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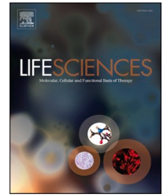
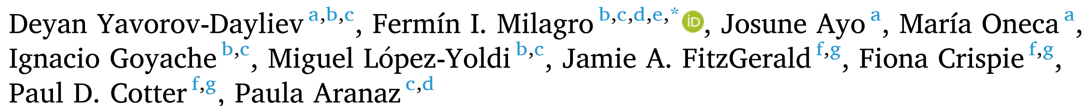


Pediococcus acidilactici CECT 9879 (pA1c®) and heat inactivated pA1c® (pA1c® HI) ameliorate gestational diabetes mellitus in mice



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A R T I C L E I N F O

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A B S T R A C T

Aims: Gestational diabetes mellitus (GDM) is the most common complication of pregnancy and is known to be associated with an increased risk of postpartum metabolic disease. Based on the important role that the intestinal microbiota plays in blood glucose regulation and insulin sensitivity, supplementation of probiotic and postbiotic strains could improve glucose metabolism and tolerance in GDM.

Main methods: 56 4-week-old female C57BL/6J-mice were divided into 4 groups ($n = 14$ animals/group): control (CNT), high-fat/high-sucrose (HFS), pA1c® alive (pA1c®) and heat-inactivated pA1c® (pA1c®HI). Serum biochemical parameters were analyzed, gene expression analyses were conducted, and fecal microbiota composition was evaluated by shot-gun sequencing.

Key findings: pA1c®- and pA1c® HI-supplemented groups presented reduced fasting blood glucose levels and reduced insulin resistance during gestation and exhibited lower visceral adiposity and increased muscle tissue, together with an improvement in intrahepatic TGs content and ALT levels. Liver gene expression analyses demonstrated that pA1c® and pA1c® HI activities were mediated by modulation of the insulin receptor, but also by an overexpression of beta-oxidation genes, and downregulation of fatty acid biosynthesis genes. Shot-gun metagenomics demonstrated that *Pediococcus acidilactici* was detected in the feces of all the pA1c® and pA1c® HI-group after the supplementation period (75 % of the microbial profile was *Pediococcus acidilactici*) in only nine weeks of supplementation, and modulated gut microbiota composition.

Significance: These results may be considered as future perspectives for the development of preventive, even therapeutic options for GDM based on hyperglycemia reduction, blood glucose regulation, hepatic steatosis attenuation and insulin resistance alleviation.

1. Introduction

Gestational diabetes mellitus (GDM) is described as a glucose intolerance on a background of chronic insulin resistance resulting in a hyperglycemia of varying severity, occurring for first time during women gestation [1]. GDM is the most frequent metabolic disorder during pregnancy that is known to be related to short- and long-term side effects for pregnant women and their offspring [2].

In the last two decades, the prevalence of GDM has skyrocketed to alarming levels. GDM affects approximately 14 % of pregnancies worldwide [3], and this number is set to increase with the escalating obesity pandemic. Some of the epidemiological factors causing this rise in GDM prevalence are the increase in obesity rates in women of reproductive age [4], the increase in maternal age [5], excessive gestational weight gain [6], ethnicity [7], genetics [8] and family history or any form of diabetes mellitus (DM) among others [9]. On the

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other hand, the main outcomes of developing GDM are increased risks of pregnancy-induced hypertension [10], type 2 diabetes (T2DM), metabolic syndrome and cardiovascular disease in the future [11], fetal growth restriction [12], and partum and postpartum problems [13] in women with GDM. Research data finds that women with GDM are 7.5 times more vulnerable to develop T2DM in the future than normal pregnant women [14]. Moreover, infants whose mothers developed GDM, are linked to a high rate of hyperinsulinemia [15], hypoglycemia [16], macrosomia [17] and DM [18]. For a female baby from a GDM mother, the risk of developing GDM in future will rises considerably with age, leading to a vicious circle affecting the health of many generations [19].

A systematic review study has established a strong relationship between the gut microbiome, its action during GDM pregnancy, and dysbiosis [20]. Throughout pregnancy, a woman's body undergoes many hormonal, immunological and metabolic changes [21]. Even though the involvement of microbiota in GDM is unclear, it is thought that pregnancy plays a role in increasing intestinal permeability, leading to an imbalance within the community of gut microorganisms, triggering dysbiosis. A plausible hypothesis is that hormones induce a change in mucosal tight junction proteins within the intestinal mucosa [22], affecting the nutrient delivery to the foetus and moulding the gut microbiota community structure and function [23]. This chain of events may result in deregulation of inflammation and immunity, and may also be associated with insulin resistance (IR) [24]. Moreover, a few studies have reported that intestinal microbiota modulated inflammation response and IR in GDM pregnancies [25,26]. In turn, microbiota may be modulated by probiotic supplementation and medical nutrition therapy with the ultimate goal of reversing the resultant dysbiosis and normalizing glucose tolerance in GDM patients [27,28]. Given that the gut microbiome is found at the epicenter of GDM, an updated knowledge and understanding of the molecular mechanisms involved between microbiota and GDM is warranted.

The anti-diabetic and microbiota-modulating potential of the probiotic *Pediococcus acidilactici* CECT 9879 (pA1c®) has already been demonstrated in studies conducted by our group in the past [29–32]. Here, we wanted to assess whether the probiotic, both alive (pA1c®) and heat-inactivated (pA1c® HI), was able to prevent the development of GDM by improving glucose metabolism, together with modulating other parameters, including body weight gain, white adipose tissue (WAT) accumulation, hepatic steatosis, cholesterol profile and gut microbiota, in a preclinical high fat/high sucrose (HFS) diet-induced GDM mouse model. The use of the heat-inactivated bacterium is related to safety and future technological issues, particularly in vulnerable patients, gestation women, and pediatric populations.

2. Material and methods

2.1. Strains and culture

Pediococcus acidilactici strain was deposited, according to the Budapest Treaty, in the Spanish Collection of Type Cultures (CECT) with identification reference CECT 9879 from the proprietary strain collection of Genbioma Aplicaciones S.L. (Polígono industrial Noain-Esquiroz, S Street, Nave 4, Navarra, 31,191, Spain) backed by an international patent "Probiotics for regulating blood glucose [PCT/EP2020/087284; WO2021/123355A1]". This species meets the criteria of qualified presumption of safety (QPS) by the European Food Safety Authority (EFSA). pA1c® was cultured as described previously [29,33–35] and was inactivated by heat during 60 min at 80 °C. Briefly, the selected probiotic strain was cultivated in a controlled medium under optimal conditions of pH and temperature, as provided by the supplier under proprietary conditions. After fermentation, the cultures were concentrated and lyophilized to ensure viability. Then, a portion of the lyophilized product was subjected to inactivation to obtain postbiotics.

2.2. Gestational diabetes C57BL/6J mice experimental design and diets

56 four-week-old female C57BL/6J mice and 28 four-week-old male C57BL/6J mice (Charles River) were used in this study. The experiment had a total duration of 10 weeks. Mice were kept in an isolated room (two different rooms, one for males and other for females) with controlled temperature (21 °C–23 °C) and humidity (50 % ± 10 %), and a 12 h:12 h artificial light/dark cycle. All mice were acclimatized to the experimental facility for one week, where all animals were fed a control diet (2014, Teklad, Global 14 % Protein Rodent Maintenance Diet). Following the acclimation period, female mice were randomly divided and allocated into four groups ($n = 14$ animals/group; Control, HFS, pA1c® and pA1c® HI) until the end of the study (ten weeks of supplementation). Animals were housed in cages of four and five animals with ad libitum access to water and controlled food intake until they became pregnant, then they were housed in individual cages until the end of the study. To induce gestational diabetes, three of the four groups were fed a HFS diet (D12451, Research Diets, 20 Jules Lane; New Brunswick, NJ, with 20 % of kcal corresponding to protein, 35 % of kcal to carbohydrates and 45 % of kcal to fat) and fructose (10 %) in the water for 6 weeks before gestation. During the three weeks of gestation and after the mouse pups were born, the HFS diet and the fructose in the water were maintained. Two of the three HFS groups were supplemented with the convenient treatment during the entire duration of the study: pA1c® group (10^{11} CFU/day/mice of the probiotic), pA1c® HI group (10^{11} CFU/day/mice of the postbiotic). HFS-control group had no supplement. The remaining control group (CNT) maintained the control diet (14 % protein) and the 10 % fructose in the water during the whole experiment. All male mice received control diet during the whole experiment, without fructose (10 %) in the water (Fig. 1).

The probiotic and postbiotic formulations were prepared every three days and were mixed with the HFS-diet. Once prepared, the mixture was provided to the mice for consumption over a 3-day period. After the mice finished the diet containing probiotics/postbiotics, a fresh batch was prepared and provided every 3 days. The amount of probiotic and postbiotic formulation were adjusted at a daily pA1c® and pA1c® HI dose of 1×10^{11} CFU per animal per day. The lactic acid bacteria (LAB) counts in the HFS diet + probiotic or postbiotic formulation were performed by plate counting on MRS agar (Scharlau, Barcelona, Spain), incubated for 48 h at 37 °C under CO_2 atmosphere (5 %).

Three days before mating the females with the males (week six of supplementation), the males were introduced in the same room as the females and some sawdust was sprinkled from the cages of the males to those of the females in order of restarting and synchronizing their menstrual cycle, which had stopped (anestrum) due to the lack of contact with males for five weeks. After these three days, on the crossing day, the males were put in contact with the females in individual cages for each couple for one day.

Body weight was checked weekly. Fasting blood glucose by venous tail puncture was measured at the beginning of the study and during gestation. All animals were euthanized by decapitation at week ten of supplementation, trunk blood was collected, and serum and plasma samples were obtained for biochemical analysis. Tissue samples from the liver, kidney, spleen, gastrocnemius muscle, white adipose tissue (WAT) depots (mesenteric, subcutaneous, epididymal, and perirenal) were isolated, weighed and immediately stored at −80 °C. All procedures were performed by following the national and institutional ethical guidelines of the Care and Use of Laboratory Animals, with the consent of the Food Safety and Environmental Health Service of the Government of Navarra, Spain. The protocol was approved by the Ethics Committee for Animal Experimentation of the University of Navarra (protocol reference 080/22).

2.3. Blood biochemical parameters

In order to determine the glucose and lipid profiles, serum total

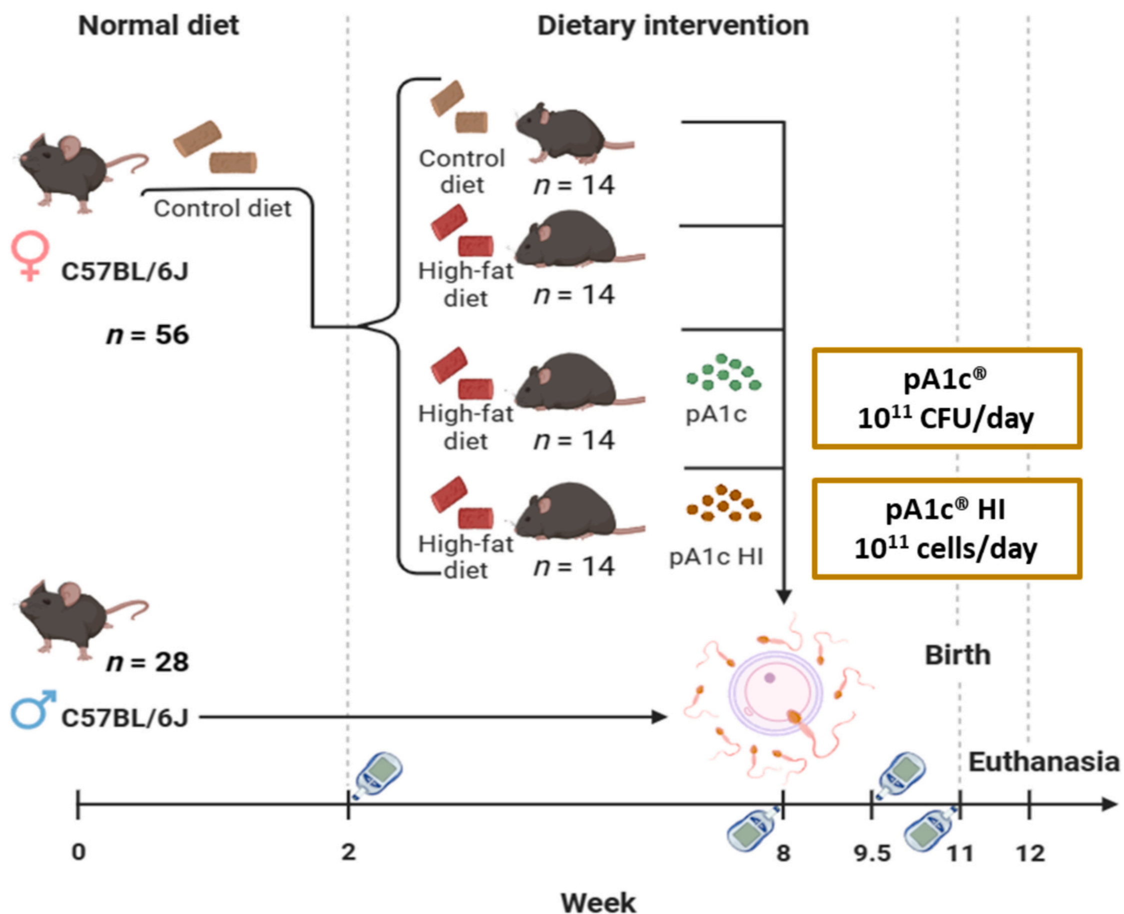

Fig. 1. Experimental design of the experiment.

cholesterol, HDL-cholesterol (HDL-Chol), total cholesterol/HDL-Chol ratio, triglycerides (TG), serum glucose, aspartate transaminase (AST), and alanine transaminase (ALT) were quantified with an HK-CP kit (ABX Pentra, Montpellier, France) adapted for a Pentra C200 analyser (HORIBA ABX, Montpellier, France). Insulin was quantified with a specific ELISA kit by following the protocol described by the manufacturer (Mercodia AB, Uppsala, Sweden). IR was evaluated by the homeostasis model of the insulin resistance index (HOMA-IR): [serum glucose levels (mmol L^{-1}) × insulin levels (mU L^{-1})] / 22. Besides, the Log (TG/HDL-Chol) ratio was also calculated to ascertain the atherogenic index of plasma (AIP).

2.4. Hepatic triglyceride content

The TG content of the hepatic samples was determined as previously described [36]. Briefly, a 100 mg aliquot of liver from each animal was dissolved in ethanol with 10 % of potassium hydroxide and incubated overnight at 55 °C with gentle shaking. At the next day, after total tissue digestion, samples were centrifuged for 5 min at 1500 rpm. Supernatants were then collected, dissolved in 50 % (v/v) ethanol and mixed. After that, samples were mixed in a proportion 1:1 with magnesium chloride 1 M, incubated for 10 min on ice and centrifuged for 5 min at 1500 rpm. The triglyceride content of these samples was quantified using the HK-CP kit adapted for the Pentra C200 analyser (HORIBA ABX, Montpellier, France).

2.5. RNA extraction and quantitative real-time PCR (qPCR) analyses

Total RNA from 100 mg of liver from control, HFS, pA1c® and pA1c® HI samples was extracted using TRIzol® RNA isolation reagent (Invitrogen Life Technologies, Paisley, UK). The concentration and purity of RNA were determined at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Then, 1 μg of RNA were treated with DNAse I (DNase I-RNase free, Invitrogen Life Technologies, USA) and reverse-transcribed into cDNA using 200 IU of M-MLV-RT (Invitrogen Life Technologies) in the presence of 40 IU of recombinant RNAsin® Ribonuclease inhibitor (Promega, Madison, WI, USA), with an incubation of 10 min at 25 °C, 50 min, at 37 °C and 15 min at 70 °C. Gene expression analyses were performed by quantitative-real time PCR (qPCR) using TaqMan Universal PCR master mix and specific probes from Life Technologies: *Acox-1*, *Cpt-2*, *Insr*, *Ppar-a*, *Scd-1* and *Srebf-1* (TaqMan™ Gene Expression Assays, primer sequences in ESM Table 1) in triplicate using a CFX384 Touch™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). Gene expression levels were normalized compared to TATA box binding protein (*Tbp-1*) gene expression as a housekeeping control. Gene expression differences between pA1c®- and pA1c®-HI-supplemented and non-supplemented samples were estimated using the relative quantification $2^{-\Delta\Delta Ct}$ method. Gene expression levels were represented as relative mRNA expression normalized against the HFS group.

2.6. Fecal sample collection and metagenomic and bioinformatic analysis

Feces of the different groups of the study were collected on week ten of supplementation and were immediately stored at −80 °C in cryotubes for future analyses. DNA isolation and bacterial DNA sequencing analyses were performed at Teagasc Food Research Centre, APC Microbiome (Fermoy, County Cork, Ireland).

Briefly, DNA was extracted from the 56 samples using the Qiagen Power Faecal Pro kit (Qiagen, China) according to the manufacturer's

instructions. DNA was quantified using the Qubit High Sensitivity Assay (Invitrogen, USA) and sequencing libraries were prepared using the Illumina DNA preparation kit from Illumina, following manufacturer's guidelines. Unique duel indices from IDT (Integrated DNA Technologies, United States) were used to index the samples. Samples were pooled and the final pool was quantified and sequenced on a NextSeq 2000, using a P2 300 cycle flow cell (Illumina, United States), again according to the manufacturer's instructions.

Mean sequencing output was 783,764 (±371,629) read-pairs per sample. Read quality was evaluated using FastQC (v 0.11.8) [37] and MultiQC (v 1.9) [38]. Low quality and artefactual sequences were removed using Trimmomatic (v0.32; PE, CROP:143, HEADCROP:21, ILLUMINACLIP:2:30:10:5, SLIDINGWINDOW:6:15, MINLEN:80) [39], while contaminants were removed via alignment to the mouse (GRCm38), human (GRCh38), and decoy (build 37d5) genomes using BowTie2 (v 2.4.4; default parameters) [40]. This provided a mean filtered output of 585,702 (±284,460) read-pairs, and removed all reads from the negative control which was not used further. Taxonomic identities were assigned using Kraken2 (v 2.1.1; confidence: 0.1, minimum hits: 5, base quality: 20) and Bracken (v 2.2; kmer length of 35, read length of 122, count threshold of 50, species-level) [41]. Microbial gene function and pathways were determined using the HUMAnN3 pipeline (v 3.6; e-value of 1, pre-screen threshold of 0.01, uniref90 v 201901b, chocophlan v 31) [42]. Features (abundance data for microbial taxa, metabolic pathways) were analyzed in R (v 4.2.1) [43]. Where noted, features were center-log ratio transformed with count-zero multiplicative replacement of zeros (zCompositions, [44]), while host parameters were z-scored (0 mean, variance of 1) for gradient analysis. Taxonomic alpha and beta diversity were explored using the vegan library [45] for NMDS (relative abundance), RDA (CLR transformed data), PERMANOVA (NMDS and RDA), and step-wise model selection for constrained RDA. Bray-Curtis dissimilarity was used for relative

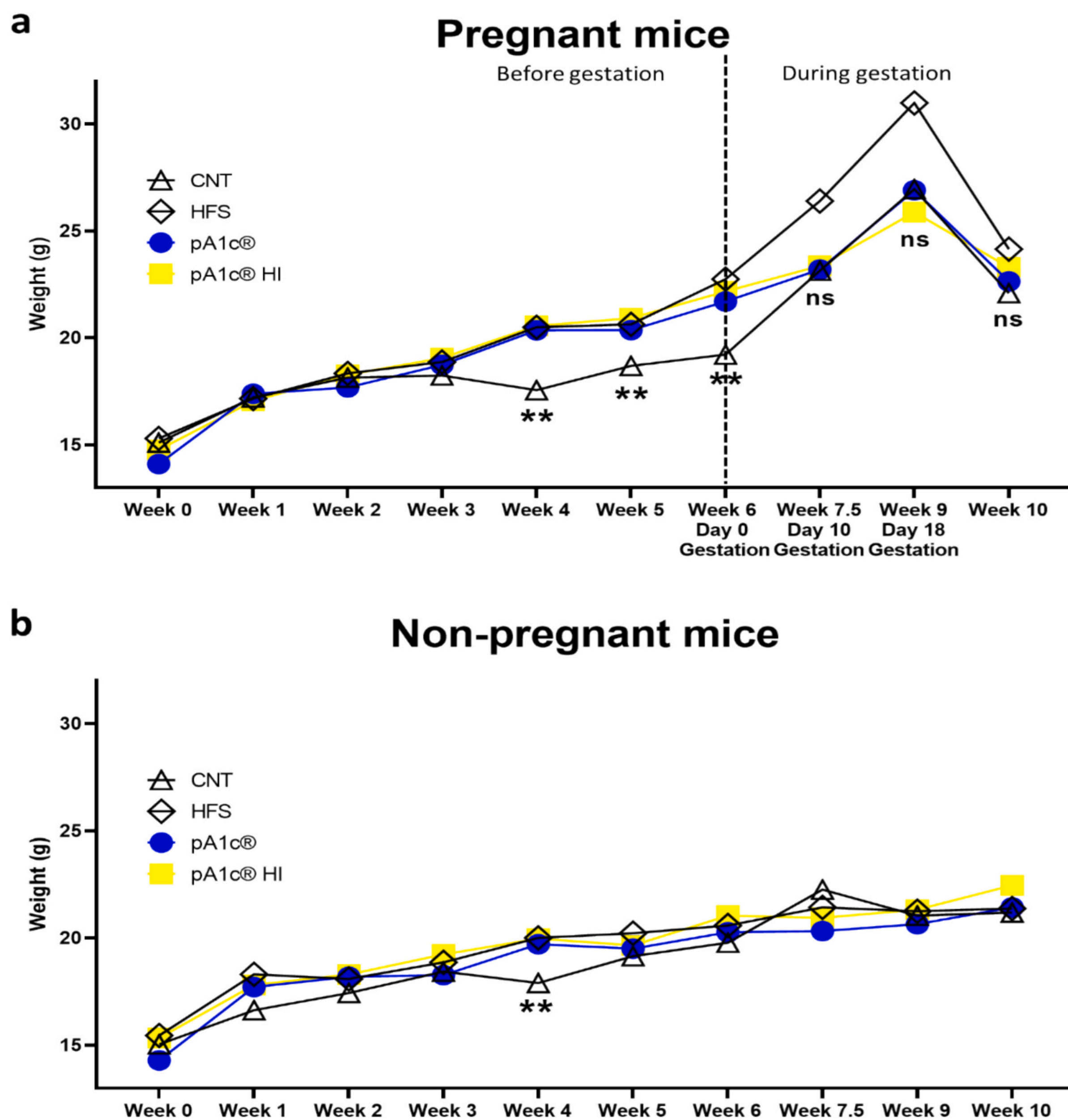

Fig. 2. Weight weekly monitoring during the experiment. Effects of pA1c® and pA1c® HI supplementation on total weight in pregnant (a) and non-pregnant (b) mice. Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test ($^{ns}p > 0.05$; $^{**}p < 0.01$).

abundances, and Euclidean distances were used for CLR. Differential testing of CLR data used a linear model of the form (feature ~ treatment * pregnancy), with contrasts between the different conditions of pregnancy and treatment made using the emmeans [46] library with a post-hoc Benjamini-Hochberg multiple testing correction (FDR) [47]. Figures were composed using the ggplot2 [48] and patchwork [49] libraries.

3. Results

3.1. pA1c® and pA1c® HI prevented gestational diabetes and type 2-diabetes in pregnant and non-pregnant female mice

During the study, weight evolution of pregnant and non-pregnant mice was monitored weekly (Fig. 2). No remarkable differences were observed in pregnant mice, except for a significant total weight gain of all the groups that received HFS in weeks four, five and six in comparison with control group, and an increase in the weight of HFS-mice during gestation (weeks six-nine) compared to the other groups, without reaching statistical significance (Fig. 2a). There were no differences between the non-pregnant mice groups (Fig. 2b).

In addition to weight monitoring, the evolution of fasting blood glucose levels in pregnant and non-pregnant mice was followed throughout the experiment (Fig. 3). Control pregnant mice had slightly lower blood glucose values than the other three groups (which had all received HFS) at week six (the day they became pregnant; day zero of gestation), but the difference was not significant (Fig. 3a). Moreover, on day ten of gestation, it was observed that the blood glycaemia of the HFS-mice continued to rise, while the glucose levels of both probiotic (pA1c®) and postbiotic (pA1c® HI) groups remained significantly lower in comparison (Fig. 3a). Further, it could be seen that pA1c® HI corresponded with a reduction in blood sugar to the same level as the control group (which had not received high-fat diet). Furthermore, on day twenty of gestation (last day of gestation), it was observed that the HFS-mice maintained very high fasting glucose levels, while pA1c®- and pA1c® HI-supplemented mice maintained low blood sugar levels (Fig. 3a). This reduction in glycaemia by pA1c® and pA1c® HI was statistically significant with respect to the HFS, and both presented similar values to the control group.

Interestingly, non-pregnant mice were found to show the same pattern as pregnant mice (Fig. 3b), but instead developed diet-induced T2DM. This is the first study to test the activity of the probiotic

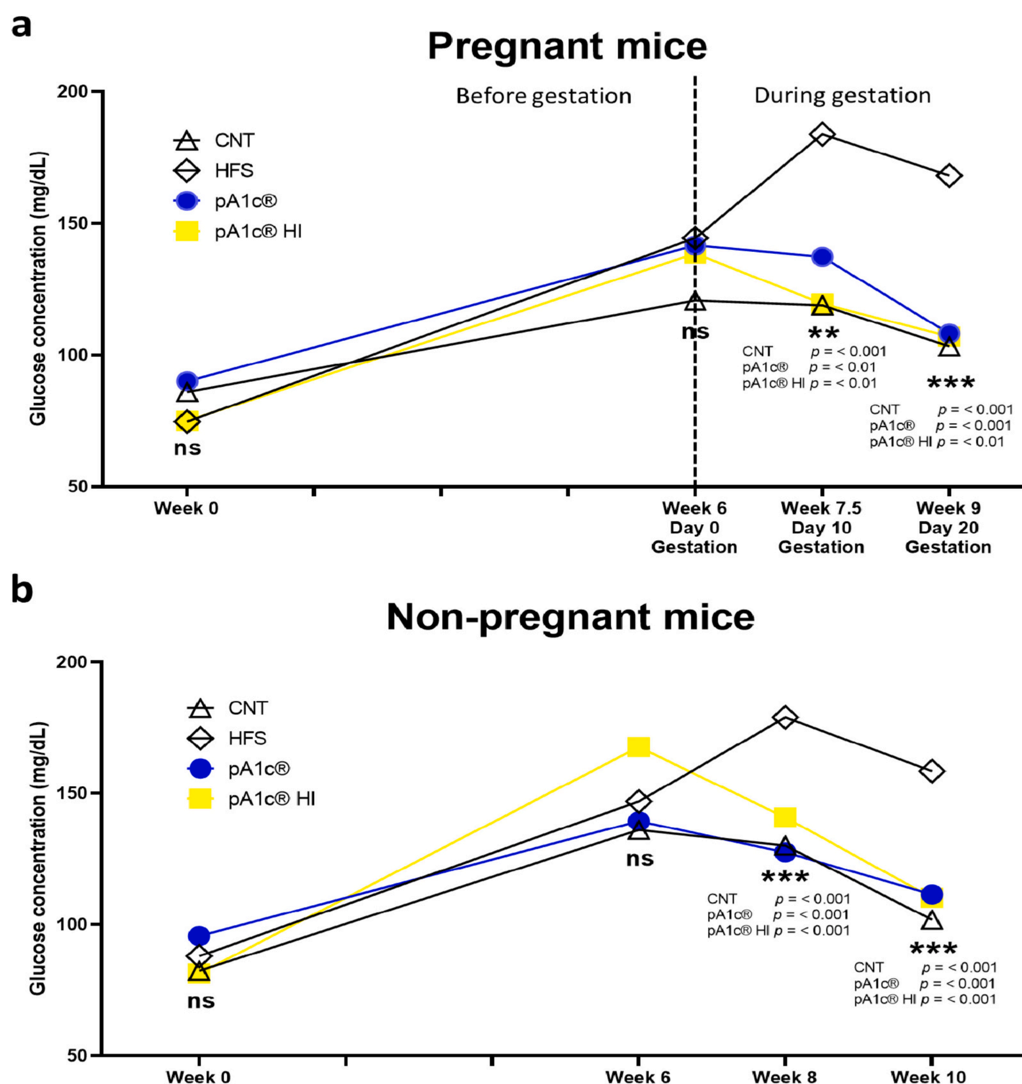

Fig. 3. Fasting blood glucose levels during the experiment. Effects of pA1c® and pA1c® HI supplementation on fasting blood glucose in pregnant (a) and non-pregnant (b) mice. Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test ($^{ns}p > 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). The p values showed below the Kruskal-Wallis analyses corresponded to the Dunn's multiple comparison test and were respect of the HFS group.

pA1c® in female mice with T2DM, and it is also the first study to test the efficacy of the inactivated probiotic pA1c® HI in T2DM. It can be observed that both pA1c® and pA1c® HI are able to normalize fasting blood glucose to control group values after week 8 of supplementation. This glucose-lowering activity by pA1c® and pA1c® HI is statistically significant compared with HFS-group (Fig. 3b).

3.2. Probiotic (pA1c®) and postbiotic (pA1c® HI) supplementation increase skeletal muscle weight and reduce total white adipose tissue

As mentioned above, no differences were observed in the weights of any of the 4 groups during gestation of the pregnant mice (Table 1), despite observation of a lower weight in the control group compared to the other three groups at day 0. This difference became blurred as they gained weight during pregnancy, until it became non-significant. In addition, no statistical differences in the weight of the main organs (liver, spleen and kidney) were found either. Although there were no differences in total weight, an increase in skeletal muscle by pA1c® and pA1c® HI was found, together with a decrease in total white adipose tissue (WAT) by pA1c® in comparison with the HFS-group.

Focusing now on the non-pregnant mice, it can be observed that there were no differences between the 4 groups in weight at the beginning and end of the study (ESM Table 2). As in the case of the pregnant mice, there were no changes in organ weights, but an increase in skeletal muscle and a decrease in WAT were found in both the pA1c® and pA1c® HI groups compared to the HFS-mice. Comparing pregnant (Table 1) and non-pregnant (ESM Table 2) mice, it was observed that the pregnant mice gained more weight during the experimental period due to gestation. However, upon euthanasia, there were no significant differences in total body weight or tissue weights, including white adipose tissue (WAT), between them.

Table 1
Tissue weights in pregnant C57BL/6J mice under normal and experimental conditions. Data are expressed as mean ± SEM*.

Pregnant	CNT (n = 7)	HFS (n = 7)	pA1c® (n = 7)	pA1c® HI (n = 6)	Kruskal-Wallis (p)
Weight (g)					
Day 0 Gestation	19.22 ± 0.4^{a}	22.75 ± 0.6^{b}	21.71 ± 0.5^{b}	22.17 ± 0.2^{b}	<0.01
Day 18 Gestation	26.96 ± 1.8	30.99 ± 1.7	26.90 ± 1.8	25.88 ± 1.8	ns
Before euthanasia	22.09 ± 1.1	24.15 ± 1.1	22.64 ± 1.0	23.30 ± 1.1	ns
Tissue weights (weight proportion related to total body weight)					
Liver	4.40 ± 0.3	4.25 ± 0.3	3.90 ± 0.1	4.45 ± 0.5	ns
Spleen	0.32 ± 0.0	0.38 ± 0.1	0.34 ± 0.1	0.38 ± 0.4	ns
Kidney	0.64 ± 0.1	0.72 ± 0.1	0.63 ± 0.1	0.67 ± 0.1	ns
Gastrocnemius muscle	0.57 ± 0.1$^{a\ b}$	0.53 ± 0.1$^{a\ b}$	0.73 ± 0.1^{a}	0.66 ± 0.1^{a}	<0.01
WAT depot weight (weight proportion related to total body weight)					
Total WAT	0.26 ± 0.1^{a}	0.48 ± 0.1^{b}	0.31 ± 0.1^{a}	0.40 ± 0.1^{b}	<0.05

*Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test ($^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$). Superscript numbers a and b designated groups in which statistically significant differences were observed (^{a}Statistically equal to the control group; ^{b}Statistically equal to the HFS group). CNT, control; HFS, high-fat-sucrose; pA1c®, *Pediococcus acidilactici*; pA1c® HI, *Pediococcus acidilactici* heat-inactivated; WAT, white adipose tissue. Total fat: sum of the mesenteric, subcutaneous, epididymal, and perirenal depots.

3.3. Supplementation with pA1c® and pA1c® HI ameliorate insulin resistance in gestational diabetes mice

The biochemical analyses performed after euthanasia confirmed the significant decrease in the glucose values of pregnant mice supplemented with the pA1c® and pA1c® HI in comparison to the glycemia of HFS mice (Table 2). These 2 groups even present lower glycemic values than the control group that had not received HFS diet. In addition, although the decrease is not significant, it can be observed that the two groups pA1c® and pA1c® HI present lower values of the HOMA insulin resistance index than the HFS mice. Moreover, pA1c® and pA1c® HI were able to normalize the total cholesterol/HDL cholesterol (Total Chol/HDL) ratio and high ALT-transaminase levels to control values in comparison to the HFS group.

Regarding to non-pregnant mice, it was observed that pA1c® did not present any difference in any of the biochemical parameters compared to the HFS mice, except for a significant reduction in the ALT liver damage biomarker (ESM Table 3). In contrast, pA1c® HI reduced fasting blood glucose to control levels. Further, pA1c® HI-mice presented lower levels of the HOMA insulin resistance index than the HFS group, although as in the case of pregnant mice, this reduction was not significant. In addition, as seen with pA1c®, pA1c® HI counteracted the rise in liver transaminase ALT due to HFS-diet, resulting in values similar to those seen in the control group.

Regarding the comparison between pregnant (Table 2) and non-

Table 2
Biochemical parameters in pregnant C57BL/6J mice under normal and experimental conditions. Data are expressed as mean ± SEM*.

Pregnant	CNT (n = 7)	HFS (n = 7)	pA1c® (n = 7)	pA1c® HI (n = 6)	Kruskal-Wallis (p)
Glucose metabolism biomarkers					
Glucose (mg dL^{-1})	117.67 ± 4.5^{a}	137.34 ± 8.2^{b}	114.88 ± 2.9^{a}	113.63 ± 5.9^{a}	<0.05
Insulin (μg L^{-1})	0.49 ± 0.1	0.49 ± 0.1	0.50 ± 0.1	0.49 ± 0.1	ns
HOMA-IR	3.33 ± 0.1	4.09 ± 0.2	3.51 ± 0.2	3.39 ± 0.2	ns
Cholesterol metabolism biomarkers					
Chol (mg dL^{-1})	107.14 ± 6.9	111.71 ± 5.9	103.14 ± 5.3	102.67 ± 4.9	ns
HDL-Chol (mg dL^{-1})	46.89 ± 2.9	40.06 ± 4.4	44.64 ± 2.8	44.09 ± 4.1	ns
Total Chol/HDL	2.29 ± 0.1^{a}	3.14 ± 0.6^{b}	2.33 ± 0.1^{a}	2.43 ± 0.2$^{a\ b}$	<0.05
TG (mg dL^{-1})	108.86 ± 10.9	88.86 ± 5.1	98.86 ± 9.8	74.00 ± 6.6	ns
AIP Index	0.36 ± 0.1	0.36 ± 0.1	0.34 ± 0.1	0.23 ± 0.1	ns
Hepatic biomarkers					
ALT (U L^{-1})	59.49 ± 4.4^{a}	88.37 ± 3.0^{b}	63.86 ± 6.6^{a}	66.50 ± 6.1^{a}	<0.05
AST (U L^{-1})	410.49 ± 60.0	493.20 ± 42.9	410.54 ± 73.1	504.53 ± 86.9	ns

*Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test ($^{ns}p > 0.05$; $^{*}p < 0.05$). Superscript numbers a and b designated groups in which statistically significant differences were observed (^{a}Statistically equal to the control group; ^{b}Statistically equal to the HFS group). CNT, control; HFS, high-fat-sucrose; pA1c®, *Pediococcus acidilactici*; pA1c® HI, *Pediococcus acidilactici* heat-inactivated; HOMA-IR, homeostasis model of the insulin resistance index, HOMA-IR index was calculated with the formula: [serum glucose levels (mmol L^{-1}) × insulin levels (mU L^{-1})]/22; Chol, total cholesterol; HDL-Chol, HDL-cholesterol; TG, total triglycerides; AIP, atherogenic index of plasma, AIP index was calculated with the formula: AIP = log(TG/HDL-Chol); ALT, alanine aminotransferase; AST, aspartate aminotransferase.

pregnant (ESM Table 3) mice, it was observed that pregnant mice exhibited significantly elevated levels of all analyzed biochemical parameters, including glucose, HOMA-IR, cholesterol, and triglycerides, compared to non-pregnant mice (ESM Table 4). Specifically, in the CNT group, pregnant mice showed glucose levels of 117.67 mg/dL, whereas non-pregnant mice had levels of 109.14 mg/dL. Similarly, in the HFS group, pregnant mice exhibited glucose levels of 137.34 mg/dL, compared to 126.60 mg/dL in non-pregnant mice. While these differences did not always reach statistical significance, the trend towards higher glucose and HOMA-IR levels in pregnant mice was consistent across both dietary groups. This suggests that pregnancy alone, even in the absence of a high-fat-sugar diet, exacerbates glucose dysregulation and insulin resistance, particularly in the context of metabolic stress. These findings further emphasize the impact of gestation on metabolic parameters, especially when combined with a high-fat diet, and highlight the utility of our model for studying gestational diabetes. Thus, it is suggested that gestational diabetes exacerbates metabolic issues, highlighting the potential risks associated with pregnancy in the context of glucose and lipid metabolism. The elevated biochemical markers suggest that pregnancy-related metabolic changes may contribute to a deterioration in overall metabolic health, warranting further investigation into the mechanisms underlying these alterations.

The first assay performed on liver tissue was the quantification of hepatic TG (Fig. 4). A statistically significant decrease in intrahepatic TG was observed in the pA1c®- and pA1c® HI-supplemented pregnant mice when compared to the HFS group (Fig. 4a). Non-pregnant mice experienced the same decrease in intrahepatic TG concentration, but in this case, it was not significant due to the large intra-group variation (Fig. 4b).

Aside from intrahepatic TG quantification, liver gene expression analysis of key genes involved in glucose metabolism and the biosynthesis and degradation of lipid metabolism, quantified by qPCR in pregnant (Fig. 5a) and non-pregnant (Fig. 5b) mice were assayed. Gene expression analyses revealed an overexpression of the gene that encodes for the insulin receptor (*Insr*) in the pA1c® HI-supplemented group in pregnant and non-pregnant mice showing presenting values even higher that control group.

Moreover, it was also shown that supplementation with the pA1c® and pA1c® HI produced an overexpression of *Acox-1*, *Cpt-2* and *Ppar-α* in pregnant mice, which are fundamental beta-oxidation genes. *Acox-1* and *Ppar-α* expression was also increased in pA1c® (only *Acox-1*) and pA1c® HI supplemented groups in comparison with HFS group in non-pregnant mice. Importantly, the supplementation with pA1c® significantly decreased the expression of *Scd-1* and *Srebf-1*, which are important genes in the biosynthesis of fatty acids, in pregnant and non-pregnant mice in comparison with HFS group. pA1c® HI also presented lower expression of these 2 genes in comparison with HFS group in pregnant and non-pregnant mice, but this reduction was not significant.

Furthermore, we have performed a comparative analysis of basal gene expression levels between the CNT and HFS groups in both pregnant and non-pregnant mice (ESM Fig. 1). The results indicated a clear trend towards elevated expression of genes involved in fatty acid biosynthesis, such as *Scd-1* and *Srebf-1*, in pregnant mice compared to their non-pregnant counterparts. Conversely, the expression of genes associated with fatty acid degradation pathways, including peroxisomal (*Acox-1*) and mitochondrial β-oxidation (*Cpt-2*), was reduced in pregnant mice. These findings suggest that gestation induces a shift in hepatic lipid metabolism, favoring lipogenesis over fatty acid catabolism, which may contribute to the exacerbated lipid accumulation and metabolic disturbances observed during pregnancy, particularly in the HFS-fed groups.

3.4. Metagenomics results

ESM Fig. 2 summarizes the microbial profile of the different groups of the trial (CNT, HFS, pA1c®, pA1c® HI) divided in pregnant and non-pregnant mice at the end of the study. The most remarkable outcome observed was that pA1c®- and pA1c® HI-groups presented very high relative abundances (almost 75 % on average) of the species *Pediococcus acidilactici* in both pregnant and non-pregnant mice (a little more abundance in the pA1c® group that in pA1c® HI) compared to the HFS group. Moreover, *P. acidilactici* species was found in the feces of 100 % of the pA1c®- and pA1c® HI-animals, while this species was not found in CNT or HFS groups, in only 9 weeks of supplementation.

The metagenomic study showed no differences in the species richness of the groups (Fig. 6a), but a statistically significant decrease in the α-diversity Shannon diversity index (Fig. 6b) and inverse Simpson index (Fig. 6c) in pA1c®- and pA1c® HI-supplemented groups compared with CNT and HFS groups. Moreover, guided β-diversity analyses revealed a strong, significant effect of treatment (pA1c®- and pA1c® HI-supplementation) in differentiating the microbial community, and a non-significant effect of pregnancy on the community composition (ESM Fig. 3).

Hence, the impact of pregnancy on specific bacterial species and metabolic pathways was investigated by using shot-gun sequencing. ESM Fig. 4 displays the functions and taxa from differences in pregnancy, comparisons being made across the multiple different conditions (CNT, HFS, pA1c® and pA1c® HI). Difference in bacterial abundance and metabolic pathways are relatively small between pregnant and non-pregnant mice, suggesting pregnancy as a minor player in shaping microbiome (at least in this dataset). Due to the fact that no major

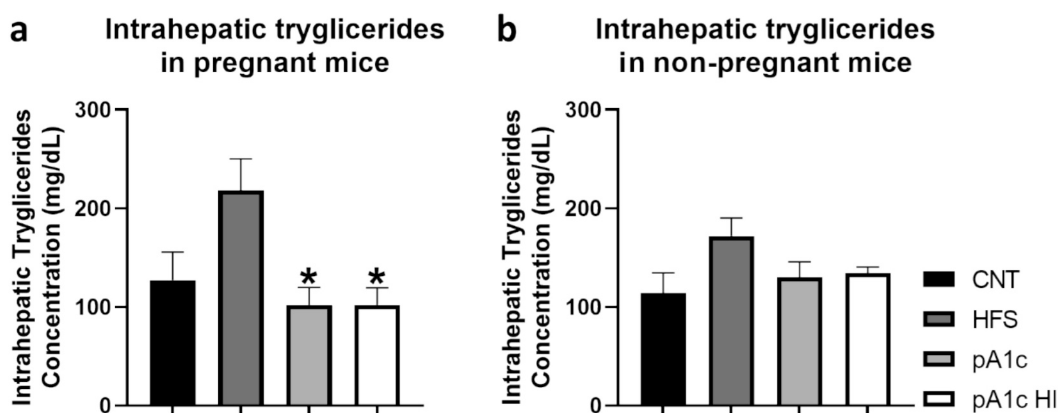

Fig. 4. Intrahepatic triglycerides levels of control and supplemented pregnant (a) and non-pregnant (b) mice. Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test (Dunn's multiple comparison test, *$p < 0.05$ with respect of the HFS group).

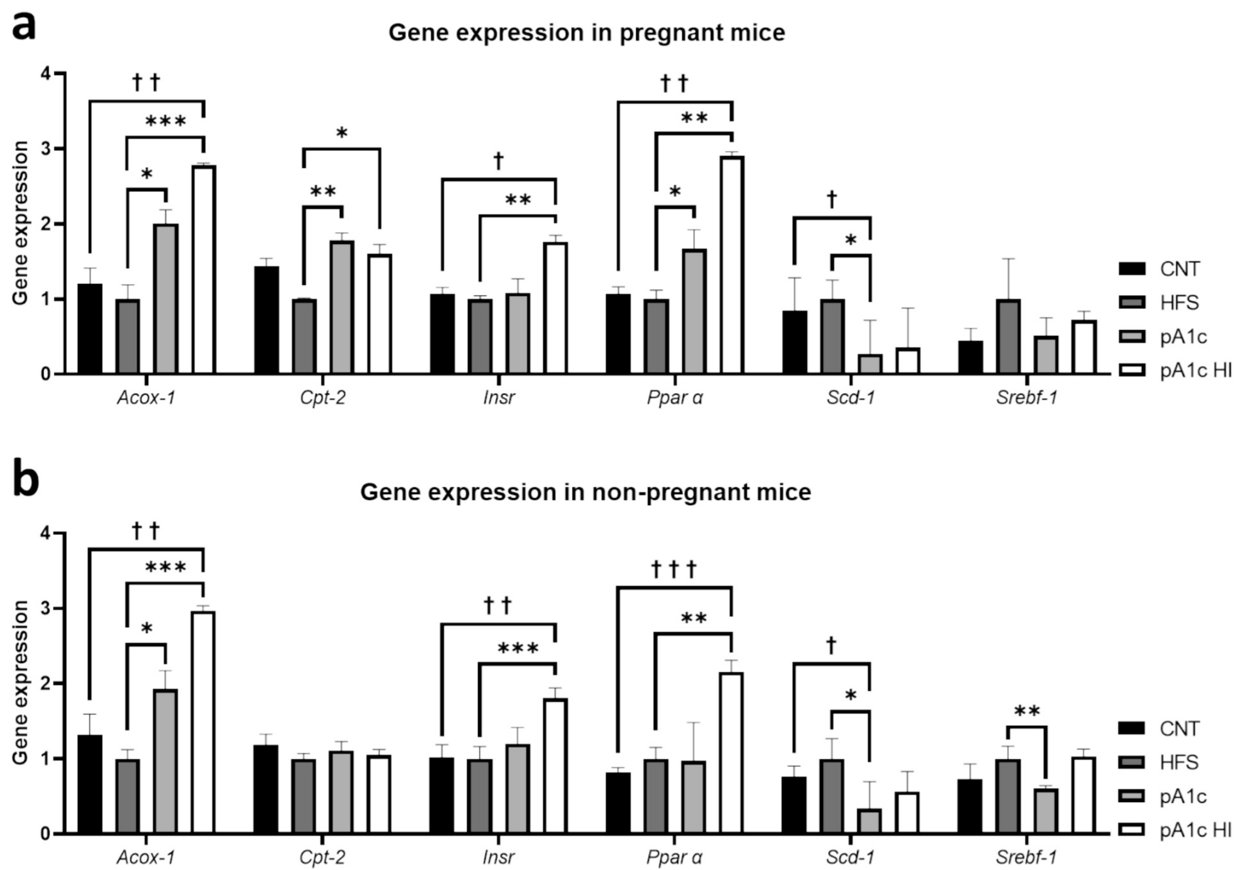

Fig. 5. Liver gene expression analysis of key genes involved in glucose metabolism and the biosynthesis and degradation of lipid metabolism, quantified by qPCR in pregnant (a) and non-pregnant (b) mice. Results are expressed as the mean ± SD relative to HFS mice ($n = 7$ per group). Gene expression levels were normalized to the housekeeping gene *Tbp-1*. Statistical analyses were performed using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test when statistical significance ($p < 0.05$) was reached in the Kruskal-Wallis test (Dunn's multiple comparison test, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs HFS group; †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.001$ vs control group).

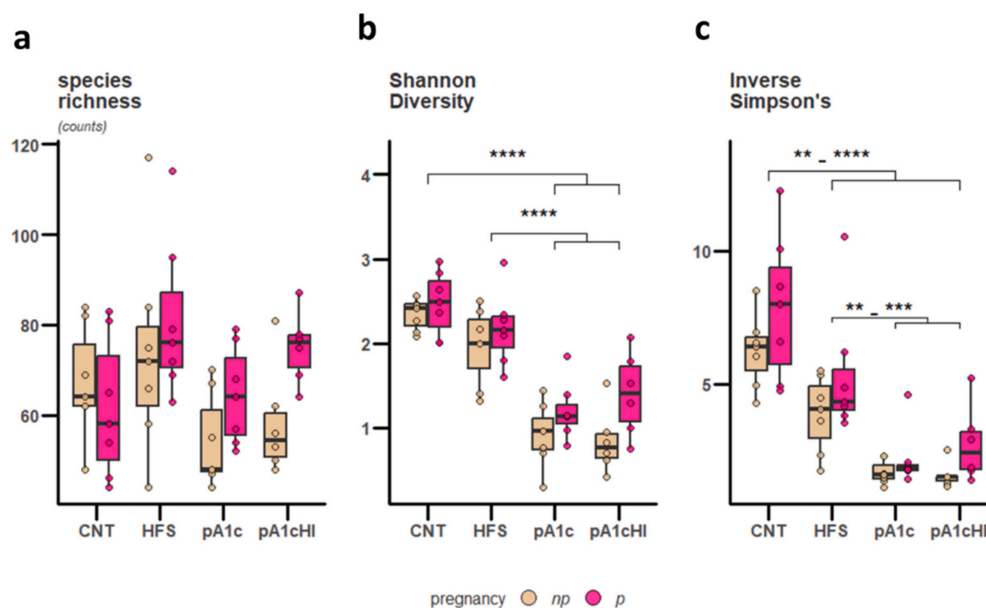

Fig. 6. Alpha-diversity analyses. Species richness of the groups (a), α-diversity Shannon diversity index (b) and inverse Simpson index (c) in pA1c®- and pA1c® HI-supplemented groups compared with CNT and HFS groups in pregnant and non-pregnant mice.

differences were observed between the abundances of pregnant and non-pregnant mice, and that pregnancy was not seen to influence the microbial composition in this study, from this moment on, it was worked with mixed models regardless of whether they were pregnant or not, to obtain greater statistical power. In addition to the effect of pregnancy, the effect of the HFS diet was assessed by shot-gun metagenomic analyses. It was compared the microbiome of the mice that received control diet with the mice that received HFS diet without the probiotic/postbiotic supplementation. ESM Fig. 5a outlines the main differences in the microbiota composition between CNT- and HFS-groups. The most outstanding result noticed was the increase in the abundance of *Akkermansia muciniphila* and some species of *Lactobacilli* in the HFS group in comparison with CNT group. Moreover, ESM Fig. 5b showed the relation between the changes in the species abundance and the metabolic pathways in which they are involved. However, only one specifical relation between a bacterial activity and a metabolic pathway was found. An increased abundance of the bacterium *Muribaculum intestinale* in control-mice was related with the folate transformations metabolic pathway.

Besides pregnancy and the type of diet, the effect of the third variable of the study (the effect of the treatment) was also evaluated. Since there were not many differences in the microbial profile between mice that received pA1c® and those supplemented with pA1c® HI (discussed below), for the analysis of the treatment effect in comparison with the HFS group, the two groups, pA1c® and pA1c® HI, were combined, obtaining a larger sample size. The most noteworthy differences between the pA1c®/pA1c® HI group and the HFS group were that supplementation with the probiotic/postbiotic increased the abundance of the LAB *P. acidilactici*, *Pediococcus pentosaceus* and three species of *Lactobacilli*, including *Lactiplantibacillus plantarum*, whereas it decreased the abundance of *Streptococcus infantarius*, *Clostridium perfringens*, *Turicibacter* and *Enterococcus faecium* (Fig. 7a).

Moreover, Fig. 7b pointed out the association between the homolactic fermentation metabolic pathway and the increased *P. acidilactici* abundance. These findings once again underscore the involvement and the importance of pA1c® and pA1c® HI in the glucose metabolism and homeostasis.

Lastly, Fig. 8a shows the differences in bacterial abundances between the group that received the alive probiotic (pA1c®) and the group that received the heat-inactivated probiotic (pA1c® HI), and Fig. 8b indicates the differences in physiological functions and metabolic pathways between the two already mentioned groups.

Although there are not many differences in the microbial profile between the two groups, 2 important changes were observed. Mice of the pA1c®-group presented an increased abundance of the bacterium *Paenibacillus polymyxa*, while exhibited a decreased abundance of *A. muciniphila* in comparison with pA1c® HI-supplemented mice (Fig. 8a). Furthermore, none of the bacteria showed a specific relationship with any functional metabolic pathway (Fig. 8b).

4. Discussion

As mentioned above, previous studies by our group have demonstrated the anti-diabetic activity of pA1c® in *C. elegans* and rodent (mice and rat) models. Therefore, our intention with this study was to test the probiotic in pregnant mice to see if it maintained the glycaemia-normalizing effect described in diabetic male mice, also in female mice with T2DM and GDM. In addition to pA1c®, pA1c® HI was also tested to study if the postbiotic had the same activity as the alive probiotic. As this was the first time for our group working with GDM-mice model, quite a few difficulties were experienced in the process of synchronizing the menstrual cycle of female mice and in mating. As a result of this, a low rate of pregnant mice was obtained, exactly 50 % per group. It was thus decided to split the study into two parts and work in parallel with GDM-female pregnant mice ($n = 7$ per group) and T2DM-female non-pregnant mice ($n = 7$ per group). Although the number of animals per group was halved, it was believed that there were still enough to continue the research and give us a general idea and an insight into the probiotic (alive and inactivated) activity in GDM. Moreover, with the splitting of this study, the effect of the probiotic pA1c® can be analyzed for the first time in T2DM-female mice (until now all studies of our group with the probiotic have been carried out in T2DM-male mice). Further, a first approach of the activity of the inactivated probiotic pA1c® HI in T2DM can be made (never tested before). Due to the division of the experiment, in addition to the probiotic supplementation, the effect of pregnancy on the gut microbiota could also be analyzed. Finally, by observing the effect of the probiotic on glycaemia, visceral fat, muscle, inflammation and cholesterol metabolism in previous experiments and also in this, it was decided to explore the role of the liver and the hepatic steatosis in the mechanism of action of the probiotic.

Regarding weight monitoring, the lack of differences in the weight of

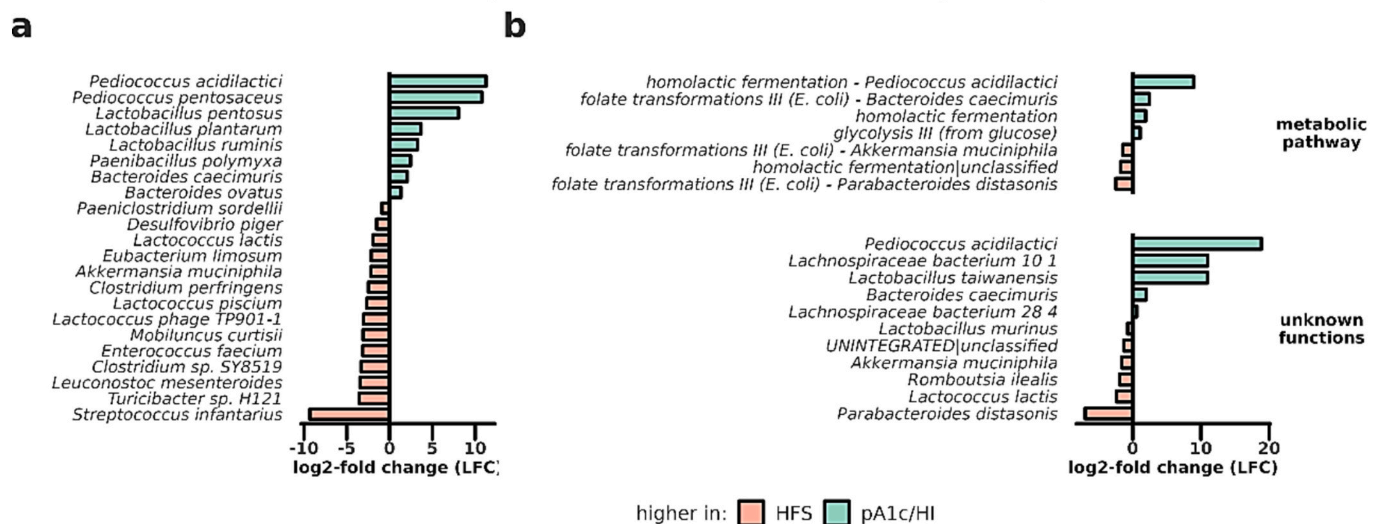

Fig. 7. Effect of the supplementation of pA1c® and pA1c® HI on the microbial abundances (a) and on the metabolic pathways involved (b) in comparison with HFS group.

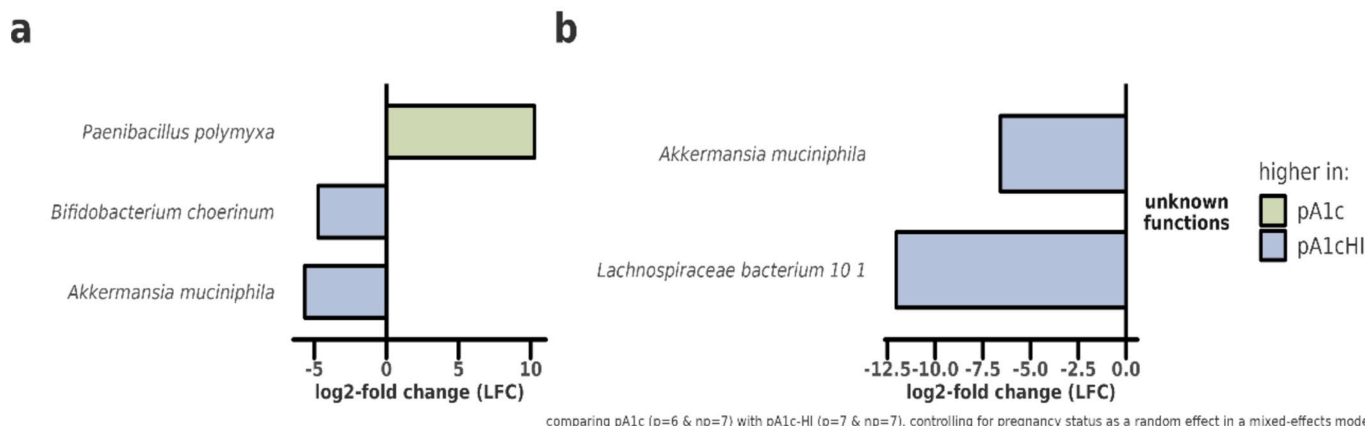

Fig. 8. Effect of the supplementation of pA1c® on the microbial abundances (a) and on the metabolic pathways involved (b) in comparison with pA1c® HI group.

the female mice is in agreement with other studies in which the non-use of female mice in metabolic research is advocated because they fail to present the long-term metabolic abnormalities consistent with obesity [50,51].

On the other hand, the results of blood glucose monitoring during gestation were of great interest, as it confirmed that the experimental model and the induction of gestational diabetes were optimally performed. Moreover, the obtained results demonstrated that at day zero of gestation all the groups with different treatments had the same blood glucose levels, so at that point, the mice had not yet developed the GDM. In addition, it was found that the increase in glucose by the HFS group was detected from day ten of gestation and was maintained until the end of pregnancy, coinciding with the general criteria for the diagnosis of GDM mentioned above (second and third trimester of pregnancy in humans) [52,53]. These outcomes showed that the high-fat diet alongside fructose in water were adequate ways to induce GDM. Although there are other ways to induce GDM (streptozotocin or knockout mice), the one induced by a high-fat diet is the one that most closely resembles GDM in humans. Taking all these results together, it can be affirmed that the probiotic and the postbiotic are capable of preventing GDM in mice. Therefore, these results may be considered as future perspectives for the development of preventive or even therapeutic options for GDM, based on hyperglycemia reduction and blood glucose regulation. In the same line, the data obtained from the glucose monitoring of the non-pregnant mice is in line with previous studies of our group where it was shown that diabetes was induced after 6–8 weeks of supplementation with a HFS diet, and that it took about 5–8 weeks to show the effectiveness of the probiotic [29,34,35].

In addition, weight tissue findings (in both, pregnant and non-pregnant mice) are consistent with previous results in which it was demonstrated that the probiotic does not affect total body weight, but it is capable of increasing lean muscle tissue and decrease visceral fat and WAT [29]. In fact, we had previously shown that one of the mechanisms of action of the probiotic was the activation the PI3K/*Akt-1* pathway by upregulating *Akt-1* expression in the gastrocnemius muscle, which in turn leads to a decline in the expression levels of *Foxo-1* and *Pdk-4* to counteract the excess of glucose and inhibit insulin resistance [29]. These outcomes are very positive as pregnancy is a time when muscle mass is lost, and fat levels increase. Therefore, the increase in skeletal muscle and the decrease in visceral fat by the probiotic can, in addition to lowering blood glucose and alleviating insulin resistance, help to prevent the possible disturbances of GDM.

The development of hepatic steatosis is characterized by an excessive TG accumulation in hepatocytes and insulin receptor deficiency [54]. This chronic disease is the most common cause of liver injuries, and is strongly linked to excessive TG, increased ALT aminotransferase levels and insulin resistance, represented by an inflammation process and high values of HOMA index [55–57]. It is established that patients with fatty liver disease are 5 times more likely to develop insulin resistance and thereby develop T2DM [58]. The obtained results in fasting blood glucose, HOMA index and ALT levels, together with low values of TG in pA1c® HI-supplemented pregnant and pA1c®-supplemented non-pregnant mice in comparison with HFS group (Table 2 and ESM Table 3), led us to further investigate the involvement of the liver in the mechanism of action of the probiotic.

Liver gene expression analyses revealed an overexpression in the *Insr* mediator. This result reaffirms once again the involvement of glucose metabolism and the insulin signaling pathway in the mechanism of action of this probiotic, and the alleviation of the hepatic steatosis, since, as mentioned above, is characterized by an insulin receptor deficiency. Further, beta-oxidation genes results are in accordance with *C. elegans* and mice data [29,33], demonstrating that the mechanism of action of the probiotic affected the fatty acid beta-oxidation metabolism, which could explain the WAT reduction observed [59]. Moreover, previous studies have reported a leading role for *Ppar-α* in fatty liver diseases [60,61]. *Ppar-α* upregulation decreased the advancement of insulin resistance in liver steatosis and inflammation [62]. Furthermore, besides inducing beta-oxidation, by controlling mitochondrial (*Cpt-2*) and peroxisomal (*Acox-1*) fatty acid degradation, and reducing insulin resistance, by reducing hepatic steatosis and inflammation, *Ppar-α* is involved in several other regulatory pathways such as breakdown of TG and lipid droplets, lipoprotein metabolism, immunity-related pathways and alleviation of dyslipidemia [63,64]. Taken together, this overexpression of *Ppar-α* could be one of the causes that could explain the better lipid and inflammatory profile of pA1c®- and pA1c® HI-supplemented mice. In addition, it could also be behind the reduction of the observed hepatic steatosis, the alleviation of the insulin resistance together with the overexpression of *Insr*, and the mitigation of the triglycerides accumulation. Finally, as in the case of the overexpression of the beta-oxidation genes, the inactivation of the de novo *lipogenesis* genes (*Scd-1* and *Srebf-1*) explains the significant reduction in WAT that pA1c®- and pA1c® HI-supplemented mice exhibited in comparison to HFS group.

Thus, fatty acid biosynthesis downregulation, the inhibition of the de novo *lipogenesis* process, together with the rise in the beta-oxidation activity, *Ppar-α*-signaling, the modulation of the insulin signaling pathway and the reduction of intrahepatic TG shed light on the mechanism by which pA1c® and pA1c® HI improve glucose metabolism, insulin resistance and hepatic steatosis, and therefore alleviate GDM.

In recent years, different metagenomic analyses have shown that gut microbiota dysbiosis is closely related to the development of GDM by modulating IR and inflammation response [25,26]. Therefore, one

modern and hopeful approach to GDM targets the restoration of gut microbiota homeostasis and neutralization of intestinal dysbiosis by probiotic- and postbiotic-supplementation. Therefore, in this study, we have analyzed the effect of pregnancy, the effect of the HFS diet and the effect of the different treatments (probiotic- and postbiotic supplementation) in diet and fructose-induced GDM-C57BL/6J mice.

ESM Fig. 2 findings (specially the high relative abundance of *P. acidilactici*) suggested that pA1c®- and pA1c® HI-supplementation may be responsible of the glucose-lowering, intrahepatic TGs-reducing and insulin signaling- and lipid metabolism-modulating observed effects. Due to the great abundance of *P. acidilactici*, the rest of the differences in the microbiota between supplemented and non-supplemented groups may be masked.

The alpha diversity (α-diversity) is the measure of the species diversity within an ecosystem [65] and beta diversity (β-diversity) is the measure of the difference in diversity among ecosystems in a given area, and is quantified as the variability in community composition (the identity of taxa observed) among samples within a habitat [66]. The decrease in the α-diversity and the no differences in the β-diversity might be attributed to the great abundance of the species *P. acidilactici* in pA1c®- and pA1c® HI-groups, which covers the 75–80 % of the pA1c®- and pA1c® HI-mice gut microbiome, displacing other species.

In addition, ESM Fig. 4 suggest that despite the dynamic changes occurring during pregnancy, the intestinal microbiota remains relatively stable, and according to our findings, the metabolic and physiological parameters exhibit minimal variation between pregnant and non-pregnant, underscoring the remarkable homeostatic mechanisms operating during this critical period, confirming pregnancy as a secondary factor in the modulation of the microbiota (at least in our results).

According to the differences between control and HFS diet, although it is well known that an increased abundance of *Akkermansia* and *Lactobacillus* is negatively correlated to the development of metabolic diseases such as obesity and T2DM, there are also studies, especially that of Tan et al., carried out in mice, in which it was observed an increase in the abundance of *Akkermansia* and *Lactobacillus* in the groups fed with high-fat diet and HFS diet compared with mice received control diet, suggesting that these species might be positively related with a pro-inflammatory status, chronic intestinal inflammation and metabolic disorders [67]. In general, there is usually a lot of inconsistency in studies conducted in preclinical models among themselves, and sometimes there is no congruence between the studies carried out in rodents and those performed in humans (in where the beneficial effect of *Akkermansia* has been certainly demonstrated [68]), since the abundance of important bacteria such as *Lactobacillus*, *Bifidobacterium* spp. and Bacteroidetes differs between humans and rodents and it is not easy to extrapolate preclinical experimental data to randomized controlled trials [69]. Further, the observed increase in *M. intestinale* in the control diet group was described in the literature as beneficial. This microorganism is a glycan-degrading bacteria and thereby increase the levels of butyrate in obese mice, alleviating glucose and lipid metabolism disturbances, in comparison with control [70]. Furthermore, other authors suggested that the increase of this bacterium may be related with anti-oxidant, anti-inflammatory and gut modulating activities [71]. All of this pointed *M. intestinale* as a potential beneficial strain in metabolic disorders via folate transformations.

Regarding the implication of pA1c® and pA1c® HI supplementation in shaping the microbiome, as it was said above, the glucose-lowering and microbiota-modulating potential of the probiotic *P. acidilactici* has already been proven in previous works of our group [29–32] and other authors [72,73]. However, there are no studies (or at least none have been found) in the literature that report beneficial effects against T2DM or GDM of the postbiotic (inactivated probiotic) *P. acidilactici* or any killed bacteria. Taken together this data and the results in the abundances of pA1c® and pA1c® HI in ESM Fig. 2, it may be suggested that the anti-GDM properties observed are due to the increased abundance of *P. acidilactici* species, both alive and inactivated. Moreover, it has been demonstrated that the other species of *Pediococcus* (*P. pentosaceus*) is also capable of alleviating the symptoms of T2DM [74,75], but there are no reports of its activity in GDM. The same happens with *L. plantarum*. It is well known that in recent years, this species has been widely studied for its application as an alternative or adjuvant treatment to those already existing against metabolic disorders. It has been demonstrated that *L. plantarum* alleviated T2DM in mice via modulating gut microbiota and regulating NF-κB and insulin signaling pathway [76]. Further, other study showed that heat-killed *L. plantarum* mitigated the effects of stress-related T2DM in mice via gut microbiome modulation [77], but no studies have been conducted evidencing a positive effect against GDM of this species, either alive or inactivated by heat. On the other hand, a randomized, double-blind, placebo-controlled trial showed that other *Lactobacilli* (*Limosilactobacillus fermentum*), which was increased by pA1c® and pA1c® HI supplementation in comparison with HFS-group, was part of a multi-strain probiotic that exhibited anti-GDM activity by affecting gene expression related to insulin and inflammation, glycemic control, few lipid profiles, inflammatory markers, and oxidative stress [78].

In contrast, focusing next on the bacteria that presented higher abundance in the HFS group compared to the pA1c® and pA1c® HI groups, it was observed that the *S. infantarius* species presented the highest values in the HFS group compared to the supplemented groups (Fig. 7a). Moreover, it was found a negative correlation between *P. acidilactici* (the supplemented probiotic and postbiotic) abundance and *S. infantarius* abundance (Spearman Rho: -0.4331, p value $= 0.0047$). Further, it was observed that *P. acidilactici* abundance was also negatively correlated with other 2 species of *Streptococcus*, *S. salivarius* and *S. suis* (Spearman Rho: -0.3472, p value $= 0.0261$; Spearman Rho: -0.3761, p value $= 0.0154$ respectively). These data are according to previous results of our group, in which was established a negative correlation between *P. acidilactici* abundance and the *Streptococcus* genus abundance, and was suggested that there may be a competition between the species *P. acidilactici* and the *Streptococcus* bacteria in the gut [79]. Furthermore, in support of these statements, other works have associated positively the genus *Streptococcus* with hypercholesterolemia, high TGs content, dyslipidemias and obesity [80,81] (known risk factors for the development of GDM). In addition, the species *Streptococcus infantis* is positively associated with the development of GDM in women [82]. Specifically, several studies have revealed the virulence potential of *S. infantarius*, which has been linked to endocarditis [83], bacteremia and infections [84], and has been found to have carcinogenic and pro-inflammatory activity [85]. Apart of *S. infantarius*, a higher abundance of harmful bacteria, such as *C. perfringens*, *Turicibacter* and *E. faecium*, was observed in the HFS group compared to the pA1c® and pA1c® HI groups (Fig. 7a). It has been reported that *C. perfringens* and *E. faecium* are pathogenic bacterium that produced intestinal diseases [86], and bacteremia [87] respectively. Further, Yang et al. have reported that supplementation with the probiotic *L. plantarum* SS18-5 reduced hyperglycemia and HbA1c in rats by decreasing the abundance of *C. perfringens* [88]. These data is in agreement with our results, since pA1c®/pA1c® HI supplementation increase *L. plantarum* and decrease the abundance of *C. perfringens* in comparison with HFS-mice. In addition, in a retrospective cohort study (assessing treatments against vancomycin-resistant *E. faecium* bacteremia), DM was the most common concomitant disease with a 43.3 % [89]. Other work, carried out in obese diabetic mice reported that in comparison with the non-diabetic group, *Turicibacter* was only present in the feces of diabetic mice, suggesting that the reduction of this microorganism would be a good strategy to revert the complications originating from T2DM and obesity [90]. Nevertheless, no studies were found relating GDM with the above bacteria.

In our study, we observed that both the probiotic (*Pediococcus acidilactici* CECT 9879, pA1c®) and the heat-inactivated postbiotic (pA1c® HI) groups showed increased levels of PPAR-α expression in the liver compared to the control group. Elevated PPAR-α is known to enhance

fatty acid oxidation and improve lipid metabolism, which are particularly relevant for managing hepatic insulin resistance. Given the context of GDM in pregnant mice, these metabolic shifts may play a role in adapting maternal metabolism to reduce hepatic insulin resistance and support glucose homeostasis during pregnancy, when insulin demands and resistance tend to increase. The supplementation with both pA1c® and pA1c® HI also led to an increase in homolactic fermentation (Fig. 7b), which may have contributed to this effect by promoting a more favorable metabolic profile. The shift towards homolactic metabolism suggests an enhanced conversion of glucose to lactate, which could reduce hepatic glucose production and thereby alleviate hepatic insulin resistance. This metabolic adaptation could be beneficial in GDM by helping to lower fasting glucose levels and improve overall insulin sensitivity. Thus, the combined effect of increased PPAR-α expression and enhanced homolactic fermentation may provide a dual mechanism that supports hepatic insulin sensitivity, potentially offering protective effects against the metabolic challenges imposed by GDM. However, further studies would be valuable to precisely delineate these mechanisms and confirm their impact on long-term maternal and fetal metabolic health.

Finally, regarding pA1c® and pA1c® HI differences, it has been reported that a microbial product from *P. polymyxa* (increased abundance in pA1c®-supplemented mice in comparison with pA1c® HI-mice), which was named enzamin, ameliorated adipose inflammation with impaired adipocytokine expression and IR in db/db mice [91]. Moreover, exopolysaccharides produced by *P. polymyxa* reduced sucrose-induced hyperglycemia [92]. Thus, *P. polymyxa* is thought to be a beneficial microorganism for suppressing the development of metabolic disorders such as T2DM and obesity, and may have anti-GDM activity, since it is able to alleviate important GDM-related disturbances as hyperglycemia, inflammation, IR and obesity, but further studies are needed. On the other hand, *A. muciniphila* (increased abundance in pA1c® HI-supplemented mice in comparison with pA1c®-mice) is an important bacterium in the regulation of glycemic metabolism [68], and it has been demonstrated that GDM women presented lower abundances of these microorganism [93]. Taken together these data, it could be suggested that in addition of the GDM-alleviating activity of the bacterium *P. acidilactici* observed in this study, pA1c® may exerted his beneficial effect by promoting *P. polymyxa*, and in turn increasing enzamin. In contrast, pA1c® HI increased the levels of *A. muciniphila* compared with pA1c®. In addition, a spearman's rank correlation test revealed a significant negative correlation between *P. acidilactici* and *A. muciniphila* in the mice receiving pA1c® (Spearman Rho: −0.5467, p value = 0.0431), while the same statistical test revealed that did not exist any significant correlation between *P. acidilactici* heat-inactivated and *A. muciniphila* (Spearman Rho: −0.1706, p value = 0.5774). Hence, it could be suggested that the alive probiotic *P. acidilactici*, might compete in the intestine in some way with *A. muciniphila*, thus explaining the low abundance of *Akkermansia* in the pA1c®-supplemented mice in comparison with HFS-group and also in comparison with the pA1c® HI-group, since the probiotic is killed, and there cannot occur competition between the two microorganisms.

As a conclusion, it is crucial to differentiate between the mechanisms of action of probiotics and postbiotics at the microbiome level. Probiotics, being live microorganisms, exert their effects by colonizing the gut, modulating the immune response, and producing various metabolites that influence microbial composition and function. In contrast, postbiotics, which are inanimate bacteria or byproducts of probiotics (including substances like short-chain fatty acids, enzymes, and other bioactive compounds), can directly interact with the gut microbiota and host tissues without the need for colonization, and exert their physiological function.

In our study, the observed differences in bacterial abundances between the groups receiving live probiotics (pA1c®) and heat-inactivated probiotics (pA1c® HI) underscore these mechanistic differences. Specifically, the increased abundance of *P. polymyxa* in the pA1c® group may enhance the production of beneficial metabolites, such as enzamin, which has been shown to ameliorate adipose inflammation and improve insulin resistance. Conversely, the higher levels of *A. muciniphila* in the pA1c® HI group suggest a different functional role in glucose metabolism regulation. This indicates that the mechanisms through which live and heat-inactivated probiotics exert their effects on the microbiome and host metabolism can differ significantly, highlighting the need for further investigation into their respective roles in metabolic health.

Overall, the safety of using both live and heat-inactivated probiotics during pregnancy is a crucial consideration, particularly in the context of potential clinical applications. While live probiotics have been studied extensively for their beneficial effects on maternal and fetal health, concerns about their safety during pregnancy, especially regarding the risk of translocation or infection persists in some cases. Heat-inactivated probiotics, on the other hand, may offer safety advantages as they do not pose the risk of live bacterial overgrowth or infection, making them potentially safer for vulnerable populations, including pregnant women.

Several studies have demonstrated the safety of heat-inactivated probiotics in general populations, with no significant adverse effects reported in short-term trials [94]. However, the evidence for their long-term effects, particularly during pregnancy, remains limited. Current literature supports the notion that heat-inactivated probiotics are less likely to trigger adverse immune responses or cause issues like bacterial translocation, but more clinical studies are needed to fully evaluate their long-term safety, efficacy, and potential side effects during pregnancy. Future research should focus on randomized controlled trials to provide further evidence supporting the clinical application of heat-inactivated probiotics in pregnant populations.

5. Conclusions

It has been demonstrated that the supplementation of the probiotic strain pA1c® and the postbiotic strain pA1c® HI have beneficial effects on blood glucose levels during pregnancy, glucose and lipid metabolisms parameters, WAT adiposity, TGs content, biomarkers of liver damage, muscle mass and inflammatory markers in high-fat/high sucrose diet-induced GDM-mice. In addition, we have shown that the main metabolic pathways by which pA1c® and pA1c® HI acts on GDM and T2DM are the activation of the insulin signaling pathway, the peroxisomal- and mitochondrial-fatty acid beta-oxidation process and inhibition of the lipogenesis. Moreover, *P. acidilactici* species was found in the feces of 100 % of the pA1c®- and pA1c® HI-animals (75 % of the pA1c®- and pA1c® HI-microbial profile was *P. acidilactici*), while this species was not found in CNT or HFS groups, in only 9 weeks of supplementation, and modulated the gut microbiota, evaluated by shot-gun sequencing. Although further research is necessary, our data suggest pA1c® and pA1c® HI as potential agents for the prevention and management of GDM and his related disturbances such as hyperglycemia, IR, inflammation and dyslipidemia.

Authors relationship and activities

JA is a shareholder of Genbioma Aplicaciones SL. JA and MO are co-authors of the patent 'Probiotics for regulating blood glucose (PCT/EP2020/087284; WO2021/123355A1)'.

CRediT authorship contribution statement

Deyan Yavorov-Dayliev: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fermín I. Milagro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal

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Declaration of competing interest

All the authors listed have contributed significantly to the work. J.A. is shareholder of the company Genbioma Aplicaciones S.L. J.A. and M.O. are co-authors of the patent cited in the manuscript [PCT/EP2020/087284]. The rest of the authors declare no conflict of interest. The data presented in this manuscript have not been published and are not under consideration for publication elsewhere.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2024.123359.

Data availability

Data will be made available on request.

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