Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *Food Funct.*, 2023, **14**, 10855

Pediococcus acidilactici (pA1c®) alleviates obesity-related dyslipidemia and inflammation in Wistar rats by activating beta-oxidation and modulating the gut microbiota†

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Due to the importance of the gut microbiota in the regulation of energy homeostasis, probiotics have emerged as an alternative therapy to ameliorate obesity-related disturbances, including cholesterol metabolism dysregulation, dyslipidemia and inflammation. Therefore, the objectives of this study were to evaluate the effect of the probiotic strain *Pediococcus acidilactici* (pA1c®) on the regulation of adiposity, cholesterol and lipid metabolism, inflammatory markers and gut microbiota composition in diet-induced obese rats. Twenty-nine four-week-old male Wistar rats were divided into three groups: rats fed a control diet (CNT group, n = 8), rats fed a high fat/high sucrose diet (HFS group, n = 11), and rats fed a HFS diet supplemented with pA1c® (pA1c®group, n = 10). Organs and fat depots were weighed, and different biochemical parameters were analysed in serum. Gene expression analyses in the adipose tissue were conducted using real-time quantitative-PCR. Faecal microbiota composition was evaluated using 16S metagenomics. Animals supplemented with pA1c® exhibited a lower proportion of visceral adiposity, a higher proportion of muscle, an improvement in the total-cholesterol/HDL-cholesterol ratio and a decrease in the total cholesterol, triglyceride and aspartate aminotransaminase (AST) serum levels, together with a decrease in several inflammation-related molecules. The expression of key genes related to adipose (*Adipoq*, *Cebpa* and *Pparg*) and glucose (*Slc2a1* and *Slc2a4*) metabolism in the adipose tissue was normalized by pA1c®. Moreover, it was demonstrated that pA1c® supplementation activated fatty acid β-oxidation in the adipose tissue and the liver. Metagenomics demonstrated the presence of pA1c® in the faecal samples, an increase in alpha diversity, an increase in the abundance of beneficial bacteria, and a decrease in the abundance of harmful micro-organisms, including the *Streptococcus* genus. Thus, our data suggest the potential of pA1c® in the prevention of obesity-related disturbances including hypercholesterolemia, hypertriglyceridemia, inflammation and gut microbiota dysbiosis.

Received 24th April 2023,
Accepted 6th November 2023

DOI: 10.1039/d3fo01651j

rsc.li/food-function

1. Introduction

Obesity corresponds to a worldwide chronic disease of multifaceted aetiology, characterized by excessive accumulation and unusual distribution of the adipose tissue that lead to physiopathological dysfunction of the human organism.[1,2] Being a complex disease, besides the affectation of the adipose tissue, other factors can accompany this pathology, such as dyslipidemia,[3,4] cholesterol dysregulation,[5] and inflammation (cytokines),[6–9] among others. Furthermore, the prevalence of obesity has been rising gradually over the last few years and is currently at unprecedented levels: more than 68% of adults in the United States are considered overweight, and 35% are obese.[10,11] This problem also has an impact on the children and adolescent population, with rising obesity rates in almost all nations in the last four decades.[12] For all these reasons, it has become vital, crucial and compulsory to develop novel and alternative therapies and treatments to reduce the progression of this pathology and deal with obesity-related disturbances, including dyslipidemia and inflammation.

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† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3fo01651j

In recent times, numerous works have shown a strong link between obesity and dysbiosis.[6,13–16] Chronic low-grade inflammation and gut microbiota dysbiosis are two of the most common obesity-related disorders, being defined as long-term inflammation lasting several months to years[17] and an imbalance in the gut microbial community that is associated with disease,[18] respectively. The gut microbiota has been considered a superorganism within our bodies that can modulate many physiological functions, including immunity development[19,20] and inflammation regulation.[21,22] For this reason, it is important to identify strategies that are able to modulate intestinal microbiota composition, to turn back the dysbiosis and to help alleviate the problems caused by obesity. Thereby, supplementation with beneficial microorganisms (probiotics) and bioactive compounds that have anti-obesogenic properties may be a good alternative to existing conventional and more invasive treatments for obesity.

Previous studies of our group demonstrated that pA1c® was able to regulate the lipid and carbohydrate metabolism in *C. elegans* through the modulation of the insulin signalling pathway.[23] Moreover, the probiotic strain pA1c® was able to regulate glucose metabolism in diabetic mice.[24] Bearing this in mind, and with the goal of continuing to explore the metabolic activities of pA1c®, we administered this probiotic strain to male Wistar rats fed an obesogenic diet, which mimics three important obesity-related disturbances, namely, fat accumulation, dyslipidemia and inflammation. We propose that pA1c®, apart from glucose metabolism regulation in diabetic mice,[24] may also be involved in the regulation of lipid metabolism and inflammation. Hence, the objectives of this study were: (1) the evaluation of the effects of the supplementation with pA1c® on the regulation of adiposity and dyslipidemia in diet-induced obese rats and (2) the investigation of the mechanism of action of pA1c® and its impact on the gut microbiota composition in diet-induced obese rats.

2. Materials and methods

2.1. Bacterial strain

Pediococcus acidilactici strain pA1c® 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, 31191, Spain) backed by an international patent "Probiotics for regulating blood glucose [PCT/EP2020/087284; WO2021/123355A1]". This bacterium was grown in deMan-Rogosa-Sharpe Agar (MRS) medium at 37 °C (facultative anaerobe). This species meets the criteria of qualified presumption of safety (QPS) by the European Food Safety Authority (EFSA) and GRAS status (FDA).

2.2. Experimental design

Twenty-nine four-week-old male Wistar rats (Charles River) were used in this study. The rats were kept in an isolated room with controlled temperature (21–23 °C) and humidity (50% ± 10%), and a 12 h : 12 h artificial light/dark cycle. All rats were acclimatized to the experimental facility for two weeks, where all animals were fed a control diet. Following the acclimation period, the animals were randomly divided and allocated into three groups until the end of the study. One group (control diet group, CNT; n = 8) was still given the control diet (2014, Teklad Global 14% Protein Rodent Maintenance Diet), and the other two groups received a high fat/high sucrose (HFS) diet (D12451, Research Diets, 20 Jules Lane; New Brunswick, NJ) for nine weeks. One of the HFS groups, named HFS (n = 11), was fed only the HFS diet, while the other group named pA1c® (n = 10) was supplemented with the probiotic strain pA1c® in addition to the HFS diet. The rats were housed in individual cages with *ad libitum* access to water and controlled food intake. Body weight was checked weekly and food intake was recorded two times per week.

All animals were euthanized by decapitation at week nine of supplementation, trunk blood was collected, and serum and plasma samples were obtained for biochemical analysis. Tissue samples from the liver, kidneys, spleen, gastrocnemius muscle, and white adipose tissue (WAT) depots (mesenteric, retroperitoneal, epididymal, and subcutaneous) were isolated, weighed and immediately stored at −80 °C. All procedures were performed 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 038-21).

2.3. Experimental diets

The control diet contained 14% of protein. The commercial HFS diet had the following energy distribution: 20% of kcal corresponding to protein, 35% of kcal corresponding to carbohydrates and 45% of kcal corresponding to fat (ESI Table 1†). In the pA1c® group, the lyophilized probiotic strain pA1c® (10^{10} CFU per day per rat) was mixed with the HFS diet and given to the animals during the whole experiment. The diet mixed with the probiotic was changed every 3 days to avoid rancidity and oxidation. The lactic acid bacteria (LAB) counts in the HFS diet + pA1c® were used as viability confirmation of the strain and were performed by plate counting on MRS agar (Scharlau, Barcelona, Spain), incubated for 48 h at 37 °C under a CO_2 atmosphere (5%).

2.4. Biochemical analyses

Total cholesterol, HDL-cholesterol (HDL-Chol), triglycerides (TG), glucose, aspartate aminotransaminase (AST), and alanine aminotransaminase (ALT) were quantified with an HK-CP kit (ABX Pentra, Montpellier, France) adapted for a Pentra C200 analyser (HORIBA ABX, Montpellier, France). Monocyte chemotactic protein-1 (MCP-1), oxidized low-density lipoprotein (ox-LDL) and interleukin-6 (IL-6) serum concentrations were quantified using specific ELISA kits (Thermo Fisher Scientific Inc., Waltham, MA, and MyBiosource, San Diego, CA). Serum insulin

was quantified with a specific ELISA kit (Mercodia AB, Uppsala, Sweden). The log (TG/HDL-Chol) ratio was calculated to determine the atherogenic index of plasma (AIP).[25]

2.5. RNA extraction and quantitative PCR analysis

Total RNA from 200 mg of mesenteric fat and liver samples from the control, HFS and pA1c® 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). Then, 1 μg of RNA was treated with DNAse I (DNase I-RNase free, Invitrogen Life Technologies) 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), with incubation for 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: *Adipoq*, *Cebpa*, *Fasn*, *Slc2a1*, *Slc2a4*, *Pparg*, *Srebf*, *Acot-8*, *Acox-1*, *Cpt-1a*, *Cpt-2*, *Hsd-17b4*, *Plin-1*, *Scp-2*, *Scd-1*, *Aaca-2*, *Hmgcl* and *Hmgcs-2* (TaqManTM Gene Expression Assays) in triplicate using a CFX384 Touch™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA). Gene expression levels were normalized compared to *TATA box binding protein* (*Tbp*) gene expression as a housekeeping control. Gene expression differences between the pA1c®-supplemented and non-supplemented samples were estimated using the relative quantification $2^{-\Delta\Delta Ct}$ method.

2.6. Fecal sample collection and metagenomic analyses

Feces of the different groups of the study were collected on week nine of supplementation and immediately stored at −80 °C. DNA isolation and bacterial DNA sequencing analyses were performed at the CIMA LAB Diagnostics, Genomics Unit of the Centre for Applied Medical Research (Pamplona, Spain). dsDNA characterization was performed using Qubit (Thermo Fisher). The regions V3 and V4 of the ribosomal 16S gene were sequenced by using Illumina protocols explained elsewhere.[26] Briefly, sequencing consists of two polymerase chain reactions (PCRs), in which the V3 and V4 regions of the 16S rRNA gene were amplified. It required the use of 16S-forward and 16S-reverse specific primers (16S Amplicon PCR forward primer = 5 0 TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGCCTACGG GNGGCWGCAG; 16S Amplicon PCR Reverse Primer = 5 0 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC; Nextera XT DNA Index Kit FC-131-1002 Illumina; San Diego, CA). The protocol followed for the first PCR was at 95 °C for 3 min and 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. After the cleansing process, 5 μl was taken from the first PCR sample to be used for the second PCR. For the second PCR, the protocol followed was 95 °C for 3 min and 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. A cleansing process was performed after each PCR to remove the primers from the sample. Afterwards, the samples were loaded into an MiSeq equipment for sequencing and quantification. The operational taxonomic unit (OTU) grouping methods were used to analyze the gut microbiome. Taxonomy was assigned using BLAST and HITdb. The alignments of the different OTUs (sequences) were conducted using the workflow of the 16S Metagenomics of Illumina database, which allows the classification at the phylum, class, order, family, genus or species level. The abundance matrices were filtered and then normalized at each level of classification. Bioinformatic analyses of the differences in the gut microbiota of the different groups were performed using MicrobiomeAnalyst. Finally, a predictive analysis of the metabolic function of the intestinal microbiota was conducted by Tax4Fun using the program MicrobiomeAnalyst.

2.7. Statistical analyses

Body related tissues and biochemical parameters, qPCR analyses and ELISAs were evaluated using the ordinary one-way ANOVA test followed by Fisher's LSD test when significant differences were obtained. 16S metagenomics (alpha-diversity and the differential abundance of families, genera and species) were analysed using Metagenome-seq of MicrobiomeAnalyst. Correlation analyses were performed using Spearman's rank correlation coefficient.

3. Results and discussion

3.1. pA1c® reduces adipose tissue accumulation and increases lean muscle in rats

No differences in the total body weight were observed between the pA1c® group and the HFS group, although there was a non-significant downward trend in the probiotic-supplemented rats (Table 1). No differences were also found in the weights of the liver, spleen and kidneys between the CNT, HFS and pA1c® groups. However, the animals supplemented with pA1c® exhibited a higher proportion of lean muscle tissue than the HFS rats. Compared with the HFS group, the rats that received the probiotic had lower weights of all the fat depots, and this weight reduction was statistically significant in the mesenteric fat (Table 1). These results confirm the findings of our previous work in which we demonstrated the fat accumulation- and lipid droplet-reducing properties of the probiotic pA1c® in *C. elegans*.[23] The tendency of the probiotic to reduce all types of visceral fat led us to think that a fattening phase (weight gain) before the experiment or a longer duration of the supplementation phase could have revealed more pronounced significant differences in the adipose tissue between the pA1c® and HFS groups. Numerous studies have linked high mesenteric fat with obesity and inflammation.[27–29] Therefore, a possible mechanism of action of the probiotic could be the reduction of mesenteric fat and thus inflammation.

3.2. pA1c® reduces cholesterol and triglyceride contents, and enhances inflammatory response in rats

pA1c® supplementation significantly reduced total cholesterol and triglyceride levels when compared with the HFS group. In

Table 1 Tissue weights in Wistar rats under normal and experimental conditions[a]

	CNT (n = 8)	HFS (n = 11)	pA1c® (n = 10)	ANOVA (p)
Weight (g)	409.83 ± 7.95	474.09 ± 7.40$^{\#\#\#}$	460.16 ± 8.92$^{\#\#\#}$	<0.001
Tissue weights (weight proportion related to total body weight)				
Liver	2.37 ± 0.06	2.48 ± 0.09	2.42 ± 0.06	ns
Spleen	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	ns
Kidney	0.27 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	ns
Gastrocnemius muscle	0.55 ± 0.01	0.47 ± 0.01$^{\#\#\#}$	0.51 ± 0.01$^{*,\#\#}$	<0.001
WAT depot weight (weight proportion related to total body weight)				
Mesenteric fat	0.59 ± 0.10	1.27 ± 0.07$^{\#\#\#}$	0.97 ± 0.05$^{**,\#\#}$	<0.001
Epididymal fat	1.78 ± 0.15	3.36 ± 0.26$^{\#\#\#}$	3.18 ± 0.20$^{\#\#\#}$	<0.001
Subcutaneous fat	1.38 ± 0.08	2.33 ± 0.13$^{\#\#\#}$	2.18 ± 0.16$^{\#\#\#}$	<0.001
Retroperitoneal fat	1.44 ± 0.15	3.02 ± 0.24$^{\#\#\#}$	2.95 ± 0.21$^{\#\#\#}$	<0.001
Visceral WAT	3.81 ± 0.37	7.66 ± 0.42$^{\#\#\#}$	7.17 ± 0.45$^{\#\#\#}$	<0.001
Total WAT	5.19 ± 0.44	9.98 ± 0.48$^{\#\#\#}$	9.35 ± 0.58$^{\#\#\#}$	<0.001

[a] Data are expressed as mean ± SEM. Statistical analyses were performed using the one-way ANOVA test followed by the Fisher's LSD test when statistical significance ($p < 0.05$) was reached in the ANOVA test. (Fisher's LSD test, $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to the HFS group; and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ with respect to the CNT group.) CNT, control; HFS, high-fat-sucrose; pA1c®, *Pediococcus acidilactici*; and WAT, white adipose tissue. Visceral fat WAT corresponds to the sum of mesenteric, epididymal and retroperitoneal fat depots; and total WAT corresponds to the sum of visceral WAT and subcutaneous fat.

Table 2 Biochemical variables in C57BL/6J mice under normal and experimental conditions[a]

	CNT (n = 8)	HFS (n = 11)	pA1c® (n = 10)	ANOVA (p)
Cholesterol metabolism biomarkers				
Cholesterol (mg dL^{-1})	89.66 ± 4.89	105.7 ± 5.58	82.88 ± 5.20**	<0.05
HDL-Chol (mg dL^{-1})	24.11 ± 0.80	23.63 ± 0.90	21.29 ± 1.06	ns
Total Chol/HDL	3.71 ± 0.13	4.46 ± 0.12$^{\#\#\#}$	3.88 ± 0.10**	<0.001
TG (mg dL^{-1})	56.50 ± 2.86	68.00 ± 6.51	48.86 ± 3.76*	<0.05
AIP	0.37 ± 0.02	0.45 ± 0.05	0.36 ± 0.01	ns
Hepatic biomarkers				
ALT (U L^{-1})	43.33 ± 1.22	47.43 ± 1.25	47.44 ± 1.16	ns
AST (U L^{-1})	172.16 ± 5.10	204.22 ± 5.35$^{\#\#}$	181.35 ± 6.67*	<0.05

[a] Data are expressed as mean ± SEM. Statistical analyses were performed using the one-way ANOVA test followed by the Fisher's LSD test when statistical significance ($p < 0.05$) was reached in the ANOVA test. (Fisher's LSD test, $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to the HFS group; and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ with respect to the CNT group.) CNT, control; HFS, high-fat-sucrose; pA1c®, *Pediococcus acidilactici*; TG, triglycerides; AIP, atherogenic index of plasma; ALT, alanine aminotransferase; and AST, aspartate aminotransferase.

fact, although without statistical significance, the pA1c® rats showed even lower values than the control group, normalizing the HFS-induced dyslipidemia (Table 2): the pA1c®-supplemented animals showed a lower ratio between total cholesterol and HDL-cholesterol than the HFS rats, indicating an amelioration of the cholesterol profile. In addition, recent studies have shown that the species *Pediococcus acidilactici* could be used as a cholesterol-reducing probiotic for preventing or managing hypercholesterolemia and dyslipidemia.[30–32] Taking all these data together, we suggest that supplementation with pA1c® could be a potential alternative or complementary strategy to traditional hypercholesterolemia treatments. However, it is necessary to go deeper into the study of the mechanism of action of the probiotic in cholesterol metabolism.

Moreover, although no statistically significant difference was found in the atherogenic index of plasma (AIP), the pA1c® group showed lower levels than the control and HFS rats. Furthermore, a significant decrease in one of the most important hepatic biomarkers of liver damage (AST) was found in rats supplemented with the probiotic in comparison with the HFS group.

Regarding inflammation, a decrease in the circulating levels of three pro-inflammatory biomarkers (MCP-1, ox-LDL and IL-6) was observed in the pA1c®-supplemented group when compared with the HFS group (Fig. 1A–C). MCP-1 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages.[33] Likewise, ox-LDL has been found to have antigenic potential and contribute heavily to atherosclerosis-associated inflammation, activating both innate and adaptive immunity.[34] In the same way, IL-6 is a pro-inflammatory cytokine closely related to obesity, inflammation and immunity.[35] Besides, it is more than proven that cytokines that are released by inflammatory cells infiltrating the obese adipose tissue, such as IL-6 and MCP-1, can act on immune cells leading to local and generalized inflammation.[36] Furthermore, the white adipose tissue of obese people is characterized by increased production and secretion of a wide range of inflammatory molecules including IL-6, which may have local effects on the same white adipose tissue physiology

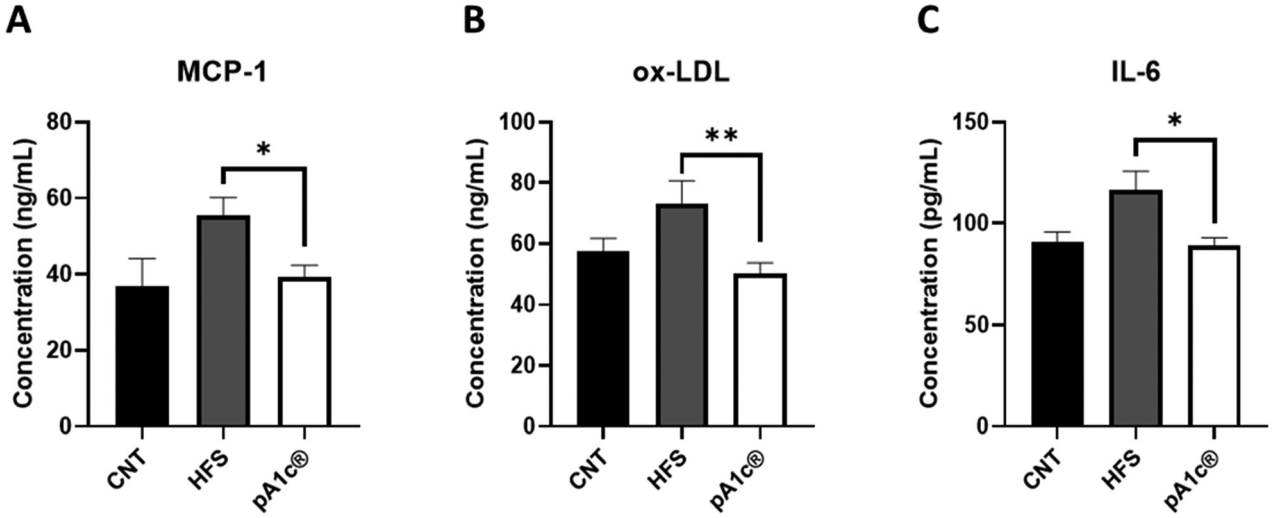

Fig. 1 The MCP-1 (A), ox-LDL (B) and IL-6 (C) levels of the control, HFS and probiotic-treated rats quantified by ELISA. Statistical analyses were performed using the one-way ANOVA test followed by the Fisher's LSD test when statistical significance ($p < 0.05$) was reached in the ANOVA test (Fisher's LSD test, *$p < 0.05$ with respect to the HFS group).

but also systemic effects on other organs such as the liver.[37] Hence, and in conjunction with the above results, we can corroborate the strong anti-inflammatory effect of pA1c®, and at the same time, we can complement the mechanism of action proposed with the reported findings. Therefore, the molecular mechanism of action by which this probiotic exerts its anti-obesogenic effect is by reducing visceral adiposity (mesenteric fat), TG, cholesterol, hepatic biomarker (AST), and the serum levels of three important pro-inflammatory molecules (MCP-1, ox-LDL and IL-6) that are usually increased in obese people.

3.3. The anti-obesogenic activities of pA1c® are mediated by anti-inflammatory pathways, adipocyte differentiation, and the activation of the insulin signalling pathway, the fatty acid beta-oxidation pathway and the lipolysis process

Gene expression analyses of the mesenteric fat were performed due to the probiotic-induced reduction of this fat depot, and of liver tissue (Fig. 2). Adiponectin (*Adipoq*) is the most abundant peptide secreted by adipocytes. Its regulation plays a key role in obesity-related diseases, regulating inflammation and energy metabolism, stimulating fatty acid oxidation, reducing plasma triglycerides and improving glucose metabolism by increasing insulin sensitivity.[38,39] Thus, its significant upregulation in the pA1c®-supplemented group compared with the HFS and CNT groups (Fig. 2A) may explain the biomolecular pathway by which the probiotic increases the anti-inflammatory response and reduces the TG serum levels. Moreover, a downregulation of *Cebpa* was found in the pA1c®-supplemented group in comparison with the HFS rats in both the mesenteric fat and liver (Fig. 2A and C). The members of the C/EBP family of transcription factors participate as critical regulators throughout the development of adipocyte differentiation where C/EBPα proteins were found to play a central role in later stages of adipocyte maturation.[40–42] This reduction in *Cebpa* is in consonance with similar obesogenic studies that have been carried out, where the authors conclude that the decreased weight gain and adipose tissue mass and the reduction of TG after a treatment were caused by the inhibition of *Cebpa*.[42,43] Furthermore, it has also been demonstrated that the reducing levels of *Cebpa* in adipose tissue could reduce insulin resistance and pro-inflammatory cytokines such as IL-6.[42,44] Thus, it is suggested that *Cebpa* may be an important mediator in the fat-reducing and inflammatory-normalizing activities of the probiotic, although further studies are needed. On the other hand, an upregulation of two important genes that govern glucose metabolism (*Slc2a1* and *Slc2a4*) was observed in the pA1c®-supplemented group when compared with the HFS group. In the case of *Slc2a1*, a significant increase was also observed when compared with the CNT group. GLUT proteins are encoded by the *Slc2* genes and are members of the major facilitator superfamily of membrane transporters. GLUTs 1–5 are the most thoroughly studied and all have well-established roles as glucose and/or fructose transporters in various tissues and cell types.[45] Inhibition of these genes appears to be closely related to insulin resistance, so their overexpression, together with that of adiponectin, may help to combat obesity and the related disturbances arising from it. Furthermore, a strong upregulation of the peroxisome proliferator-activated receptor gamma (*Pparg*) in the pA1c® group was noticed. Several studies linked the overexpression of *Pparg* with an insulin resistance- and dyslipidemia-alleviating effect and an anti-inflammatory activity,[46–48] so this finding supports the anti-inflammatory and dyslipidemia-alleviating role of pA1c® in obesity. No differences were found in *Fasn* and *Srebf* expression in mesenteric fat. Interestingly, these 2 genes, together with *Scd-1*, were found significantly downregulated in the liver with respect to the HFS group, and even with the control in the case of *Fasn* and *Scd-1* (Fig. 2C). *Srebf*,

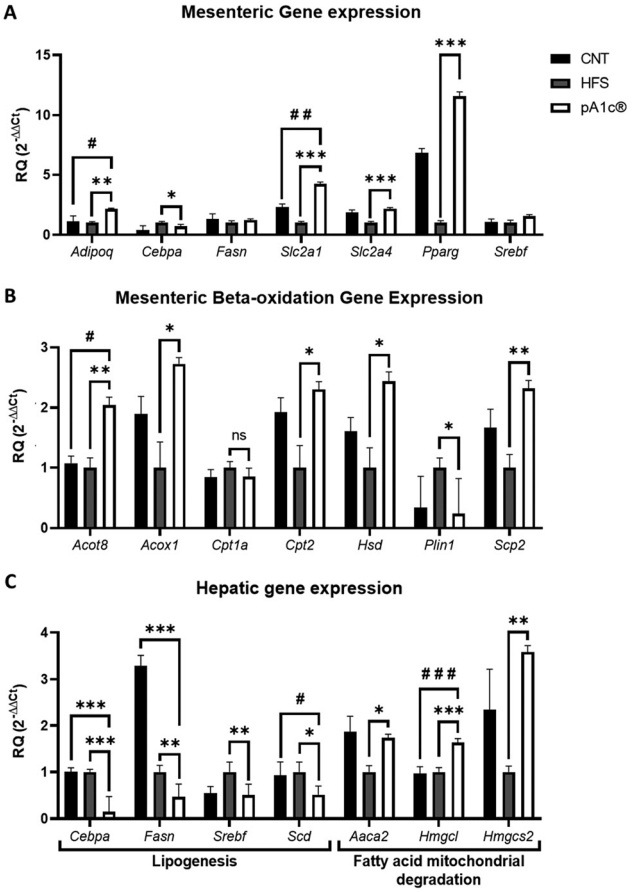

Fig. 2 Mesenteric fat gene (A), fatty acid beta-oxidation and lipolysis (B) and liver (C) gene expression quantified by real-time PCR (qPCR) in Wistar rats. The gene expression levels were normalized to the housekeeping gene (*Tbp*-1). Data are expressed using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using the one-way ANOVA test followed by the Fisher's LSD test when statistical significance ($p < 0.05$) was reached in the ANOVA test (Fisher's LSD test, $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ with respect to the HFS group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ with respect to the CNT group).

Scd-1 and *Fasn* are well-known lipogenic genes, and are involved in the synthesis of fatty acids and adipogenesis.[49–51] In addition, they are highly interconnected with each other, since upregulated *Srebf* levels are capable of activating *Fasn*.[52] Beysen *et al.* demonstrated the importance of *Fasn* in the process of lipogenesis, which inhibited alleviated hepatic steatosis, non-alcoholic fatty liver disease (NAFLD) and obesity.[53] Moreover, Qin *et al.* reported that dietary fibre alleviates hepatic fat deposition *via* inhibition of lipogenic gene expression of *Srebf*.[54] Hence, these findings support the anti-lipogenic role of pA1c® in the liver of individuals with obesity and are in agreement with previous studies conducted by our group on *C. elegans* and mice.[23,55]

The expression analyses of beta-oxidation genes in mesenteric fat showed an upregulation of the fatty acid degradation process in the pA1c®-supplemented group, through the activation of the peroxisomal (by the upregulation of the *Acot-8*, *Acox-1*, *Hsd-17b4* and *Scp-2* genes in mesenteric fat) and mitochondrial (by the upregulation of *Cpt-2*) fatty acid beta-oxidation, in comparison with the HFS group (Fig. 2B). This increase in fatty acid oxidation would be confirmed again in liver tissue, where we observed a significant upregulation of the *Aaca-2*, *Hmgcl* and *Hmgcs-2* genes (Fig. 2C). The triggering of fatty acid beta-oxidation in adipose and liver tissues after the probiotic supplementation could be one of the mechanisms by which mesenteric and visceral fat reduction occurred. Importantly, this rise in beta-oxidation, together with the overexpression of the glucose metabolism genes *Slc2a1* and *Slc2a4*, is in concordance with our previous studies on *C. elegans* and mice, where we demonstrated that the mechanism of action of pA1c® affected the insulin signalling and beta-oxidation pathways.[23,55] Looking more closely at Fig. 2B reveals a downregulation of perilipin-1 (*Plin-1*), which is a gene that encodes lipid droplet surface proteins named perilipins, in the pA1c® rats compared with the HFS rats. Perilipins sequester lipids by protecting lipid droplets from a lipase action modulating and governing the lipolysis process.[56] Associated studies have established a strong relationship between high levels of *Plin-1* in the white adipose tissue and excessive fat accumulation and adiposity, and the development of chronic inflammation and obesity.[57,58] Therefore, the reduction of *Plin-1* by the probiotic activated the lipolysis process, which, combined with the increase in beta-oxidation, could be a promising alternative approach for therapy against obesity. Probably, if we had extended the study a few more weeks, we would have been able to see these changes in gene expression reflected at the phenotypic level (significant reduction in the total weight of the pA1c®-supplemented rats compared with the HFS rats not supplemented with pA1c®).

3.4. pA1c® modulates increasing alpha diversity and the abundance of beneficial bacteria and decreasing harmful micro-organisms in the gut microbiota

In recent years, numerous works have established a strong correlation between the gut microbiota dysbiosis and the appearance of obesity, and its associated health problems such as dyslipidemia, metabolic syndrome, cardiovascular disease, atherosclerosis or the development of chronic inflammation.[16,59,60] Hence, one alternative viewpoint of obesity treatment might be the modulation of the intestinal microbiota with bioactive compounds or live organisms (probiotics), with the main objective of restoring gut microbiota balance and neutralization of intestinal dysbiosis.

The metagenomic study showed a statistically significant increase in the alpha-diversity (Chao-1 diversity index, $p = 0.028$; and observed sample diversity index, $p = 0.009$) in the pA1c®-supplemented group compared with the HFS group (Fig. 3A). There is evidence that supports a relationship between lower alpha-diversity and higher body mass index,[61] and between high alpha-diversity and weight loss.[62] Furthermore, there are studies that have linked an increase in alpha-diversity with an improvement in cholesterol regulation and the prevention of hyperlipidemia and dyslipidemia.[63] Therefore, we can assume that the probiotic supplementation, apart from increasing and

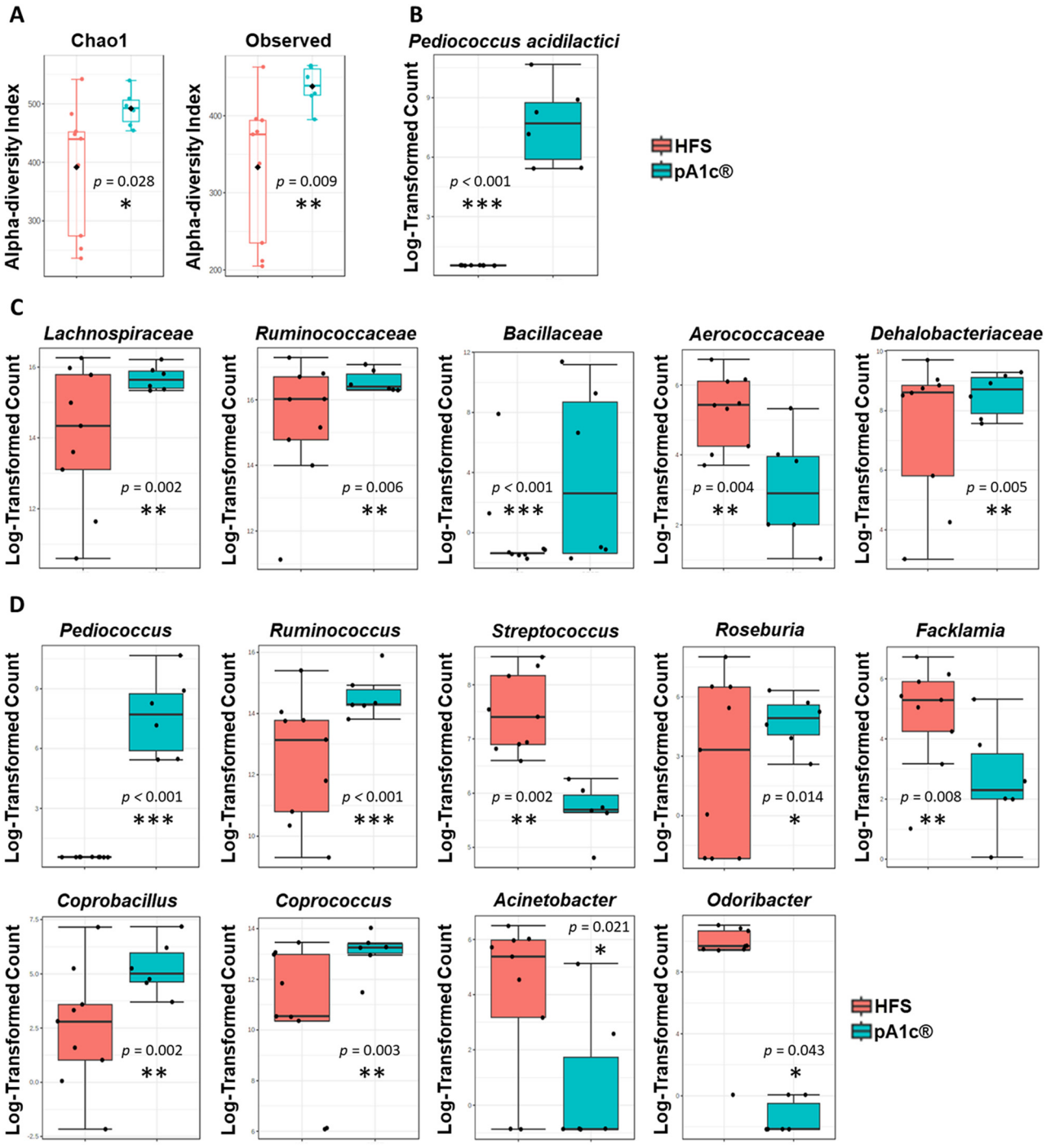

Fig. 3 Alpha-diversity analyses (A), presence of the supplemented probiotic *Pediococcus acidilactici* pA1c® (B), and statistically significant differences in families (C) and genera (D), between the pA1c®- and HFS-supplemented groups. Metagenomic statistical analyses and the abundance of the bacteria were calculated using Metagenome-seq, with log-transformed bacterial 16S rRNA gene copy counts (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ with respect to the HFS group).

improving the species richness of the intestinal microbiota, may directly contribute to a reduction of body weight and the normalization of the cholesterol profile, thus contributing to avoid the onset of dyslipidemia.

A significant increase in the abundance of *Pediococcus acidilactici* (species to which the probiotic strain pA1c® administered to the supplemented rats belongs) was detected in the pA1c® group compared to the HFS group (Fig. 3B). This species appeared in the feces of all the pA1c®-supplemented rats. Hence, these findings support the idea that pA1c® is able to colonize the colon and settle in the intestine with 100% effectiveness in only nine weeks of supplementation.

Microbiota analyses performed through 16S evidenced significant differences in the abundance of five families between the pA1c®- and HFS-supplemented groups. pA1c® increased the abundance of the Lachnospiraceae, Ruminococcaceae, Bacillaceae and Dehalobacteriaceae families and decreased that of Aerococcaceae (Fig. 3C). It is well established that the Lachnospiraceae and Ruminococcaceae families are butyrate-producing bacteria and have been negatively correlated with obesity.[64,65] Moreover, different studies correlate increased abundance of these bacterial families with an alleviation of obesity in high fat rodents by enhancing gut barrier function, reducing inflammation, ameliorating weight gain, mitigating liver function damage and alleviating insulin resistance.[64,66–68] On the other hand, it is still unclear whether the Bacillaceae family plays a role in obesity and, if it does, what function it exerts. However, in recent years there has been a boom in the use of probiotics from the Bacillaceae family as an alternative treatment for obesity.[69–72] Therefore, an increase in the abundance of this family might be beneficial for obesity-related disturbances. Furthermore, obesity has been related with a decreased abundance of several groups within the class Clostridia, including Dehalobacteriaceae.[73] Additionally, previous studies of our group have revealed that the family Dehalobacteriaceae was involved in inflammation, being one of the main overexpressed families in subjects with low-inflammatory index compared with individuals with high-inflammatory index,[74] so its increase after pA1c® supplementation would evidence the anti-obesogenic and anti-inflammatory capacity of pA1c® against chronic inflammation and obesity. Other scientific investigations have reported Aerococcaceae as a pathogenic micro-organism that may be involved in human infections;[75] thus, its abundance decline after pA1c®-supplementation should be understood as a protective mechanism of defence of the gut microbiota against external infections.

Regarding genus, the nine-week pA1c®-supplementation increased the abundance of *Pediococcus*, *Ruminococcus*, *Roseburia*, *Coprobacillus* and *Coprococcus*, and decreased the levels of the *Streptococcus*, *Facklamia*, *Acinetobacte*r and *Odoribacter* genera when compared with the HFS group (Fig. 3D). Over the last few years, probiotics derived from the *Pediococcus* genus, including the *Pediococcus acidilactici* strains, have been studied in the context of obesity. Numerous studies have reported that an increase in the abundance of *Pediococcus* was associated with lower fat accumulation, lower serum TG and cholesterol levels, reduced liver damage, lower inflammation and ameliorated obesity-related dyslipidemia through the modulation of the gut microbiota.[23,30–32,76–79] Besides, butyrate has emerged with vast beneficial effects on energy metabolism, intestinal homeostasis and immune response regulation that seems to play an important role in the development and alleviation of obesity. Butyrate is a small-chain fatty acid (SCFA) organic metabolite produced by the fermentation of dietary fibre and resistant starch. Several studies have provided a protective role of butyrate against inflammation, hypercholesterolemia, obesity and obesity-related diseases.[80,81] Thus, the overexpression of butyrate-producing bacteria, such as the genera *Ruminococcus*, *Roseburia* and *Coprococcus*,[82–84] could be beneficial in relation to dyslipidemia, inflammation and obesity. Moreover, aside from the butyrate-producing effect, the literature describes *Roseburia* as a genus that is negatively correlated with obesity and liver steatosis[85] while *Coprococcus*, together with *Coprobacillus*, have been reported as bacteria that correlate negatively with serum biochemical and cholesterol metabolic parameters,[86] shedding some light on the hepatic biomarkers, TG and cholesterol parameter reduction observed above. In contrast, *Streptococcus* is positively correlated with obesity, inflammation and weight gain, and is found to be decreased in the HFS diet-induced obese mice through the effect of SCFA,[87,88] while *Facklamia* is an opportunistic genus that tends to increase in abundance in obesity-related problems such as type-2 diabetes and insulin resistance.[89] Furthermore, different authors have established a positive relationship between the abnormal increase in body weight, visceral fat, fat mass, and the abundance of *Acinetobacter.*[88,90] Additional authors have reported that a *Sanghuangporus vaninii* mixture ameliorated obesity related type-2 diabetes and altered intestinal microbiota by reducing the abundance of *Odoribacter.*[91]

Finally, a predictive analysis of the metabolic function of the intestinal microbiota was performed using Tax4Fun (ESI Table 2†). According to our gene expression analysis data, the prediction confirmed differences in the signalling pathways related to lipid and carbohydrate metabolism.

3.5. pA1c® correlates negatively with *Streptococcus*, cholesterol parameters, mesenteric fat, triglycerides and liver damage

In order to demonstrate whether the presence of *Pediococcus acidilactici* in animal feces could be related to the improvements observed in biochemical and physiological parameters, as well as to the presence or absence of other bacteria in the gut, we carried out different correlation analyses (ESI Fig. 1†). As expected, a statistically significant negative correlation was observed between pA1c® and cholesterol metabolism-related parameters: between pA1c® and total cholesterol (Spearman Rho: −0.7516, p value = 0.008), between pA1c® and total chol/HDL ratio (Spearman Rho: −0.7227, p value = 0.012), between pA1c® and TG levels (Spearman Rho: −0.7585, p value = 0.007), and between pA1c® and AIP (Spearman Rho: −0.6880, p value = 0.019). Moreover, a negative correlation was also observed between pA1c® and AST (Spearman Rho: −0.6302, p value = 0.039), and mesenteric fat (Spearman Rho: −0.7400, p value = 0.009). In contrast, a positive correlation was found between pA1c® and the gastrocnemius muscle (Spearman Rho: −0.7400, p value = 0.009). Therefore, these results confirm once again the involvement of pA1c® in cholesterol metabolism, lipid regulation (fat accumulation in mesenteric fat) and metabolic diseases such as obesity and NAFLD (characterized by high TG content and liver damage).

Interestingly, we found a strong negative correlation between pA1c® and the genus *Streptococcus* (Spearman Rho: −0.8018, p value = 0.0003, Fig. 4A). Curiously, *Streptococcus*

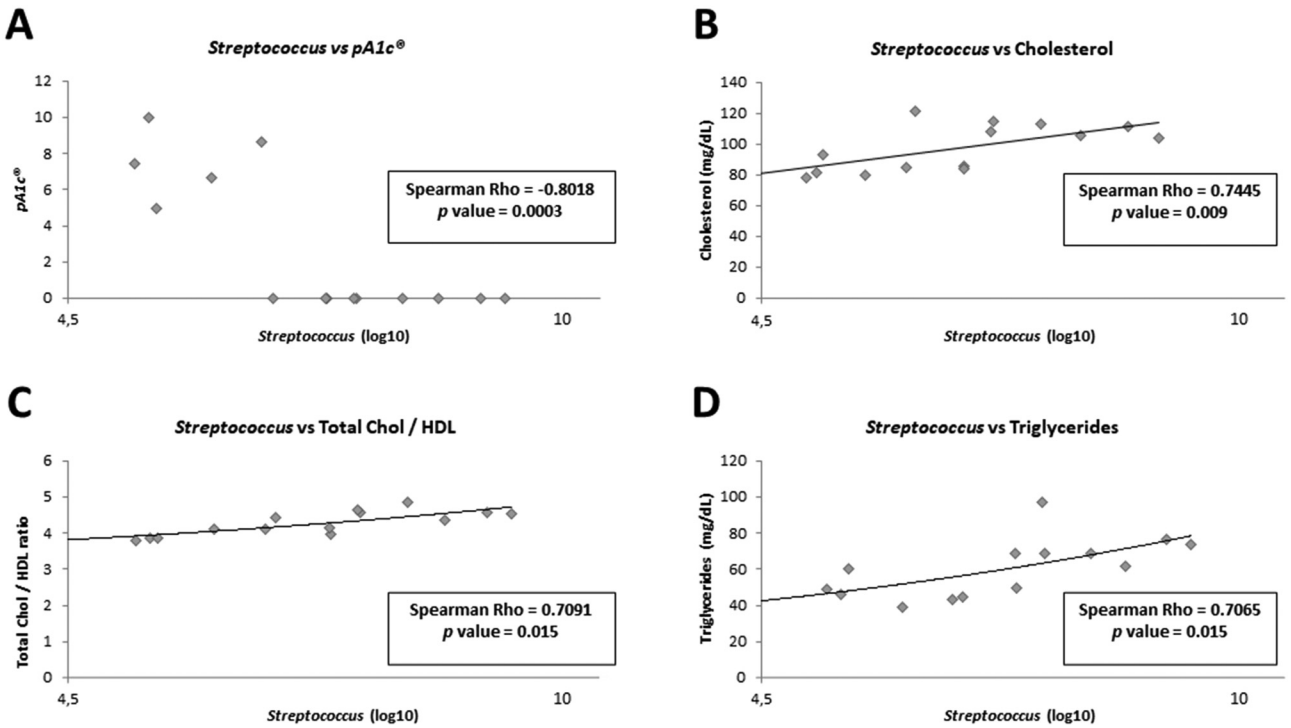

Fig. 4 Spearman correlations between *Streptococcus* and pA1c® (A), between *Streptococcus* and cholesterol (B), between *Streptococcus* and total chol/HDL ratio (C), and between *Streptococcus* and triglycerides (D).

demonstrated a significant positive correlation with total cholesterol (Fig. 4B), total chol/HDL (Fig. 4C), and TG levels (Fig. 4D), just the opposite of pA1c®. Similar to our findings, recent studies have positively associated the genus *Streptococcus* with hypercholesterolemia,[92] high TG level,[93] dyslipidemia[94] and obesity.[92–94] Furthermore, certain works have revealed the potential use of *Pediococcus acidilactici* in the food industry, since it is able, through the bacteriocins it produces, to inhibit the growth of pathogenic microorganisms such as *Streptococcus pyogenes*[95] and *Streptococcus mutants*,[96] highlighting once again the negative association between both. Interestingly, Renye *et al.* have reported the contrary effect. They have found that one *Streptococcus* species produced a bacteriocin with anti-pediococcal activity, inhibiting the growth of *Pediococcus*.[97] Therefore, it was hypothesized that there may be a competition between the species *Pediococcus acidilactici* (already described its anti-obesogenic, anti-inflammatory and anti-hypercholesterolemic activity) and the *Streptococcus* genus in the intestine; and depending on which bacterium is predominant, the host will have more (*Streptococcus* is predominant) or less (*Pediococcus acidilactici* is predominant) risk of developing metabolic diseases, such as obesity, hypercholesterolemia or hypertriglyceridemia.

4. Conclusions

We have demonstrated that the administration of the probiotic strain pA1c® has beneficial effects on TG and cholesterol metabolism parameters, visceral adiposity, biomarkers of liver damage, and inflammatory markers in high-fat diet-induced obese rats. In addition, we have shown that the main metabolic pathways by which pA1c® acts on obesity are the activation of the peroxisomal- and mitochondrial-fatty acid beta-oxidation process and inhibition of lipogenesis, which led to a reduction in inflammation. Furthermore, pA1c® would modulate the gut microbiota by increasing the microbial diversity, favouring SCFA-producing bacteria and competing with obesity and dyslipidemia-associated bacteria, such as species of the genus *Streptococcus*. Although further research is necessary, our data suggest pA1c® as a potential agent for the management of obesity and related comorbidities such as chronic inflammation, dyslipidemia, and dysbiosis.

Author contributions

Conceptualization: Deyan Yavorov-Dayliev, Fermín I. Milagro, Miguel López-Yoldi, and Paula Aranaz; data curation: Deyan Yavorov-Dayliev, Miguel López-Yoldi, and Paula Aranaz; formal analysis: Deyan Yavorov-Dayliev; funding acquisition: Fermín I. Milagro; investigation: Deyan Yavorov-Dayliev, Fermín I. Milagro, Miguel López-Yoldi, Josune Ayo, María Oneca, and Paula Aranaz; methodology: Deyan Yavorov-Dayliev, Fermín I. Milagro, Miguel López-Yoldi, and Paula Aranaz; project administration: Fermín I. Milagro; resources: Deyan Yavorov-Dayliev, Fermín I. Milagro, Miguel López-Yoldi, Iñigo Clemente, José Ignacio Riezu-Boj, Josune Ayo, María Oneca,

and Paula Aranaz; software: Deyan Yavorov-Dayliev, Iñigo Clemente, and José Ignacio Riezu-Boj; supervision: Fermín I. Milagro, Josune Ayo, and Paula Aranaz; validation: Fermín I. Milagro and Paula Aranaz; visualization: Fermín I. Milagro and Paula Aranaz; writing – original draft: Deyan Yavorov-Dayliev; writing – review & editing: Fermín I. Milagro, Josune Ayo, María Oneca, and Paula Aranaz.

Conflicts of interest

Josune Ayo is a shareholder of the company Genbioma Aplicaciones S.L., and Josune Ayo and María Oneca are co-authors of the patent "Probiotics for regulating blood glucose [WO2021/123355A1]". The rest of the authors declare no conflicts of interest. Deyan Yavorov received a predoctoral grant from the Government of Navarra (Ayudas para la contratación de doctorandos y doctorandas "Doctorados industriales 2020") [Reference: 0011-1408-2020-000010].

Acknowledgements

The authors thank Goyo Sanzol and Pentabiol S.L for the deposit of the *P. acidilactici* pA1c® strain. The authors acknowledge the partners of Genbioma Aplicaciones S.L. for their constructive criticism in the development of this manuscript, as well as Luís Gosálbez (Sandwalk BioVentures S.L.).

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