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Pasteurized *Akkermansia muciniphila* ameliorates insulin resistance by reducing placental inflammation in GDM mouse model

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ABSTRACT

Gestational diabetes mellitus (GDM) is a common and serious complication during pregnancy. Depleted next-generation probiotic, Akkermansia muciniphila (AKK) in GDM women indicates its potential on GDM prevention. However, the functions and mechanisms of AKK on GDM remain unclear. Due to the limited strategies for GDM therapy, combined with the anaerobic properties of AKK, herein, we reported pasteurized AKK functions as novel postbiotics which ameliorated glucose intolerance and insulin resistance in the GDM mouse model induced by high-fat diet (HFD) feeding combined with STZ. The oral administration of pasteurized AKK enhanced glucose homeostasis and alleviated placental inflammation in the GDM mouse model. Specifically, placental macrophage polarization was transferred by AKK treatment. In addition, the outer membrane protein of AKK, Amuc_1100, mimicked anti-inflammatory properties and improvement of GDM, which served as an effector protein. These findings demonstrate that oral AKK supplementation alleviated placental inflammatory responses through modulating macrophage polarization. Mechanically, we uncovered that a heat-stable outer membrane protein of AKK, Amuc_1100, mimics the anti-diabetic properties of pasteurized AKK through oral administration. Taken together, our findings demonstrated an effective treatment of GDM from the perspective of potential probiotic agents.

1. Introduction

Gestational diabetes mellitus (GDM) is a common complication during pregnancy [1]. According to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) diagnostic standard, the prevalence of GDM among Chinese pregnant women reached 15.6 % in 2024 [2], which is attributable to multiple factors, including the adoption of revised diagnostic criteria, progressive Westernization of lifestyle, and an increasing proportion of advanced maternal age pregnancies [2]. Prevention and management of GDM in China continue to face substantial challenges, such as non-uniform diagnostic standards, regional imbalance in health-care resources, and intrinsic limitations of conventional treatment paradigms [2,3]. In the long term, GDM also significantly increases the risk of type 2 diabetes (T2D), obesity, and other metabolic diseases of the mother and child [4], showing an "intergenerational effect" in clinical practice [5]. Hence, GDM is a great threat to female reproductive health and causes an economic burden for society. Current effective strategies involve lifestyle, dietary intervention, exercise guidance and insulin administration under specified conditions [6]. It should be considered that there are still adverse reactions, such as hypoglycemia and ketonuria. Therefore, an urgent medical need exists for GDM patients and their offspring.

In the second and third trimesters in metabolic diseases, the placenta is considered not only a strong secretory organ, but also a source and target of multiple pathological stimuli [7]. Previous researches attributed chronic inflammation and insulin resistance during pregnancy to multiple hormones and cytokines derived from the placenta [8–10]. Recently, Huang et al. explored the association between placental inflammation (reflected by TNF-α levels in the placenta, umbilical veins and arteries) and abnormal glucose homeostasis in GDMs, which indicated the causal role of placental inflammation in the alleviation of GDM-related insulin resistance [11]. As the main producers of TNF-α in

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the placenta, macrophages are one of the largest leukocyte groups at the maternal-fetal interface, which crucially remodel placental development and inflammation [12–14]. Mrizak et al. found severe infiltration of proinflammatory M1 macrophages and reduced anti-inflammatory M2 macrophages in GDM placentae, accompanied by excessive placental and circulating proinflammatory cytokines (IL-6, TNF-α), indicating deteriorated immune infiltration, macrophage phenotype changes and inflammatory status involve in GDM [15].

Akkermansia muciniphila (AKK) is a member of the *Verrucomicrobia* and is a common gut symbiont, which is considered as " next-generation probiotics" [16]. Studies have shown that AKK increases thermogenesis and secretion of glucagon-like peptide-1 (GLP-1) in mice induced by a high-fat diet (HFD), reduces blood glucose, and improves insulin resistance [17]. In addition, AKK alleviates inflammation in T2D mice, thereby improving diabetic symptoms [18]. It is noted that the intestinal AKK abundance in GDM women is sharply reduced, indicating its potential biological functions in GDM therapy [19]. Based on anaerobic activity, pasteurized AKK exhibits stronger effects in improving glucose tolerance and insulin resistance, as well as reducing energy absorption in mice, compared to live AKK. Therefore, heat-killed AKK is considered better clinical safety and prospects for clinical application. Amuc_1100 is a specific protein isolated from the outer membrane of AKK, which retains biological activity at pasteurization temperature and exerts beneficial effects [20,21]. Exploring the functions and mechanisms of action of pasteurized AKK in glucose homeostasis maintenance has become an attractive issue for seeking breakthroughs in GDM prevention and therapy.

The present study uncovered a novel therapeutic strategy of pasteurized AKK in improving GDM. Functionally, oral supplementation effectively lowered blood glucose, improved insulin resistance and recovered the immuno-inflammatory response of placental macrophages in GDM mice. Furthermore, we identified purified outmembrane protein of AKK, Amuc_1100, mimics the anti-diabetic properties of heat-killed AKK through oral administration. Taken together, we demonstrated an effective treatment of GDM from the perspective of potential probiotic agents.

2. Materials and methods

2.1. Culture and pasteurization of AKK

AKK (ATCC BAA-835) was cultured in brain heart infusion (BHI) broth under strict anaerobic conditions. This culture was diluted with anaerobic PBS containing 2.5 % glycerol to a dose of 1.5×10^8 CFU/100 μL. AKK was inactivated by pasteurization for 30 min at 70°C. After pasteurization, no viable AKK could be recovered in culture.

2.2. Expression and purification of Amuc_1100

The PCR product of a His-tagged Amuc_1100 was cloned into the pET23a-N-His vector (OriGene Technologies, Wuxi, China) to construct the expression plasmid. The plasmid pET-23a-Amuc_1100 was transformed into Rosetta (DE3) pLysS competent cells, cultured in LB broth with ampicillin (50 μg/mL) at 37°C with 220 rpm shaking. Isopropyl β-D-1-thiogalactopyranoside (0.5 mM) was added to the LB broth to induce Amuc_1100 expression at the logarithmic growth phase. The bacteria were harvested when the OD_{600} was over one and then lysed by sonification, the centrifugal supernatant was used to purify the Amuc_1100 protein with Ni-16NTA His·Bind. Amuc_1100 protein was stored at −80°C until use.

2.3. Animals

C57BL/6 J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All animal procedures were approved by the Nanjing Medical University Committee on the Care and Use of Animals (permit number IACUC-2011052). The mice were maintained in a temperature- and humidity-controlled environment with a 12-h light-dark cycle. To establish the GDM model, 8-week-old female mice were continuously fed a high-fat diet (HFD) for 4 weeks. Then, female and male mice were bred overnight in a 2:1 ratio, and successful mating was evidenced by the presence of a vaginal mucous plug in females the next morning, which was regarded as gestation day G0.5d. After 16-hour starvation, female mice were injected intraperitoneally (i.p.) with either sodium citrate buffer or streptozotocin (STZ, 30 mg/kg) for three consecutive days. After fasting overnight on G18d, all the pregnant female mice were sacrificed, the blood and placental samples were harvested for subsequent detections. Animals were sacrificed at indicated time points, and blood and tissue samples were harvested for molecular biology, histology, and biochemistry analyses.

For AKK or Amuc_1100 administration, according to the previous study [20], after 3-week HFD feeding, female mice in the GDM group were gavaged sterile PBS (200 μL), pasteurized AKK (2×10^8 CFU) and purified Amuc_1100 (3 μg) every day, respectively.

2.4. In vivo imaging of purified Amuc_1100 in mice

To monitor the biodistribution of purified Amuc_1100 in mice, Amuc_1100 were labeled with DiR fluorescence dyes (DiR lodide, Yeasen, 40757ES25) and administered by oral gavage. Briefly, Amuc_1100 (3 μg) was incubated with DiR (30uM) at 37°C for 30 min. Mice were anesthetized and imaged over a 24-hour period using IVIS Spectrum In Vivo Imaging System (PerkinElmer, Boston, USA). Afterward, organs were collected and imaged using IVIS. The PBS was administered to mice as control.

2.5. Cell culture and polarization stimulation

RAW 264.7 and THP-1 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). For RAW 264.7, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, Waltham, MA, USA) and 1 % antibiotics (penicillin/streptomycin, KeyGen Biotech, Nanjing, China) at 37 ℃ in a humidified atmosphere containing 5 % CO2. Cells were pretreated with or without BAY 11–7082 (5 μM) for 30 min, and were differentiated into M1 by 20 ng/mL Interferon-γ (IFN-γ, proteintech, Chicago, IL, USA) and 100 ng/mL lipopolysaccharide (LPS, sigma, Louis, MO, USA) for 24 h. For THP-1 cells, cells were cultured in RPMI1640 medium and differentiated into M0 by 40 ng/mL Phorbol-12-Myristate-13-Acetate (PMA, sigma, Louis, MO, USA) for 24 h. M0 THP-1 cells were further differentiated into M1 by 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 h. M1 RAW 264.7 and THP-1 cells were incubated of pasteurized AKK bacterium (AKKs: cells=10:1) and purified Amuc_1100 (5 μg/mL) for 24 h.

2.6. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For the GTT analysis, mice were fasted for 16 h and then i.p. injected with glucose (1 g/kg body weight) at G13d. For ITT analysis, mice were fasted for 6 h and i.p. injected with insulin (0.75 U/kg body weight) at G15d. Blood glucose was measured before the injection and 15, 30, 60, 90 and 120 min after the *i.p.* injection with a glucose monitor (Roche Diagnostics, Indianapolis, IN, USA). Areas under curve (AUC) was calculated and statistically analyzed by Origin8 (Version 8.6, OriginLab, Northampton, MA, USA).

2.7. Detection of serum insulin and homeostasis model assessment for insulin resistance (HOMA-IR)

Serum samples were collected by centrifugation at 4000 rpm for 10 min at 4 ℃, insulin level was detected using a Mouse Insulin ELISA

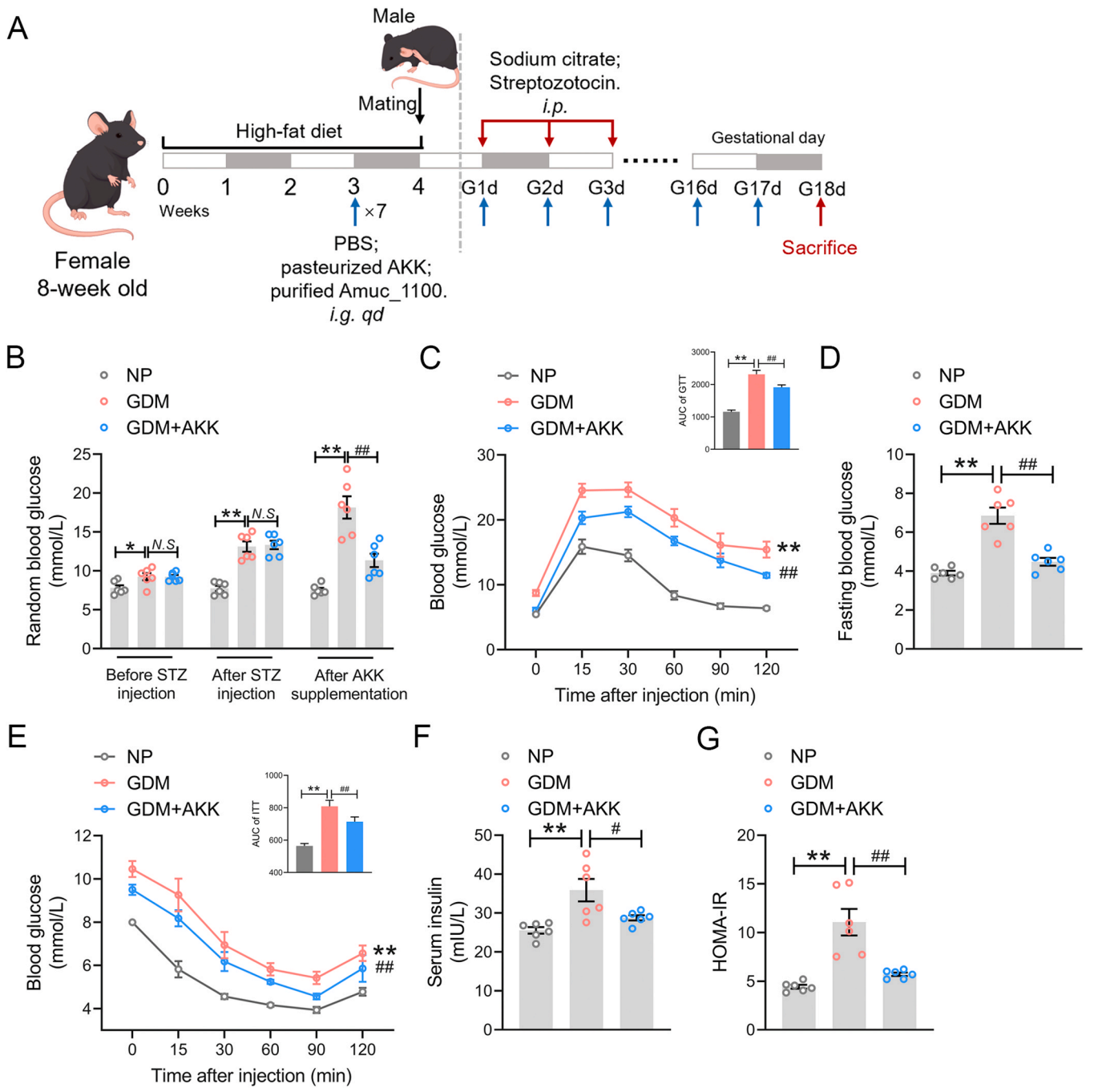

Fig. 1. Pasteurized AKK improves disordered glucose homeostasis in GDM mice. HFD-fed female mice were administrated with STZ to establish the GDM model, and then pasteurized AKK was given by oral supplementation (n = 6 per group). (A) Experimental flow chart (B) Random blood glucose (RBG) before STZ injection, RBG after STZ injection and RBG after one-week gavage of pasteurized AKK; (C) Glucose tolerance test (GTT) and AUC; (D) Fasting blood glucose (FBG) after one-week gavage of pasteurized AKK; (E) Insulin tolerance test (ITT) and AUC; (F) Serum insulin level; (G) HOMA-IR index. Data are presented as mean ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus NP; $^{\#}P < 0.05$ and $^{\#\#}P < 0{\cdot}01$ versus GDM, unpaired two-tailed Student's *t*-test.

Kit (Cat no., ml001983, Shanghai Enzyme-linked Biotech., Shanghai, China), following the manufacturer's protocol. Fasting blood glucose was determined with a glucometer at G18d after 16-hour starvation before blood collection.

The HOMA-IR was calculated according to the following formula: HOMA-IR = Fasting insulin (mIU/L) × Fasting glucose (mmol/L)÷22.5.

2.8. Enzyme-linked immunosorbent assay (ELISA)

IL-6 (ml098430 for mouse, ml058097 for human), TNF-α (ml002095 for mouse, ml0378734 for human), leptin (ml028544 for mouse, ml028534 for human), adiponectin (ml107260 for mouse, ml106105 for human) and IL-10 (ml037873 for mouse, ml064299 for human) levels were measured by corresponding ELISA Kits (Shanghai Enzyme-linked Biotech., Shanghai, China), following the manufacturer's protocol.

2.9. Immunofluorescent staining

Frozen placentae were cut into 6-μm transverse sections. The sections were treated overnight at 4 ℃ with a negative control reagent (5 % goat serum, AR0006, Boster, Wuhan, China) or diluted primary antibodies. The antibodies against iNOS (Cat no., GB123965–100) and

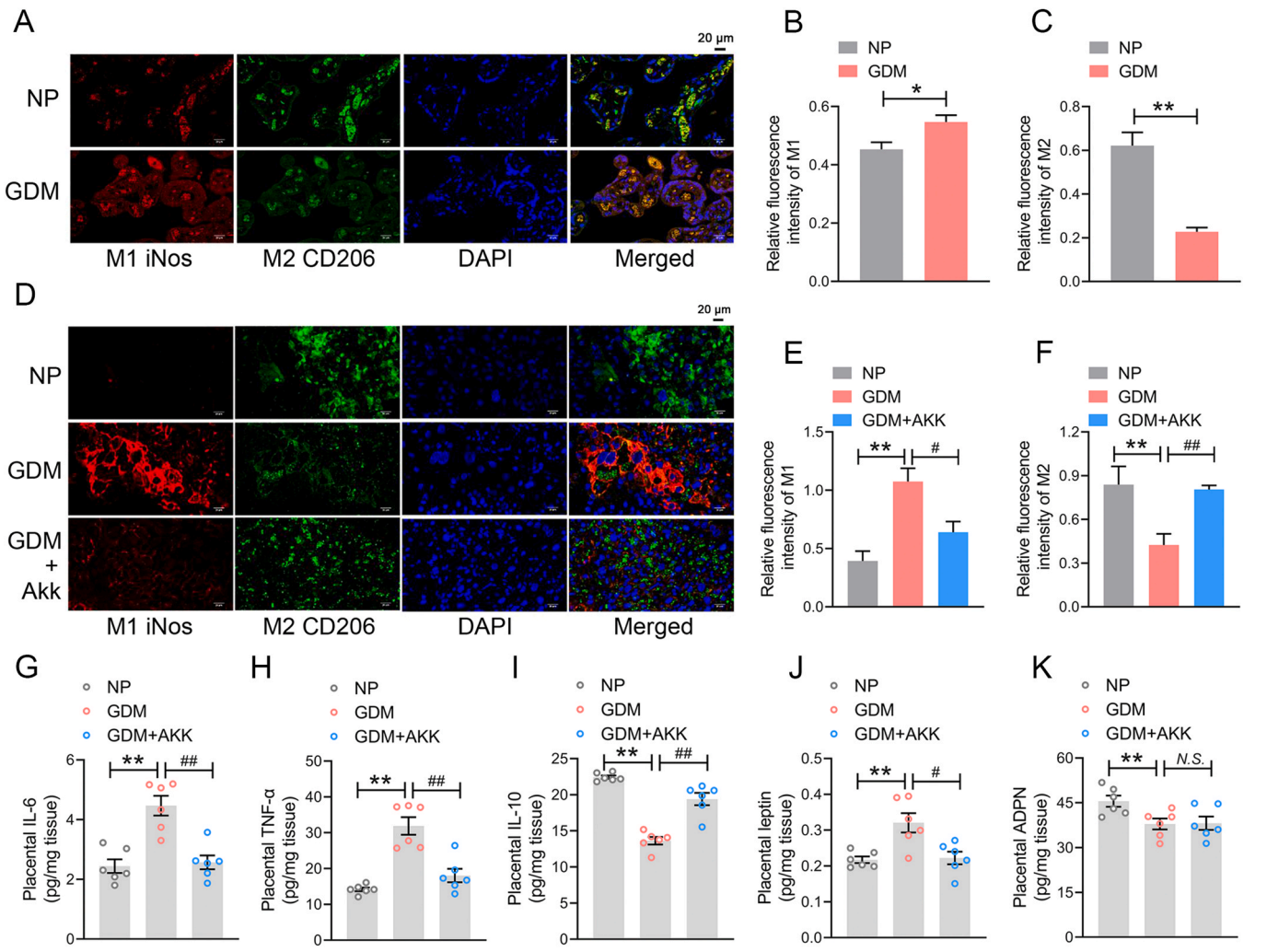

Fig. 2. Pasteurized AKK reduces placental inflammation in GDM mice. (A) Representative images from immunofluorescent staining in human placental sections. HFD-fed female mice were administrated with STZ to establish the GDM model, then pasteurized AKK was given by oral supplementation (n = 6 per group); (B) Relative fluorescence intensity of M1 in human placental sections. (C) Relative fluorescence intensity of M2 in human placental sections; (D) Representative images from immunofluorescent staining in mouse placental sections; (E) Relative fluorescence intensity of M1 in mouse placental sections; (F) Relative fluorescence intensity of M2 in mouse placental sections; (G) Placental IL-6 level; (H) Placental TNF-α level; (I) Placental IL-10 level; (J) Placental leptin level; (K) Placental adiponectin level. Data are presented as mean ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus NP; $^{\#}P < 0.05$ and $^{\#\#}P < 0{\cdot}01$ versus GDM, unpaired two-tailed Student's *t*-test.

CD206 (Cat no., GB113497–100) were purchased from Servicebio (Wuhan, China, 1:400 dilution). Next, the slides were incubated with anti-mouse and anti-rabbit immunofluorescent (Alexa Fluor 555/488 Conjugate) secondary antibodies (Cell Signaling Technology, Danvers, CO, USA).

2.10. Human subjects

3 normal pregnant women and 3 GDM pregnant women hospitalized for delivery in Nanjing Women and Children's Healthcare Hospital from October 2022 to October 2023 were selected. Placenta tissues were collected, washed in PBS and immediately stored in 4 % paraformaldehyde. This study was approved by the Ethics Committee of Nanjing Women and Children's Healthcare Hospital (2018KY-007), all the patients have signed the informed consent.

2.11. Statistical analysis

Data were analyzed using GraphPad Prism 8.0 software by analysis of unpaired two-tailed Student's *t*-test for two groups. Statistical significance was defined as $^{*}P < 0.05$ and $^{**}P < 0.01$ versus NP; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus GDM. All the data are presented as mean ± SD.

3. Results

3.1. Pasteurized AKK improves disordered glucose homeostasis in GDM mice

To evaluate the biological potential of pasteurized AKK in GDM, HFD-fed female mice were administrated with STZ to establish the GDM model according to flow chart (Fig. 1A). As shown in Fig. 1B, there were slightly significant differences in random blood glucose (RBG) between NP and GDM groups before the STZ injection ($0.01 < P < 0.05$); the administration of STZ elevated RBG up to 11.1 mmol/L in mice ($P < 0.01$), indicating that the GDM mouse model was successfully established; after oral supplementation of pasteurized AKK, RBG was dramatically lower than the GDM group, suggesting that AKK intervention relieved glucose metabolic disorder in GDM mice.

Since glucose tolerance and insulin sensitivity reflect glucose

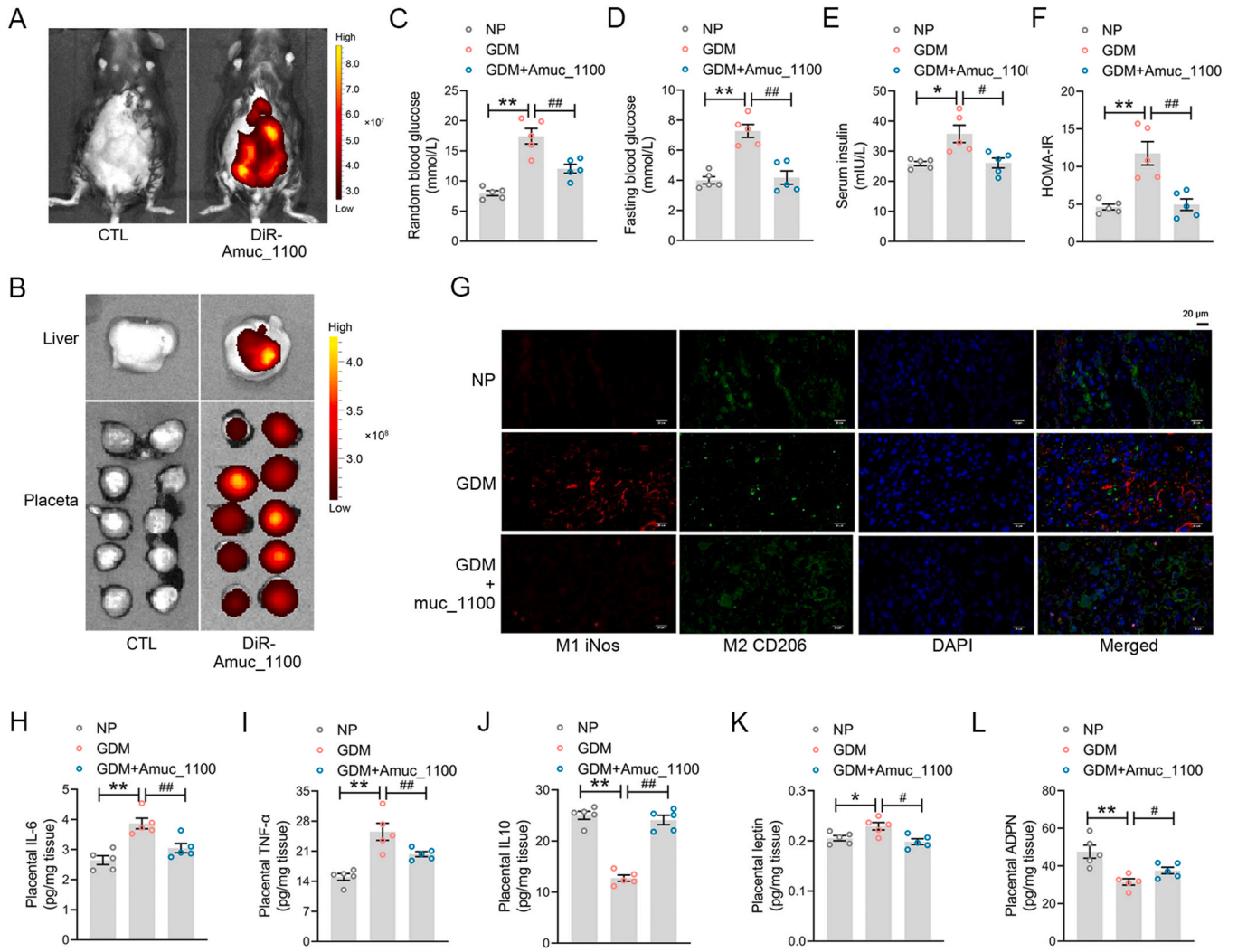

Fig. 3. Purified Amuc_1100 maintains glucose homeostasis and reduces placental inflammation in GDM mice. *In vivo* imaging analysis after oral gavage of Amuc_1100 labeled with DiR fluorescence dyes, (A) Whole-body distribution of Amuc_1100; (B) Distribution of Amuc_1100 in liver and placenta. HFD-fed female mice were administrated with STZ to establish the GDM model, then purified Amuc_1100 was given by oral supplementation (n = 5 per group). (C) RBG; (D) FBG; (E) Serum insulin level; (F) HOMA-IR index; (G) Representative images from immunofluorescent staining in mouse placental sections; (H) Placental IL-6 level; (I) Placental TNF-α level; (J) Placental IL-10 level; (K) Placental leptin level; (L) Placental adiponectin level. Data are presented as mean ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus NP; $\#P < 0.05$ and $\#\#P < 0.01$ versus GDM, unpaired two-tailed Student's *t*-test.

homeostasis, we performed glucose tolerance test (GTT) at G13d. Compared with the GDM group, pasteurized AKK-treated mice exhibited higher glucose tolerance and reduced fasting blood glucose (Fig. 1C and D), indicating that AKK administration enhances glucose clearance in GDM individuals. Maternal and fetal risks in GDM are mainly due to severe insulin resistance of the mother, insulin tolerance test (ITT) at G15d suggested a higher insulin sensitivity after AKK gavage (Fig. 1E). Furthermore, we also observed improved excessively high serum insulin level and HOMA-IR (homeostasis model assessment of insulin resistance) (Fig. 1F and G). Collectively, oral supplementation of pasteurized AKK enhances glucose homeostasis in the GDM mouse model.

3.2. *Pasteurized AKK reduces placental inflammation in GDM mice*

Placental-derived inflammation is reported to be associated with abnormal maternal glucose metabolism in GDMs. We performed immunofluorescence co-localization of macrophage markers in the human placenta, as shown in Fig. 2A-C, GDM status induced human placental M1 polarization ($iNOS^{+}$) and reduced M2 polarization ($CD206^{+}$), which suggests a higher inflammatory environment in GDM placentae. Accordingly, a similar tendency of macrophage polarization was observed in mouse placentae, accompanied by reversed placental proinflammatory alteration induced by pasteurized AKK (Fig. 2D-F). These data suggested that AKK alleviated placental inflammatory responses through modulating macrophage polarization.

During pregnancy, the placenta functions as a temporary secretory organ, mainly produce IL-6 and TNF-α. In GDM mice, placental IL-6 and TNF-α contents were abnormally elevated, which could be dramatically suppressed by pasteurized AKK (Fig. 2G-H). Consistently, pasteurized AKK also regulated serum inflammatory cytokines (Supplementary Figure S1A-B). Besides, we also observed rescue effect of AKK on placental and serum IL-10 concentration, which is recognized as M2 type-specific cytokines (Figs. 2I and Supplementary Figure S1C). Several placentae-derived hormones reflect insulin resistance and inflammation, such as prolactin, placental growth hormone, leptin, adiponectin, etc. We detected circulating and placental levels of leptin and adiponectin, and found that oral AKK intervention reversed abnormal leptin secretion, but not adiponectin (Figs. 2J-K and Supplementary Figure S1D-E). In summary, the results indicated that pasteurized AKK influenced the secretory function of placentae, which is involved in inflammation and insulin resistance.

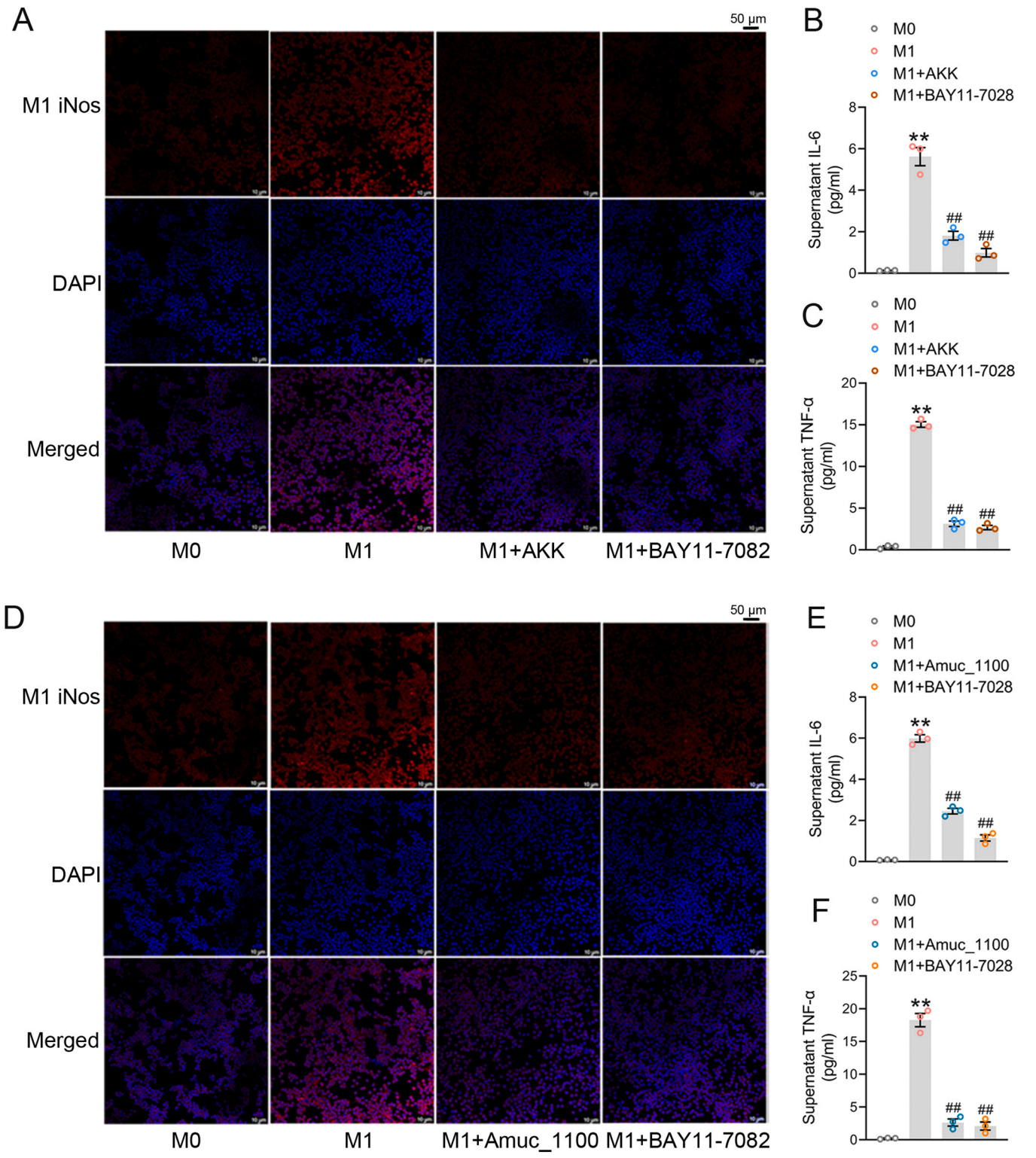

Fig. 4. Pasteurized AKK or purified Amuc_1100 regulates RAW 264.7 macrophage polarization. RAW264.7 cells were pretreated with BAY 11–7082 (5 μM) for 30 min, followed by stimulation with 20 ng/mL IFN-γ and 100 ng/mL LPS for 24 h. Cells were incubated with pasteurized AKK after differentiation (n = 3 per group). (A) Representative images from immunofluorescent staining in RAW264.7 macrophages; (B) Supernatant IL-6 level; (C) Supernatant TNF-α level. Cells were incubated with purified Amuc_1100 after differentiation. (D) Representative images from immunofluorescent staining in RAW264.7 macrophages after Amuc_1100 incubation; (E) Supernatant IL-6 level; (F) Supernatant TNF-α level. Data are presented as mean ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus NP; $\#P < 0.05$ and $\#\#P < 0.01$ versus GDM, unpaired two-tailed Student's *t*-test.

3.3. Purified Amuc_1100 maintains glucose homeostasis and reduces placental inflammation in GDM mice

To monitor the biodistribution and ensure stability of purified Amuc_1100 in gastrointestinal environment, we performed oral supplementation of Amuc_1100 labeled with DiR fluorescence dyes in pregnant mice. *In vivo* imaging revealed a widespread distribution of Amuc_1100, with pronounced accumulation in the liver and placenta tissue (Fig. 3A-B), substantiating stability in the circulation and capacity that reaches the placenta of Amuc_1100 to exert potential biological effects. To further explore whether purified Amuc_1100 mimics the biological functions of pasteurized AKK, we performed oral supplementation of Amuc_1100 in GDM mice. RBG and FBG were both reduced in the Amuc_1100 group (Fig. 3C-D). Meanwhile, increased fasting serum insulin level and HOMA-IR were also reversed by purified Amuc_1100 (Fig. 3E-F). The results clarified that Amuc_1100 serves as a hypoglycemic agent in the GDM model. Similarly, in mouse placentae, oral Amuc_1100 treatment significantly reduced $iNOS^+$ M1 macrophages and induced $CD206^+$ M1 macrophages (Figs. 3G and Supplementary Figure S2), suggesting Amuc_1100 protein mimics anti-inflammatory properties of AKK gut bacteria in placental microenvironment.

Moreover, IL-6, TNF-α and IL-10 were significantly rescued by Amuc_1100 administration (Fig. 3H-J), with reduced circulating levels (Supplementary Figure S3A-C). In GDM mouse placentae and serum, we observed elevated leptin and suppressed adiponectin concentrations, and Amuc_1100 supplementation triggered the reversed tendency of placental and circulating hormones (Fig. 3K-L and Supplementary Figure S3D-E).

3.4. Pasteurized AKK or purified Amuc_1100 regulate macrophage polarization

Since we have confirmed pasteurized AKK and purified Amuc_1100 altered the placental inflammatory microenvironment *in vivo*, we selected RAW 264.7 and THP-1 cell line to dissect the direct anti-inflammatory effects of pasteurized AKK and Amuc_1100. AKK incubation (AKKs: cells=10:1) robustly inhibited M1 polarization ($iNOS^+$) (Fig. 4A and Supplementary Figure S4A), and reduced supernatant IL-6 and TNF-α (Fig. 4B-C and Supplementary Figure S4B-C). Similarly, incubation of Amuc_1100 also repressed M1 polarization and excessive inflammatory cytokine secretion of RAW 264.7 and THP-1 cells (Fig. 4D-F and Supplementary Figure S4D-F).

4. Discussion

GDM is the most common metabolic disorder that occurs during pregnancy, and its incidence is continuously increasing worldwide, along with elevated risks of adverse pregnancy outcomes for both mother and child [22]. Current clinical strategies for GDM mainly involve improving lifestyle habits (diet, exercise, etc.), with insulin supplementation therapy necessarily [6]. However, limited therapeutic effects spawn significant demands for clinically exploring novel therapy for GDM. In the present study, we identified pasteurized AKK as a therapeutic involved in GDM improvement. The oral administration of pasteurized AKK enhanced glucose homeostasis and alleviated placental inflammation in the GDM mouse model. Specifically, placental macrophage polarization was transferred by AKK treatment. In addition, the outer membrane protein of AKK, Amuc_1100, mimicked anti-inflammatory properties and improvement of GDM, which served as an effector protein.

Accumulating evidence has confirmed that GDM has emerged as a widespread health issue, imposing significant challenges on global health systems and economies [1]. Over recent decades, the incidence of GDM has been on an upward trend, suggesting that it will persist in the coming years. Metabolic disorders that arise from imbalances of gut microbiota are considered as crucial predisposition of GDM [23]. Balanced diversity and community of gut microbiota are linked to metabolic diseases, including T2D and obesity, etc. [24]. Defective glucose tolerance is always accompanied by increased risks of GDM in overweight and obese pregnant women [25,26]. Relationships between the gut microbiome and GDM status were extensively discovered, which indicates that detection in microbial composition is potentially employed to identify individuals at risk for GDM [27].As a keystone commensal that colonizes the intestinal mucus layer; AKK abundance serves as a barometer of gut microecological homeostasis. Previous study demonstrated that AKK degrades mucin into acetate, propionate, and other short-chain fatty acid (SCFA) precursors, thereby supplying cross-feeding substrates for butyrate-producing taxa such as *Faecalibacterium* and *Roseburia* [28]. AKK supplementation augments SCFA production, reinforces the epithelial barrier, restrains inflammatory responses and modulates gut microbiota [29–32]. Consequently, AKK functions both as an “ecosystem engineer” for butyrate producers and as a “natural brake” on opportunistic pathogens, and its fluctuation directly sculpts the metabolic and immunological landscape of the gut microbiota.

AKK has long been considered a potential next-generation probiotic from bench to bedside. Recently, Yang et al. reported a novel strategy in the prevention of GDM from the perspective of functional food, xylooligosaccharides ameliorate insulin resistance in GDM mice, through improving gastroenterological dysfunction and enriching intestinal AKK abundance [27], which implies potential biological functions of AKK probiotics on preventing GDM. Consistent with the evidence above, pasteurized AKK was directly supplemented in the present study, which provides a novel and preclinical strategy for preventing GDM. As anaerobic probiotics initially cultured from human gut microbiota, active AKK is susceptible to the external environment (such as temperature, stomach acid, bile and antibiotics administration, etc.), resulting in instability in preservation, transportation, consumption and absorption [33]. However, the application of pasteurized probiotics partially avoids disturbance by external factors, and infection risk that may exist in live bacteria, which extends marketing values in the development of microecological agents. Furthermore, inactivated probiotics naturally avoid potential mutations, resistance gene transfer and other risk factors that exist in alive bacteria growth and reproduction, which indicates that inactivated probiotics are safer and more suitable for hypo-immunity populations, such as pregnant women [34]. Therefore, in the present study, pasteurized AKK is an optimal and more suitable option for improving GDM, compared with alive AKK.

The application of pasteurized AKK breaks the mainstream opinion that only oral administration of live probiotics is beneficial for therapeutic use, such as alleviation of inflammation, obesity, T2D, and non-alcoholic fatty liver disease, which makes pasteurized AKK at the forefront of translational application research. In the previous study, Clara et al. published the first clinical trial of AKK, indicating that supplementation of inactivated AKK significantly improves multiple metabolic indicators in overweight/obese human volunteers without any intolerant and adverse responses [35]. Together with other associated studies, concepts and application potentials of postbiotics are extensively explored and discussed. In 2021, the International Scientific Association for Probiotics and Prebiotics (ISAPP) issued a consensus statement on the definition and scope of postbiotics [36]. Based on postbiotics, specific inactivated bacteria or active components have become research hotspots. Amuc_1100, a heat-stable outer membrane protein derived from AKK, has exhibited remarkable biological effects on resisting metabolic disorders [35]. Recent animal research illustrated that oral Amuc_1100 supplementation facilitated lipolysis and thermogenesis through activating the AC3/PKA/HSL pathway, substantially improving obesity [37]. Amuc_1100 was also reported to promote the biosynthesis and extracellular availability of intestinal 5-HT (serotonin) through the TLR2 signaling pathway, as well as modulate the composition of intestinal flora and tryptophan metabolism, indicating infected

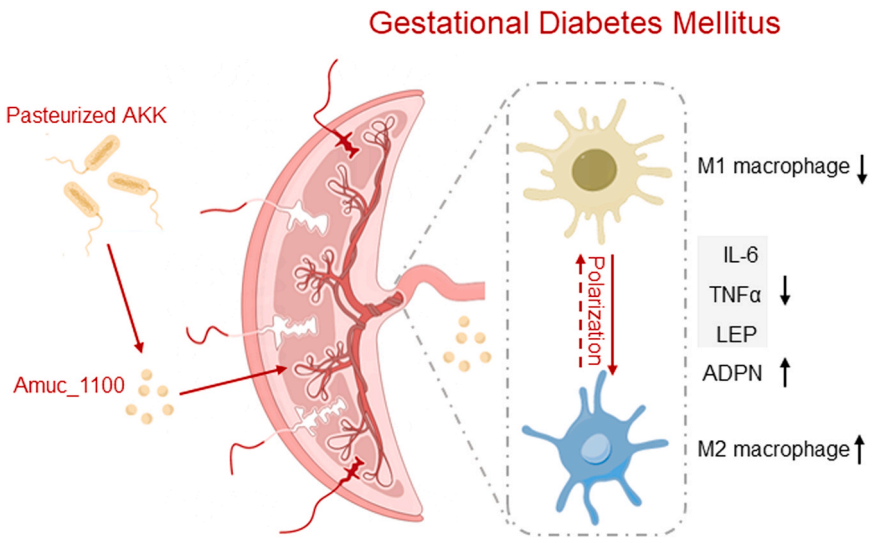

Fig. 5. The functional model illustrating the mechanism by which pasteurized AKK ameliorates GDM.

gastroenteric function and homeostasis [38]. Explorations of Amuc_1100 provide perspective for explaining why pasteurized AKK shows biological functions on physiological homeostasis. In this study, we found that Amuc_1100 mimics the anti-diabetic properties of heat-killed AKK through oral administration in a GDM mouse model.

Mechanically, low-grade systemic inflammation occurs during pregnancy, which initiates insulin resistance [39]. As an endocrine organ, the placenta functions as a source and target of multiple pathological stimuli in the second and third trimesters in metabolic diseases, which secretes multiple inflammatory cytokines, exacerbating chronic inflammatory responses and maternal insulin resistance [40]. Previous studies have attributed insulin resistance during pregnancy to the increased production of placental tumor necrosis factor-α (TNF-α) [41, 42]. When compared with normal pregnant women, a higher ratio of M1 macrophages and a lower ratio of M2 macrophages were observed in the placenta of GDM cases [43], suggesting that placental inflammation may serve as an essential factor of GDM, and providing a potential target for GDM therapy. Consistent with these findings, we also observed increased pro-inflammatory M1 macrophages and decreased anti-inflammatory M2 macrophages in the placenta of GDM patients, indicating a placental inflammatory state during GDM. Our *in vivo* and *in vitro* experiments illustrated direct beneficial effects of pasteurized AKK and purified Amuc_1100 on placental macrophage polarization.

5. Conclusions

Based on the constructed GDM mouse model, we functionally explored that oral supplementation of pasteurized AKK and Amuc_1100 effectively lowered blood glucose, improved insulin resistance and recovered the immune-inflammation of placental macrophages (Fig. 5). In summary, we identified pasteurized AKK and its outer membrane protein, Amuc_1100 as potential placental anti-inflammatory probiotic and prebiotics. However, as a limitation of this study, how Amuc_1100 penetrates the placenta is necessary to be addressed by more extended exploration. Furthermore, long-term metabolic homeostasis of offspring and molecular pathways influenced by pasteurized AKK and Amuc_1100 administration could be explored and identified through further multi-omics analysis.

Author contributions

Design: SYJ, LL and CXW. Conduct: WY, ZYT, CY and FJL. Analysis: ZH, SYJ and LL. Writing manuscript: SYJ, LL and CXW.

Consent for publication

All authors listed in this manuscript have provided their consent for publication.

Ethical approval

All animal procedures were approved by the Nanjing Medical University Committee on the Care and Use of Animals (permit number IACUC-2011052).

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Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.repbio.2025.101073.

Data Availability

The data and material that support the findings of this study are available from the corresponding author upon reasonable request.

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