) TNF- $\alpha$ ; (L) IL-6. The dotted lines represent the 95% confidence intervals of the regression curves.

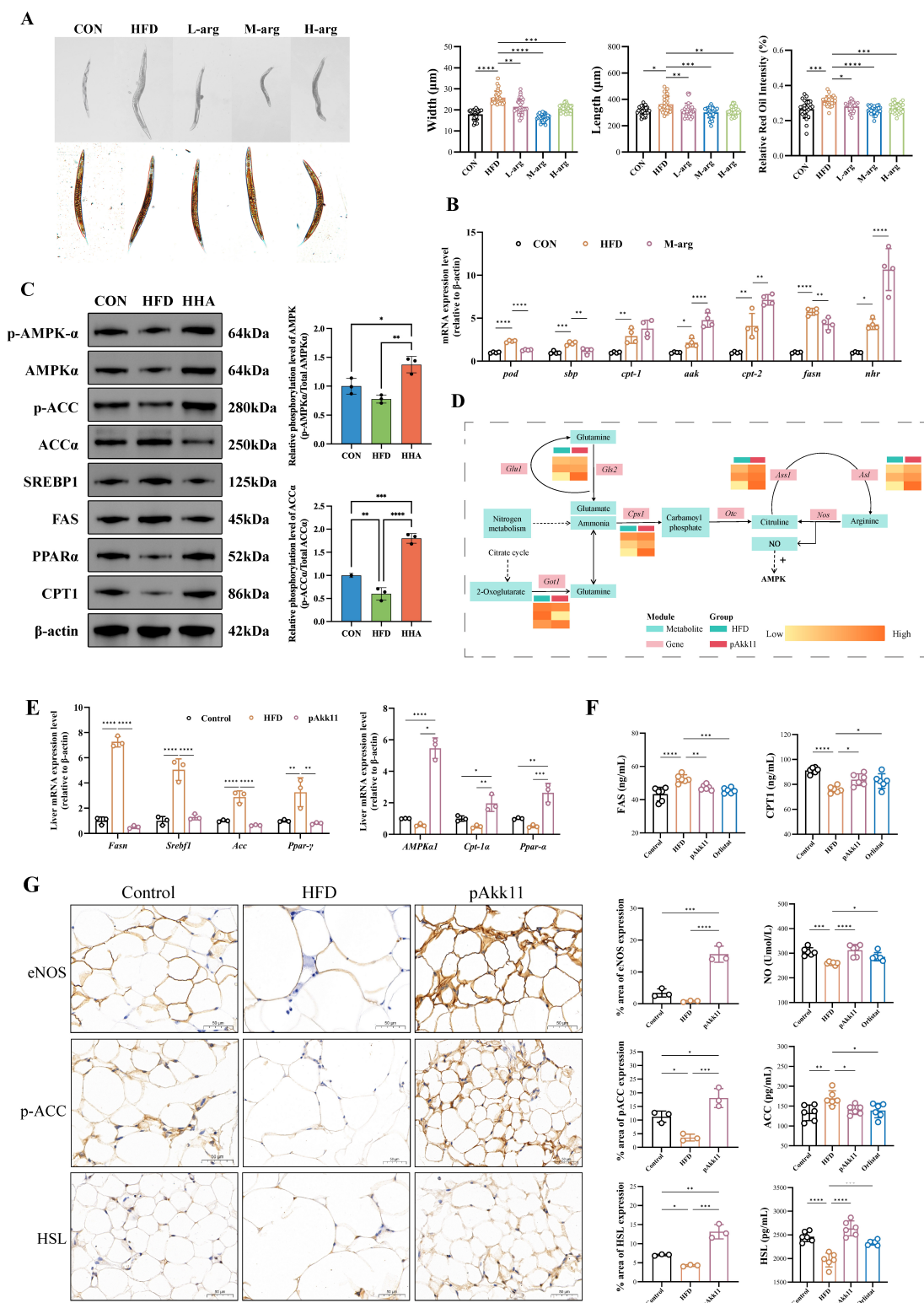
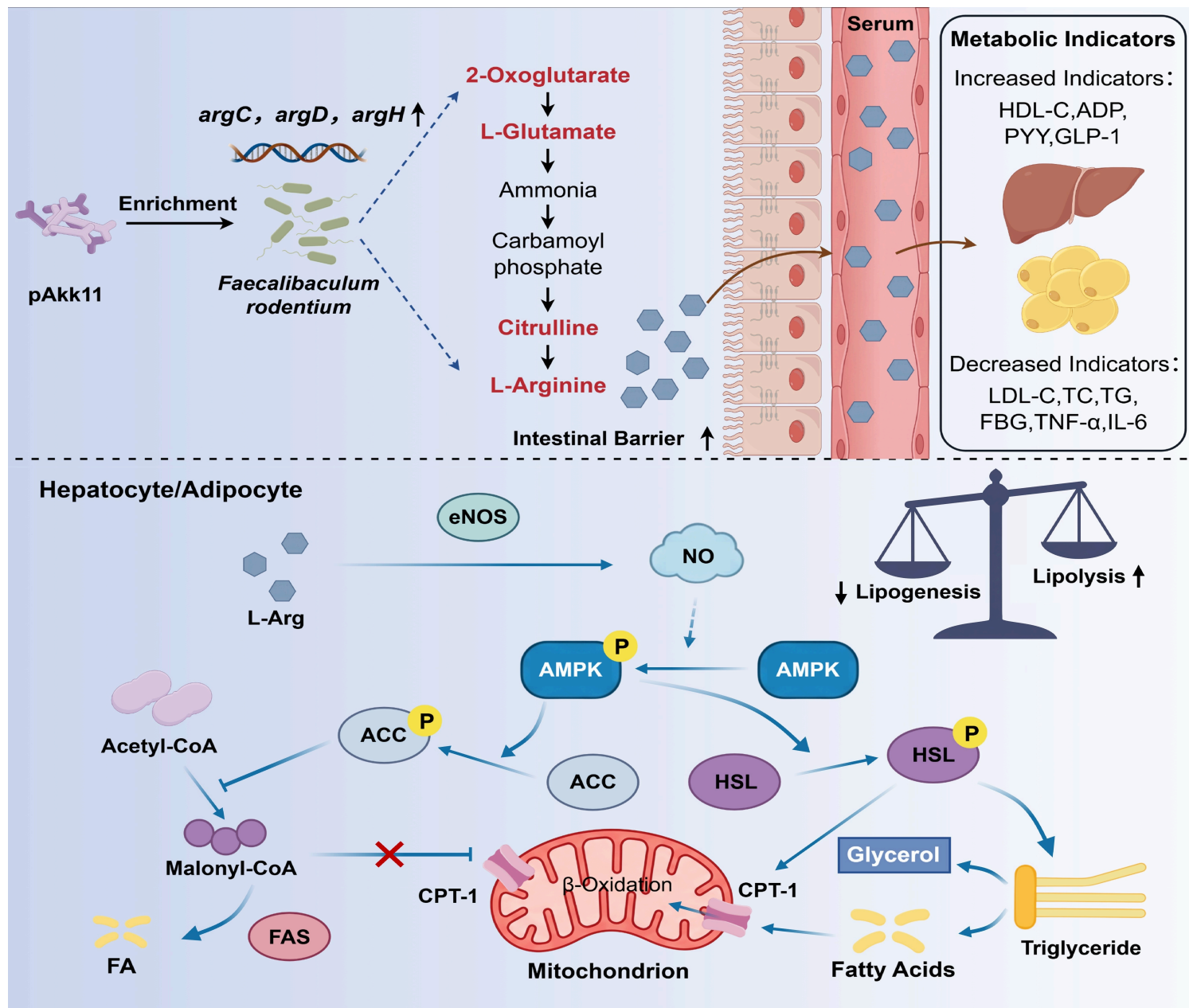


**Figure 9**

**L-arginine regulates lipid metabolism via the AMPK pathway.** (A) Representative images of nematode morphology, Oil Red O staining for lipid droplets, and quantitative analysis of body width, body length, and relative Oil Red O intensity in *C. elegans*; (B) Relative mRNA expression levels of lipid metabolism-related genes (*pod*, *sbp*, *cpt-1*, *aak*, *cpt-2*, *fasn*, *nhr*) in *C. elegans*; (C) Metabolic process of L-arginine; (D) Expression of key genes in the liver (normalized to Control group, with  $\beta$ -actin as internal reference); (E)

Changes in the levels of lipid metabolism-related indicators in serum (FAS, CPT1, NO, ACC, HSL); (F) Immunohistochemical analysis of adipose tissue (eNOS, p-ACC, HSL).



### Figure 10

### Schematic diagram illustrating the mechanism underlying pAkk11-mediated amelioration of obesity

## Supplementary Files

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