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Companilactobacillus alimentarius CNTA 209 alleviates diet-induced obesity in mice through adipose tissue browning and gut barrier modulation

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The use of probiotics with health-promoting effects has emerged as a promising therapeutic strategy for managing obesity and metabolic syndrome. In this study, we characterized the probiotic properties of a novel strain, *Companilactobacillus alimentarius* CNTA 209, and investigated its potential anti-obesity effects and safety in rodent models. *C. alimentarius* exhibited sensitivity to all tested antibiotics, resistance to simulated gastric and intestinal conditions *in vitro*, and functional activities including β-galactosidase activity and short-chain fatty acid (SCFA) production. *C. alimentarius* supplementation mitigated liver damage induced by a high-fat, high-fructose diet and significantly reduced adiposity in obese C57BL/6 mice by enhancing brown adipose tissue metabolic activity. Metagenomic analysis revealed a beneficial modulation of gut microbiota composition, associated with improved intestinal barrier function. A comprehensive toxicological assessment conducted in Wistar rats confirmed the safety of the strain at a dose of 1×10^9 CFU per animal per day for oral administration. This study provides the first documented evidence of anti-obesity and metabolic benefits conferred by a strain of *C. alimentarius*, positioning CNTA 209 as a novel and safe candidate for the development of probiotic-based interventions targeting obesity and related metabolic disorders.

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Introduction

Obesity is a chronic, complex and multifactorial condition associated with a range of metabolic disturbances, including excessive adipose tissue accumulation, low-grade systemic inflammation, insulin resistance, dyslipidemia and liver steatosis. These complications significantly increase the risk of developing other chronic conditions, such as type 2 diabetes, cardiovascular diseases or metabolic dysfunction-associated liver disease (MASLD), underscoring the urgent need for effective preventive and therapeutic strategies.[1] In this regard, the gut microbiota has emerged as a critical modulator of host metabolism, immune function, and intestinal integrity, positioning it as a potential target in the management of the pathophysiology of obesity and its related pathologies.[2]

In recent years, the use of specific probiotic strains has garnered increasing attention as a promising therapeutic approach for modulating the composition and activity of gut microbiota, improving intestinal barrier integrity, and regulating host metabolic responses. These effects contribute to mitigating the obesity-associated complications, including the improvement of insulin sensitivity, attenuation of low-grade systemic inflammation and improvement of cholesterol levels, among others.[3]

Among probiotics, different Lactic Acid Bacteria (LAB) have emerged as key modulators of host metabolism and gut microbiota composition. The widespread presence of LAB in the human intestinal tract, along with their established use in the food industry, provides a strong genetic and safety background, making them globally recognized and widely used probiotics.[4]

However, the properties, functionality and potential health-promoting activities of probiotics are unique and strictly strain-specific and cannot be inferred from other strains, even

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of the same species, without appropriate experimental evidence.[5] According to the European Food Safety Authority (EFSA), this includes the genomic characterization of the proposed probiotic, the absence of transferable antibiotic resistance, and toxicological studies in appropriate animal models. Furthermore, a detailed understanding of the underlying mechanisms is essential for validating their therapeutic potential for clinical use.

Companilactobacillus alimentarius (*C. alimentarius*, and formerly *Lactobacillus alimentarius*) is a facultatively heterofermentative Gram positive LAB present within the microbiota of various traditional fermented foods, which exerts several functions in fermentative or ripening processes, making it one of the options when developing possible new probiotics.[6] Moreover, *C. alimentarius* species has been proven to constitute effective starters in LAB-induced fermentation, improving the digestibility of wheat-derived foods.[7] Nonetheless, its potential application in preventing or treating metabolic disturbances linked to obesity has not yet been explored.

In this study, we report the *in vitro* characterization of a novel LAB strain, *Companilactobacillus alimentarius* CNTA 209, isolated from homemade chorizo. We further evaluate its therapeutic potential in mitigating key metabolic complications associated with diet-induced obesity in C57BL/6 mice, with particular emphasis on elucidating its underlying mechanisms of action and assessing its safety profile for potential future human application.

Material and methods

Bacterial strain and growth conditions

The strain used in this study is *Companilactobacillus alimentarius* CNTA 209, which was derived from CNTA culture collection and was collected from homemade chorizo. This strain is deposited at the "Colección Española de Cultivos Tipo (CECT)" under accession number CECT 30970.

C. alimentarius CNTA 209 was grown in MRS (Merck) at 37 °C under anaerobic conditions. Over a 48-hour incubation period, bacterial growth was quantified at set intervals by plating serial dilutions on MRS agar to determine cell counts log10 (CFU per mL) and by direct pH measurement using a pH meter Basic 20+ (Crison Instruments S. A., Barcelona, Spain).

In vitro probiotic characterization tests

The growth rate, antibiotic resistance and resistance to gastrointestinal fluids, biofilm formation, short chain fatty acids (SCFA) and β-galactosidase production were assessed *in vitro* following the methods described previously.[8]

The kinetic data of *C. alimentarius* CNTA 209 were modelled using the online tool Python 3.9 (Python Software Foundation. Python Language Reference, version 3.9.13) according to the four-parameter Gompertz Model.[9] For the assessment of the potential antibiotic resistance of the probiotic, minimal inhibitory concentrations (MICs) of nine antibiotics (all antibiotics were purchased from Sigma-Aldrich, St Louis, MO, USA) were determined according to the ISO 10932/IDF 223 standard (2010)[10] using the microdilution method on a 96-well microtiter plate using susceptibility test media. The MIC values were calculated in three independent experiments.

The resistance to simulated gastric and intestinal fluids of *C. alimentarius* CNTA 209 was determined as previously described.[8] Briefly, the original bacterial loads were 6,5 log cycles, and microbial inactivation cycles were calculated by subtracting the initial microbial count from the count obtained after 2 hours of treatment in gastric simulant or after an additional 2 hours of treatment in intestinal simulant.

To analyse the production of SCFAs, the previously described methodology was used.[11] Uncontrolled-pH batch cultures of *C. alimentarius* CNTA 209 were grown anaerobically for 48 hours in a defined medium. The medium was supplemented with either 2% (w/v) of the prebiotics synergy 1 (FOS), P95 (FOS), or glucose (positive control); a negative control with no carbon source was also included. Samples taken at 0 and 48 hours were centrifuged, and the filtered supernatants were analysed for SCFAs by GC-MS. All cultures were performed in triplicate.

The β-galactosidase activity of *C. alimentarius* CNTA 209 was assessed as previously described by dos Santos *et al.*[12] A colony of *C. alimentarius* CNTA 209 was incubated with an ONPG disk rehydrated in 0.1 mL of phosphate-buffered saline (PBS; 0.85% w/v NaCl). A positive reaction was indicated by the development of a yellow chromophore, resulting from the release of *o*-nitrophenol. This assay was performed in triplicate across two independent experiments.

The ability of *C. alimentarius* CNTA 209 strain to form biofilms was determined as previously described.[8]

Adhesion, exclusion and competence assays in Caco-2 cells

E. coli O157:H7 enterohemorrhagic strain 19206 was obtained from DSMZ (Germany). *C. alimentarius* 209 was cultured in MRS broth (Merck KGaA, Darmstadt, Germany) at 30 °C under anaerobic conditions and *E. coli* was cultured in a TSB + YE broth (Scharlab, S.L. Barcelona, Spain) at 37 °C under an aerobic atmosphere.

The Caco-2 human colon adenocarcinoma cell line was used as a model for intestinal cells. The cell line was obtained from DSMZ (ref. ACC 169, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM 10569 Gibco high glucose + GLUTAMAX + sodium pyruvate, Thermo Fisher Scientific) supplemented with 20% fetal bovine serum (fetal bovine serum, qualified, heat inactivated, Brazil, A310500064, Teknovas) and 1% penicillin–streptomycin (P0781, Sigma-Aldrich, Saint Louis, MI, USA) at 37 °C under a 5% CO_2 atmosphere.

In adhesion assays, 24-well tissue culture plates (Costar 24-well Clear TC-treated Multiple Well Plates, Individually Wrapped, Sterile, Corning) were seeded with 3×10^4 Caco-2 cells per well. Then, the cells were cultured for approximately 2 weeks until a monolayer culture formed. After seeding, the medium without antibiotics was used to avoid any interference. *E. coli* from 18 h culture in TSB + Ye and *C. alimentarius* 209 from 19 h cultures in MRS broths for lactic

acid bacteria were washed once with distilled water containing 0.1% peptone (Merck KGaA, Darmstadt, Germany). Bacterial dilutions were prepared in DMEM for the different tests. After washing the Caco-2 cells twice with phosphate buffered saline (DPBS without calcium or magnesium, pH = 7.3 (A314190094, Teknovas)), 0.5 ml of DMEM with bacterial suspension was added to each well at a concentration of 1×10^7 UFC per mL and incubated for 60 min at 37 °C. The final ratio resulting after incubation was 1 : 10 cells per bacterium. Unattached bacteria were removed by 3-fold washing with DPBS containing no calcium or magnesium.

In competition assays, *C. alimentarius* was added simultaneously with *E. coli* 14. To test a possible exclusion or/and displacement of *E. coli* by *C. alimentarius* or *vice versa*, the incubation of Caco-2 cells with one strain was followed by washing off unattached bacteria, then the second bacterial strain was added and incubated 60 minutes later. Approximately, 1 : 10 cell : bacterial concentration of *C. alimentarius* was tested. The bacterial concentrations tested were *C. alimentarius* ($7.0 \times 10^6 \pm 2.4 \times 10^6$ CFU per well) and *E. coli* ($2.4 \times 10^7 \pm 1.3 \times 10^7$ CFU per well). The % of bacterial adhesion in competition, exclusion and displacement was calculated relative to the total adhesion of each bacterial strain individually onto Caco-2 cells. The exact number of viable *C. alimentarius* and *E. coli* in the bacteria suspensions used in assays was determined by plate counting. In each experiment, plate counting was performed on MRS for lactic bacteria and VRBD agar media for *E. coli* (Merck, Germany). MRS agar plates were incubated for 2 days in anaerobiosis and VRBD agar plates for 24 h in aerobiosis, both at 37 °C. Caco-2 cells were harvested from the plates using 0.05% trypsin–EDTA (Sigma Chemical Co., St Louis, MO, USA) for 5 min, and adherent *C. alimentarius* and *E. coli* were counted (cfu per well) in the same medium and conditions of bacterial suspensions. The % of *E. coli* adhesion in competence, exclusion and displacement assays is calculated from the individual adhesion of *E. coli* onto Caco-2 cells.

C57BL/6 mice experimental design and diets

Thirty 4-week-old C57BL/6J male mice were purchased from Envigo (Envigo Research Models and Services, Indianapolis, IN). All procedures were performed by following the national and institutional ethical guidelines of the Care and Use of Laboratory Animals, with the consent of the Food Safety and Environmental Health Service of the Government of Navarra, Spain. The protocol was approved by the Ethics Committee for Animal Experimentation of the University of Navarra (protocol reference E3-24(100-21E2)).

The experiment had a total duration of 14 weeks (Fig. S1). Mice were kept in an isolated room with controlled temperature (21–23 °C) and humidity (50 ± 10%) and a 12 h artificial light–dark cycle. All mice were acclimatised to the experimental facility for three days and fed with a control diet (2014, Teklad, Global 14% Protein Rodent Maintenance Diet, ENVIGO RMS SPAIN, Barcelona, Spain). Then, 22 mice were randomly divided and allocated into 2 groups ($n = 11$) until the end of the study. Mice were housed 5–6 per cage with *ad libitum* access to water and controlled food intake. These 2 groups were fed an HFS diet (D12451; Research Diets, NJ, USA; with 20% of energy corresponding to protein, 35% to carbohydrates and 45% to fat) and fructose (10% wt/vol) in water for 3 weeks to induce a pre-obesity and pre-diabetes stage. The remaining 8 animals were housed four per cage and maintained with the control maintenance diet during the whole experiment as the non-obese control group (CNT).

After three weeks, fructose was maintained in water, but the HFS was supplemented with the following treatment for 10 weeks: *C. alim.* group (fed with HFS diet supplemented with 1×10^9 CFU of probiotic per mouse per day), and HFS group (fed with HFS diet with no supplement). The bacterial formulations were prepared every 3 days, and food intake was controlled twice per week. Body weight was checked weekly. All mice were euthanised by decapitation at week 10 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, white adipose tissue (WAT) depots (mesenteric, retroperitoneal, epididymal and subcutaneous) and brown adipose tissue (BAT) depots were isolated, weighed and immediately stored at −80 °C.

Blood biochemical analyses

Serum total cholesterol, HDL-cholesterol, triacylglycerides (TAGs), glucose, aspartate transaminase (AST), and alanine transaminase (ALT) were quantified with the HK-CP kit adapted for the Pentra C200 analyser (HORIBA ABX, Montpellier, France). Specific ELISA kits were used to quantify plasma concentrations of monocyte chemotactic protein-1 (MCP-1) (Thermo Scientific) and zonulin-1 (ZO-1). Serum insulin was also quantified with a specific ELISA kit (Mercodia AB, Uppsala, Sweden). Insulin resistance was evaluated by the homeostasis model of insulin resistance (HOMA-IR) formula: [serum glucose levels (mmol L^{-1}) × insulin levels (mU L^{-1})]/22.5. Finally, the quantification of the hepatic triglyceride levels was performed as previously described.[13]

Glucose tolerance test

An intraperitoneal glucose tolerance test (IGTT) was performed at week 9 of the study. For this experiment, food was withheld 15 h before the test, leaving the animals with only access to water. Before the test, mice were weighed and D-glucose (1.5 g kg^{-1}) was intraperitoneally administered. Glycaemia was quantified using a glucometer and blood glucose test strips (Optium Plus, Abbott Diabetes Care, Witney, Oxon, UK) by venous tail puncture before (baseline) and after glucose administration (at 20, 40, 60, 90 and 150 min). Glucose content (mg dL^{-1}) was used to calculate the area under the curve (AUC), as previously described.[13]

RNA extraction and quantitative PCR

Total RNA from liver, mesenteric fat and BAT was extracted using Trizol® RNA isolation reagent (Thermo Fisher Scientific, Paisley, UK) following the manufacturer's instructions. RNA

purity was determined by measuring the absorbance at 260/280 nm in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was treated with DNAse (Ambion™ DNase I, RNase-free; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions and reverse transcription was performed as described in previous works.[13]

Gene expression analyses were performed by quantitative real-time PCR (qPCR) using TaqMan Universal PCR master mix and specific probes from Applied Biosystems Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA) and Integrated DNA Technologies Inc. (Coralville, IA, USA). All reactions were performed using a CFX384 TouchTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The expression level of each gene was normalized compared to the expression of the *Tbp* gene from Life Technologies (Mm01277042_m1, TaqMan Gene Expression Assays, Carlsbad, CA, USA), which was used as the housekeeping gene control. Gene expression differences between treated and untreated worms were analyzed using the relative quantification $2^{-\Delta\Delta Ct}$ method.[14]

Fecal sample collection and metagenomic analyses

The day before the sacrifice, fresh fecal samples were collected from each animal, ensuring their precedence and stored at −80 °C. The following bacterial DNA isolation and sequencing analysis were performed by the CimaLab diagnostic genomics unit of the Center for Applied Medical Research (CIMA, Pamplona, Spain). The comparison with a curated Illumina database allowed for a characterization of the sequenced 16S genetic regions, grouped in operational taxonomic units (OTUs), and data were normalized by Centered Log-Ratio (CLR) normalization. The DEseq2 method[15] was used for differential expression analysis of metagenomic data between the relative abundance of *C. alimentarius* CNTA 209-treated mice and the non-supplemented HFS samples at the phylum, family, genus and species levels. A statistical *p*-value cutoff set at 0.01 and FDR < 0.005 was established, using the tool available at https://www.microbiomeanalyst.ca/.

Toxicological evaluation of *C. alimentarius* CNTA 209

In addition to the functional study in obese mice, a toxicological study was conducted to determine the potential toxicity of *C. alimentarius* CNTA 209. For this, male and female Wistar rats fed with the control diet were supplemented with the same dose of the prebiotic as previously examined (10^9 CFU per animal per day) for 10 weeks. This *in vivo* toxicological experiment was approved by the Ethics Committee on Animal Experimentation of the University of Navarra (protocol GLP like DDUNAV 012/22). Wistar rats (n = 20, 10 male and 10 female) were purchased from Envigo (Envigo Research Models and Services, Barcelona, Spain). Rats were distributed in four groups: control diet male rats (C–M, n = 5), control diet female rats (C–F, n = 5), *C. alimentarius* male rats (T–M, n = 5), and *C. alimentarius* female rats (T–F, n = 5). Animals were housed in Makrolon® cages in groups of 5 animals per cage. After an acclimation period to the environmental conditions (12 h day/night cycle, temperature 22 ± 2 °C, relative humidity 50 ± 10%, and water *ad libitum*), all animals consumed a control diet (2014, Teklad Global 14% Protein Rodent Maintenance Diet) during the whole experiment. Both male and female groups of *C. alimentarius* received a daily dose of 10^9 CFUs of *C. alimentarius* CNTA 209 embedded in a dense paste obtained after mixing the powder diet with water. The control groups (both males and females) received only the vehicle. Food intake was evaluated weekly and *ad libitum* water was fed 7 days a week for 10 weeks.

The study included a general evaluation of viability/mortality, food consumption and general symptoms. A total of ten Irwin complete tests were carried out weekly during the whole experiment. A more in-depth study of serum biochemical and haematological parameters was performed the day before the sacrifice using a Cobas c111® analyser (Hoffmann-La Roche; Basel, Switzerland) and a Sysmex XT-1800i® apparatus (Roche 2011, Kobe, Japan), respectively. The day before the sacrifice, animals were housed individualized and kept in metabolic cages to determine daily volume, appearance, colour and smell of the urine, together with an evaluation of biochemical parameters using a Cobas u411® analyser (Hoffmann-La Roche; Basel, Switzerland). After sacrifice, all animals were subjected to a macroscopic anatomopathological study, including general state, detection of wounds or external abnormalities, an "*in situ*" evaluation of the organs, and an individualized exam of each extracted organ. From each animal the following organs were extracted: spleen, heart, adrenal glands, liver, pancreas, kidneys, testicles, thymus, and the whole gastrointestinal tract. Histological samples were stored in 4% formaldehyde except for testicles, which were fixed in Davidson's fixing solution and preserved in 70% ethanol after 48 h. All samples were sent to Patconsult.LAB.SL (Barcelona, Spain) for further processing and macroscopic and microscopic evaluation.

Statistical analyses

Cell culture assays were performed as three independent biological replicates with three technical replicates each. For statistical analysis, GraphPadPrism V.8 was used. First, the Shapiro–Wilk test was performed to assess the normality of the data. Then, depending on the result, either ANOVA or Kruskal–Wallis tests were applied.

Animal data were evaluated by the one-way ANOVA test followed by Duncan's method as a multiple comparisons test when statistical significance ($p < 0.05$) was reached in the ANOVA test. Toxicological analyses were performed by 2 × 2 ANOVA, comparing the effect of treatment (*C. alimentarius* CNTA 209 supplementation), sex and the interaction between these two factors.

Results and discussion

C. alimentarius CNTA 209 exhibits probiotic properties

Every bacterial strain that might be claimed for use as a food supplement with health-promoting activities should possess

specific properties that qualify it as a suitable probiotic candidate. These properties include resistance to gastric and intestinal acidity, sensitivity to antibiotics, short chain fatty acid production, adherence to intestinal cells, and ability to colonize, among others.[16] Furthermore, a strain should be non-pathogenic and safe for human consumption. It must be taken into account that these probiotic properties, together with the potential health benefits of its consumption, are strain specific, so the characterization and functional evaluation of each specific strain as a probiotic candidate is mandatory. In the case of *C. alimentarius*, some specific strains have been reported to exhibit probiotic properties with very diverse origins, such as Chinese fermented foods,[17] kimchi[18] or sausages.[19,20]

Genomic comparison of our strain CNTA 209 with all *Companilactobacillus alimentarius* genomes from the NCBI database (n = 3) revealed an Average Nucleotide Identity (ANI) of over 96% with the type strain, conclusively identifying it as *C. alimentarius*. Therefore, the complete genome sequence of *C. alimentarius* CNTA 209 was deposited in the NCBI database under the accession number JBPARW000000000.1.

The initial step in the *in vitro* analysis of the candidate probiotic strain *C. alimentarius* CNTA 209 involved characterizing its growth parameters in the standard LAB medium, MRS broth. Under anaerobic conditions at 37 °C over a 48-hour period, bacterial growth was monitored by plating samples collected at predefined times. As shown in Fig. 1A, the resulting growth data were accurately modeled using the Gompertz function, demonstrating an excellent fit with an R value of 1 and an RMSE of 0.05 (Table S1).

The use of microbial feed additives must avoid exacerbating the proliferation of antimicrobial resistance (AMR) by introducing additional resistance genes into the gut microbial community or by accelerating their spread. For this reason, *C. alimentarius* CNTA 209 was tested to evaluate potential resistance to antibiotics. No resistance to antibiotics was found through bioinformatics analysis of the probiotic genome sequence. To confirm this, the MICs of different antibiotics were determined in triplicate by the broth microdilution method, and the results were interpreted using the breakpoints recommended by the European Food Safety Authority (Table 1).

C. alimentarius CNTA 209 showed susceptibility to all 9 antibiotics tested, according to the MIC breakpoint values for *Lactobacillus* facultatively heterofermentative recommended by EFSA.[21] Further bioinformatic analyses did not detect virulence factors in the genomic sequence of *C. alimentarius* CNTA 209. In summary, according to the EFSA, this strain is a suitable candidate for a microbial feed additive.

The ability of a probiotic to provide health benefits depends on its resistance to gastrointestinal conditions and its successful colonization of the colon, which requires a minimum concentration of 6 log CFU per g or CFU per mL in the ileum.[22] The study demonstrated that *C. alimentarius* CNTA 209 has good resistance to simulated gastric and intestinal fluids (Fig. 1B). It survived for 2 hours in gastric fluid with minimal loss of viability (less than 1 log cycle) and maintained a high population after 2 hours in the intestinal fluid (6 log CFU per mL).

Short-chain fatty acids (SCFAs), organic monocarboxylic acids with 2–6 carbon atoms produced by specific gut bacteria, have multiple demonstrated benefits for human health. Reduced levels of SCFAs have been associated with gut barrier dysfunction, low-grade inflammation and altered glucose, lipid and energy homeostasis.[23] In this study, the GC-MS analysis determined that *C. alimentarius* strain CNTA 209 did not produce propionic, butyric, valeric, caproic, or isobutyric acids. However, it generated acetic acid in all tested media (ranging from 33.33 to 899.67 μg mL^{-1}) and isovaleric acid only when carbohydrates like glucose or FOS were added (Table 2).

The presence of β-galactosidase in Lactic Acid Bacteria (LAB) is crucial for dairy production and for metabolizing lactose in the gut, thereby alleviating intolerance.[24] Here, the production of β-galactosidase by *C. alimentarius* CNTA 209 was evaluated throughout the semi-qualitative *in vitro* test, applying sterile filter paper disks impregnated with ONPG. The change in color indicated the ability of this strain to break

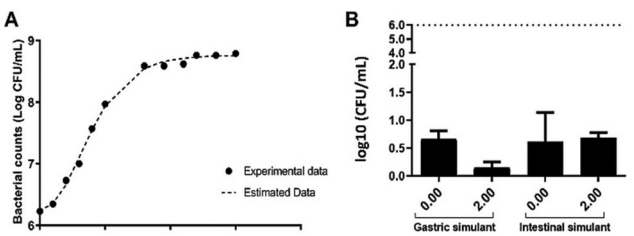

Fig. 1 (A) Growth curve of *C. alimentarius* CNTA 209 through the incubation time at 37 °C in MRS broth. Experimental values and estimation using the Gompertz model. (B) Gastric and intestinal fluid tolerance of *C. alimentarius* CNTA 209 (log cycle reduction).

Table 1 Antibiotic susceptibilities of *C. alimentarius* CNTA 209

	MIC values against antibiotics (mg L^{-1})							
	Gm	Km	Sm	Tc	Cl	Cm	Am	Em
CNTA 209	2	64	8–16	2–4	0.032–0.063	2	2	0.016
MIC cut-off values[a]	≤16	≤64	≤64	≤8	≤1	≤4	≤4	≤1

The result shows an average of 3 replicates. Gm, gentamycin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Cl, clindamycin; Cm, chloramphenicol; Am, ampicillin; Em, erythromycin. [a] Established MIC breakpoint values for *Lactobacillus* facultative heterofermentative (EFSA).

Table 2 SCFA production (μg mL^{-1}) by *C. alimentarius* CNTA 209

Medium	Acetic acid	Isovaleric acid
Defined medium without adding a carbon source	80.00 ± 26.46	nd
Defined medium with glucose (2%)	33.33 ± 35.12	2.12 ± 1.39
Defined medium with synergy 1 (2%)	363.33 ± 60.23	6.27 ± 4.93
Defined medium with P95 (2%)	866.67 ± 130.51	9.96 ± 3.53

nd: not detected.

down the disaccharide lactose into its monosaccharide components, glucose and galactose, demonstrating the β-galactosidase activity of *C. alimentarius* CNTA 209 (Fig. S2).

The biofilm-forming ability of certain LAB strains offers multiple health benefits by creating a protective layer that shields against pathogens, enhances microbial balance, and increases resilience to harsh gastrointestinal conditions like pH changes and bile salts. This promotes better survival and sustained colonization in the gut, which in turn improves nutrient absorption through the controlled release of metabolites, supports immune function, and contributes to overall gastrointestinal health and well-being.[25] In this work, the potential biofilm-forming capability of the strain was evaluated *in vitro*. Thus, *C. alimentarius* CNTA 209 displayed a very strong biofilm-forming capacity on an abiotic surface (Fig. 2A), with absorbance values higher than 0.5, ranging from 1.81 ± 0.69 (24 h) to 6.00 ± 0.00 (48 h).

Adhesion of probiotic bacteria to the intestinal wall is a desirable characteristic when selecting a strain, since it is a marker of persistence and durability in the intestine. In our study, we investigated the potential effect of *C. alimentarius* modifying the adhesion of pathogenic *E. coli* O157:H7 onto Caco-2 cells as a model of the intestinal barrier.[26] Here, the individual adhesion capacity of *E. coli* O157:H7 was initially assessed as a normalization parameter for competence, exclusion and displacement assays. We observed that, when *C. alimentarius* and *E. coli* were incubated at the same time onto Caco-2 cells (competence assays), *E. coli* was reduced by about 50% (Fig. 2B). Moreover, when *C. alimentarius* was exposed prior to *E. coli* (exclusion assays), the pathogenic bacteria were significantly reduced by approximately 75%. Finally, when *E. coli* is incubated with Caco-2 cells, then washed and incubated with our probiotic strain (exclusion), the adhesion of *E. coli* is drastically reduced by almost 90%. In these assays, *C. alimentarius* was also quantified. Compared to its own adhesion, *C. alimentarius* remained almost intact in competence assays, but was drastically reduced in exclusion and displacement assays. Altogether, these results indicate that *C. alimentarius* is capable of excluding and displacing *E. coli.* Therefore, it is possible to speculate that *C. alimentarius* can have a preventive effect on pathogenic bacteria and is capable of detaching bacteria that are already present in the intestine. It has been previously shown that several strains of *L. plantarum* have a high autoaggregation capacity, which also correlates with the ability to co-aggregate with pathogenic bacteria, preventing or inhibiting their adhesion.[27] Such behavior could explain why our *C. alimentarius 209* is capable of drastically reducing the adherence of *E. coli* in exclusion and displacement assays. In exclusion assays, *C. alimentarius* is almost depleted after incubation with *E. coli*, but the pathogenic bacteria are also reduced compared to their individual adhesion. This would support the theory that *C. alimentarius* could co-aggregate with *E. coli*, preventing its attachment to intestinal cells. In this context, different LAB strains have been studied in recent years for their capacity to "fight" pathogens like *Salmonella*, *Escherichia coli* and *Clostridium*.[28] It has also been shown that IBD patients display an increased proportion of bacteria from the Enterobacteriaceae family and a decreased concentration of bacteria from the phylum Firmicutes.[29] Moreover, the microbiome of patients with Crohn's disease generally contains an anomalous amount of adherent invasive *E. coli*. (AIEC). In our displacement assays, *E. coli* adhesion is greatly reduced after *C. alimentarius* incubation, and a portion

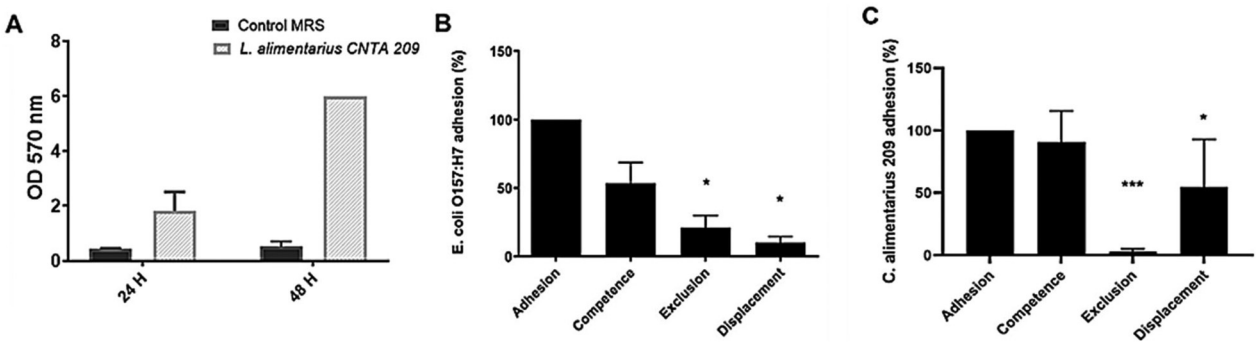

Fig. 2 (A) *C. alimentarius* CNTA 209 exhibited the property of biofilm formation. The figure shows the biofilm of *C. alimentarius* CNTA 209. The data have been presented as mean ± SD of three independent experiments, each performed in triplicate. (B) Adhesion of *E. coli* on Caco-2 cells in competence (*C. alimentarius* + *E. coli*), exclusion (*C. alimentarius*/*E. coli*) and displacement (*E. coli*/*C alimentarius*) assays. (C) Adhesion of *C. alimentarius* CNTA209 on Caco-2 cells in competence, exclusion and displacement assays. The data presented are the means ± standard deviation of 4 independent assays with 3 wells per assay. Statistical test: one way ANOVA, *post hoc* Bonferroni. * Indicates the means which were significantly different ($p < 0.05$) from the control; data for adhesion of *E. coli* alone.

of *C. alimentarius* remains in the intestine. These results could indicate that the probiotic strain is capable of displacing an already attached bacterium, either by occupying the same binding site or producing molecules that would result in *E. coli* detachment. Further studies would be needed to elucidate the specific mechanism by which *C. alimentarius 209* modulates *E. coli* adhesion to intestinal cells.[22]

In summary, our *in vitro* tests demonstrated that *C. alimentarius* CNTA 209 was able to resist gastric and intestinal acidity, showed sensitivity to the entire spectrum of antibiotics analyzed, ability to form biofilms, β-galactosidase activity, and produced SCFAs (acetic and isovaleric acids) *in vitro.* Moreover, our *in vitro* cell culture tests evidenced that this strain was able to counteract the adhesion of *E. coli* O157:H7 onto Caco-2 cells in exclusion and displacement environments. All these properties demonstrate the suitability of *C. alimentarius* CNTA 209 as a probiotic candidate.

C. alimentarius CNTA 209 reduces adiposity by stimulating BAT activity

The main consequence of the consumption of high HFS diets is the increase in body weight and enhanced adiposity, specifically visceral fat.[30] This excess of adipose tissue poses a metabolic and cardiovascular risk, since WAT releases fatty acids, hormones and pro-inflammatory cytokines that can interfere with glucose maintenance, contributing to the development of insulin resistance.[31,32] For this reason, we evaluated how our probiotic could be modulating the effect of the high fat-high sugar diet on body weight, weight gain, and the accumulation and distribution of adipose tissue.

As a result of the diet, animals fed with HFD and 10% of fructose in water (HFS group) exhibited a significantly higher body weight in comparison with the control (CNT group) mice from week 3 to the end of the study (Fig. S3A). No differences were observed in the body weight of the *C. alimentarius* CNTA 209 (*C. alim*) and HFS group animals throughout the experiment. Thus, only control diet-fed animals exhibited a significantly reduced body weight gain in comparison with the HFS group (Fig. S3B). Regarding HFS-fed animals, no differences were observed in the daily and total diet intake between HFS and *C. alim* groups (Table S2). These data suggest that the probiotic supplementation does not affect body weight management nor diet intake. Moreover, the treatment did not affect the weights of the liver, spleen and kidneys (Table S3).

Regarding body composition, no differences were observed in the muscle mass proportion, quantified by the weight of gastrocnemius muscle between the *C. alimentarius* and HFS groups, related to total body weight (Fig. 3A). However, significant differences were observed in the case of WAT depots, where animals supplemented with *C. alimentarius* CNTA 209 exhibited a significant reduction in the retroperitoneal (Fig. 3C) and mesenteric fat (Fig. 3D) depots in comparison with the HFS group.

This effect led to a significant reduction in total visceral fat mass, considered as the sum of epididymal, retroperitoneal and mesenteric fat mass, in the probiotic-treated group, in comparison with the untreated-HFS animals (Fig. 3F).

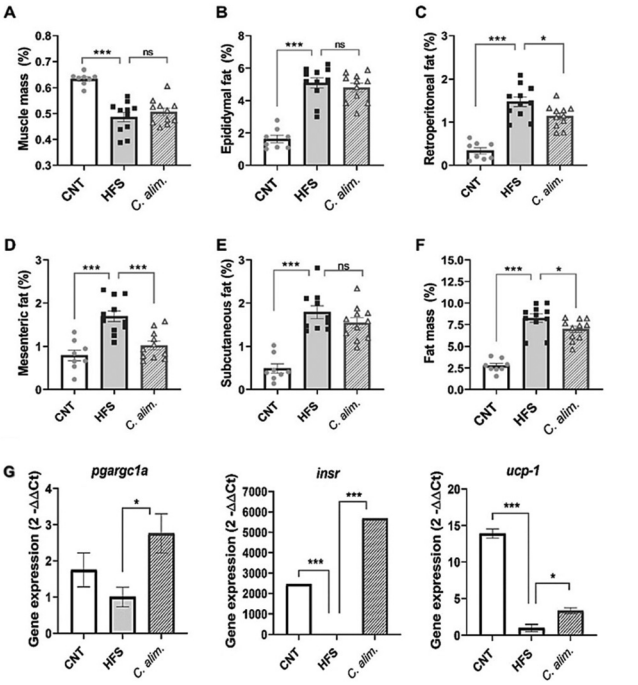

Fig. 3 Gastrocnemius muscle and white adipose tissue depots, expressed as the weight proportion relative to the total body weight, of the different groups of the study. Mean ± SEM. (A) Gastrocnemius muscle. (B) Epididymal fat. (C) Retroperitoneal fat. (D) Mesenteric fat. (E) Subcutaneous fat. (F) Total white adipose tissue proportion. (G) Gene expression levels of *Ppargc1a*, *Insr* and *Ucp1*, relative to the HFS group, in mesenteric fat samples. Results are expressed as the mean ± SEM. Statistical differences were analysed by the one-way ANOVA test followed by Fisher's LSD test for multiple comparisons when ANOVA $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, with respect to the HFS group). CNT: control diet group; HFS: high-fat-fructose diet group; *C. alim*: *C. alimentarius* CNTA 209 group.

This finding suggests that, despite no observed differences in the total body weight, supplementation with *C. alimentarius* CNTA 209 counteracted the excess adiposity induced by the HFS diet, modulating body composition. In order to determine the molecular pathways involved in this anti-adipogenic effect, different gene expression analyses were performed. Mesenteric fat tissue was selected for these analyses, as it represents a highly proinflammatory visceral fat that is more metabolically active than subcutaneous and retroperitoneal adipose tissues, playing important roles in the development and pathogenesis of insulin resistance.[33,34]

Gene expression analyses revealed no differences in the expression of lipogenesis-key genes, such as *Pparg*, *Srebf2*, *Fabp4*, *Scd1* or *Plin1*, between *C. alimentarius* and HFS mesenteric adipocytes. However, samples from the probiotic group exhibited a significant upregulation of *Ppargc1a*, *Insr* and *Ucp1* genes in comparison with HFS samples (Fig. 3G). Overexpression of *Ppargc1a*, coding for the peroxisome proliferative activated receptor gamma coactivator 1 alpha or Pgc-1a,

is a transcriptional coactivator that increases mitochondrial activity, enhancing fatty acid oxidation and energy expenditure. However, it also promotes browning of WAT by inducing the expression of thermogenic genes, including uncoupling protein 1 gene (*Ucp1*), also significantly upregulated in *C. alim*-supplemented animals, in comparison with HFS mice. Overexpression of UCP1 in white adipocytes can improve glucose uptake and potentially enhance insulin sensitivity by promoting the browning of this adipose tissue.[35] It is also remarkable that the probiotic induces the overexpression of *Insr* in the control-diet group. The expression of the insulin receptor gene improves insulin sensitivity and promotes glucose uptake in adipocytes, but it has also been previously reported to play a crucial role in the regulation of WAT browning through activation of UCP1 and PGC-1a.[36,37] Thus, our results would suggest that the fat-reducing effect of *C. alimentarius* would be explained by the enhanced metabolic expenditure of white adipocytes, promoting a "browning" effect, turning them into "brown-like" adipocytes.

In order to confirm this hypothesis, we analysed different browning-related genes in BAT samples from the three groups. Although no differences were observed in the BAT proportion among groups (Fig. 4A), *C. alim*-supplemented animals exhibited a significant up-regulation of *Cpt2* (Fig. 4B), in comparison with non-supplemented HFS mice. The overexpression of the carnitine palmitoyltransferase 2 (*Cpt2*) gene suggests an increased capacity for mitochondrial fatty acid oxidation in BAT mitochondria, a metabolic signature of the increased energy demand for promoting thermogenesis in this tissue.[38] Moreover, the increased expression of *Slc2a4* (Fig. 4C) in *C. alim*-treated BAT in comparison with untreated HFS samples might be related to an increased glucose uptake in this tissue. The overexpression of these thermogenesis-key genes in BAT, including *Pnpla2* (Fig. 4D), *Pparg* (Fig. 4E), *Ppargc1a* (Fig. 4F), *Prdm16* (Fig. 4G) and *Ucp1* (Fig. 4H), suggests the activation of overall metabolism and the enhancement of the mitochondrial thermogenic (heat-generating) response, managing metabolic dysfunction induced by the HFS diet.

Thus, our results confirm the previous observation in mesenteric fat tissue and highlight the hypothesis that *C. alimentarius* CNTA 209 modulates adiposity by increasing BAT metabolic activity and promoting browning of WAT. In fact, dietary, pharmaceutical, and microbial strategies that promote adipose tissue browning by the activation of key-browning genes such as PGC-1a, UCP1 and PRDM16,[39,40] might represent a promising and targeted means of reducing visceral fat and improving metabolic function. Although the role of probiotics in regulating mitochondrial thermogenesis remains largely unexplored, previous works have reported a similar anti-obesity mechanism. Thus, Gu *et al.* observed that supplementation with an aqueous extract of fermented barley

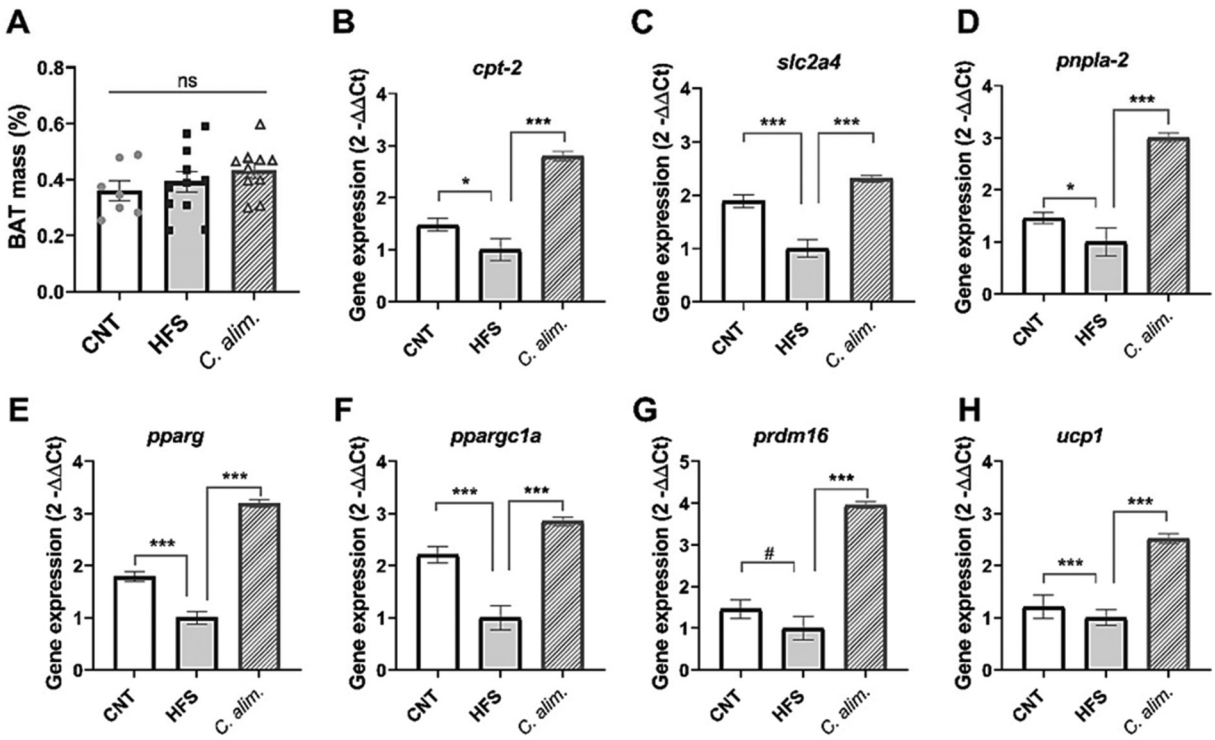

Fig. 4 (A) Brown adipose tissue (BAT) depot, expressed as the weight proportion relative to the total body weight, of the different groups of the study. (B–H) Expression levels of *Cpt2* (B), *Slc2a4* (C), *Pnpla2* (D), *Pparg* (E), *Ppargc1a* (F), *Prdm16* (G) and *Ucp1* (H) in BAT, relative to the HFS group, in brown fat samples. Results are expressed as the mean ± SEM. Statistical differences were analysed by the one-way ANOVA test followed by Fisher's LSD test for multiple comparisons when ANOVA $p < 0.05$ (# $p < 0.1$, *$p < 0.05$, ***$p < 0.001$ with respect to the HFS group). CNT: control diet group; HFS: high-fat-fructose diet group; *C. alim*: *C. alimentarius* CNTA 209 group.

with *Lactobacillus plantarum* dy-1 (LFBE) decreased obesity in rats partly by increasing the BAT mass and the energy expenditure by activating BAT thermogenesis and WAT browning in a UCP1-dependent mechanism.[41]

C. alimentarius CNTA 209 improves liver health in diet-induced obese C57BL/6 mice

Metabolic syndrome is characterized by a cluster of biochemical abnormalities, prominently including insulin resistance and dyslipidemia. Insulin resistance impairs the normal cellular uptake of glucose, leading to compensatory hyperinsulinemia and contributing to the development of fatty liver disease.[1,42]

In the case of rodents subjected to high-fat diet (HFD) feeding, apart from excess adiposity, these models reliably develop key features of metabolic syndrome, including insulin resistance and impaired glucose uptake, which promotes hepatic overproduction of very low-density lipoproteins (VLDL), inducing liver steatosis. Consequently, HFD-fed rodents exhibit a dyslipidemic profile characterized by elevated plasma triglycerides, reduced HDL cholesterol, and an increased proportion of small, dense LDL particles. These biochemical alterations not only serve as markers of metabolic dysfunction but also contribute to the progression of cardiovascular and hepatic complications observed in diet-induced obesity.[43,44] For this reason, in order to determine whether probiotic supplementation could be modulating glucose homeostasis, a week previous to the sacrifice, the animals were subjected to a glucose tolerance test. As can be observed in Fig. S4A, all animals showed an increase in the serum glucose levels after the injection of the glucose overload (1.5 mg kg^{-1} animal).

However, the recovery time to normal blood glucose levels was significantly lower in the animals in the CNT group compared to the mice in the HFS group, as observed by the lower blood glucose levels at points 20, 40, 60 and 90 minutes, which induced a significantly reduced area under the curve (Fig. S4B). In the group supplemented with *C. alimentarius*, although there were no differences in the AUC, these animals were able to reduce glycemia at the 90 min point ($p < 0.05$), in comparison with the HFS group, suggesting an improvement in the recovery of glucose levels.

After sacrifice, no differences were observed in the levels of glucose, insulin and HOMA index between groups (Table 3). Regarding lipid metabolism, although HDL levels were significantly higher in HFS *vs.* CNT animals, the high total cholesterol levels in the HFS group evidenced the hypercholesterolemic effect of a high-fat, high-fructose diet. This finding indicates that the rise in HDL did not offset the overall increase in circulating cholesterol and suggests a relative enrichment of non-HDL. This is in line with a recent review on the metabolic impact of high-fat diets in murine models, which highlights the lack of consistency in cholesterol and HDL lipoprotein metabolism responses. Specifically, mixed outcomes have been reported, with some studies showing decreased or unchanged HDL levels following HFD consumption, while a comparable proportion of studies reported a significant increase in the HDL levels.[45]

Unlike humans, mice lack cholesteryl ester transfer protein (CETP), which limits the transfer of cholesteryl esters from HDL to apoB-containing lipoproteins and favors the accumulation of cholesterol within the HDL fraction.[46] Consequently, increases in total cholesterol in mice are frequently accompanied by elevations in HDL cholesterol, reflecting a compensatory response to the excess of lipid availability rather than an improvement in HDL functionality or cardiovascular protection.[45,47] Thus, we evaluated the relative proportion of these HDL molecules through the HDL/total cholesterol ratio, which evidenced a reduction in this proportion in obese mice in comparison with the control diet-fed animals, supporting the presence of a dysregulated lipid metabolism associated with high-fat feeding.

Interestingly, despite being fed the same diet, animals supplemented with *C. alimentarius* showed a tendency to reduce the total cholesterol levels ($p = 0.094$) in comparison with the HFS group, without affecting the HDL/total cholesterol ratio. No differences were also observed in the levels of the inflammatory marker MCP-1 among groups.

However, animals supplemented with the probiotic exhibited significantly reduced levels of the hepatic transaminase ALT (Fig. 5A), with no differences with the control diet-fed animals (CNT). Although a similar trend was observed in the case of AST, no statistically significant differences were reached (Fig. 5B). Moreover, an analysis of intrahepatic trigly-

Table 3 Biochemical parameters of the different groups of the study

Biochemical parameter	CNT ($n = 8$)	HFS ($n = 11$)	*C. alim* ($n = 11$)	ANOVA
Glucose (mg dL^{-1})	116.98 ± 11.97	142.10 ± 7.23	129.11 ± 7.64	ns
Insulin (μg L^{-1})	0.48 ± 0.08	0.68 ± 0.12	0.49 ± 0.06	ns
HOMA-IR	3.94 ± 0.90	6.15 ± 1.32	3.93 ± 0.59	ns
Cholesterol (mg dL^{-1})	117.00 ± 7.96***	166.00 ± 7.91	149.33 ± 3.53	0.0002
HDL cholesterol (mg dL^{-1})	53.22 ± 5.78**	67.98 ± 2.84	61.24 ± 2.48	0.0314
HDL-c/total cholesterol	0.47 ± 0.02*	0.41 ± 0.01	0.40 ± 0.02	0.0337
Triglycerides (mg dL^{-1})	115.25 ± 7.48*	139.20 ± 5.00	142.09 ± 9.06	0.0477
MCP-1 (pg mL^{-1})	56.06 ± 4.55	59.16 ± 8.29	52.67 ± 6.37	ns

Results are expressed as the mean ± SEM. Statistical analyses were performed using the one-way ANOVA test followed by Fisher's LSD test when statistical significance ($p < 0.05$) was reached (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ with respect to the HFS group).

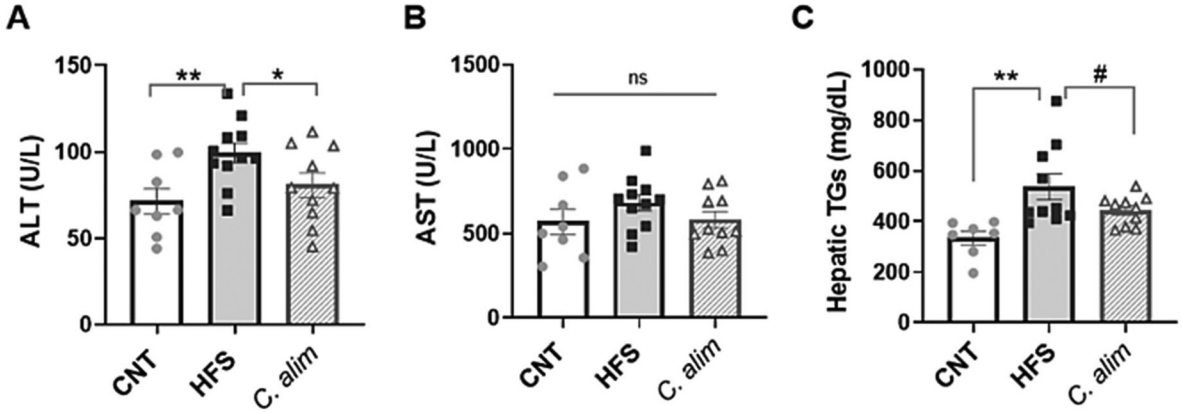

Fig. 5 Alanine aminotransferase (ALT, A) and aspartate aminotransferase (AST, B) for each group of the study. (C) Quantification of the intrahepatic triglyceride levels (mg dL^{-1}). Results are expressed as the mean ± SEM. Statistical differences were analysed by the one-way ANOVA test followed by Fisher's LSD test for multiple comparisons when ANOVA $p < 0.05$ (# $p < 0.1$, *$p < 0.05$, **$p < 0.01$ with respect to the HFS group; ns: not significant). CNT: control diet group; HFS: high-fat-fructose diet group; *C. alim*: *C. alimentarius* CNTA 209 group.

ceride content was performed on the liver samples, showing that animals supplemented with the probiotic exhibited a tendency toward reduced liver steatosis (determined by the triglyceride content) in comparison with the non-supplemented HFS group ($p = 0.075$), with no difference from the control diet-fed group ($p > 0.05$, Fig. 5C).

Both the reduction in the transaminase levels and in the intrahepatic triglycerides suggested a protective effect of *C. alimentarius* against liver steatosis induced by the HFS diet. In order to determine the molecular mechanisms underlying these effects, we performed gene expression analysis through qPCR on the liver samples.

Animals supplemented with *C. alimentarius* CNTA 209 exhibited a significant reduction in the gene expression of two key regulators of glucose metabolism in the liver: *Foxo1* (Fig. 6A) and *Slc2a4* (Fig. 6B) genes. FOXO-1 promotes gluconeogenesis and suppresses glycolysis in the liver, so its down-regulation might indicate a reduction in the hepatic glucose production. Moreover, FOXO1 can influence the expression of *Slc2a4* (also known as Glut-4), encoding a transporter for the glucose uptake, so its down-regulation suggests a reduction in hepatic glucose production and glucose uptake, normalizing the levels to the control diet-fed group. A similar trend was observed for *Ppara* (Fig. 6C). In the same line, the probiotic induced a significant increase in ppargc1α gene expression (Fig. 6D), which encodes for PGC-1α protein specially involved in energy metabolism and mitochondrial function. Overexpression of this gene in the liver has been related to increased fatty acid oxidation and reduced liver steatosis.[48]

Chronic impairment of lipid metabolism is closely related to oxidative stress, which plays a crucial role in the development of fatty liver disease.[42] For this reason, we also evaluated if the reduction in transaminase content could be accompanied by a modulation of detoxifying enzyme expression in this organ. Thus, we observed that those animals supplemented with the probiotic exhibited a significant reduction in the expression of catalase (*Cat*), catechol-*O*-methyltransferase (*Comt*), glutathione S-transferase mu 1 (*Gstm1*), and glutathione S-transferase theta 1 (*Gstt1*) in the liver (Fig. 6E–H). All these genes encode for detoxifying proteins related to the elimination of products from oxidative stress, so their down-regulation in the probiotic group might be explained by a lower oxidative stress environment in the liver as a consequence of greater glucose uptake and better insulin sensitivity in this organ, normalizing their expression towards the control diet-gene levels.

All these data suggest that *C. alimentarius* CNTA 209 supplementation improves liver health and ameliorates hepatic steatosis and oxidative stress induced by the HFS diet. The liver protecting function of certain probiotics, including those of the *Lactobacillus*, *Bifidobacterium* and *Streptococcus* genera, has already been proposed.[49,50] Thus, specific probiotic strains from the species *Bifidobacterium adolescentis* and *Lacticaseibacillus rhamnosus* have been shown to alleviate non-alcoholic fatty liver disease induced by a high-fat, high-cholesterol diet.[51] However, in some human intervention trials, no differences in non-alcoholic fatty liver disease (NAFLD) markers have been observed after supplementation with other probiotic strains, including multi-strain studies.[52] To our knowledge, our study reports the first evidence implicating a *C. alimentarius* strain as a probiotic with hepatoprotective effects against diet-induced steatosis and oxidative stress.

Supplementation with *C. alimentarius* CNTA 209 modulates gut microbiota

The mechanisms by which a probiotic strain may influence host metabolic health include the modulation of the composition and activity of the intestinal microbiota by restoring microbial diversity, promoting the growth of beneficial commensals, inhibiting pathogenic species, or producing metabolites such as SCFAs. Through these strategies, probiotics contribute to the improvement of intestinal barrier integrity, the

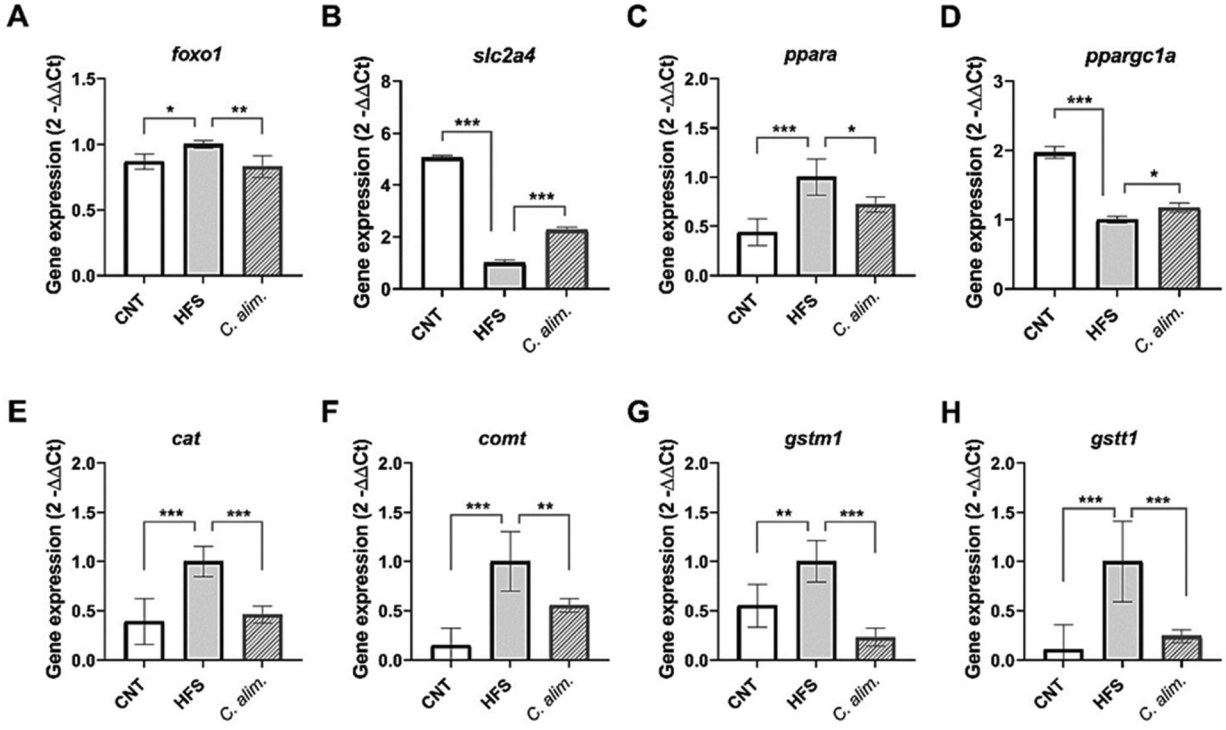

Fig. 6 Gene expression levels of *Foxo1* (A), *Slc2a4* (B), *Ppara* (C), *Ppargc1a* (D), *Cat* (E), *Comt* (F), *Gstm1* (G) and *Gstt1* (H) in the liver, relative to the HFS group. Results are expressed as the mean ± SEM. Statistical differences were analysed by the one-way ANOVA test followed by Fisher's LSD test for multiple comparisons when ANOVA $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ with respect to the HFS group). CNT: control diet group; HFS: high-fat-fructose diet group; *C. alim*: *C. alimentarius* CNTA 209 group.

maintenance of intestinal homeostasis and the attenuation of low-grade systemic inflammation. These effects collectively contribute to the improvement of metabolic functions, positioning probiotics as a promising tool in the prevention and management of various gut-related and systemic disorders.[53]

Thus, one of the objectives of this work was not only to describe the physiological and metabolic changes induced by *C. alimentarius* CNTA 209, but also to describe its ability to modulate the gut microbiota composition through 16S metagenomic analyses.

First, we evaluated the influence of both diet and probiotic supplementation on alpha and beta diversities, considering the metagenomic data from the CNT, HFS and *C. alim* groups. No differences were observed in the alpha diversity among groups, evaluated by the Simpson and Shannon indices. However, both PCA (Fig. 7A) and PCoA (Fig. 7B) revealed a significant difference in the beta-diversity (Permanova $p < 0.001$) between the three groups. However, no differences were observed at the phylum level between the three groups (Fig. S5).

In order to identify the specific changes that the probiotic supplementation induced at the family, genera and species levels, only the HFS and HFS–*C. alim* groups were included in the analyses. *C. alim*-supplemented animals exhibited a significant increase in the families Sutterellaceae (Fig. S6A), Rhodospirillaceae (Fig. S6B), Elusimicrobiaceae (Fig. S6C) and Bacillales incertae sedis (Fig. S6D). In contrast, probiotic supplementation induced a significant reduction in Helicobacteriaceae (Fig. S6E) and Veillonellaceae (Fig. S6F) families. In the case of Veillonellaceae, species from this family have been shown to affect gut permeability,[54] inducing a low-grade systemic inflammation and alterations in lipid metabolism, predisposing individuals to obesity,[55,56] so its reduction could be associated with the metabolic improvements observed with the probiotic.

At the genus level, *C. alimentarius*-supplemented mice exhibited a significant increase in some specific genera (Fig. 7C), including *Parasutterella*, *Anaerobaculum*, *Fodinicurvata*, *Macellibacteroides*, *Elusimicrobium*, *Weisella* and *Eggerthella*. This latter has been found to be a genus reduced in individuals with obesity[57] and NAFLD.[58] In contrast, animals supplemented with the probiotic showed a significant amelioration of the genera *Gemella*, *Spirosoma*, *Helicobacter* and *Fibrisoma* (Fig. 7D). These changes at the genus level correspond to the modulation of some specific bacterial species (Fig. 8). Thus, the increase in the *Parasutterella* genus (Fig. 7C) induced by *C. alim* might be explained by the higher abundance *of Parasutterella excrementihominis* (Fig. 8A) in this group, in comparison with non-supplemented HFS samples. This species has been recently found to be increased after treatment with obeticholic acid in mice with NAFLD, due to the ability of this species to modulate host bile acids and improve liver health.[59]

Importantly, the analyses of the specific species demonstrating the presence of the DNA corresponding to *L. alimentarius* (or *C. alimentarius*) in the faeces of *C. alim*-sup-

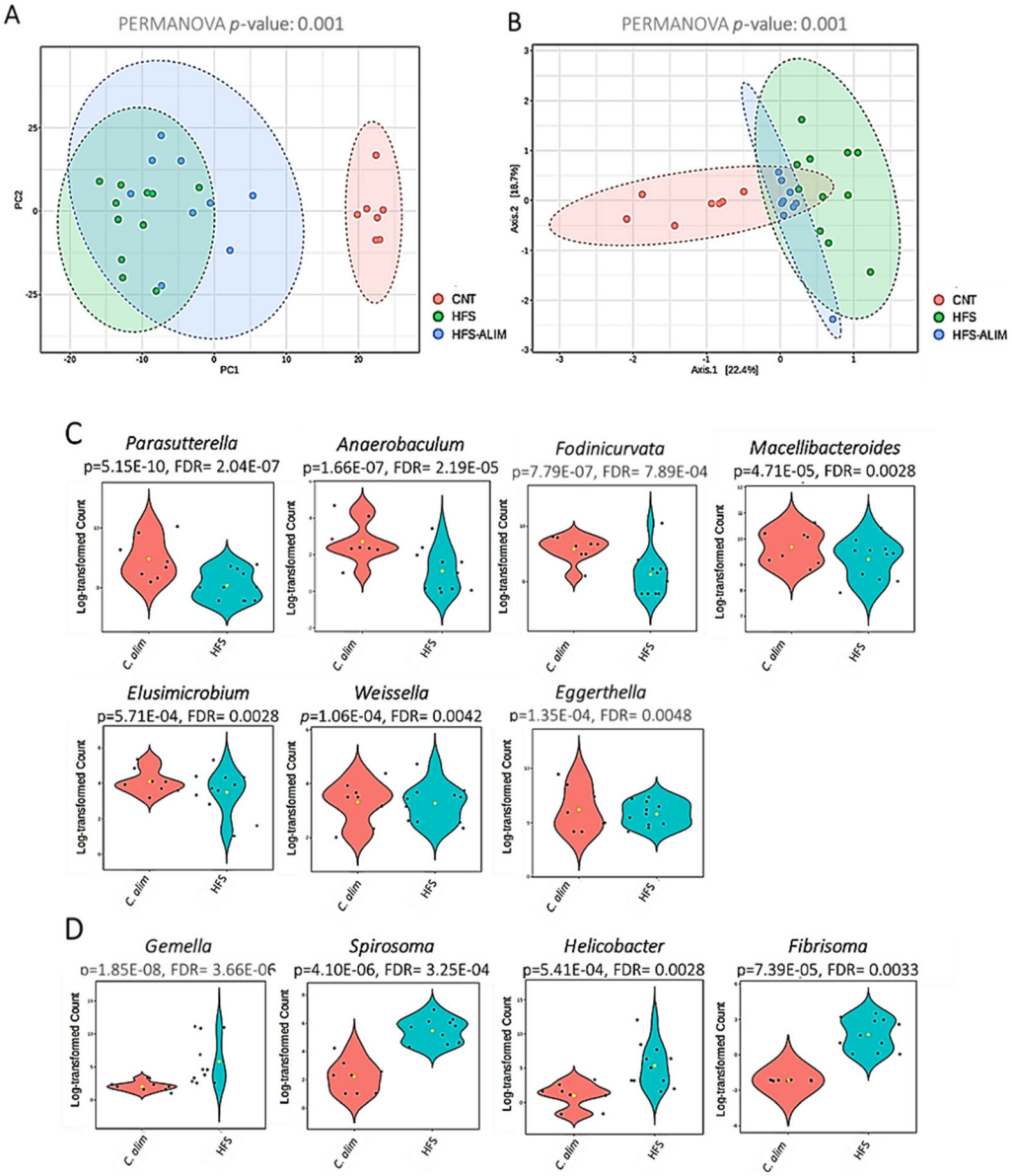

Fig. 7 (A and B) Representation of the beta-diversity by PCA (A) and PCoA (B). (C) Representation of the normalized data of those genera that are significantly more abundant in the *C. alim* than in the HFS group. (D) Representation of the normalized data of those genera that are significantly less abundant in *C. alim* in comparison with the HFS group. Metagenomic statistical analyses were performed using Deseq2, considering a minimum *p* value <0.01, and FDR < 0.005.

plemented animals, but its absence in the HFS group, demonstrated the presence of the probiotic in the mice gut and the lack of contamination among groups. Interestingly, *Lactobacillus taiwanensis* was more abundant in the probiotic-supplemented group. Moreover, *C. alim*-samples were enriched in three different *Bacteroides* species (*Bacteroides caecigallinarum*, *Bacteroides acidifaciens* and *Bacteroides helcogenes*) (Fig. 8A). Although the relationship between *Bacteroides* and obesity is controversial in humans, some recent works have reported the potential anti-obesity effect of specific species of this genus in rodents, such as *Bacteroides acidifaciens*,[60,61] so the role of this genus in mice still needs further studies.

In the case of species significantly decreased by probiotic supplementation (Fig. 8B), two *Desulfovibrio* species (*Desulfovibrio arcticusarcticus* and *Desulfovibrio aespoeensis*) were significantly reduced in *C. alim*-supplemented faeces, in comparison with HFS. This reduction has already been observed after supplementation with other *Lactobacillus*-related probiotics, such as *Limosilactobacillus reuteri* (*L. reuteri*) or *Lacticaseibacillus rhamnosus* GG. Interestingly, supplementation with a mixture of these two LAB species with *Bifidobacterium animalis* subsp. *lactis* BB12 improved hepatic steatosis and fibrosis in HFD-fed mice, which was related to the decreased abundance of *Desulfovibrio* in faeces. A sub-

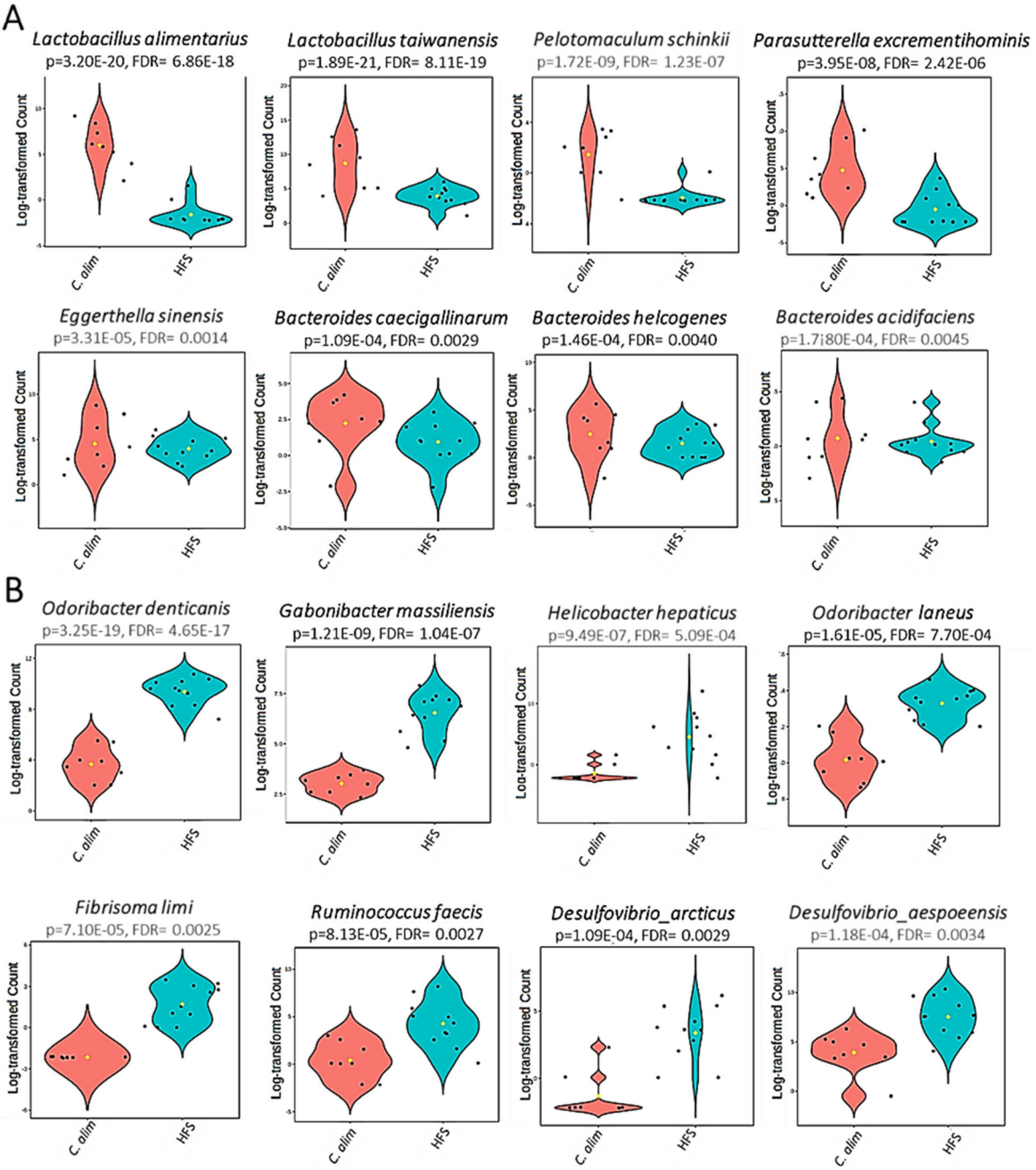

Fig. 8 Representation of the normalized data of those species significantly more (A) and less (B) abundant in *C. alim* in comparison with the HFS group. Metagenomic statistical analyses were calculated using Deseq2, considering a minimum *p* value <0.01, and FDR < 0.005.

sequent study evaluating the effect of supplementation with a species of this genus induced the development of fatty liver disease, suggesting the modulation of *Desulfovibrio* as a potential target to combat NAFLD.[62]

Finally, probiotic supplementation induced a significant reduction in the proportion of *Helicobacter* (Fig. 7D), and specifically in the *H. hepaticus* species (Fig. 8B). This species is considered a known murine hepatic pathogen. Thus, some specific treatments such as rifaximin have been shown to ameliorate fatty liver disease by modulating the presence of *H. hepaticus*.[63]

Thus, the significant reduction in the *H. hepaticus* and *Desulfovibrio* species and the increase in the abundance of *Eggerthella* and *Parasutterella* might be related to the hepatic protective effect previously observed by *C. alimentarius* CNTA 209 and contributes to demonstrating the ability of this probiotic to modulate the gut microbiota towards a metabolically healthier composition.

Supplementation with the probiotic *C. alimentarius* CNTA 209 does not induce any toxic effect.

While many probiotics, including LAB such as *C. alimentarius*, are classified as Generally Recognized As Safe (GRAS) or qualify for the Qualified Presumption of Safety (QPS) status in Europe, these designations are not automatically granted and require robust scientific evidence before human consumption.[64–66] In fact, besides the previously described properties that a probiotic strain should compete for being considered a potential food additive according to EFSA, the strain specificity involves the need for toxicological studies in appropriate animal models to demonstrate the safety of each specific probiotic.[67] In this sense, Wistar rats are commonly used for such preclinical investigations, as they provide valuable insights into potential systemic toxicity, organ-specific effects, and local gastrointestinal tolerance. These studies are essential to support the safety of novel probiotic candidates, fulfill regulatory requirements, and ensure consumer protection.

For this reason, a parallel toxicological analysis was performed to demonstrate the safety of oral administration of *C. alimentarius* CNTA 209. Both male and female standard diet-fed Wistar rats were used in this experiment, which were supplemented with the probiotic strain (dose of 1×10^9 CFU per animal per day) or vehicle (water) for 10 weeks. No differences were observed in the diet intake between treated and untreated rats along the study (Table S4), which represents an intake of the administered dose of the probiotic (or vehicle) close to 100% for each group and week (Table S5). Body weight (Table S6) and body weight change (Table S7) did not differ between the groups supplemented with *C. alimentarius* and controls, for both sexes. At the end of the study, none of the animals exhibited lethality or alteration of the general symptoms (determined by the weekly Irwin test).

After 10 weeks of supplementation, the hematological parameters (Table S8), differential blood cell counts (Table S9) and coagulation markers (Table S10) did not show differences between groups for both sexes, with all obtained data in the normal range for Wistar rats. The biochemical analysis (Table S11) did not reveal any effect on renal function-related parameters (creatinine and urea) or the metabolic parameters (glucose, cholesterol, triglycerides) between animals supplemented with the probiotic and controls. Regarding liver function, no differences were observed in the levels of hepatic transaminases. However, male rats supplemented with the probiotic exhibited significantly reduced levels of bilirubin, being within the normal range, which could be related to the previously mentioned improvement in liver function by *C. alimentarius* CNTA 209. Interestingly, female rats supplemented with the probiotic showed reduced levels of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) enzymes in comparison with untreated controls. High levels of both CPK and LDH have been related to cardiovascular damage,[68] so their reduction might suggest a reduction in tissue damage induced by the probiotic.

On the other hand, the biochemical analyses of urine samples (Table S12), including the general characteristics (volume, aspect, color and odor), together with the biochemical parameters, did not evidence relevant findings between supplemented and control groups.

Finally, the general external examination, as well as the pathological study of the abdominal and thoracic cavities and the removed target organs, did not show any macroscopic alterations between the group treated with *C. alimentarius* and the controls. No differences were observed in the weight of the organs between treated and untreated animals, both for male (Table S13) and female (Table S14) rats. Additionally, a histological evaluation was performed for the spleen, heart (atrium and ventricle), esophagus, stomach, lymph nodes, mesenteric, adrenal glands, liver, small intestine (duodenum, jejunum and ileum), large intestine (cecum, colon and rectum), pancreas, Peyer's patches, lungs, kidneys, testes, ovaries and thymus. None of the analyses revealed possible alterations due to the treatment with the probiotic, and all microscopic studies were within the normal range for Wistar rats of that age and corresponding sex. Additionally, no changes in gastrointestinal symptoms or histological alterations were detected in the examined sections of the gastrointestinal tract—including the esophagus, stomach, mesenteric lymph nodes, Peyer's patches, small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum)—indicating good local tolerance. With all this information, the toxicological analyses evidenced that a dose of 1×10^9 CFU per animal per day can be considered the non-observed adverse effect level (NOAEL).

C. alimentarius CNTA 209 supplementation modulates gut permeability

Although the pathogenesis of obesity and metabolic syndrome-related diseases is complex and multifactorial, the link between these metabolic diseases and gut microbiota dysbiosis, intestinal permeability, and inflammation has long been acknowledged.[69]

Tight junction proteins—including ZO-1, occludin, and claudins—are essential elements of the intestinal epithelial barrier, where they regulate paracellular transport and maintain the selective permeability of the gut lining.[70] This barrier plays a fundamental role in blocking the passage of luminal antigens, pathogens, and endotoxins into systemic circulation. When tight junction integrity is compromised, intestinal permeability increases—a condition commonly known as "leaky gut"—allowing pro-inflammatory molecules such as lipopolysaccharides (LPS) to enter the bloodstream.[71,72] This can initiate chronic low-grade inflammation, a key factor in the onset and progression of obesity and associated metabolic disorders, including insulin resistance and NAFLD.[73] In fact, excessive consumption of simple carbohydrates, saturated fats, and processed foods appears to be directly linked to dysbiosis, which can lead to intestinal hyperpermeability and leaky gut syndrome.[72] Thus, maintaining the structure and function of the intestinal barrier is essential for controlling inflammation and reducing obesity-related metabolic risks.

In this context, probiotics have shown the potential to strengthen gut barrier function through multiple mechanisms

that support tight junction stability. Some strains promote the expression of tight junction proteins like ZO-1, occludin, and claudins, thereby reinforcing epithelial cohesion, but also regulating pro-inflammatory cytokines.[74] Additionally, probiotics produce metabolites such as SCFAs, including butyrate and propionate, that serve as energy sources for colonocytes and stimulate the assembly of tight junctions. Moreover, probiotics can inhibit pathogenic bacteria and limit their adhesion to the intestinal lining, thereby reducing the likelihood of toxin-induced barrier disruption.[75] Taken together, some probiotics might contribute to improving gut permeability, restoring the mucosal balance, and protecting against systemic inflammatory responses.

In our study, we investigated the potential of *C. alimentarius* for improving gut health by analyzing the expression of selected key genes related to intestinal function. For this purpose, RNA was extracted from colon samples obtained from both male and female Wistar rats used in the toxicological study, allowing us to assess whether the effects were consistent across sexes. Although no differences were observed in the gene expression of *Cldn1* (coding for claudin 1) and *Ocln* (coding for occluding) genes between treated and untreated samples (Fig. 9A and B), male Wistar rats supplemented with the probiotic exhibited a significantly reduced expression of the C–C motif chemokine ligand 2 (*Ccl2*) gene, suggesting a reduced inflammatory state (Fig. 9C). This effect was accompanied by a significant increase in the levels of *Tjp1* (p = 0.0014, Fig. 9D) and a tendency in the case of *Tjp2* (p = 0.0607, Fig. 9E) in both male and female rats subjected to the probiotic treatment, in comparison with the control rats. These two genes code for the tight junction proteins 1 and 2 (or ZO-1 and ZO-2, respectively), structural proteins that play a crucial role in maintaining intestinal health and regulating gut permeability through the epithelial cells. Under normal conditions, its overexpression in the colon means a good barrier function.[69,71] However, under inflammatory conditions and intestinal barrier disruption, the expression of these proteins decreases in the gut, as these proteins are released to the circulation, increasing their blood levels.[76,77]

For this reason, we have also quantified the levels of ZO-1 (zonula occludens-1, encoded by *Tjp-1*) in blood samples of these animals. This analysis demonstrated that supplementation with the probiotic induced a significant reduction in the levels of circulating ZO-1 in male rats (Fig. 9F), in comparison with the controls. This result suggests that *C. alimentarius* CNTA 209 protects against leaky gut, increasing gut function and reducing inflammation. A previous study reported the potential role of *C. alimentarius* strains in restoring gut permeability. For example, Zhao and colleagues demonstrated that supplementation with *Lactobacillus alimentarius* NKU556,

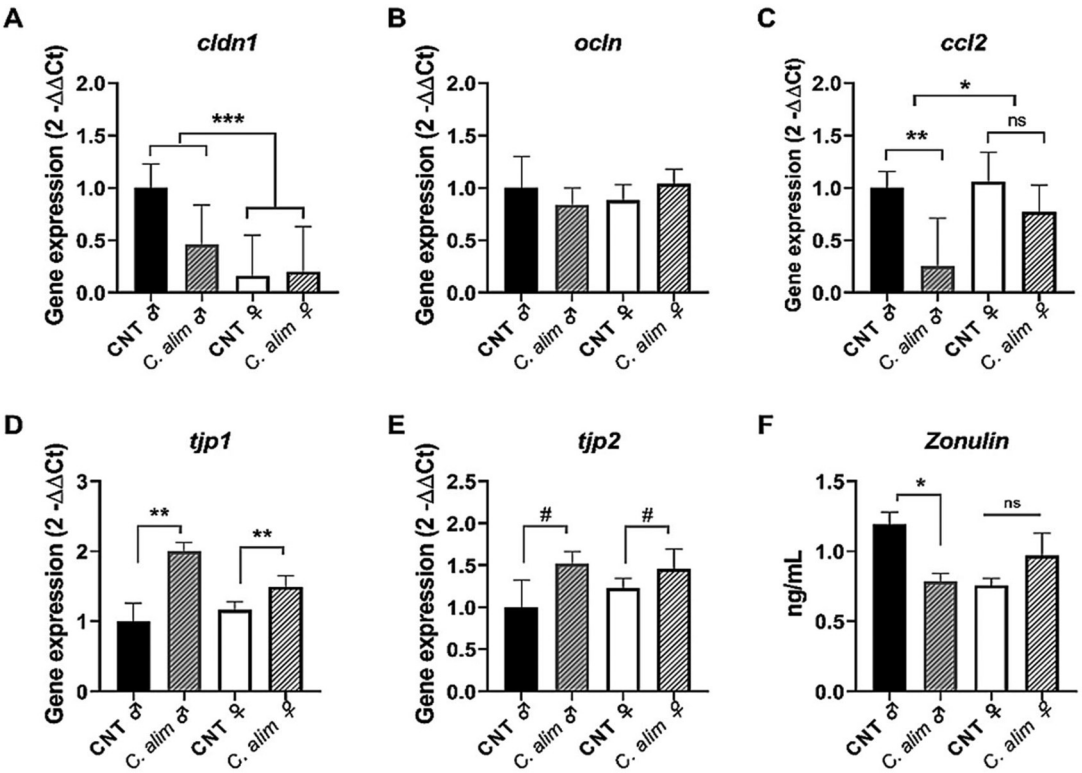

Fig. 9 Gene expression levels of *Cldn1* (A), *Ocln* (B), *Ccl2* (C), *Tjp1* (D) and *Tjp2* C (E) in supplemented and non-supplemented control animals, in both male (♂) and female (♀) rats. Expression relative to the corresponding CNT group. (F) Circulating levels of zonulin (ZO-1, ng mL^{-1}). Results are expressed as the mean ± SEM. Statistical differences were analysed by 2-way ANOVA (#p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001, with respect to the CNT group). CNT: control diet group; *C. alim*: *C. alimentarius* CNTA 209 group.

isolated from traditional Chinese fermented food, significantly increased the expression of tight junction proteins and effectively reduced pro-inflammatory cytokines and oxidative stress in a DSS-induced colitis mouse model.[78,79] Other probiotic strains from *Lactobacillus* and *Bifidobacterium* species have also been shown to enhance intestinal mucosal immune function, which has been associated with the amelioration of fatty liver disease in patients with NAFLD.[78,79]

Despite the obtained results, it is worth mentioning that the evaluation of the probiotic's effect on gut health was performed on samples from the Wistar rat toxicology study, which limits the ability to extrapolate these results to a diet-induced obesity model, such as the C57BL6 mice. However, our observations about the improvement of intestinal barrier integrity may represent a key mechanism underlying the observed physiological benefits of *C. alimentarius* CNTA 209, including protection against the development of fatty liver.

Conclusions

In conclusion, our study provides the first evidence of a *C. alimentarius* strain, CNTA 209, exhibiting probiotic properties *in vitro*. These include sensitivity to all tested antibiotics, resistance to simulated gastric and intestinal conditions, β-galactosidase activity, and the ability to produce SCFAs. Supplementation with this probiotic (1×10^9 CFU per day per animal) significantly reduced white adiposity in obese C57BL/6 mice by promoting adipose tissue browning, as indicated by the upregulation of key thermogenic genes in WAT and BAT, such as *Ppargc1a*, *Prdm16*, and *Ucp1*. Moreover, *C. alimentarius* CNTA 209 ameliorated liver steatosis and oxidative stress induced by a HFS diet. Metagenomic analysis revealed a beneficial modulation of gut microbiota composition, characterized by a reduction in obesity-associated taxa and improved intestinal barrier function. Importantly, a toxicological evaluation conducted in both male and female Wistar rats confirmed the safety of the administered dose (1×10^9 CFU per animal per day), supporting the protective role of *C. alimentarius* in maintaining intestinal barrier integrity. Overall, this study presents the first scientific evidence identifying a *C. alimentarius* strain as a promising probiotic candidate for the management of obesity-related conditions, including chronic inflammation and fatty liver disease.

Author contributions

Conceptualization: R. V., L. V.-V., F. M. and P. A.; methodology: *in vitro* characterization of the probiotic properties: L. V.-V., N. L.-G. and A. S.-V.; *in vitro* cellular experiments: A. S.-V. and L. V.-V.; functional evaluation in C56BL6 mice: I. G., M. L.-Y., N. S. and P. A.; toxicology: A. G.; validation: R. V., L. V.-V., A. G., P. A. and F. M.; formal analysis: A. S.-V., L. V.-V., I. G., A. G., and P. A.; investigation: R. V., L. V.-V., N. L.-G., A. S.-V., I. G., M. L.-Y., N. S., A. G., P. A. and F. M.; resources: R. V., L. V.-V., F. M. and P. A.; writing – original draft preparation: L. V.-V., I. G., and P. A.; writing – review and editing: R. V., L. V.-V., A. S.-V., N. L.-G., M. L.-Y., F. M., I. G., and P. A.; project administration: R. V., L. V.-V., F. M., and P. A.; funding acquisition: R. V., L. V.-V., and P. A. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Companilactobacillus alimentarius CNTA 209 is deposited at the "Colección Española de Cultivos Tipo (CECT)" under accession number CECT 30970, and its potential uses are registered in the patent EP25382189.6, entitled: COMPANILACTOBACILLUS ALIMENTARIUS STRAIN AND USES THEREOF. I. G., L. V.-V., R. V., F. I. M. and P. A. are authors of this patent.

Data availability

All data are presented in the manuscript and its supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5fo04242a.

Moreover, additional data will be made available on request.

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