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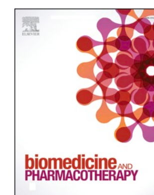

The melatonergic agonist agomelatine ameliorates high fat diet-induced obesity in mice through the modulation of the gut microbiome

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A B S T R A C T

Background and purpose: Melatonin has shown beneficial effects on obesity, both in humans and experimental models, via regulating the altered circadian rhythm and thus ameliorating the gut dysbiosis associated with this metabolic condition. However, its clinical use is limited, mostly due to its short half-life. Agomelatine is an agonist of the melatonin receptors that could be used to manage obesity and offer a better profile than melatonin.
Experimental approach: Male C57BL/6 mice were fed a high fat diet and orally treated for five weeks with agomelatine, or melatonin or metformin, used as control drugs. Metabolic profile, inflammatory status, vascular dysfunction and intestinal microbiota composition were assessed.
Key results: Agomelatine lessened body weight gain, enhanced glucose and lipid metabolisms, and improved insulin resistance. It also reduced the obesity-associated inflammatory status and endothelial dysfunction, probably linked to its effect on gut dysbiosis, consisting of the restoration of bacterial populations with key functions, such as short chain fatty acid production.
Conclusions and implications: Agomelatine can be considered as a novel therapeutic tool for the management of human obesity and its associated comorbidities.

1. Introduction

Obesity is highly prevalent, with 1.9 billion overweighted adults, being 650 million obese. It represents a major worldwide public health challenge that reduces life expectancy by 0.9–4.2 years and causes the death of 3.4 million adults per year [1].

Obesity is typically associated with 'poor' diets, high in fat and low in dietary fibre. Mechanistically, this is related to insulin resistance,

Abbreviations: AUC, area under the curve; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; HFD, high fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; IMCs, immature myeloid cells; JNK, jun N-terminal kinase; LDL, low-density lipoprotein; *Mcp-1,*, monocyte chemotactic protein 1; MDSCs, myeloid-derived suppressor cells; NAFLD, non-alcoholic fatty liver disease; NF-κB, nuclear factor-κB; PICRUSt, phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PCoA, principal coordinates analysis; STAT, signal transducer and activator of transcription.

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resulting in dysregulation of glucose and lipid metabolisms, together with development of low-grade systemic inflammation. Infiltration of activated immune cells in metabolic tissues, including fat, liver and gut, leads to proinflammatory cytokine secretion and re-programming of immunoregulatory cells. These alterations raise the risk of comorbidities, like metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and/or cardiovascular diseases [2]. Development of obesity and the associated inflammation, insulin resistance and comorbidities have been closely related to dysbiosis, modifications in the composition and/or function of the intestinal microbiota [3]. This has also been linked to the impairment of gut permeability and energy homeostasis [4]. Therefore, alleviating gut dysbiosis may be an effective treatment for obesity and its related disorders [5].

In addition, the circadian rhythm and the lipid metabolism are nowadays accepted to cross-regulate through different mechanisms that include hormones like melatonin, leptin or glucocorticoids, intestinal microbiome and energy metabolism. Its dysregulation may be also associated with the risk of obesity, which may subsequently worsen circadian clocks [6].

In this regard, melatonin, which is an important regulator of the circadian rhythm [7], oxidative stress [8], immune function [9], and glucose and lipid metabolism [10], has been described to also prevent obesity in high fat diet-fed mice. This effect was associated with the modulation of gut microbiota, by restoring *Firmicutes/Bacteroidetes* ratio and raising *Akkermansia* abundance, in line with the role of gut microbiome in the pathogenesis of obesity [11]. In fact, commensal microorganisms and host intestinal cells are an abundant source of melatonin [12], regulated by diet rather than by circadian rhythms [13,14]. Therefore, given its important role in the permeability, motility, barrier function and immune protection at the intestine [15–20], therapeutic manipulation of the intestinal melatonin system could lead to a significant improvement of the metabolic syndrome. The relevance of melatonin for intestinal homeostasis is already highlighted by studies in inflammatory bowel disease, reporting a negative correlation of disease progression with local and systemic melatonin pathways [21], but its impact in obesity is understudied.

The extremely short half-life displayed by melatonin (under 30 min) has encouraged the development of novel melatonin agonists with better pharmacokinetics. Among these, agomelatine is an agonist of the melatonin receptors MT1 and MT2, but also an antagonist of serotonin receptors 5-HT2B and 5-HT2C [22]. Agomelatine shows potent oral antidepressant and anxiolytic properties and is licensed for the management of major depressive disorders in adults [23]. Recently, it has been reported its anxiolytic/antidepressant effect in obese rats, maybe due to an amelioration of the high fat diet (HFD)-associated alterations [24]. Moreover, the capacity of agomelatine to prevent diabetes in a murine model has been explored [25]. However, a comprehensive study of the effects of agomelatine across the different organs involved would be necessary to consider its therapeutic value in these conditions. With this aim, agomelatine was assayed in an experimental model of obesity induced by a HFD in mice. Its effect was compared to that of melatonin and metformin, which has been used as a weight loss drug [26] and their impact on the metabolic profile, vascular dysfunction, inflammatory response, and gut microbiota composition was evaluated in order to identify the pathological pathways targeted by oral agomelatine treatment.

2. Materials and methods

2.1. Materials

All the chemicals were purchased from Sigma-Aldrich Quimica SL (Madrid, Spain), unless otherwise specified.

2.2. Animals and experimental design

The study was performed in agreement with the ‘Guide of the Care and Use of Laboratory animals’ as promulgated by the National Institute of Health and all procedures were approved by the Ethics Committee of Laboratory Animal of the University of Granada (Spain) (Ref. No. 28/03/2016/030). Eight-week-old male C57BL/6 mice obtained from Janvier labs (St. Berthevin, Cedex, France) were housed in makrolon cages, maintained under controlled light-dark cycle (12 h light/dark cycle), temperature and relative humidity (22 ± 1ºC, 55 ± 10 %) and provided with a free access to tap water. Mice were fed with either a standard chow diet (13 % calories from fat, 20 % calories from protein and 67 % calories from carbohydrate; Global diet 2014; Harlan Laboratories, Barcelona, Spain) or a high fat diet (HFD) (59 % calories from fat, 13 % calories from protein and 28 % calories from carbohydrate; Purified diet 230 HF; Scientific Animal Food & Engineering, Augy, France). They were randomly divided in seven groups ($n = 8$): control diet, HFD and five HFD-treated groups. Mice were daily treated by oral gavage with melatonin (15 mg/kg), agomelatine (10, 25 and 50 mg/kg) or metformin (250 mg/kg) dissolved in water for 31 days (more detail in supplementary). Animal body weight and food and water intake were regularly controlled, and energy efficiency was calculated as the ratio of body weight gain (g) to caloric intake (kcal). One week before the sacrifice, a glucose tolerance test was performed. At the end of the experiment, mice were fasted overnight, a blood sample was collected by cardiac puncture under isoflurane anaesthesia and then sacrificed by cervical dislocation (Fig. 1).

2.3. Glucose tolerance test

At week 4, mice fasted for 8 h were given a 50 % glucose solution in water (2 g/kg of body weight) by intraperitoneal injection, and a blood sample was taken from the tail vein before glucose administration and 15, 30, 60 and 120 min after. Blood glucose was determined with a handheld glucometer (Contour XT, Ascensia Diabetes Care, S.L., Barcelona, Spain).

2.4. Plasma determinations

Blood samples were taken on the day of sacrifice in heparin blood collection tubes and centrifuged for 20 min at 5000 g at 4ºC. The plasma was frozen at − 80ºC until further analysis. Plasma glucose, LDL (low-density lipoprotein)-cholesterol and HDL (high-density lipoprotein)-cholesterol concentrations were measured by colorimetric methods using Spinreact kits (Spinreact, S.A., Girona, Spain). Plasma insulin levels were quantified using a mouse insulin ELISA kit (Alpco Diagnosis, Salem, NH, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was computed with the formula: fasting glucose (mM) × fasting insulin (μU/mL) / 22.5.

2.5. Morphological variables

After sacrifice, liver and abdominal and epididymal fat were collected, cleaned, and weighed. Fat/weight index was estimated by dividing body weight by tibia length. The samples were then frozen in liquid nitrogen and stored at − 80 °C.

2.6. Vascular reactivity studies and NADPH oxidase activity

Descending thoracic aortic rings were dissected to assess obesity-associated vascular dysfunction by measuring acetylcholine vaso-relaxant ability and NADPH oxidase activity.

For the vascular reactivity study, the organ chamber was filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 and glucose 11) at 37 °C and gassed with 95 % O_2 and 5 % CO_2 (pH 7.4). Length-tension characteristics were

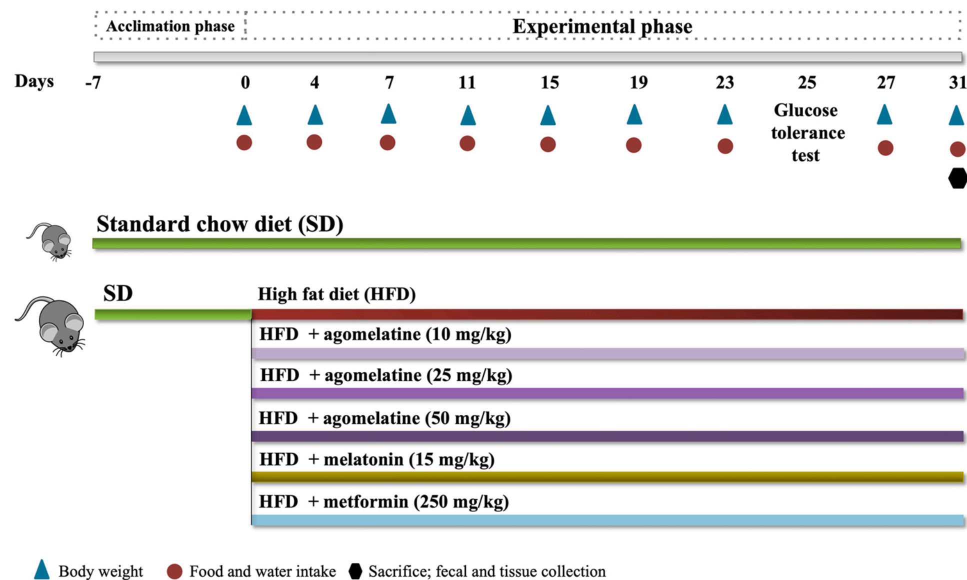

Fig. 1. Schematic diagram of experiment study design showing mice groups, chronogram, dietary and treatment intervention.

obtained via the myograph software (Myodaq 2.01 (Danish Myo-technologies, Denmark)) and the aortae were loaded to a tension of 5 mN. After the stabilization period, cumulative concentration-response curves to acetylcholine (10^{-9} M-10^{-5} M) were performed in intact rings pre-contracted by U46619 (10^{-8} M). Relaxant responses to acetylcholine were expressed as a percentage of pre-contraction.

The evaluation of NADPH oxidase activity in aortic rings was performed by lucigenin-enhanced chemiluminescence assay. Aortic rings were incubated for 30 min at 37 °C in HEPES-containing physiological salt solution (pH 7.4; in mM: NaCl 119, HEPES 20, KCl 4.6, $MgSO_4$ 1, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 1, $CaCl_2$ 1.2 and glucose 5.5). To stimulate the aortic production of O^{2-}, the rings were incubated with NADPH (100 µM). Consequently, the aortic rings were then placed in tubes containing physiological salt solution, with or without NADPH and lucigenin was injected automatically at a final concentration of 5 µmol/L. NADPH oxidase activity were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic ring.

2.7. Analysis of protein expression by Western blot

Representative liver samples (n = 3) were processed to assess MAPK, AKT, AMPK and IL-6 expression by western blotting as described before [27]. Proteins were separated in a 10 % SDS-PAGE and transferred to a PVDF membrane (GE Healthcare Life Sciences, Marlborough, MA, USA). Membranes were blocked and probed overnight at 4 °C with anti-AMPK (1:2000 dilution), anti-p-AMPK (1:1000 dilution), anti-AKT (1:1000 dilution), anti-p-AKT (1:1000 dilution), anti-p38 MAPK (1:1000 dilution), anti-p-p38 MAPK (1:1000 dilution), anti-IL6 (1:500 dilution) (Cell Signalling, Danvers, MA, USA); followed by 1 h of incubation with peroxidase-conjugated anti-rabbit IgG antibody (1:5000 dilution) (Sigma-Aldrich, Madrid, Spain); and β-actin (1:1000 dilution) (Santa Cruz Biotechnology, Inc., Heidelberg, Alemania) The target proteins were detected by Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Spain SL, Madrid, Spain). Densitometry-based semi--quantification of protein levels was performed using ImageJ software (Free Software Foundation Inc).

2.8. Analysis of gene expression by RT-qPCR

Total RNA was extracted from liver and fat samples using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's recommendations, and was reverse transcribed into cDNA using oligo(dT) primers (Promega, Southampton, UK). Real time quantitative PCR (qPCR) amplification and detection was carried out on optical-grade 48 well plates in EcoTM Real time PCR System (Illumina, San Diego, CA, USA) with 20 ng of cDNA, the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) with specific primers (Table 1). The mRNA relative quantitation was calculated using the ΔΔCt method and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as housekeeping gene.

2.9. Flow cytometry

The cells from adipose and liver tissues were collected following the procedure previously described with some modifications [28]. CD45 +CD11b+ and CD11b+Ly6C+ populations were determined. For this, both tissues were isolated excised, washed and cut into small pieces ($\leq$ 3 mm^3). Fat minced tissue was digested with HBSS solution containing 1 mg/ML collagenase Type I in a 37 °C water bath for 30 min. The digested tissue solution was filtered using 100 µm cell strainers, washed and red blood cell lysis was performed for 5 min at room temperature. The single cell suspension was counted, and cells were stained with phenotyping markers (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, unless otherwise stated) including: FcγR blocking, Viability Dye (eFluor 780, Ref. 65–0865-14; eBioscience, San Diego, USA), anti-CD3 (AmCyan, Clone 145–2C11), anti-CD4 (APC-Vio 770, Clone REA1211), anti-CD11b (APC-Vio 770, Clone REA592), anti-CD11c (FITC, Clone N418) and LyC6 (PE-Vio770, Clone 1G7. G10) for 15 min at 4 °C in the dark. The cells were then fixed with the Flow Cytometry Fixation Buffer (Catalog #FC004, R&D Systems®, Abingdon, UK). Data collection was performed using a flow cytometer CANTO II (BD Biosciences, New Jersey, USA) and analyzed with Flowjo (Flowjo LLC, Ashland, Oregon, USA).

2.10. DNA extraction and Illumina MiSeq sequencing

Faecal DNA was isolated as described[29]. The resultant sequences were quality-filtered, clustered, and taxonomically allocated against the SILVA database with 97 % similarity threshold using the QIIME software

Table 1
qPCR primer sequences.

Gene	Organism	Sequence 5'- 3'	Annealing T °C
Gapdh	Mouse	FW: CCATCACCATCTTCCAGGAG RV: CCTGCTTCACCACCTTCTTG	60
Adiponectin	Mouse	FW: GATGGCAGAGATGGCACTCC RV: CTTGCCAGTGCTGCCGTCAT	64
Ampk	Mouse	FW: GACTTCCTTCACAGCCTCATC RV: CGCGCGACTATCAAAGACATACG	60
Glut 4	Mouse	FW: GAGAATACAGCTAGGACCAGTG RV: TCTTATTGCAGCAGCGCCTGAG	62
Il-1®	Mouse	FW: TGATGAGAATGACCTCTTCT RV: CTTCTTCAAAGATGAAGGAAA	55
Il-6	Mouse	FW: TAGTCCTTCCTACCCCAATTTCC RV: TTGGTCCTTAGCCACTCCTTCC	60
Jnk-1	Mouse	FW: GATTTTGGACTGGCGAGGACT RV: TAGCCCATGCCGAGAATGA	60
Leptin	Mouse	FW: AGATCCCAGGGAGGAAAATG RV: TGAAGCCCAGGAATGAAGT	60
Leptin R	Mouse	FW: GCTATTTTGGGAAGATGT RV: TGCCTGGGCCTCTATCTC	60
Mcp-1	Mouse	FW: CAGCTGGGGACAGAATGGGG RV: GAGCTCTCTGGTACTCTTTTG	62
Muc-2	Mouse	FW: GCAGTCCTCAGTGGCACCTC RV: CACCGTGGGGCTACTGGAGAG	60
Muc-3	Mouse	FW: CGTGGTCAACTGCGAGAATGG RV: CGGCTCTATCTCTACGCTCTCC	60
Occludin	Mouse	FW: ACGGACCCTGACCACTATGA RV: TCAGCAGCAGCCATGTACTC	56
Tff-3	Mouse	FW: CCTGGTTGCTGGGTCCTCTG RV: GCCACGGTTGTTACACTGCTC	63
Tlr4	Mouse	FW: GCCTTTCAGGGAATTAAGCTCC RV: AGATCAACCGATGGACGTGTAA	60
Tnf-α	Mouse	FW: AACTAGTGGTGCCAGCCGAT RV: CTTCACAGAGCAATGACTCC	60
Zo-1	Mouse	FW: GGGGCCTACACTGATCAAGA RV: TGGAGATGAGGCTTCTGCTT	56

package (Version 1.9.1) (Knight Lab, San Diego, CA, USA). The resulting abundance was used to compute the total bacterial diversity in an equivalent manner.

DNA from fecal contents was isolated following the procedure described by Rodríguez-Nogales et al., 2017 [29]. Total DNA from faecal samples was PCR amplified using primers targeting regions flanking the variable regions 4–5 of the bacterial 16S rRNA gene (V4–5), gel purified, and analyzed using multiplexing on the Illumina MiSeq machine. PCR reactions from the same samples were cleaned and then normalized using the high-throughput Invitrogen SequalPrep 96-well Plate kit. Later, a library from the samples was made fluorometrically to be quantified fluorometrically before sequencing.

The resulting sequences were completed, quality-filtered, clustered, and taxonomically assigned on the basis of 97 % similarity level against the RDP (Ribosomal Database Project) by the QIIME software package (Version 1.9.1) (Knight Lab, San Diego, CA, USA). Sequences were selected to estimate the total bacterial diversity of the DNA samples in a comparable manner and were trimmed to remove barcodes, primers, chimeras, plasmids, mitochondrial DNA and any non-16S bacterial reads and sequences < 150 bp.

Alpha diversity (α-diversity) indices and bacterial abundance data were compared using Kruskal–Wallis test followed by pairwise Mann–Whitney U comparison. Resulting p-values were corrected by the Bonferroni method. Analysis of α-diversity was performed on the output normalized data, which were evaluated using Mothur. The biomarkers for both species taxonomic analysis and functional pathways via calculation of the linear discriminant analysis (LDA) score among different phenotype groups were calculated by LEfSe (linear discriminatory analysis (LDA) effect size) (Version 1.0). Principal coordinate analysis (PCoA) was performed to identify principal coordinates and visualize β-diversity in complex multidimensional data of bacteriomes from different groups of mice. Differences in β-diversity were tested by permutational multivariate analysis of variance (PERMANOVA) using the web-based algorithm tool Microbiome Analyst [30]. The data are expressed as the mean ± standard error of the mean (SEM). Experimental data were analysed in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) by one-way or two-way ANOVA or Pearson correlation. Data with $p < 0.05$ were considered statistically significant. Metabolic phenotypes were obtained by genera classification according to their primary fermentation products as acetate, butyrate, lactate, or other producers using Bergey's Manual of Systematic Bacteriology[31]. The genera with unknown or ambiguous fermentative products were excluded. Major genera were classified according to the dominant fermentation end-product(s).

Hierarchical clustering and heat maps depicting the metabolic parameters, patterns of abundance and log values were constructed within the "R" statistical software package (version 3.6.0; https://www.r-project.org/) using the "pheatmap", "heatmap.2" and "ggplots" packages. Spearman's correlations of bacterial taxa with metabolic parameters and KEGG metagenomic functions were calculated in "R". Co-occurrence networks between taxa and functions were calculated by using the open-source software Gephi (https://gephi.org/) to find differential associations caused by similar alterations in the proportion of different taxa and their predicted functions between different groups of mice. Modularity-based co-occurrence networks were analysed at a Spearman's correlation cut off 0.7 and $p < 0.01$; the selected correlation data were imported into the interactive platform, Gephi (version 0.9.2; https://gephi.org), and the following modularity analyses and keystone node identification were conducted within Gephi.

2.11. Statistics

All results are expressed as the mean ± SEM. Differences between means were assessed for statistical significance with a one-way analysis of variance (ANOVA) and post-hoc least significance tests. Differences between proportions were examined with the chi-squared test. All statistical analyses were performed with the GraphPad 8 software package (GraphPad Software, Inc., La Jolla, CA, USA), with statistical significance set at $p < 0.05$.

3. Results

3.1. Agomelatine reduces body weight and ameliorates the altered plasma biochemical profile in HFD-fed mice

The administration of agomelatine (10, 25 and 50 mg/kg) to HFD-fed mice significantly lessened body weight gain compared to untreated control obese mice (Fig. 2A). Agomelatine did not present an anorexigenic effect since it did not modify the total energy intake but lowered the energy efficiency (Fig. 2B) and consequently reduced epididymal and abdominal fat deposits (Fig. 2C). Similar effects were seen in obese mice treated with melatonin (15 mg/kg) or metformin (250 mg/kg) (Fig. 2A-C).

Mice receiving agomelatine also showed lower plasma glucose concentrations at all time points compared to untreated HFD-fed control mice (Fig. 3A), thus resulting in significant reductions in the area under the curve (AUC) (Fig. 3A). Accordingly, these mice displayed significantly reduced values of the HOMA-IR index (Fig. 3B), a marker of insulin intolerance calculated with the fasting insulin (Fig. 3B) and glucose values (Fig. 3B). In fact, the mice treated with 50 mg/kg of agomelatine showed similar HOMA-IR index to control mice. Likewise, melatonin and metformin ameliorated the glucose intolerance status (Fig. 3B).

Regarding the lipid profile, the untreated HFD-fed group presented a hypercholesterolemic status, with higher levels of total cholesterol, LDL- and HDL-cholesterol than control diet fed mice. As expected, metformin

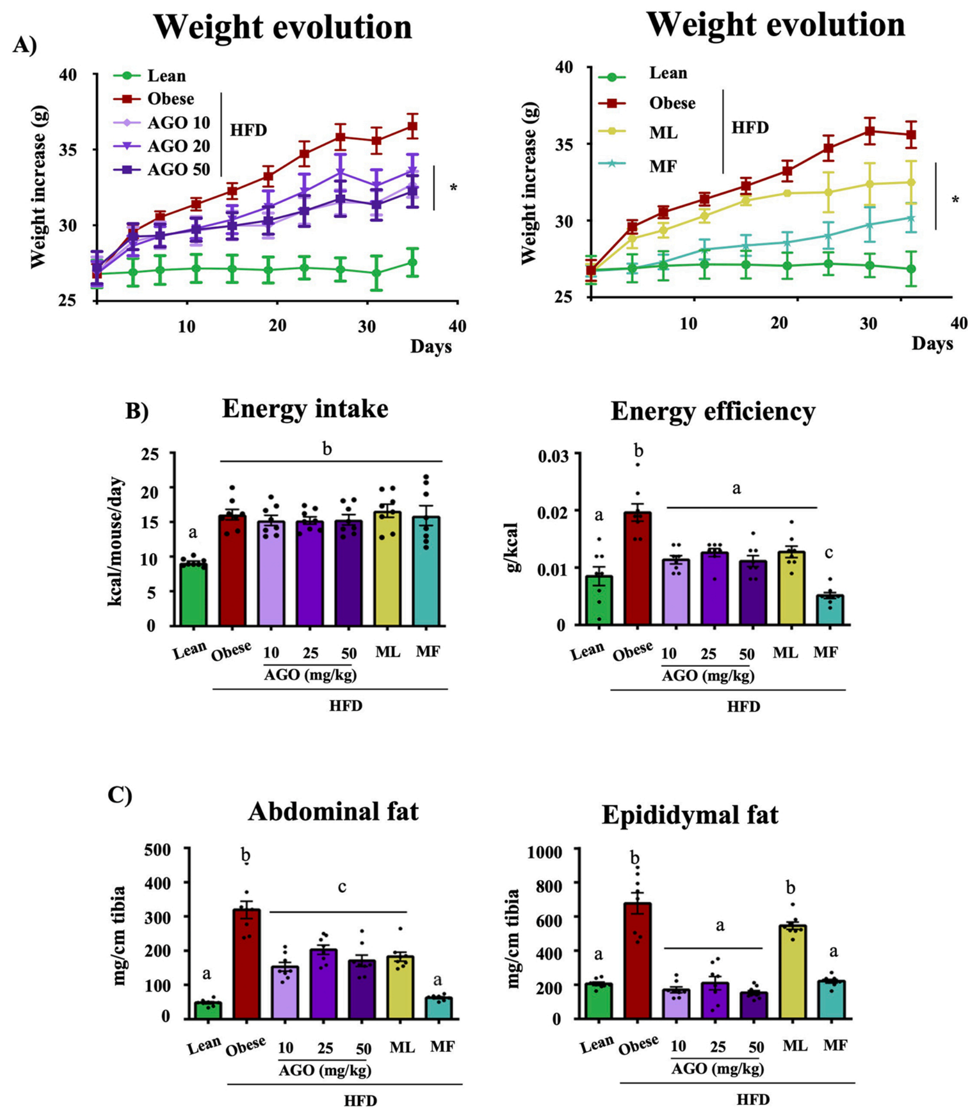

Fig. 2. Effects of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on (A) body weight evolution; (B) energy intake and energy efficiency, and; (C) epididymal and abdominal fat in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

treatment significantly ameliorated the alterations in the cholesterol profile, reducing total and LDL-cholesterol (Fig. 3C). Interestingly, agomelatine treatment significantly improved the cholesterol profile in the same way as metformin, whereas melatonin had no effect on LDL-cholesterol and only significantly reduced HDL-cholesterol (Fig. 3C).

3.2. *Agomelatine treatment improves the inflammatory status in obese mice*

Cardiovascular problems are one of the main comorbidities associated with obesity. The endothelium-dependent vasodilator response to acetylcholine of aortic rings was measured to analyse the functionality of the endothelium. The results showed a reduction in vasodilatory responses in the HFD group in comparison with control mice (Fig. 4A). Improved endothelial relaxation could be observed in mice treated with the highest doses of agomelatine, as well as with melatonin and metformin. Since this could be related to the oxidative stress, the activity of the enzyme NADPH, the main source of reactive oxygen species in this location, was measured. Obese mice exhibited a more pronounced activation than the lean ones. The highest doses of agomelatine, melatonin and metformin downregulated it (Fig. 4B).

Expression of *Glut-4* in fat of HFD-fed mice was decreased in comparison with non-obese mice (Fig. 5A). Melatonin treatment did not show any effect but agomelatine (50 mg/kg) and metformin were able to increase its expression (Fig. 5A).

Non-treated HFD-fed mice displayed a reduced fat expression of *Ampk* while agomelatine and metformin treatments reverted it (Fig. 5A). Elevated fat expression of leptin in the HFD group (Fig. 5B) was linked to a decreased expression of leptin receptor (Fig. 5B). Regarding adiponectin, obese mice presented a lower expression in adipose tissue, which was significantly increased by agomelatine and metformin treatments (Fig. 5B). The adipose tissue of non-treated HFD mice displayed an amplified expression of *Tnf-α*, *Il-1β*, *Il-6* and monocyte chemotactic protein 1 (*Mcp-1*) (Fig. 5C), compared to control mice. Only agomelatine reduced the expression of pro-inflammatory mediators in fat tissue (Fig. 5C).

When immune cell infiltration in fat tissue was evaluated by flow cymetometry, obese mice showed a significant infiltration of $CD45^{+}CD11b^{int}$ cells. Treated mice showed no significant differences compared to the lean ones. Moreover, agomelatine, at the highest dose, significantly reduced the number of this cell type (Fig. 5D).

In liver, HFD-fed mice showed a higher expression of phosphorylated

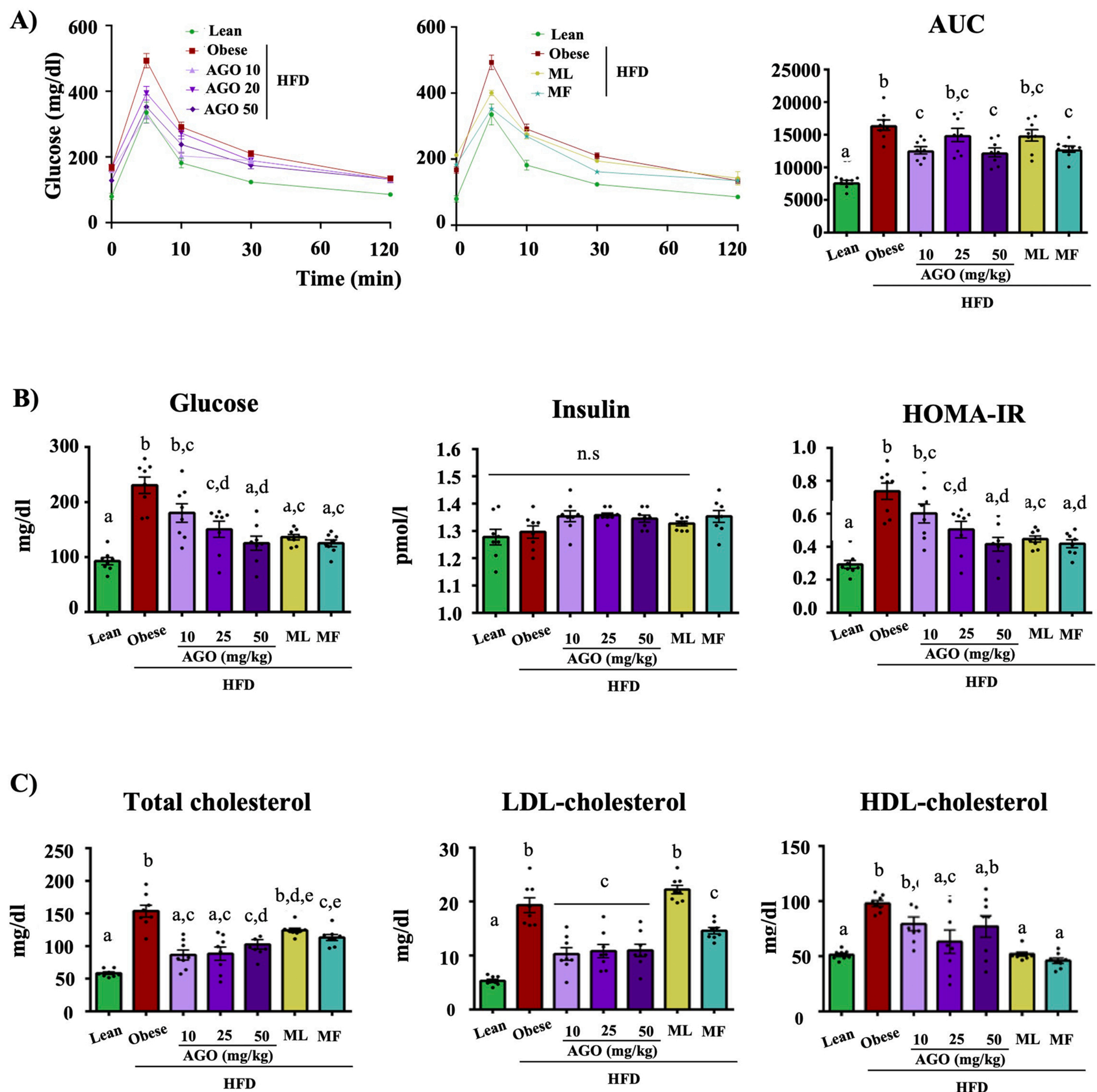

Fig. 3. Result of the administration of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) on (A) glucose tolerance test and area under the curve (AUC); (B) basal glucose, insulin levels, and HOMA-IR index; and (C) Total, LDL- and HDL- Cholesterol plasma levels in high fat diet (HFD) group. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

p38 MAPK and a reduced expressión of phosphorylated AMPK and AKT, in comparison with lean mice, which is associated with the alterations of glucose and lipid metabolisms described above and the production of pro-inflammatory mediators. However, agomelatine and metformin treatments counteracted this effect (Fig. 6A). The expression of *Jnk-1* was also significantly up-regulated in the liver of untreated obese mice, whereas the expression of the receptor for leptin (*Leptin R*) was significantly decreased, in comparison with non-obese ones, which were significatnly reverted by all treatments. (Fig. 6B). Obese miced also displayed a higher expression of *Tlr-4,* which was downregulated by all the treatments (Fig. 6B). Consequently, non-treated obese mice displayed an amplified liver expression of *Il-6* (its protein expression is also shown in Fig. 6A), *Tnf-α*, *Il-1β*, and monocyte chemotactic protein 1 (*Mcp-1*) (Fig. 6C), compared to control mice. All the treatments reduced the expression of those pro-inflammatory mediators (Fig. 6C). The flow cymetometry analysis of liver immune infiltration showed that the total number of myeloid-derived suppresor cells (MDSCs) ($Ly6C^+CD11b^+$) was increased in obese mice compared to control mice. All treatments restored their accumulation (Fig. 6D). Regarding the macrophages, obese mice showed a significant infiltration of $CD45^+CD11b^{int}$ cells. However, no significant differences were observed in the treated mice in comparison with the lean ones. Moreover, agomelatine, at all doses, significantly reduced the number of this cell type in the liver, normalizing it (Fig. 6D).

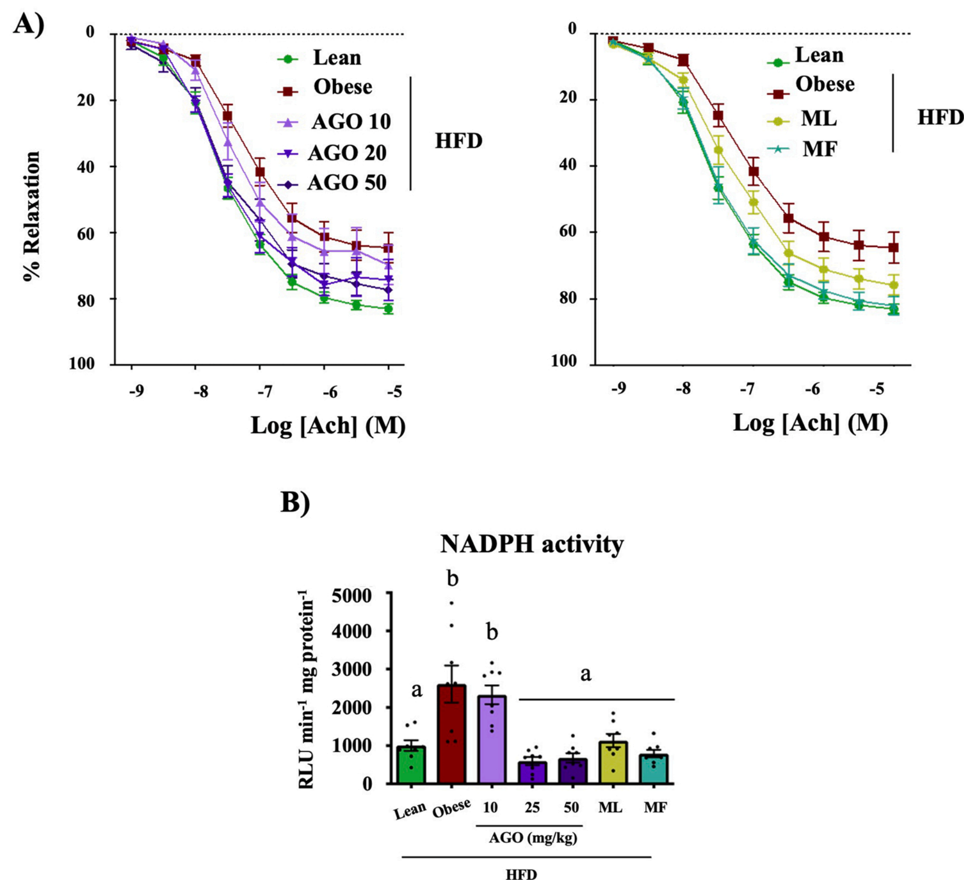

Fig. 4. Effect of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on (A) aortic endothelial function, and; (B) aortic NADPH activity in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

3.3. Agomelatine treatment ameliorates intestinal barrier dysfunction

Next, we evaluated the effect of HFD and the treatments on the intestinal homeostasis. Mucosal integrity appeared compromised in untreated HFD-fed mice, demonstrated by a down-regulation of intestinal epithelial markers, such as *Muc-2*, *Muc-3*, *Occludin*, *Tff-3* and *Zo-1* (Fig. 7A). The administration of agomelatine and melatonin was able to increase the expression of these markers (Fig. 7A). In addition, *Tlr-4* expression in the liver appeared augmented in HFD-fed mice, which is a sign of LPS exposure due to increased intestinal permeability [32] (Fig. 6B). In this case, agomelatine, but also melatonin and metformin, were able to normalize its expression (Fig. 7B).

3.4. Agomelatine treatment restores the gut dysbiosis in obese mice

This study explores for the first time the effect of agomelatine on gut microbiota, just a previous work has described the effect of a synthetic analog of agomeline (N-(2-(7-methoxy-3,4-dihydroisoquinolin-1-yl) ethyl)acetamide hydrochloride (NMDEA) has been described to diminish depression-related behaviours in mice through different mechanisms that include regulation of gut microbiota [33]. Only the highest dose of agomelatine (50 mg/kg) was evaluated since it was the one that showed a greater effect in the macroscopic and biochemical parameters.

Several ecological features were determined, including Chao1 richness (diversity estimation), observed operational taxonomic units (OTUs) (count of unique OTUs in each sample) and Shannon diversity (a richness and evenness estimator). Microbial richness, evenness and diversity were significantly decreased in the HFD group compared to non-obese mice. Although all treatments were able to increase these ecological parameters, only agomelatine significantly restored all of them (Fig. 8A). The principal coordinates analysis (PCoA) showed evident differences between control diet- and HFD-fed groups, indicative of extremely different gut environments (Fig. 8B). Interestingly, the treatments displayed marked shifts in the obese gut microbiome (Fig. 8B). Further analysis at phylum level (Fig. 8C) revealed that HFD induced a dramatic change in the two most abundant phyla when compared to non-obese mice, significantly increasing *Firmicutes* and reducing *Bacteroidetes* (Fig. 8C). Agomelatine significantly restored to baseline F/B ratio (Fig. 8C). *Verrucomicrobia* phylum was also reduced in the HFD-fed group but agomelatine restored it (Fig. 8C).

At order level, untreated HFD mice presented a reduced proportion in the sequences of *Bacteroidales, Verrucomicrobiales* and *Lactobacillus* whilst *Erysipelotrichales*, *Clostridiales* and *Lachnospirales* abundance were significantly increased (Fig. 9). Agomelatine treatment showed a similar profile than non-obese mice while metformin only counteracted some of the altered orders (Fig. 9). When the functional-gene profile was assessed, our results showed a clear difference between non-obese mice and un-treated HFD ones (Fig. 10A). Interestingly, the treatments also produced an important change in this profile, which was more accentuated in the case of agomelatine and metformin (Fig. 10A). Thus, while metformin increased the bacterial genes associated with tryptophan, fructose and mannose metabolism and with glycosaminoglycan degradation, agomelatine up-regulated the genes involved in glycolysis, gluconeogenesis and lipid metabolism (Fig. 10A), and decreased genes involved in the transport (including ABC transporter), bacterial secretion, PPAR signalling, fatty acid biosynthesis, motility and sugars assimilation, along with others, which appeared increased in HFD-mice

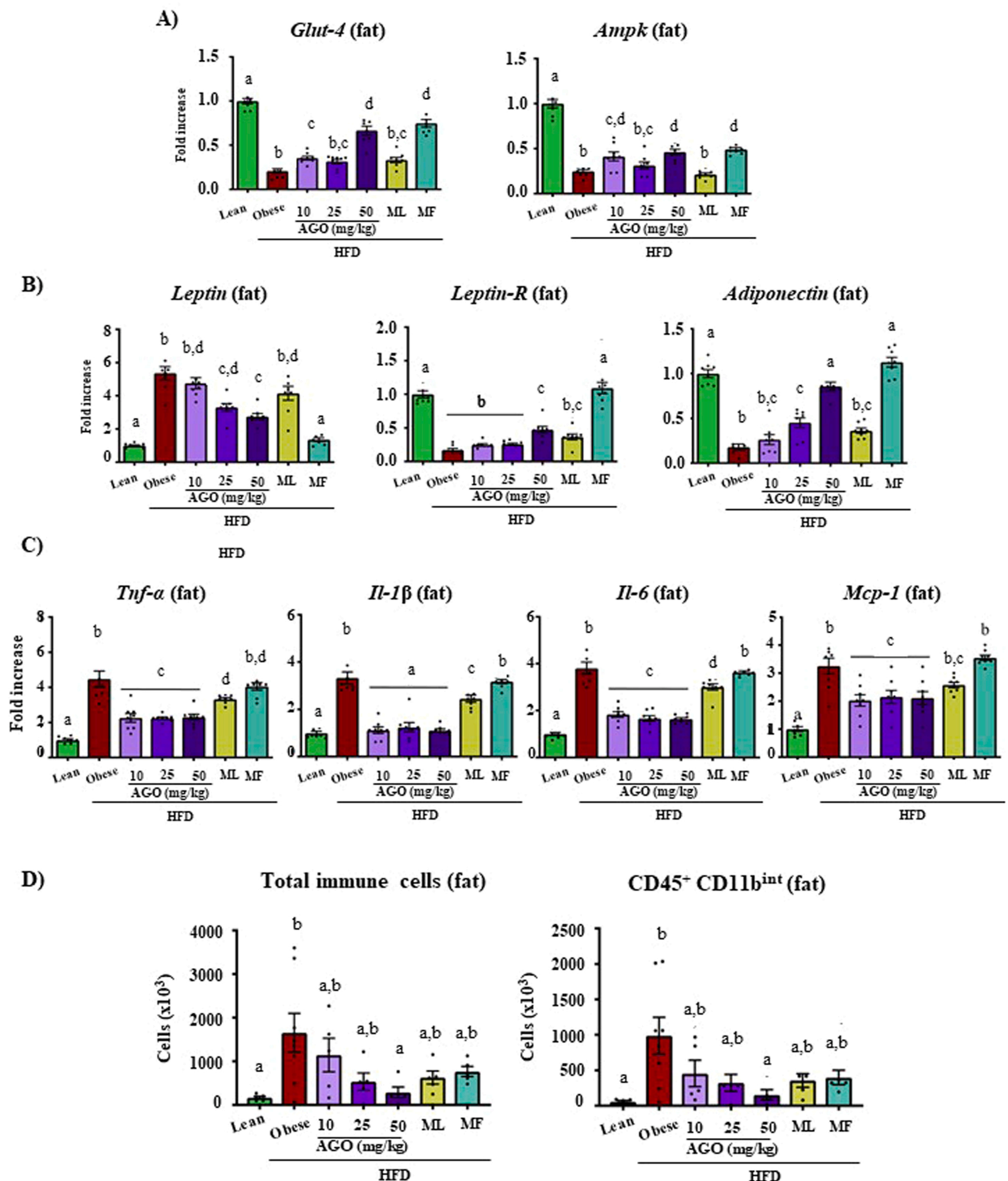

Fig. 5. Impact of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on inflammatory status. (A) Fat gene expression of *Glut-4* and *Ampk*; (B) Fat gene expression of *Leptin*, *Leptin-R* and *Adiponectin*; (C) fat gene expression of *Tnf-α*, *Il-1β, Il-6* and *Mcp-1;* (D) total and $CD45^{+}CD11b^{int}$ immune-cell populations in adipose tissue in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

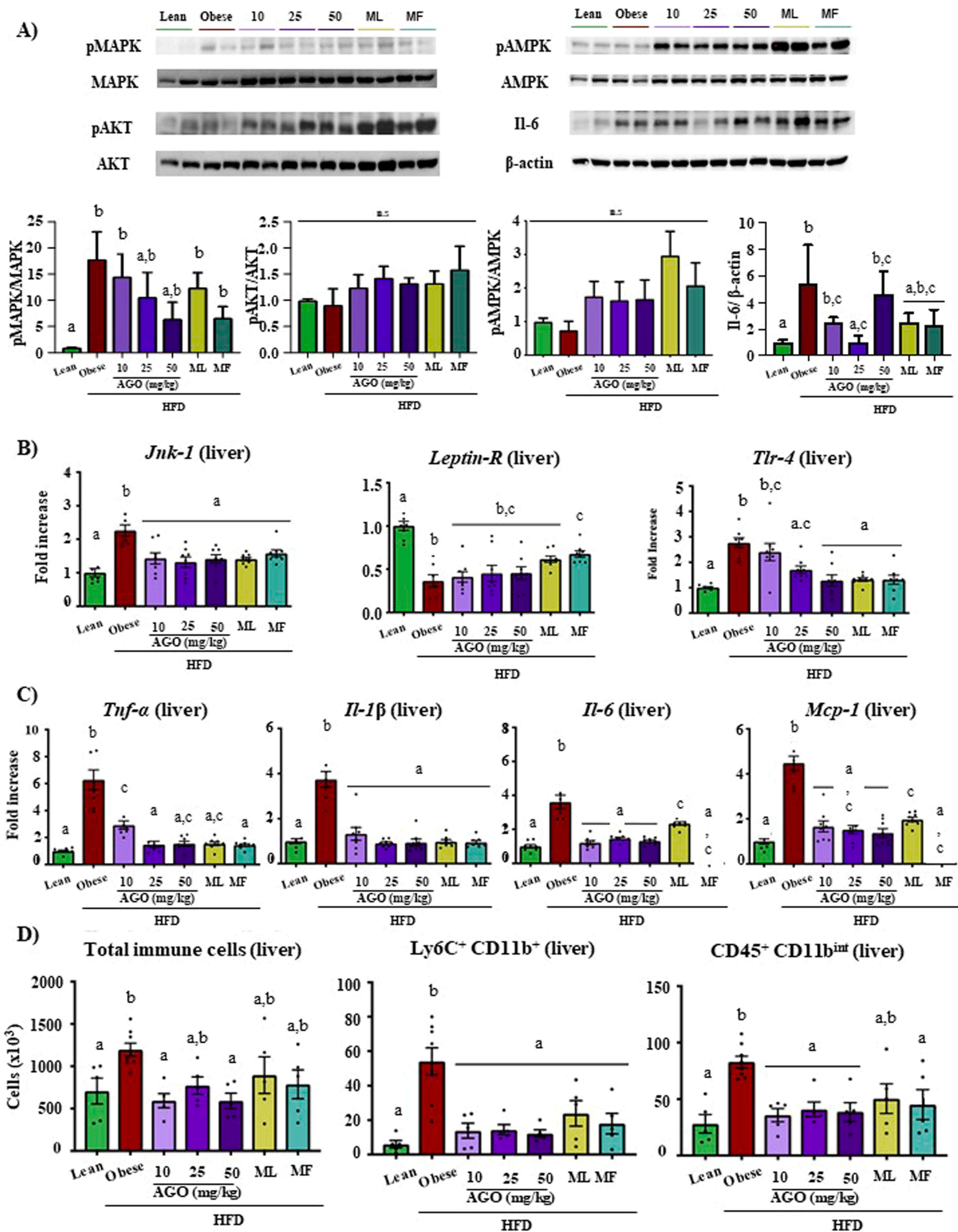

Fig. 6. Effects of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on (A) liver protein expression of pMAPK/MAPK, pAKT/AKT, pAMPK/AMPK, IL-6 and β-actin; (B) liver gene expression of *Jnk-1*, *Leptin-R* and *Tlr-4*; and (C) *Tnf-α*, *Il-1β, Il-6* and *Mcp-1*; (D) hepatic Total, $Ly6C^{+}CD11b^{+}$ and $CD45^{+}CD11b^{int}$ immune-cell populations, in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ (*p < 0.05*).

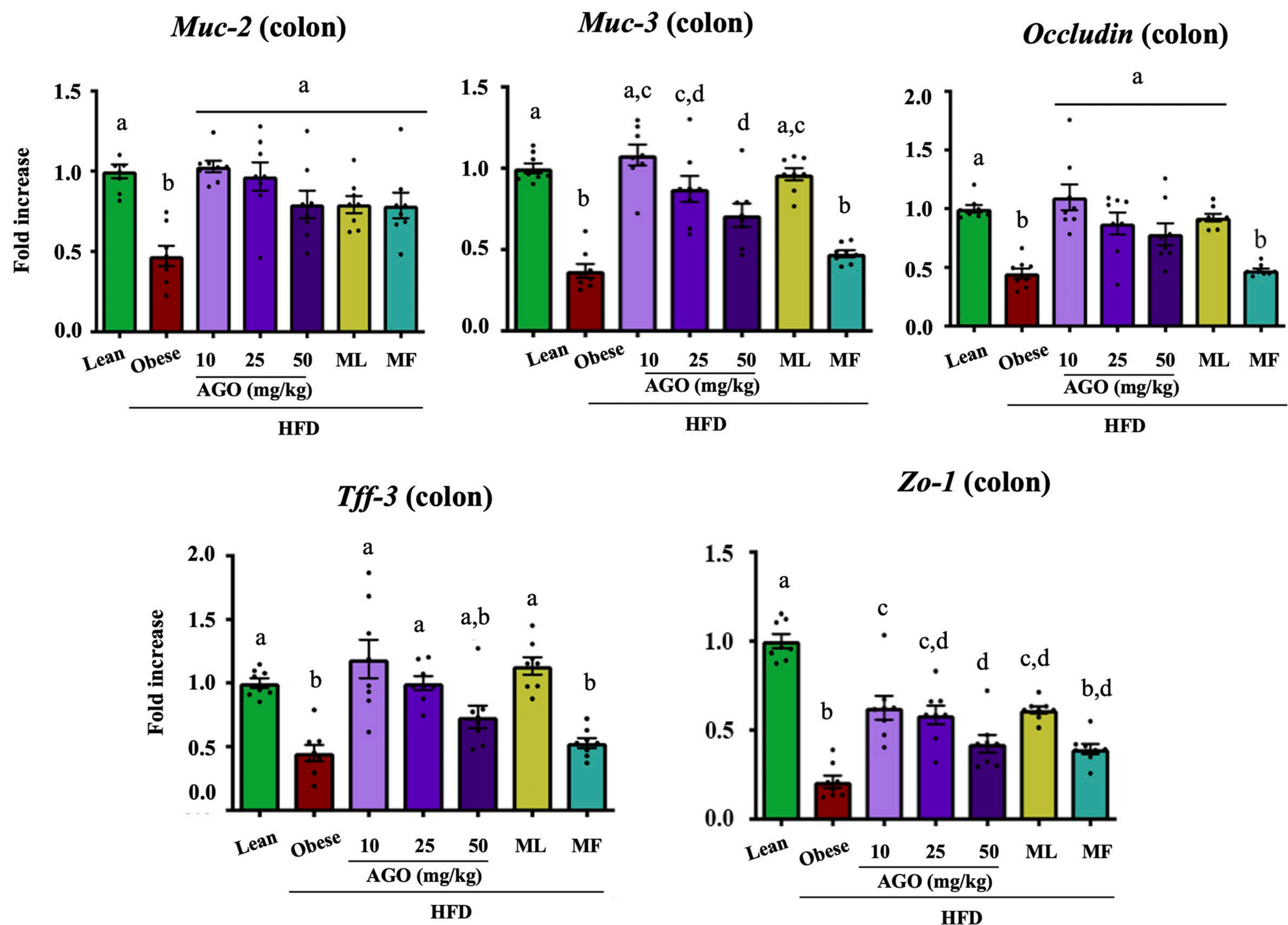

Fig. 7. Influence of agomelatine (AGO) (10, 25 and 50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on colonic gene expression of *Muc-2*, *Muc-3*, *Occludin*, *Tff-3* and *Zo-1*. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

(Fig. 10A).

Agomelatine also had a clear effect on short chain fatty acids (SCFA)-producing bacteria. Non-treated HFD-fed mice showed a decrease in butyrate-producing bacteria abundance in comparison with non-obese mice (Fig. 10B), but oral administration of agomelatine and metformin significantly increased the relative abundance of butyrate-producing bacteria (Fig. 10B). Propionate-producing bacteria were also reduced by HFD intake (Fig. 10B) although all treatments increased the abundance of these bacteria, being agomelatine the most relevant (Fig. 10B). Interestingly, agomelatine also augmented the abundance of *A. muciniphila* (Fig. 10C), a propionate producing bacteria [34].

4. Discussion

Dysregulation of circadian rhythm homeostasis has been associated with various disorders of lipid metabolism, including obesity [7]. In this sense, melatonin has been explored as a treatment for obesity and other metabolic disorders. Since conflicting results have been reported [35, 36], a more in-depth investigation is required to develop innovative therapeutic strategies. With this aim, we evaluated the effect of agomelatine, a melatonergic agonist, in experimental obesity in mice, and compared it to melatonin and metformin, the most prescribed antidiabetic drug.

Our results show that agomelatine treatment lowered body weight gain and fat accumulation in a similar manner to previously observed for melatonin[37] and metformin[38], as well as improved obesity-associated glucose intolerance. This confirms previous experimental observations that associate weight decrease and enhanced insulin sensitivity with the regulation of metabolic clock and/or the increase of the energy expenditure/intake ratio [39–41]. Although some preclinical studies have indicated that melatonin lowers body weight and visceral fat accumulation [37,39], this has not been confirmed in humans [42]. However, it has been observed that agomelatine administration in patients with night eating syndrome reduces body weight, related to restoration of sleep patterns and sleep-related eating disorders [43], and its anti-obesogenic has been recently reported in HFD-fed rats [41], in agreement with our observations.

Regarding the lipid profile, whereas melatonin only reduced HDL-cholesterol, agomelatine supplementation showed a significant improvement in the cholesterol profile. This effect could be related to the direct effect of the circadian rhythms regulating dietary lipid absorption in intestinal enterocytes [44]. Metformin treatment also ameliorated the hypercholesterolemic status induced by HFD, an effect widely described in humans and mice [45,46], lightening the severity of high fat induced hepatic steatosis. Cardiovascular problems are frequent comorbidity in obesity and its altered biochemical profile. In this regard, all the alterations described in obesity, and in this experimental model, such as insulin signalling impairment, systemic inflammation, and also intestinal dysbiosis can also affect endothelial function, as observed in this experimental model. The increase in NADPH enzyme activity, and the subsequent production of reactive oxygen species, inactivates nitric oxide, contributing to impaired endothelial function and the pathogenesis of the metabolic syndrome [47]. The decreased enzyme activity observed with agomelatine treatment could explain the enhanced

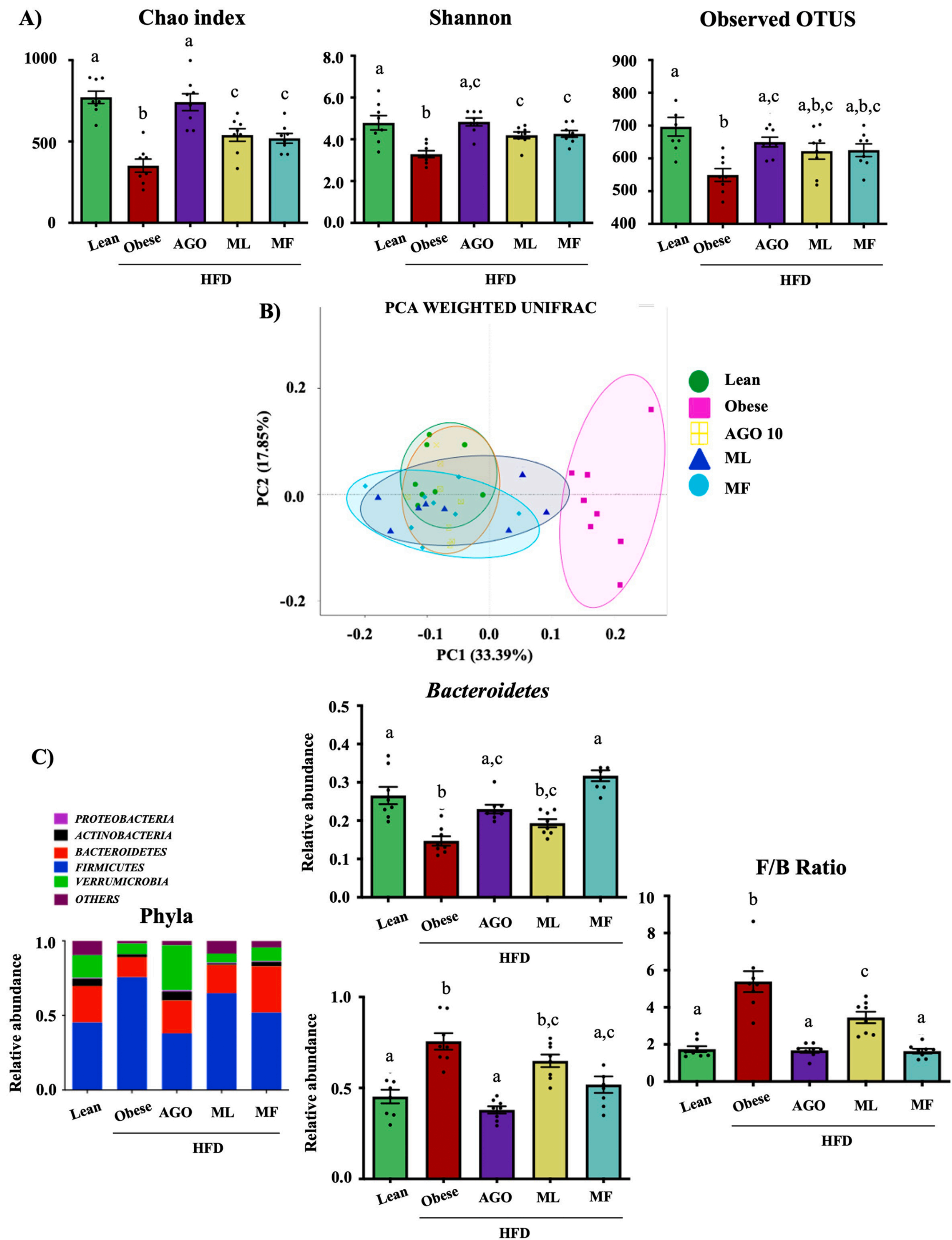

Fig. 8. Impact of the administration of agomelatine (AGO) (50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) on (A) microbiota diversity (Chao1, Shannon index and Observed OTUs); (B) beta-diversity by principal coordinate analysis score plot, and; (C) bacterial phyla and *F/B* ratio in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

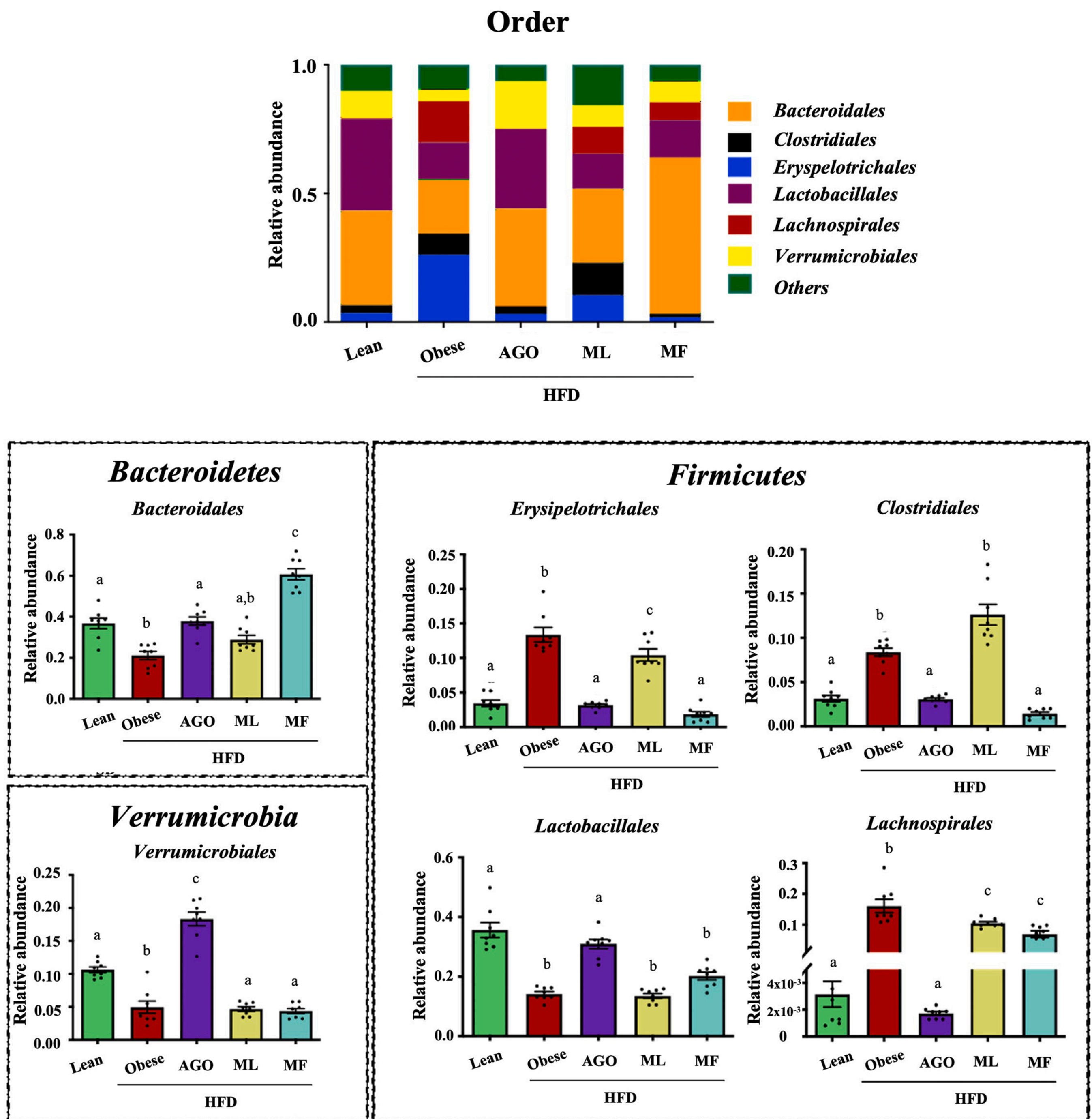

Fig. 9. Effect of agomelatine (AGO) (50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) administration on microbiota diversity at the order level in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

endothelial relaxation, as well as the general improvement of the underlying condition. Considering the above, agomelatine shows antioxidant properties that may contribute to its capacity to restore obesity-associated vascular dysfunction, similarly to the mechanism previously proposed for its protection of the renal damage observed in an experimental model of high fat-induced obesity in rats [41]. In fact, it has been suggested that the melatonergic activation by agomelatine can result in the production of antioxidant enzymes that scavenge reactive oxygen species [48,49].

The accumulation of fat tissue in obesity is linked to a chronic low-grade inflammation, with elevated circulating proinflammatory mediators, such as TNF-α and IL-6 secreted by the liver and fat tissue [50]. This drives immune cell recruitment and activation of inflammatory signalling pathways, such as the c-Jun N-terminal kinase (JNK)-related signalling, which interferes with insulin signalling, and therefore, with the glucose metabolism [51]. Consistently, HFD led to augmented expression of different pro-inflammatory mediators, including *Tnf-α*, *Il-1β*, *Il-6* and *Mcp-1*, in adipose tissue and liver. While all the treatments reduced their expression in the liver, only agomelatine reduced them in fat tissue. Likewise, all treatments significantly lowered HFD-induced *Jnk-1* up-regulation, in line with previous results [39,52]. Nevertheless, this is the first report of such anti-inflammatory activity for agomelatine treatment in obese mice, which could participate in the improvement of insulin signalling and glucose metabolism.

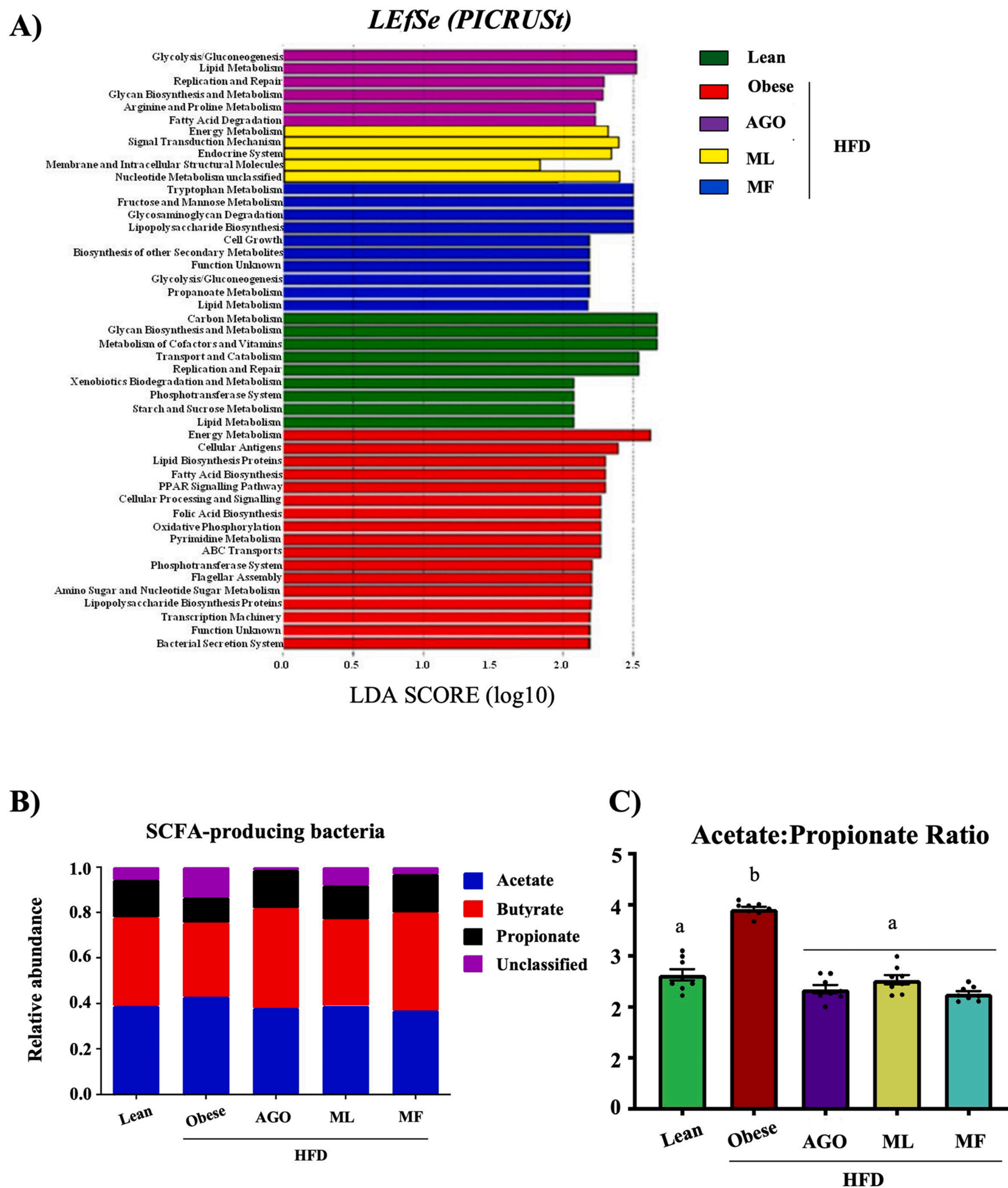

Fig. 10. (A) Metagenomic functional features predicted by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) that were differentially abundant and drove differences in control, untreated and agomelatine, melatonin or metformin treated HFD-fed mice. Effect of the administration of agomelatine (AGO) (50 mg/kg), melatonin (ML) (15 mg/kg) and metformin (MF) (250 mg/kg) on the (B) Relative abundance of short fatty acid (SCFA)-producing bacteria, and on the (C) Acetate:Propionate ratio in high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n = 8). Groups with different letters statistically differ ($p < 0.05$).

The excessive accumulation of adipose tissue can also produce an alteration in adipokine levels, such as leptin and adiponectin. Leptin, apart from suppressing appetite, is considered a pro-inflammatory mediator associated with insulin resistance [53]. In obesity, decreased expression of the leptin receptor in liver and fat lead to leptin resistance and excessive leptin release [53]. Conversely, adiponectin is an anti-inflammatory and insulin-sensitizing mediator that suppresses hepatic glucose production [54]. Altered leptin and adiponectin expression profiles were observed in this study. Lower fat expression of adiponectin in obese mice, which agrees with previous studies [55], was only significantly upregulated by agomelatine and metformin. Whilst metformin and melatonin have been widely assessed for their impact on adipokine production in experimental and human studies of obesity or diabetes [56,57], this is the first evidence of beneficial impact of agomelatine on adiponectin and leptin expression.

Besides, obesity-related insulin resistance implies intracellular glucose uptake impairment due to reduced GLUT-4 expression. Such effect was observed in HFD-fed mice and counteracted by agomelatine and metformin, but not melatonin, which neither has previously shown a significant effect on pinealectomized animals[58]. Also related with insulin resistance in obesity is the role of AMPK, involved in the translocation of GLUT-4 transporters to the membrane and the inhibition of liver gluconeogenesis and inflammatory pathways [58]. Antidiabetic drugs, including metformin, act as insulin sensitizers through AMPK

activation[59]. Hence, *Ampk* expression in liver and fat was partially restored by metformin, but also agomelatine. These results, together with the decreased glycaemia, confirm the capacity of the agomelatine treatment to improve insulin sensitivity and glucose homeostasis facilitated by central and peripheral target tissues. In addition to energy metabolism, AMPK is also recognized as a regulatory node for immune responses [60]. AMPK activation inhibits two major immune signalling pathways, nuclear factor-κB (NF-κB) and signal transducer and activator of transcription (STAT), reducing proinflammatory cytokines expression [61]. This anti-inflammatory effect was also evidenced mainly in agomelatine-treated mice, reducing cytokine expression and immune cell infiltration. The present results support previous studies that have also reported the involvement of AMPK activity in the mechanism of action of agomelatine [62]. Interestingly, obesity-associated conditions, like diabetes, are characterized by reduced autophagy linked to lower AMP/ATP ratio and AMPK expression [63] and agomelatine has been shown to ameliorate impaired diabetes-associated autophagy in high fat-fed rats via enhancement of AMPK phosphorylation. This effect may be mediated by its antioxidant effects through the activation of melatonin receptors [64]. MAPK and AKT are also essential for lipid and glucose energy metabolism, although they have not been targeted for the development of drugs to treat diabetes or obesity [65,66]. It is interesting to note that agomelatine, but also metformin and melatonin, ameliorate the dysfunction caused by the HFD.

Under pathological conditions, like obesity, the pro-inflammatory milieu stimulates the proliferation of immature myeloid cells (IMCs) and blocks the differentiation into mature myeloid populations, causing the accumulation of MDSCs ($Ly6C^{+}CD11b^{+}$). The liver is the major organ where IMCs accumulate [67], and, in agreement with previous studies[68], we observed an increase of MDSCs in obese mice. HFD could lead to immune activation and recruitment, as shown above by increased IL-6 liver expression, and reported for NAFLD patients, explaining the impaired myeloid differentiation [69]. Interestingly, all treatments restored its accumulation as well as *Il-6* expression levels in this experimental model, which has also previously been observed for metformin in vivo [70] and in vitro[71].

Macrophages are key regulators of the inflammatory process, reacting to a wide variety of stimuli, including metabolic signals. It is well known that the accumulation of inflammatory macrophages in the liver and in the fat contributes to the deregulation of glucose homeostasis, obesity-induced inflammation, and hepatic fibrosis. The hepatic macrophage population ($CD45^{+}CD11b^{int}$) was increased in obese mice and, both melatonin and metformin reduced it, as expected from other studies[37,72]. Interestingly, agomelatine showed an even stronger effect at the highest dose, and it also restored macrophage population in the adipose tissue, confirming the improvement of the inflammatory response in association with the metabolic status.

Obesity has also been associated with an increased gut permeability [5], which positively correlates with HOMA-IR index and is aggravated by liver injury [73].

Given the direct impact of the gut and the microbiome in the function of the liver and the immune system, we evaluated the effect of the treatments on the alterations in this important axis. In particular, given the effect of diet on the intestinal melatoninergic pathways [13], its impact on permeability [15,17], and the modulation of microbial composition upon agomelatine treatment appreciated in other experimental models, we believe that this mechanism could play an important role in the therapeutic benefit of agomelatine in obesity.

In line with this hypothesis, we observed a down-regulation of intestinal epithelial markers in obese mice, which could enable the access of bacterial components, such as LPS, into the circulation, contributing to the underlaying inflammation [74]. Agomelatine and melatonin increased the expression of these markers. The enterohepatic circulation makes the liver the first organ affected by increased permeability, being liver *Tlr-4* expression a good marker for LPS plasma levels [75,76]. The effect of the treatment counteracted the upregulation of liver *Tlr-4* expression, associating the beneficial effect of agomelatine with improved inflammatory response and glucose and lipid homeostasis in connection with intestinal permeability.

In line with this, obesity-associated dysbiosis may contribute to metabolic endotoxemia and thus low-grade systemic inflammation, being highly informative to evaluate microbiota composition in order to improve our understanding of the crosstalk between microbiome, gut and other metabolic organs. Interestingly, it has been previously shown that melatonin and metformin treatments can also reverse gut dysbiosis associated with metabolic endotoxemia in animal models of obesity [77–79]. In our study, we observed a decrease in microbial richness, evenness and diversity associated with HFD intake. Agomelatine has shown for the first time to produce marked shifts in the obese gut microbiome and restore the balance between *Firmicutes* and *Bacteroidetes,* of interest for the management of the metabolic syndrome and obesity [5]. The increase in *Firmicutes/Bacteroidetes* (F/B) ratio has been associated with a more efficient hydrolysis of non-digestible polysaccharides and an increased caloric use in obese individuals [5]. Other alterations described in obese patients, such as reduced *Verrucomicrobia* phylum[80], were also observed in our model and restored by agomelatine treatment. At lower taxonomic levels, agomelatine also normalized the composition of microbiota whilst metformin only had a partial effect. It is particularly interesting the increase in *Verrumicrobiales* containing *Akkermansia muciniphila*, a mucin-degrading bacterium whose abundance is inversely associated with body weight in obese mice and type 2 diabetes[11]. Treatments that stimulate its growth have shown to alleviate HFD-induced metabolic disorders [11,81], which points this as an interesting mechanism that could underlie agomelatine's beneficial effects.

The "dialogue" between the intestinal microbiota and the host primarily relies on their biochemical pathways and metabolites produced, finding an altered functional profile with HFD, which was evidenced in our study. Imputed gene expression and pathway analysis showed that agomelatine treatment correlates with an increase in glycolysis, gluconeogenesis and lipid metabolism, and underrepresentation of genes involved in the transport (including ABC transporter), bacterial secretion, PPAR signalling, fatty acid biosynthesis, motility and sugars assimilation [82,83]. Of note, agomelatine, together with metformin, increased the abundance of butyrate-producing bacteria, which have been describe to protect from HFD-induced obesity, attenuating fat gain and insulin resistance [84,85]. These results could be associated with the modification of the *Bacteroidetes* and *Lactobacillus* abundance, which participate in butyrate generation via lactate production [86], whilst the increase in propionate-producing bacteria could relate to *A. muciniphila,* a propionate producer bacteria [34]. Moreover, propionate plays a key role in counteracting cholesterol synthesis, being the ratio acetate/propionate crucial for cholesterol and lipid metabolism regulation [86].

These results support the beneficial effect observed with agomelatine and highlight its therapeutic potential for the modulation of the gut microbiota in obesity. While our study identifies the pathways that associate with its therapeutic effect, elucidating a defined mechanism would require more specific studies, although the antioxidant properties ascribed to this compound seem to play a key role. However, refining a single mechanism may also not be possible given the polypharmacological profile of agomelatine. In fact, we believe that this may be an important advantage of agomelatine over other treatments considering the numerous alterations and organs involved in the metabolic syndrome, as the results of this evaluation have shown.

In conclusion, the melatonergic agonist agomelatine improves glucose intolerance, insulin resistance, lipid metabolism and inflammatory status associated with HFD-induced obesity. Moreover, it has shown the ability to ameliorate the gut dysbiosis that characterizes this condition. These properties may support the use of agomelatine as a novel therapeutic tool to manage human obesity, which displays a better pharmacokinetic profile than melatonin and more global effects than this and metformin, the most used drug nowadays.

Author contributions

Patricia Diez-Echave, Teresa Vezza, Francesca Algieri, Antonio Jesús Ruiz-Malagón, Laura Hidalgo-García, Rocio Moron and José Garrido-Mesa performed the experiments and contributed to the acquisition and analysis of data. Marta Toral, Miguel Romero, Manuel Sanchez and Juan Duarte performed the vascular reactivity studies and NADPH oxidase activity experiment as well as their analysis. Alba Rodríguez-Nogales, María Elena Rodríguez-Cabezas, Julio Galvez and Federico Garcia contributed to the analysis and interpretation of data of microbiome analysis. María Elena Rodríguez-Cabezas, Julio Galvez, Jose Garrido-Mesa and Alba Rodríguez-Nogales designed the project and wrote the manuscript. All authors contributed to the revision.

Data Availability

Data available on request from the authors.

Acknowledgments

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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