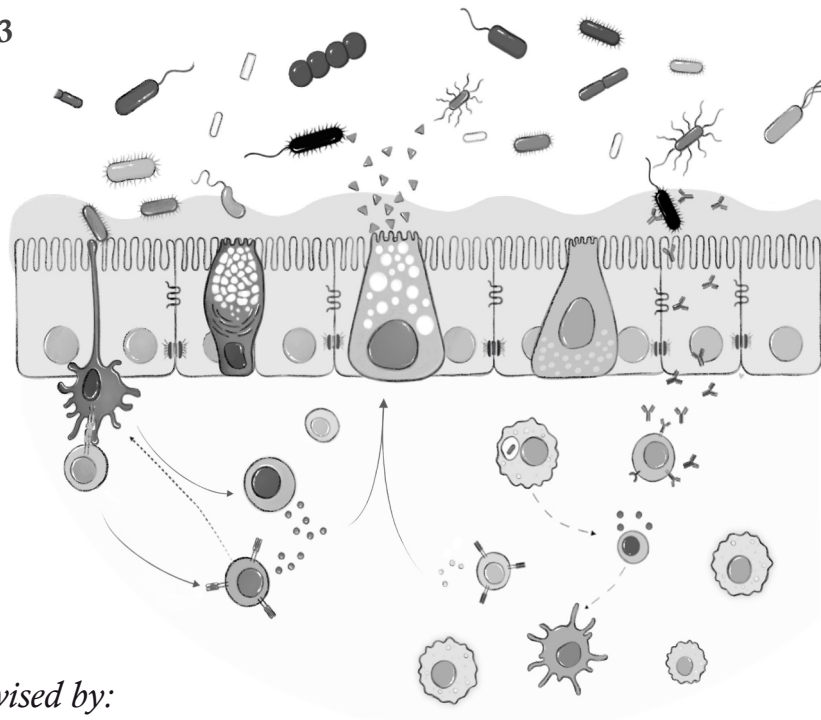


Effect and mechanisms of action of intestinal bacteria and bioactive compounds on the immune system and metabolism in obesity models

Rebeca Liébana García

Valencia, October 2023



Doctoral Thesis supervised by:
Dr. Marta Olivares Sevilla
Dr. Yolanda Sanz Herranz



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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Doctoral Thesis presented by:

Rebeca Liébana García

To the Biotechnology Department of the Polytechnical
University of Valencia

**Effect and mechanisms of action of intestinal bacteria and
bioactive compounds on the immune system and
metabolism in obesity models**

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Las Doctoras Marta Olivares Sevilla (Investigadora postdoctoral del CSIC) y Yolanda Sanz Herranz (Profesora de Investigación del CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA) de Valencia

CERTIFICAN:

Que la Graduada en Biología, **Rebeca Liébana García**, ha realizado bajo su dirección el trabajo con título "*Effect and mechanisms of action of intestinal bacteria and bioactive compounds on the immune system and metabolism in obesity models*", y autorizan su presentación para optar al grado de Doctor.

Y para que así conste, expiden y firman el presente certificado en Valencia, octubre de 2023.

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“Se ha hecho lo que se ha podido...”

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ABSTRACT

Obesity is a major public health challenge due to its high prevalence, and association with metabolic comorbidities, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disorders. Modifiable environmental factors and life style are major contributors to obesity making this disorder largely preventable and reversible. However, the unstoppable rise in obesity incidence highlight the need for more effective management measures. Hypercaloric diets are known to overactivate the intestinal immune system and disrupt the microbiome, ultimately causing detrimental metabolic effects. The loss of intestinal immune homeostasis is considered an early step preceding the development of systemic low-grade inflammation associated with obesity and metabolic complications. In this regard, extensive evidence supports that the gut microbiome may be modified favorable and, thus, help to ameliorate these conditions. Hence, identifying factors triggering the low-grade inflammation and microbiome-base solutions to reduce the obesity burden represent promising avenues of research.

This Doctoral Thesis aims to advance the knowledge and provide novel probiotics and dietary strategies to combat the burden of obesity based on their immunomodulatory properties to shape the metabolic response to the diet.

In the First Chapter, we have investigated the anti-obesogenic potential of propyl propane thiosulfinate (PTS), an organo-sulfur compound derived from *Allium* species, at two different doses (0.1 or 1 mg/kg/day) using a murine model of diet-induced obesity (DIO). Our preclinical findings showed the protective effects of PTS against obesity, reducing body weight gain and maintaining glucose homeostasis, thus suggesting its potential to ameliorate the impact of obesogenic diets. In the adipose tissue and the liver, PTS was able to prevent the increased inflammation and the aberrant lipid metabolism caused by the obesogenic diet. Moreover, PTS promoted thermogenic activity in the brown adipose tissue by increasing fatty acid oxidation markers (*Ppara*, *Cpt1a*, *Prdm16*) and mitochondrial activity. In the intestine, the highest dose of PTS normalized the IL-22 levels and gut barrier function markers (*Cldn3*, *Muc2*, *Reg3g*, *DefA*) altered by the obesogenic diet. In view of the modest changes in the microbial ecosystem, we concluded that the effects of PTS were not notably mediated by the gut microbiota.

In the Second Chapter, we have evaluated the anti-obesogenic potential and the mechanism of action of the new intestinal strain, *Phascolarctobacterium faecium* DSM 32890, isolated in our laboratory from a healthy volunteer. To that aim, we have performed different *in vitro* and *in vivo* experiments, including the use of different types of cell cultures (bone marrow-derived macrophages and group 1 of innate lymphoid cells (ILC1s)) and three DIO murine models (wild-type

C57BL/6J, *Rag1*^{-/-} mice and wild-type mice treated with the GW2580 inhibitor to block the polarization towards anti-inflammatory M2 macrophages). When administered to mice, we observed that *P. faecium* improved the glucose tolerance and reduced adiposity and body weight gain, regardless of its viability. We attributed these benefits to the immunomodulatory properties of the bacterium. Specifically, the bacterium increased the intestinal M2 macrophages, which in turn counteracted the obesity-induced increase in pro-inflammatory ILC1s. Accordingly, the metabolic benefits of *P. faecium* were cancelled when co-administered with a M2 inhibitor. Moreover, *P. faecium* reinforced the gut barrier function through the raise of secretory IgA and the expression of antimicrobial peptides genes (*Reg3g*, *Pla2g2a*) and IL-22, ultimately preventing diet-derived systemic inflammation. Additionally, a large metagenomic study including 6,361 human subjects was performed and found a higher abundance of *P. faecium* in non-obese healthy adults regardless of nationality, sex or age, suggesting that this bacterial species is associated with metabolic health safeguard.

In the Third Chapter, we have investigated the involvement of gut-resident ILC1s in obesity progression and metabolic disruption. To address this goal, we evaluated longitudinally, in a DIO murine model, the ILC1s response to an obesogenic diet and the consequences of the ILC1s depletion. In the intestine, ILC1s depletion blunted the increases in M1 macrophages and ILC2s. Additionally, ILC1s depletion promoted the ILC3-IL22 pathway, increasing mucin production, the expression of antimicrobial gut peptides (*Reg3g*, *Pla2g2a*), and the number of neuroendocrine cells. Moreover, ILC1s depletion restored microbial and metabolomic profiles, resembling those associated with a healthy symbiotic state. The improvements in gut homeostasis were linked to a higher gut hormone secretion, and reduced insulinemia and adiposity.

In summary, in this Doctoral Thesis we have evaluated the interest of two novel approaches for obesity management in preclinical models that, if confirmed in humans, might constitute new adjuvant solutions for obesity. The organo-sulfur compound PTS protects from obesity by reducing inflammation and increasing thermogenesis. Whereas *P. faecium* is a potential next generation probiotic with interesting immunomodulatory effects on M2 macrophages the are key mediators of its anti-inflammatory effects on obesity. Additionally, we have provided new knowledge demonstrating the role that gut-resident ILC1s play in obesity as an upstream activator of inflammatory signals derived from obesogenic diets.

RESUMEN

La obesidad representa un importante reto para la salud pública debido a su elevada prevalencia y a sus comorbilidades, como la diabetes tipo 2 (T2D, por sus siglas en inglés), la enfermedad del hígado graso no alcohólico (NAFLD, por sus siglas en inglés) y patologías cardiovasculares. Los factores ambientales y el estilo de vida contribuyen significativamente a la obesidad, siendo en gran medida prevenible y reversible. Sin embargo, el imparable aumento de su incidencia evidencia la necesidad de medidas más eficaces para el manejo de la obesidad. Las dietas hipercalóricas activan el sistema inmunitario intestinal y alteran la microbiota intestinal causando, en última instancia, daños metabólicos en el organismo. De hecho, la pérdida de homeostasis inmunológica a nivel intestinal se considera un evento que precede a la aparición de la inflamación sistémica de bajo grado asociada a la obesidad y sus complicaciones metabólicas. Dado que la microbiota intestinal es un factor modificable, por ejemplo, con intervenciones dietéticas, su modulación puede convertirse en una oportunidad para reducir el impacto de la obesidad. Es por ello que la identificación de los factores que participan en la respuesta inflamatoria a las dietas obesogénicas y la búsqueda de alternativas terapéuticas basadas en la microbiota constituyen una vía de investigación prometedora para combatir la obesidad.

Esta Tesis Doctoral pretende evaluar el potencial de nuevos probióticos y estrategias dietéticas para combatir la obesidad basadas en sus propiedades inmunomoduladoras.

En el Primer Capítulo hemos investigado el potencial anti-obesogénico del propil propano tiosulfonato (PTS), un compuesto organosulfurado derivado de la especie *Allium*, en dos dosis diferentes (0,1 o 1 mg/kg/día) utilizando un modelo murino de obesidad inducida por la dieta. Nuestros hallazgos demostraron los efectos protectores de PTS frente a la obesidad, ya que su administración redujo el peso corporal y mejoró la homeostasis de glucosa, lo que sugiere su potencial para reducir los efectos adversos de las dietas hipercalóricas. En el tejido adiposo y el hígado, PTS previno el aumento de la respuesta inflamatoria y las alteraciones del metabolismo lipídico causadas por la dieta hipercalórica. Además, PTS incrementó la actividad termogénica en el tejido adiposo marrón aumentando los marcadores de oxidación de ácidos grasos (*Ppara*, *Cpt1a*, *Prdm16*) y la actividad mitocondrial. Por último, a nivel intestinal, la dosis más alta de PTS normalizó los niveles de IL-22 y de los marcadores de la función barrera intestinal (*Cldn3*, *Muc2*, *Reg3g*, *DefA*) alterados por la dieta. Sin embargo, los cambios modestos en la composición de la microbiota intestinal indicarían que los efectos observados no estaban mediados de forma significativa por la microbiota.

En el Segundo Capítulo hemos evaluado el potencial anti-obesogénico y el mecanismo de acción de una nueva bacteria llamada *Phascolarctobacterium faecium* DSM 32890, aislada de un voluntario sano en nuestro laboratorio. Para ello, hemos realizado diferentes experimentos *in vitro* e *in vivo* usando diferentes cultivos celulares (macrófagos derivados de médula ósea y de células linfoides innatas intestinales del grupo 1 (LC1s)) y tres modelos murinos de obesidad inducida por la dieta (ratones C57BL/6J, ratones *Rag1*^{-/-} y ratones tratados con un inhibidor para evitar la polarización de macrófagos M2). Cuando administramos esta bacteria a ratones alimentados con una dieta hipercalórica, observamos que *P. faecium* mejoraba la tolerancia a la glucosa, y reducía la adiposidad y el aumento de peso corporal independientemente de su viabilidad. Atribuimos estos beneficios a las propiedades inmunomoduladoras de *P. faecium*. Concretamente, a su capacidad para incrementar la proporción de los macrófagos M2 en el intestino que, a su vez, contribuían a inhibir el aumento de ILC1 pro-inflamatorias causado por la dieta hipercalórica. En este sentido, los efectos beneficiosos de *P. faecium* se anularon cuando se coadministró con un inhibidor de la polarización de macrófagos M2. Además, *P. faecium* reforzó la función barrera intestinal mediante el aumento de la IgA, la expresión de péptidos antimicrobianos (*Reg3g*, *Pla2g2a*) y la citoquina IL-22 evitando, en última instancia, la inflamación sistémica causada por la dieta hipercalórica. Por último, realizamos un amplio estudio con datos metagenómicos de 6.361 personas que mostró una relación inversa entre *P. faecium* y la obesidad, independientemente de la nacionalidad, el sexo o la edad, lo que sugiere la asociación de esta bacteria con la salud metabólica.

En el Tercer Capítulo, hemos investigado la implicación de las ILC1 residentes en el intestino en la progresión de la obesidad y las alteraciones metabólicas asociadas. Para ello, hemos evaluado longitudinalmente la respuesta de las ILC1s a una dieta obesogénica y las consecuencias de la depleción de ILC1s en un modelo murino de obesidad. En el intestino, el bloqueo de ILC1s evitó el aumento de macrófagos M1 e ILC2s. Además, la depleción de ILC1s promovió la activación de la vía ILC3-IL22, aumentando la producción de mucina, la expresión de péptidos antimicrobianos (*Reg3g*, *Pla2g2a*) y el número de células neuroendocrinas. Además, el bloqueo de ILC1s restableció el perfil de la microbiota y el metaboloma, acercándose al perfil asociado con la salud metabólica. En última instancia, estas mejoras en la homeostasis intestinal se asociaron con una mayor secreción de hormonas intestinales, y una reducción de la insulinemia y la adiposidad.

En resumen, en esta Tesis Doctoral hemos evaluado el interés de dos nuevas estrategias para el manejo de la obesidad en modelos preclínicos que, de confirmarse en humanos, podrían constituir nuevas soluciones para tratar la obesidad. Por un lado, hemos visto que el compuesto organosulfurado PTS protege frente a la obesidad mediante una reducción en la inflamación y la inducción de la actividad termogénica. Por su parte, *P. faecium* puede

considerarse un potencial probiótico de nueva generación con efectos protectores sobre la obesidad a través de la modulación de los macrófagos M2, que son claves mediadores del efecto anti-inflamatorio que ejerce en la obesidad. Además, hemos aportado nuevo conocimiento sobre la función que las ILC1 residentes en el intestino desempeñan en la obesidad como activadoras de las señales inflamatorias que desencadenan las dietas hipercalóricas.

RESUM

L'obesitat representa un repte important per a la salut pública a causa de la seua elevada prevalença i a les seues comorbiditats, com la diabetis tipus 2 (T2D, per les seues sigles en anglés), la malaltia del fetge gras no alcohòlic (NAFLD, per les seues sigles en anglés) i les patologies cardiovasculars. Els factors ambientals i l'estil de vida contribueixen de manera significativa a l'obesitat, per la qual cosa aquesta és en gran mesura prevenible i reversible. No obstant, l'imparable augment de la incidència de l'obesitat evidencia la necessitat de mesures més eficaces per al maneig d'aquesta patologia. Les dietes hipercalòriques activen el sistema immunitari intestinal i alteren la microbiota intestinal causant, en última instància, danys metabòlics en l'organisme. De fet, la pèrdua d'homeòstasi immunològica a escala intestinal es considera un esdeveniment primerenc que precedeix l'aparició de la inflamació sistèmica de baix grau associada a l'obesitat i les seues complicacions metabòliques. Atés que la microbiota intestinal és un factor modificable, per exemple, amb intervencions dietètiques, la seua modulació pot convertir-se en una oportunitat per a reduir l'impacte de l'obesitat. És per això que la identificació dels factors que participen en la resposta inflamatòria a les dietes obesogèniques i la recerca d'alternatives terapèutiques basades en la microbiota constitueixen una via d'investigació prometedora per a combatre l'obesitat.

Aquesta Tesi Doctoral pretén avaluar el potencial de nous probiòtics i estratègies dietètiques per a combatre l'obesitat basada en les seues propietats immunomoduladores.

En el Primer Capítol hem investigat el potencial anti-obesogènic del propil propà tiosulfat (PTS), un compost organosulfurat derivat de l'espècie *Allium*, en dues dosis diferents (0,1 o 1 mg/kg/dia) utilitzant un model murí d'obesitat induïda per la dieta. Els nostres resultats han demostrat els efectes protectors de PTS enfront a l'obesitat, ja que la seua administració va reduir el pes corporal i va millorar l'homeòstasi de glucosa, la qual cosa suggereix el seu potencial per a reduir els efectes adversos de les dietes hipercalòriques. En el teixit adipós i el fetge, PTS va prevenir l'augment de la resposta inflamatòria i les alteracions del metabolisme lipídic causades per la dieta hipercalòrica. A més, PTS va incrementar l'activitat termogènica en el teixit adipós marró augmentant els marcadors d'oxidació d'àcids grassos (*Ppara*, *Cpt1a*, *Prdm16*) i l'activitat mitocondrial. Finalment, a escala intestinal, la dosi més alta de PTS va normalitzar els nivells d'IL-22 i dels marcadors de la funció barrera intestinal (*Cldn3*, *Muc2*, *Reg3g*, *DefA*) alterats per la dieta. No obstant, observem canvis modestos en la microbiota intestinal, concloent que els efectes observats no estan mediatos de manera significativa per la microbiota.

En el Segon Capítol hem avaluat el potencial anti-obesogènic i el mecanisme d'acció d'un nou bacteri anomenat *Phascolarctobacterium faecium* DSM 32890,

aïllat d'un voluntari sa en el nostre laboratori. Amb aquest objectiu, hem realitzat diferents experiments *in vitro* i *in vivo* utilitzant diferents cultius cel·lulars (macròfags derivats de medul·la òssia i de cèl·lules limfoides innates intestinals del grup 1 (LC1s) i tres models murins d'obesitat induïda per la dieta (ratolins C57BL/6J, ratolins *Rag1*^{-/-} i ratolins tractats amb un inhibidor per a evitar la polarització de macròfags M2). Quan administrarem aquest bacteri a ratolins que consumien una dieta hipercalòrica, observarem que *P. faecium* millorava la tolerància a la glucosa, i reduïa l'adipositat i l'augment de pes corporal independentment de la seua viabilitat. Atribuïrem aquests beneficis a les propietats immunomoduladores de *P. Faecium*, concretament a la seua capacitat per a incrementar la proporció dels macròfags M2 en l'intestí que, al seu torn, contribuïen a inhibir l'augment d'ILC1 proinflamatòries causat per la dieta hipercalòrica. En aquest sentit, els efectes beneficiosos de *P. faecium* es van anul·lar quan es va coadministrar amb un inhibidor de la polarització de macròfags M2. A més, *P. faecium* va reforçar la funció barrera intestinal mitjançant l'augment de l'IgA, l'expressió de pèptids antimicrobians (*Reg3g*, *Pla2g2a*) i la citocina IL-22, evitant, en última instància, la inflamació sistèmica causada per la dieta hipercalòrica. Finalment, realitzarem un ampli estudi amb dades metagenòmiques de 6.361 persones que va mostrar una relació inversa entre *P. faecium* i l'obesitat, independentment de la nacionalitat, el sexe o l'edat, la qual cosa suggereix l'associació d'aquest bacteri amb la salut metabòlica.

En el Tercer Capítol, investiguem la implicació de les ILC1 residents en l'intestí en la progressió de l'obesitat i les alteracions metabòliques associades. Avaluem longitudinalment la resposta de les ILC1s i les conseqüències de la depleció de ILC1s en un model murí d'obesitat. A l'intestí, el bloqueig de ILC1s va evitar l'augment de macròfags M1 i ILC2s. A més, la depleció de ILC1s va promoure l'activació de la via ILC3-IL22, augmentant la producció de mucina, l'expressió de pèptids antimicrobians (*Reg3g*, *Pla2g2a*) i el nombre de cèl·lules neuroendocrines. El bloqueig de ILC1s també va restablir el perfil de la microbiota i el seu metaboloma, similar a l'estat saludable. En última instància, aquestes millores en l'homeòstasi intestinal es van associar amb una major secreció d'hormones intestinals, i una reducció de la insulínemia i l'adipositat.

En resum, en aquesta Tesi Doctoral hem avaluat l'interés de dues noves estratègies per al maneig de l'obesitat en models preclínics que, en cas de confirmar-se en humans, podrien constituir noves solucions per al maneig de l'obesitat. Hem demostrat que el compost organosulfurat PTS protegeix de l'obesitat mitjançant una reducció en la inflamació i estimula l'activitat termogènica. A més, *P. faecium* pot considerar-se un potencial probiòtic de nova generació amb efectes protectors sobre l'obesitat a través de la modulació dels macròfags M2, que són mediadors claus de l'efecte antiinflamatori que exerceix el bacteri en l'obesitat. Finalment, hem aportat nou coneixement sobre la funció que les ILC1 residents de l'intestí exerceixen en l'obesitat com a activadores dels senyals inflamatoris que desencadenen les dietes hipercalòriques.

INTRODUCTION

1. OBESITY AND ASSOCIATED METABOLIC DISORDERS

DEFINITION AND PREVALENCE

Obesity is one of the most common preventable disorders, and it is defined as excessive fat accumulation, mainly resulting from a maintained positive energy balance, that represents a risk to health ¹. The body mass index (BMI) is an anthropometric measure used as first approach for the categorization of overweight and obesity, based on the weight and height of a person. In adults, overweight and obesity are defined by a BMI equal to or greater than 25 Kg/m² and 30 Kg/m², respectively ². However, the BMI index does not reflect the body composition, and the same BMI value may not correspond to the same degree of fatness among individuals ². For this reason, it is recommended the use of additional anthropometric measurements (such as the waist circumference) or tools such as bioelectric impedance and dual-energy X-ray absorptiometry which assesses total body fat composition ^{3,4}.

According to the World Health Organization (WHO), the prevalence of obesity has nearly tripled since 1975s, with more than 650 million adults and 340 million children and adolescents affected ⁵. The highest prevalence rates of obesity are found in high-income countries, traditionally adhered to unhealthy dietary habits and sedentary lifestyles. However, nowadays obesity prevalence is also rapidly increasing in low-income countries, where it coexists with undernutrition (**Figure 1**). Justified by its high prevalence rates, fighting obesity is one of the priorities of the 2030 Agenda for Sustainable Development adopted by the United Nations Member States ⁶.

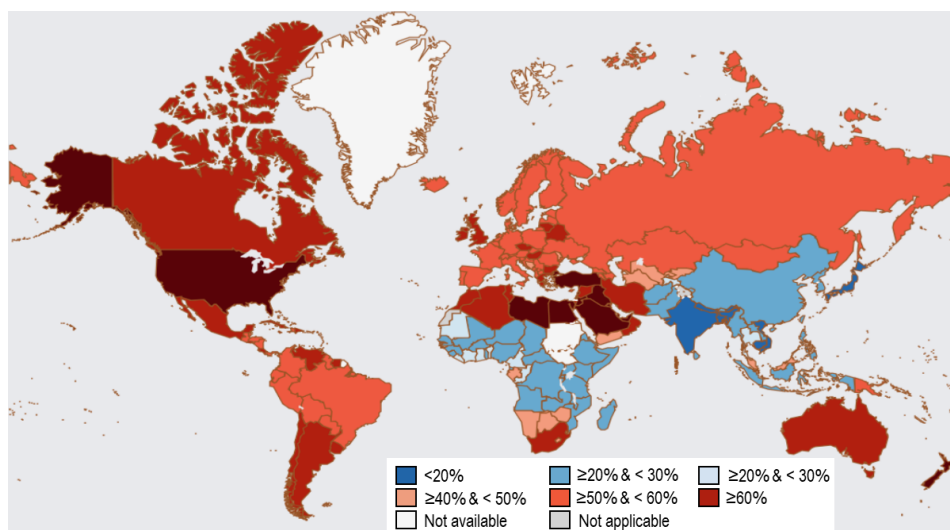


Figure 1. World overweight in adults over the age of 18 in 2016. Source: Worldwide organization. Department of non-communicable diseases. <https://ncdportal.org/>

PATHOPHYSIOLOGY OF OBESITY AND ASSOCIATED METABOLIC DISORDERS

Obesity is a complex multifactorial condition triggered by a combination of multiple interconnected genetic, environmental, and lifestyle factors. The rapid increase in obesity incidence over the past decades is mainly explained by environmental and lifestyle changes, including the excessive consumption of high caloric foods and sedentary lifestyle, rather than by genetic mutations ^{7,8}. Importantly, obese subjects show a greater risk of developing many non-communicable diseases, such as type 2 diabetes (T2D), cardiovascular disease, fatty liver, dyslipidemia, some forms of cancer, and chronic kidney disease ⁹. Additionally, the stigma associated with obesity reduces the self-confidence of the person and, sometimes, causes discrimination in the social and professional environment ¹⁰. Therefore, this stigma not only negatively impacts the physical health of the affected subjects, but also mental health and social integration ¹⁰. All these reasons justify the consideration of obesity as one of the major health and social burden of the 21st century.

The pathophysiology of obesity involves complex metabolic and molecular processes, which are not yet fully understood ¹¹. The accumulation of excessive fat in the adipose tissue (AT) is considered the main trigger of obesity complications ¹¹. Indeed, excess fat deposition in the visceral region (the so-called abdominal obesity) is consistently related to greater cardiometabolic risk ¹¹. The AT, besides serving for energy storage, acts as an active endocrine organ that contributes to the regulation of energy balance, glucose homeostasis, lipid metabolism and immunity ¹². The uncontrolled enlargement of the adipocytes, resulting from malnutrition by excess, instigates aberrant AT functioning. This includes uneven distribution of lipid mobilization and storage pathways, increases of insulin-resistant adipocytes, dysregulation of adipokine production, and recruitment of pro-inflammatory cells, that together contribute to the worsening of obesity and the associated metabolic disorders ^{12,13}. In this sense, research continues widening our understanding of the molecular basis of obesity. One of the most important mechanisms contributing to metabolic dysfunction and obesity development is the alteration of immunity. Indeed, inflammation is directly link to the aberrant functioning of metabolic-active organs ¹⁴. For instance, macrophage infiltration at the AT promotes lipolysis pathways through the secretion of cytokines such interleukin (IL)-6 ¹⁵. Similarly, hepatic inflammation is associated with increased lipid accumulation (known as ectopic fat deposition) and impaired glucose tolerance ^{16,17}.

Several authors have suggested that metabolically active organs, specially the AT, are major instigators of the chronic systemic inflammation linked to obesity ¹⁸. In the AT and liver, an increased nutrient influx, like occurs in obesity, causes stress in the endoplasmic reticulum, promoting the production of reactive oxygen and nitrogen species (ROS and RNS), ultimately activating pro-inflammatory

pathways and increasing oxidative stress ^{19,20}. Also, high levels of circulating glucose and free fatty acid from the diet can activate inflammasomes, toll-like receptors (TLRs) and thus, NF-kB mediated pathways that end in the release of pro-inflammatory cytokines (e.g., and tumor necrosis factor-alpha (TNF α), producing interferon-gamma (INF γ), IL-6, IL-1, monocyte chemoattractant protein (MCP)-1, etc) ^{14,21}. Additionally, the uncontrolled upregulation of adipokines, such as resistin and leptin, secreted by the AT in obesity can contribute to the induction of pro-inflammatory pathways and insulin resistance (IR) systemically ^{22,23}. In summary, obesity courses with an increased in the inflammatory tone that takes part in the development of some of the metabolic features of obesity.

Newly, some authors point to the intestine as the starting point of these inflammatory cascade triggered by unhealthy diets that ends in systemic inflammation and organ dysfunction ^{24–27}. As a matter of fact, the intestine is the first barrier of defense against dietary insults, harmful antigens and pathogens. Continued exposure to obesogenic diets impacts on the gut microbiota and the barrier function, impairing the gut defensive role ^{24,28}. The production of mucus and antimicrobial peptides (AMPs) by Goblet and Paneth cells are among the primary mechanisms involved in gut homeostasis, both impaired by high-fat diets (HFD) ²⁹. Additionally, obesogenic diets promote degradation and switch of epithelial tight junctions proteins like Claudins and Occludins ³⁰. Thus, this intestinal damage facilitates the penetrability and leakage of intestinal luminal components, like lipopolysaccharides (LPS), from the gut to the circulation, unleashing the pro-inflammatory tone in obesity ^{31,32}. Such pro-inflammatory signals can then target organs, like the liver, promoting inflammation and aberrant functioning ²⁷. Obesogenic diets also deeply compromises the functioning of the intestinal immune system as described hereinafter in the next section. In short, generally high-caloric diets lead to a continued and maintained increased infiltration of immune cells, such as macrophages and T cells into the intestinal mucosa ²⁴. Moreover, the consumption of obesogenic diets impairs the functioning of the enteroendocrine system partly due to the induced intestinal inflammation. For instance, in obesity there is a disruption in the production of intestinal hormones like gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1), commonly known as incretins ³³. These intestinal hormones are of a great interest, not only for their well-known function in regulating glucose homeostasis, but also because of their contribution to pro- and anti-inflammatory processes (respectively) ^{34,35}, barrier function enhancement (in the case of GLP-1 and GLP-2) ³⁵, and fat accumulation (in the case of GIP) ³⁶.

Despite scientific advances, it remains challenging to determine whether is the excessive fat storage or the impairment of the intestinal homeostasis the primary instigator of the obesity-associated systemic inflammation and metabolic dysfunction, and to what extend these factors act in a mutually reinforcing manner. Still today, the identification of the critical factor orchestrating these pro-

inflammatory cascade of events remains elusive. In this sense, evidence suggest that the gut microbiota plays a significant influence being a key player in obesity pathophysiology^{37,38}. In fact, prospective studies have shown that changes in the gut microbiota occurring on early-life might persist into maturity and contribute to a higher obesity risk in the adulthood^{24,39}. The gut microbiota strongly impacts on many of the obesity features (e.g., the inflammatory state, the intestinal barrier disruption, and the altered endocrine response to nutrients)^{40,41}. Studies like the ones performed within this Doctoral Thesis aim to expand our knowledge regarding the potential of the gut microbiota to counteract obesity through the modulation of immune response to hypercaloric diets.

CURRENT TREATMENTS AND LIMITATIONS

The global prevalence of obesity has sparked the urgency to find and implement new solutions to combat obesity. Indeed, the unstoppable rise in the incidence of obesity and overweight highlights the paucity of efficient treatments and preventative strategies⁴².

Interventions based on the promotion of healthier lifestyle, concerning diet and physical activity, are the only approach to prevent weight gain or achieve weight loss without compromising safety⁷. However, the adherence to lifestyle changes is usually sub-optimal and, consequently, their efficacy is low in the long-term. There are also some pharmacology treatments to promote satiety, such as the liraglutide (a GLP-1 analog), or to inhibit dietary fat absorption, like the orlistat (a lipase inhibitor). These drugs are normally prescribed along with lifestyle modifications and cause significant improvements, but unfortunately, their chronic use is often associated with side effects⁴³. There are also invasive strategies, such as bariatric surgery or the insertion of an intragastric balloon (IGB). Bariatric surgery is a widely effective treatment but only recommended to individuals with BMI equal or greater than 35 Kg/m²⁴⁴, and not exempted of risks and complications⁷. Conversely, IGB represents a safer alternative for individuals ineligible for bariatric surgery. However, a meta-analysis concluded that IGB does not show convincing evidence of weight loss improvement compared to traditional weight management⁴⁵.

Considering the significant contribution of inflammation to obesity complications, anti-inflammatory strategies have been suggested as promising alternatives^{46,47}. In this sense, natural immunomodulators compounds, such as some of the bioactive compounds found in vegetables, represent new options given their favorable effects with minimal side-effects^{48,49}. Also, the modulation of the gut microbiota is considered a promising tool to curb obesity development^{50,51}. Indeed, the microbes colonizing human intestine are potent immunomodulators of the immune response and inflammation^{29,50}. The horizon of the use of these novel immunomodulatory solutions in the context of obesity is discussed in detail in the third section.

2. THE IMMUNE SYSTEM AND ITS ROLE IN OBESITY AND METABOLIC DISORDERS

OVERVIEW OF THE IMMUNE SYSTEM AND ITS FUNCTIONS

The immune system is an intricate and interconnected net of cells, tissues, and organs that work together as an army against threats to ensure the internal homeostasis. The immune system is divided into two main branches, the innate and the adaptive ⁵². The innate immune system acts as a steadfast sentinel, providing immediate, non-specific protection against pathogens and other threats. It relies on the recognition of conserved microbe-associated molecular patterns (MAMPs), such as LPS or flagellin, and damage-associated molecules (DAMPs). The innate immunity is composed of several host defense molecules, such as AMPs and mucins, and immune cells, like macrophages, neutrophils, dendritic cells (DCs), and the innate lymphoid cells (ILCs) ⁵³. Likewise, the adaptive immunity provides a targeted, highly specific and long-lasting response. It is defined by the ability to recognize and react to particular antigens that have previously encountered and introduced by antigen-presenting cells. The adaptive immunity is mainly composed by lymphocytes B and T, that once activated, generate memory cells providing protection against future re-infections or threats ⁵⁴.

In this Doctoral Thesis, we have focused on the role of innate immunity in obesity, particularly in macrophages and ILC1s. ILCs are tissue-resident cells described as the innate counterparts of T lymphocytes that lack of rearranged antigen receptors despite their lymphoid morphology. ILCs are found in most tissues throughout the body (e.g. intestine, adipose tissue, lungs), being particularly abundant in mucosal surfaces ⁵⁵. However, their prevalence is extremely low comprehending $\leq 1\%$ of lymphocytes ⁵⁵. The nomenclature recently revised establishes five subsets: natural killer (NKs) cells, ILC1s, ILC2s, ILC3s and lymphoid tissue-inducer (LTi) cells ⁵⁶. However, immunologists commonly gather them into three main groups: group 1 (comprising ILC1s and NKs cells), group 2 (ILC2s), and group 3 (comprising ILC3s and lymphoid tissue-inducer (LTi) cells). This classification is based on their transcription factors (T-bet, GATA3, and ROR γ t, respectively), and the effector cytokines (**Figure 2**). ILC1 group participates in type 1 immune responses, producing IFN γ and TNF α , thus promoting pro-inflammatory responses to fight infections and clear pathogens and host dying cells. ILC3 group produces IL-17 and IL-22 (type 3 immune response) contributing to the maintenance of intestinal barrier function and the protection against extracellular pathogens ⁵⁶. ILC2s produce type 2 cytokines, such as IL-5 and IL-13, important for the defense against helminths and allergens, and for the regulation of tissue repair and metabolism ⁵⁶. It is worth mention that these immune cells show great plasticity which enables them to polarize and respond to changing environmental cues. For instance, under the influence of IL-

18 and IL-12, ILC3 can shift into ILC1-like cells, whereas IL-23 and IL-1 β can make ILC1s to become ILC3-like cells ^{57,58}.

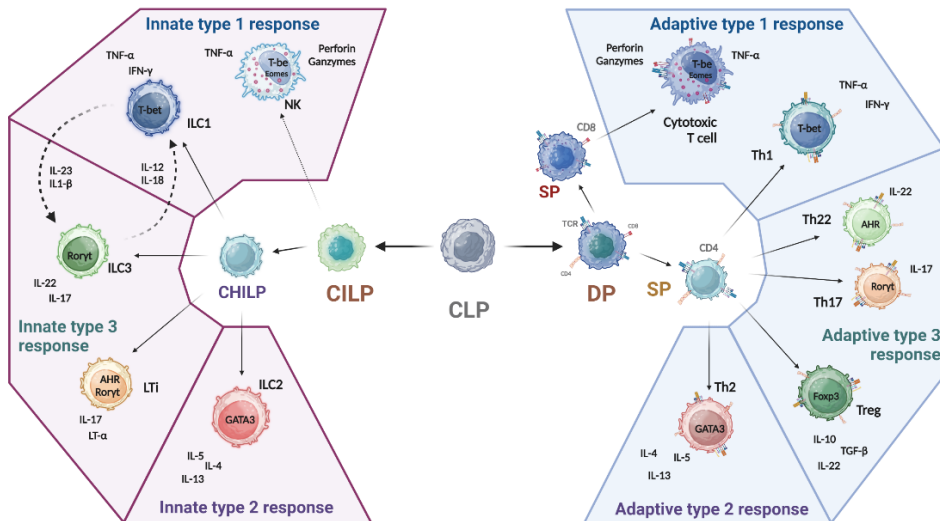


Figure 2. Schematic representation of the innate lymphoid cells (ILCs) and adaptive T-lymphocytes. This figure illustrates the similarities and differences between ILCs and T cells, two important cell types involved in immune responses belonging to complementary branches of the immune system. Both ILCs and T cells originate from a common precursor known as common lymphoid progenitor (CLP) in the bone marrow. Subsequently, CLPs give rise to two distinct lineages: the early T cell progenitor (non-represented) and the common ILC progenitor (CILCP). ILCs arise from an early common innate lymphoid progenitor (CILP) which further differentiate into a common helper-like ILC progenitor (CHILPs) giving rise to the different ILCs subsets upon cytokine stimulation. While T cells originates from the CD4⁺CD8⁺ double positive progenitor (DP) which differentiate into a CD4⁺ or CD8⁺ single positive (SP) progenitor. The development of different T cell and ILCs is regulated in a mirror fashion by specific master transcription factors, namely T-bet, GATA3, and RORγt. Each subtype exhibits a unique cytokine signature participating in a different type of immune response. Created with BioRender.com (Agreement number: WS25OA9R4W).

Macrophages are highly plastic cells presenting a wide functional diversity vital for the maintenance of tissue homeostasis ⁵⁹. Macrophages are formed mainly from monocytes originated in the bone marrow ⁵⁹. Therefore, in contrast to ILCs, these cells migrate through the blood vessels, as monocytes, towards sites of infection or damaged. Besides, there are also tissue-resident macrophage populations embryonically or monocyte-derived ⁶⁰. The functionality of macrophages is commonly resumed in two opposite phenotypes: M1 and M2. M1 macrophages present a pro-inflammatory phenotype highly cytotoxic, expressing markers like CD80/86, CD14 and inducible nitric oxide synthase (iNOs), and secreting pro-inflammatory cytokines and chemokines such, IL-6, MCP-1, IL-12 or TNF α , to fight and eliminate potential threats ⁶¹. Alternative activated or M2 are characterized by an anti-inflammatory phenotype marked by the expression of CD163, CD206, CD36 and arginase-1 (Arg-1) and the secretion of cytokines such

as IL-10, IL-4 or TGF- β , thus contributing to tissue repair, and tolerogenic responses ⁶¹. However, *in vivo* the polarization of macrophages is complex existing a continuum spectrum of macrophage polarization in between ⁶¹.

Generally, the immune system has several key functions, including the recognition and elimination of pathogens and abnormal cells, regulation of immune responses to prevent self-host damage, and maintenance of tissue integrity and repair ⁵². The intestine is considered as the largest immune organ of the body, and relies heavily on the immune system given its constant exposure to a vast and diverse array of microorganisms and other external threats. The intestinal immune system is composed by immunocompetent cells located along the intestinal epithelial surface and the lamina propria which work in cooperation with intestinal epithelial cells (IECs). In fact, IECs are now considered important components of innate immune system ⁶². IECs, as ordinary immune cells, present a variety of receptors known as pattern recognition receptors (PRRs), such as TLRs, or nucleotide-binding oligomerization domain-like receptors (NLRs), which sense MAMPs, contributing to the detection of microbes in homeostatic and disease conditions. In addition, IECs also play a crucial role in host defense by secreting AMPs, mucus, chemokines and cytokines ^{62,63}. The dialog between immune cells and IECs in the intestine is crucial for maintaining gut barrier integrity and regulating a proper host-microbe cross-talk ²⁹. Accordingly, the dysregulation of immune function has been linked to a variety of intestinal and metabolic disorders, including inflammatory bowel disease (IBD), obesity, and T2D ⁵³. Therefore, the study and discovery of mechanisms underlying the immune homeostasis breakdown and abnormal immune functioning might be the key to understand the underlying pathogenic mechanism of various conditions.

IMMUNOLOGICAL ALTERATIONS IN OBESITY AND METABOLIC DISORDERS

The immune response requires a finely tuned regulation to maintain the balance between the ability to trigger a rapid defensive immune response and, at the same time, promote self-discrimination and tolerance to commensal microorganisms ⁶⁴. The ingestion of obesogenic diets compromises this harmonious balance leading to immune dysregulation, as illustrated by chronic low-grade inflammation associated with obesity.

Macrophages are one of the immune populations that has been thoroughly researched for its involvement in obesity-related inflammation. Indeed, an increased macrophage activation and recruitment are considered a characteristic event in obesity ^{13,14}. In the AT, obese subjects presented high immune-cell infiltration, where almost half of the total cells are macrophages exhibiting a pro-inflammatory or M1-like phenotype ⁶⁵. Under stable circumstances, this percentage will not override the 10% and macrophages would be alternatively activated or M2-like ¹⁴. Accordingly, M1 increases are directly correlated to obesity and insulin resistance (IR) ^{27,65}. This increment in macrophages has been observed in other organs such as the muscle, the gut or the liver ⁵³. Particularly,

the gut is the largest macrophage reservoir in the body where these cells differentiated into a pro-inflammatory phenotype in obesity. The M1 macrophages of the intestine are thought to prime downstream inflammation in the visceral AT, a significant driver of IR ⁶⁶. Moreover, a raise in macrophages and related cytokines in the liver also increments the risk of developing additional pathologies linked to obesity, such non-alcoholic fatty liver disease (NAFLD) ^{14,67}. Interestingly, targeting macrophage offers therapeutic opportunities as some authors have shown that blocking chemoattractant receptors, like CCR2 (MCP-1/CCL-2 receptor) or suppressing pro-inflammatory cytokines produced by M1 macrophages, such as TNF α , is associated with a reduction in food intake, weight gain, and an improved insulin sensitivity ^{68,69}.

Other innate immune cells impacted by high-caloric diet and obesity include DCs, neutrophils and eosinophils. DCs play an important role in the obesity. These cells present a wide heterogenicity and are classically divided in 4 major subsets based on their molecular and cellular ontogeny: monocyte-derived DCs, type1 conventional DCs, type 2 conventional DCs and plasmacytoid DCs ⁷⁰. While some authors have positively correlated conventional type 2 DCs and plasmacytoid DCs to tissue inflammation, BMI increase and IR ^{71,72}, others have revealed type1 DCs and tolerogenic DCs can mitigate diet-induced metabolic dysregulations. For example, enhanced tolerogenic DC lifespan is translated into a phenotypic resistance to a high-caloric diet and the associated metabolic alterations ⁷³. As well, intestinal conventional type 1 DCs promote gut barrier function by facilitating colonization by gut commensals such as *Akkermansia muciniphila* ⁷⁴. Neutrophils are one of the most prevalent white blood cells and are the main responders in acute infections and tissue damage. The increases found in this immune cell subset in obesity led to hypothesized that neutrophils may be involved in obesity-related inflammation ⁵³. Specifically, neutrophils serve a part in the stimulation and recruitment of monocytes as they release TNF α and MCP-1 (or CCL-2) ⁵³. However, their contribution to obesity still remains controversial ⁷⁵. By contrast, eosinophils are reported to have a protective role in obesity and their numbers are typically decreased in this condition. Eosinophils are believed to promote thermogenesis, insulin sensitization and contribute to M2 macrophages enhancement through the secretion of IL-4 and IL-13 ⁵³.

Some studies have confirmed the importance of ILCs in obesity. The ILC1s from the fat pads have been the most researched in the context of obesity and T2D ⁷⁶⁻⁷⁹. Specifically, adipose ILC1s has been associated with the promotion of an pro-inflammatory response by instigating the M1 recruitment and polarization ^{77,79}. Additionally, increases in ILC1s in the liver have been associated with metabolic pathologies, such as NAFLD ⁸⁰. Nonetheless, the potential implication of ILC1s from other tissues, as the intestine, in obesity development remain much less understood. Other authors have described that intestinal ILC3s play a protective role in obesity ^{41,81}. For instance, in animal models, high-calorie diets caused a

decline of the intestinal type 3 responses carried out by ILC3s, and its adaptive counterpart, resulting in a decrease of IL-22 and IL-17 levels. This immune response was linked to reduced expression of AMPs and mucosal layer, and thus, to the increment of intestinal permeability described in obesity ^{41,53}. More recently, Sasaki et al. by performing a series of mechanistical experiments in mice have shed light regarding the impact of ILC2s in obesity ⁸². Specifically, these authors described a tissue-specific role of ILC2s. Whereas the AT ILC2s were decreased in obesity and seem to play a protecting role, the small intestinal resident ILC2s seemed to contribute to the obesity-development ^{53,82}.

Obesogenic diets also affect the functioning of adaptive immunity. In mice, high-caloric diets reduced the IgA secretion by B cells at the intestine, which contributes to gut barrier impairment ¹⁴. Also, in obesity, there is an increased lymphocyte recruitment and a shift towards pro-inflammatory phenotypes. It has been described that T helper (Th) 1 and CD8⁺ T cells are increased at tissue level (e.g., AT, intestine or liver), whereas T regulatory (Treg) and Th17 cells are diminished ^{14,83}. The reduction of intestinal Th17 has been associated to detrimental metabolic disturbances caused by the diet. Mice lacking one or both Roryt alleles, subsequently presenting decreased IL-22 and IL-17 responses, are more vulnerable to the effects of obesogenic diets in view of the greater glucose intolerance and body weight gain ⁸³. Interestingly, the decreases in Th17 and IgA production, mainly at intestinal level, have proven to be tightly linked to specific changes in gut microbiota derived from fat-rich diet consumption ^{83,84}.

MODULATING THE IMMUNE SYSTEM FOR MANAGING OBESITY

The immune system represents an attractive target for obesity management that could allow reducing the associated inflammatory tone and the incidence of comorbidities. In fact, the anti-inflammatory effects of several anti-diabetic drugs could be involved in their metabolic benefits ⁸⁵. One example is the thiazolidinedione, a PPAR γ nuclear receptor agonist, which also inhibits NF- κ B and restores the M2 phenotype in macrophages ^{85,86}. Similarly, metformin inhibits ROS production and TNF α activation ^{87,88}. Other strategies block the recruitment of macrophages or T cells or their pro-inflammatory shift, for instance through pharmacological inhibition of chemokine receptors or by immunometabolic reprogramming ^{89,90}. Newly, the role of ILCs in obesity offers a new avenue of therapeutic options ⁹¹. Future strategies might include the blocking of pro-inflammatory ILC1s as this subset acts as an up-stream activator of the inflammatory cascade, or the modulation of the ILC phenotype differentiation towards a 3 type considering the ILCs plasticity ⁹¹.

The boost of intestinal defense mechanisms has also been conceived as potential strategies for obesity treatment. For example, strategies increasing AMPs secretion, mucus production or the induction of anti-inflammatory cytokines such as IL-10 and IL-22 have proven to improve glucose homeostasis, reduce intestinal atrophy and reduce body weight gain in preclinical models of obesity

^{25,41,92}. Besides, the modulation of the immune system through the gut microbiota is another possible strategy. In fact, as commented above, the gut microbiota is crucial for controlling the immune system functioning and an altered microbiota influences obesity-related inflammation and the associated metabolic disorders ^{29,37}. Indeed, several authors have demonstrated that the intestinal microbiota is causally involved in the intestinal immune alterations observed in high-fat diet fed mice by performing fecal transplants ^{83,93,94}. One of the objectives of this Doctoral Thesis has been to investigate the effects of new interventions directed to alleviate the obesity-driven inflammation throughout the modulation of gut microbiota.

3. THE GUT MICROBIOTA AND METABOLIC HEALTH

DEFINITION AND FEATURES OF THE GUT MICROBIOTA

The gut microbiota refers to the diverse community of microorganisms that inhabit the gastrointestinal tract of humans and other animals. The gut microbiota composition is highly dynamic and can be affected by a variety of factors like age, diet, drugs (narcotics and xenobiotics), genetics, life-style and other environmental variables ^{95,96} (**Figure 3**). The term gut microbiome refers not only to the collection of microorganisms but also to their genes and active products (“theatre of activity”) ⁹⁵ and it is widely used in literature.

The microbiota accounts for almost 2 kilograms of our total body mass ⁹⁷. Despite sharing common microbes, individuals could be identify by their microbial metagenomic codes, using microbiota like a personal fingerprint, which is also strongly determined by body sites (e. g., oral cavity, skin, gut, or genitals) ^{98,99}. The gut harbors the densest microbial biomass where bacteria are the most abundant microorganisms, and thus the most widely studied ¹⁰⁰. Classically, over 1,000 different bacterial species are thought to reside in the human intestinal tract ¹⁰¹. However, current advances and new sequencing technologies are allowing to increasing this number to thousands of novel species that make up the human gut microbiota ¹⁰². The majority of intestinal bacteria (~90%) belongs to two major phyla, the Firmicutes and Bacteroidetes, followed by Actinobacteria and Proteobacteria ¹⁰³. Formally these phyla have been re-named as Bacillota, Pseudomonadota, Actinomycetota and Bacteroidota, respectively, according to the rules of the international code of nomenclature for prokaryotes ¹⁰⁴. However, in this Thesis former nomenclature is utilized to facilitate reference to previous literature and consistency with the chapters. In addition to bacteria, the gut microbiota also contains a smaller proportion of other microorganisms. Archaea, a group of single-celled prokaryotic microorganisms known to produce methane and other gases as metabolic products. Fungi, which are eukaryotic organisms, that are suggested to play a role in the regulation of the immune system in

emerging investigations ¹⁰⁵. Viruses and protozoa are also starting to be considered as important players in the regulation of the gut microbiota and its functions ¹⁰⁶. For example, bacteriophages (viruses that infect bacterial cells) can contribute to modulating the whole microbiota structure ¹⁰⁷.

The microbiome definition included the genes (metagenome), structural microbial components (e.g., membrane proteins, etc.) and microbial-derived metabolites ⁹⁵. The metagenome has a coding capacity superior by far to that of the human genome, and, accordingly, it has been referred as our 'second genome'. The metagenome, analyzed through shut-gun sequencing, encodes enzymes involved in the fermentation of dietary substrates, degradation of xenobiotics and the production of nutrients, among other functions ¹⁰⁸, complementing those encoded by the human genome. Although metagenome data provide valuable information of the potential functionality of genes and pathways encoded by the microbiota of a subject, still one third of the genes encoded by the gut metagenome are not annotated ¹⁰⁹. In this regard, functional omics (metatranscriptomics, metaproteomics and metabolomics) represent a more precise measurement of the actual known transcripts, proteins and metabolites, respectively, expressed or produced by the microbiota of a specific individual ¹¹⁰. For instance, metabolomics can reveal alterations of key microbial metabolic pathways or specific metabolites generated by the microbiota and involved in obesity and its metabolic complications ¹¹¹. The most studied metabolites of microbial origin and related to obesity are the short-chain fatty acids (SCFAs) produced mainly from dietary fibers, and secondary bile acids (BAs) derived from primary BAs ^{112,113}. Other metabolites such as indoles derived from tryptophan or imidazole propionate produced from histidine have also been recently tied to obesity ^{114,115}. In summary, changes in microbiota composition, functionality and metabolite production are the result of the complex interactions between different microbial species, the diet and the host and can be crucial for defining the metabolic phenotype of the subject as exposed below.

INTERACTIONS BETWEEN THE GUT MICROBIOTA AND THE HOST METABOLISM

The gut microbiota modulates human health by three main pathways of communication (endocrine, neural, and immune) and that are summarized in **Figure 3**.

The endocrine pathway involves stimulating the production of enteroendocrine hormones in response mainly to microbial metabolites. For example, SCFAs and secondary BAs produced by the microbiota interact with G protein-coupled receptors (GPCRs) promoting the secretion of cholecystokinin (CCK), GIP, GLP-1 and peptide YY (PYY) by intestinal neuroendocrine cell (NECs) ^{116,117}. These hormones regulate gut motility, appetite, glucose metabolism, fat accumulation and insulin secretion ³⁸. Besides, certain SCFAs, like butyrate, can cross the

intestinal wall and reach the circulation, and the AT or the brain, where it regulates, respectively, the release of adipokines or hypothalamic signaling ¹¹⁸.

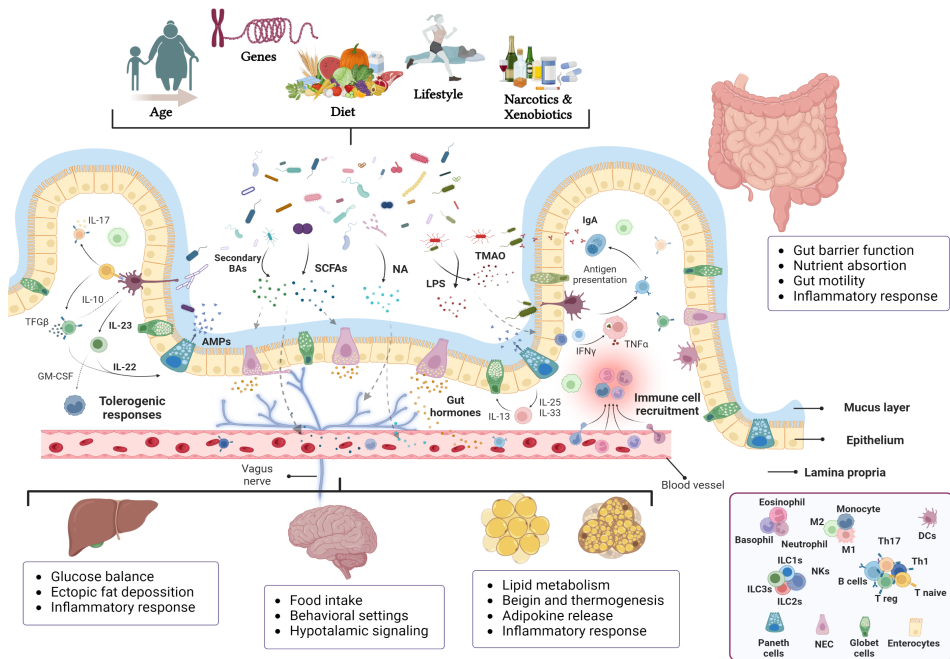


Figure 3. Microbial-host communication pathways. Factors that shape the composition of gut microbiota and how the microbiota impact on the host metabolism through three main pathways: endocrine, neural and immune. Endocrine: gut microbiota can influence host metabolism through the production of metabolites like SCFAs or secondary BAs that stimulate gut hormone secretion by NEC cells and may act on other tissues. Neural: some bacteria produce NA compounds that can reach the brain or activate vagal afferences modulating the host eating behavior. Immune: gut microbiota trains and shapes the immune system maintaining a balanced immune response that eliminates harmful bacteria and promotes tolerance towards commensal bacteria. BAs: bile acids; NEC: Neuroendocrine cells; NA: neuroactive compounds; SCFAs: Short-chain fatty acids; LPS: Lipopolysaccharide; TMAO: trimethylamine N-oxide; AMPs: Antimicrobial peptides. Created with BioRender.com (Agreement number: CB250A9DRT).

The neural pathway involves communication between the gut microbiota and the enteric and central nervous system (CNS) in the so-called gut-brain axis. The gut microbiota also influences the signaling and sensitivity of the vagal nerve and contributes to the production of neuroactive (NA) metabolites like serotonin, dopamine, and gamma-aminobutyric acid (GABA) that can act on the periphery and, in some case, cross the blood-brain barrier and directly influence the activity of the CNS ¹¹⁹. As a result, the gut microbiota impacts on various CNS functions, including energy balance and eating behavior ¹²⁰. For example, at hypothalamic level, the increments in microbially produced NA compounds, such as GABA or glutamate, affect to the secretion of anorexigenic peptides, and thus the regulation of food-behavior and metabolism ¹²¹.

Lastly, the immune pathway involves the interaction between the gut microbiota and the host immune system¹²². Gut microbiota shapes both innate and adaptive immune responses, which can positively or negatively impact on host health and metabolism. For instance, bacterial structural components, such as LPS, peptidoglycan, or flagellin, are recognized by TLRs on IECs and professional immune cells (like macrophages and DCs), triggering downstream inflammatory cascades¹²³. Similarly, bacterial metabolites, such trimethylamine N-oxide (TMAO), produced from dietary choline and carnitine, drives the M1 polarization, thus, prompting a pro-inflammatory response¹²⁴. In contrast, specific commensals promote protective responses through the TLRs. One example is Amuc_1100, a membrane protein of *A. muciniphila* that exerts protective effects of the gut barrier via TLR2¹²⁵. Additionally, bacterial metabolites, such as indoles or some SCFAs, stimulate regulatory immune cells such as ILC3s, Tregs or tolerogenic DCs to produce IL-22 and IL-10, among others, that can control inflammation and help maintain the gut barrier^{41,63,92}.

MICROBIOTA ALTERATIONS AND RELATIONSHIP TO METABOLIC HEALTH

Imbalances between microbial pathobionts and symbionts or their functions within the gut microbiota (often termed dysbiosis) have been related to many diseases¹²⁶. These alterations may cause an aberrant host-microbe communication contributing to the disease^{96,103}. These include metabolic-based conditions (e.g., obesity, T2D), autoimmune diseases, (e.g., rheumatoid arthritis, multiple sclerosis, celiac disease) and mental disorders (e.g., depression, alcohol dependence)^{119,127}. In the context of obesity, dysbiotic microbiota was first associated with decreases in microbial richness and the Bacteroidetes to Firmicutes ratio in obese mice and humans^{128,129}. Weigh lost due to caloric restriction or gastric bypass were found to restore this microbial ratio^{129,130}, and therefore, pointing Bacteroidetes/Firmicutes ratio as a plausible biomarker for obesity. However, contradictory results soon emerged^{131–133}, and nowadays the use of this ratio as obesity determinant is rather obsolete and discouraged^{134,135}. Indeed, comparison of changes at the phylum level is just an oversimplification of the complex taxonomic signature of obesity. Mainly when it comes to phyla such as Firmicutes and Bacteroidetes that contain a wide number of families and species, with really different functional attributes within them.

Recent studies have observed several bacterial species to be increase in obese subjects when compare to controls such as *Bilophila* spp., *Escherichia/Shigella* spp., *Lactobacillus reuteri* or *Desulfovibrio piger*, and were associated with unhealthier metabolic status^{136–139}. In contrast, others such *A. muciniphila*, *Bacteroides uniformis*, *Bifidobacterium* spp., *Fecalibacterium prausnitzii*, and *Parabacteroides distasonis* have been consistently related with obesity protection and, in some cases, shown to exert beneficial effects (e.g. body weight reduction, glucose balance, alleviation of hepatic steatosis and insulinopenia) mainly in pre-clinical trials^{133,140–142}. Some of these species such as, *A. muciniphila*, have been

proposed as a potential biomarkers of metabolic health ¹⁴³. Additionally, alterations in microbial-metabolic functions associate with diverse conditions ¹³⁴. For example, a reduction of butyrate levels associated to the detrimental impact of the diet in butyrate producers, such as *F. prausnitzii* or *Roseburia* spp., has been associated with obesity in some studies ^{144,145}. Besides, alterations in other microbially produced metabolites, like increases in BAs and reductions in indoles have also been related to obesity ^{113,146}. Additionally, increases in other metabolites, like imidazole propionate or kynurenine, have been recently linked to the onset of IR and the chronic inflammation in obesity ^{114,146}.

Despite the lack of consensus on the precise microbiota signatures of obesity, it is widely accepted that microbiota alterations, partly due to hypercaloric diets, are causally involved in obesity and the metabolic syndrome ¹³⁴. As a matter of fact, the transference of whole human fecal microbiota from obesity discordant twins to germ-free mice led to the reproduction of donor obese or lean phenotype in the recipient mice ⁵¹. The obese-associated gut microbiota was first proposed to contribute to metabolic dysfunction by increasing the extraction and absorption of calories from food ¹⁴⁷. Shortly after, it was described that the aberrant microbiota of obese mice induced inflammation in the gut, increasing intestinal permeability and causing metabolic endotoxemia ¹⁴⁸. In this sense, for example, administering *Enterobacter cloacae* B29, a bacterium isolated from a morbidly obese volunteer, to HFD-fed germ-free mice proved the existence of a causative link between the bacterium and the obesity onset ¹⁴⁹. Specifically, the bacterium synergized with the HFD feeding in increasing circulating levels of LPS and systemic inflammation, leading to obesity and IR development, otherwise not observed ¹⁴⁹. Lastly, the role of a fecal microbiota transplantation, from lean donors to metabolic syndrome patients, improving insulin sensitivity was shown, although effects on body weight were not detected ¹⁵⁰.

PRE-CLINICAL EVIDENCE OF THE POTENTIAL OF PREBIOTICS, FOOD BIOACTIVE COMPOUNDS AND PROBIOTICS TO COMBAT OBESITY.

Given the important role played by the gut microbiota in human health, there is a growing interest in the use of dietary strategies that modulate its composition and functionality. Dietary changes consisting of a general increases in fiber intake and reduced energy and added sugars can promote a healthy gut microbiota ¹²⁶. There are also more specific gut microbiota-based strategies, of which, the most investigated are the administration of probiotic bacteria, prebiotics or their combination (synbiotics) ¹⁵¹. Some bioactive compounds of vegetables and fruits have also been shown to modulate gut microbiota composition, counteracting the deleterious effects of obesogenic diets ^{92,152}.

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP) prebiotic is “a substrate that is selectively utilized by host microorganisms conferring a health benefit” ¹⁵³. Currently, there is a wide list of

prebiotic substances, mostly present in vegetables, fruits and whole cereals consumed on our daily basis. Some of the most studied are inulin, fructo-oligosaccharides (FOS), human milk oligo-saccharides (HMOs), galacto-oligosaccharides (GOS) and arabinoxylans (AX)^{153,154}. The diet is also source of other bioactive compounds such as polyphenols, carotenoids, terpenoids, vitamins, organo-sulfur compounds and plant sterols. Even if there is no consensus term, bioactive compounds can be defined as “*compounds which have the capability and the ability to interact with one or more component(s) of a living tissue presenting a wide range of probable effects*”^{155,156}. Some of these bioactive compounds may exert beneficial effects through the gut microbiota modulation. This is the case of polyphenols whose protective effects depend on the changes evoked on the microbial composition (e.g., stimulation or inhibition of the growth of specific bacteria) or on their biotransformation by the microbial activity¹⁵⁷. Besides, pre-clinical evidence have demonstrated the anti-inflammatory, anti-adipogenic, anti-oxidant and cardioprotective properties of polyphenols and other bioactive compounds, playing an important role in obesity^{26,158,159}. For instance, indigo (a plant-derived extract rich in polyphenols) exerts anti-obesogenic effects through the induction of IL-10 and IL-22 induction, acting as agonist of the aryl hydrocarbon receptor (AhR)²⁵. Similarly, the *Allium*-derived compound, propil-propane thiosulfonate (PTSO), counteracts obesity development by reinforcing gut barrier integrity, reducing immune cell infiltration and systemic inflammation¹⁶⁰.

Another dietary strategy is the administration of specific probiotic microorganisms^{161,162}. Probiotics are defined as “*live microorganisms which when administered in adequate amounts confer a health benefit on the host*”¹⁶³. To the date, probiotics have shown to modulate immune function and confer potential health benefits in a wide range of pathologies such obesity, IBD or T2D^{164,165}. The classic probiotic strains evaluated in obesity have belonged to the genera *Bifidobacterium* and *Lactobacillus* and are reported to improve glucose homeostasis and reduce inflammation, BMI and, even abdominal adiposity^{162,166}. Their use as probiotics is broadly extended in foods and as supplements given their safety recognition by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA)¹⁶⁷. Currently, novel bacterial strains isolated from the human indigenous microbiota are emerging as alternatives for the prevention and treatment of different conditions, including obesity and metabolic diseases, known as the next-generation probiotics¹⁶⁸. This is the case of strains of the species *A. muciniphila*, *B. uniformis*, *Holdemanella bififormis*, *Dysosmobacter welbionis* or *F. prausnitzii*^{169–173}. *A. muciniphila* probably is the most widely studied in obesity as it has been subjected to several preclinical trials demonstrating its anti-obesogenic effect (e.g., decreasing obese parameters, improving insulin sensitivity, reducing inflammation and maintaining intestinal homeostasis)¹⁷⁴. Similarly, pre-clinical studies performed in our team in DIO mice treated with *B. uniformis* have shown its ability to alleviate primarily the

inflammatory tone and contribute reducing body weight gain, improving oral glucose tolerance and restoring systemic hormonal and biochemical alterations (leptin and triglyceride levels)¹⁷⁵. However, the effects of *H. biformis* in DIO mice were related to its ability to enhance GLP-1 secretion, restoring glucose metabolism¹⁷¹. Alternatively, the benefits of *D. welbionis* have been associated with the preservation and promotion of brown adipose tissue (BAT) functionality, whereas *F. prausnitzii* induced anti-inflammatory effects partially attributed to the butyrate production capacity¹⁷³. Here, it is important to highlight that the effects of probiotics vary depending on the specific bacterial strain, dose, and host factors. Then, different individuals may respond differently to probiotic interventions due to variations in their diet, baseline microbiota composition, and immune status, among others^{176,177}. All these considerations should be included in the corresponding clinical study, that ideally should be a randomized double-blind placebo-controlled trial, devoted to prove the effectiveness and tolerance of the potential probiotics before being transferred into the market as new strategies to combat obesity.

CLINICAL EVIDENCE OF THE EFFECTS OF MICROBIOME-BASED STRATEGIES ON OBESITY AND METABOLIC DISORDERS

The success of some microbiome-based strategies in pre-clinical studies and, in some cases, in human intervention studies, illustrate their potential as promising adjuvants in the management of obesity and associated disorders. Even if the transference to the clinic is still of a great challenge, to the date, there are several successful cases next presented that encourage further research in this area.

A pioneer double blind intervention's study in obese women with inulin-type fructans showed that improved metabolic markers associated with changes of the gut microbiota composition¹⁷⁸. Specifically, inulin-type fructans increased the abundance in faeces of *Bifidobacterium* and *F. prausnitzii*, and decreased *Bacteroides intestinalis*, *Bacteroides vulgatus* and *Propionibacterium* spp. These changes were correlated with decreased LPS serum levels and fat mass, among others¹⁷⁸. In another study, the administration of oligofructose-enriched inulin to overweight or obese children successfully reduced weight, fat mass and IL-6 levels and, at the same time, increased *Bifidobacterium* fecal numbers¹⁷⁹. In another study, oligofructose-enriched inulin has proved to reduce the appetite sensation and consequently the energy intake in overweight and obese children¹⁸⁰. As a last example, in overweight adults the administration of GOS paled markers of metabolic syndrome, as it reduced pro-inflammatory cytokines, circulating lipids, and plasmatic insulin^{181,182}.

Diets rich in fruits, berries and vegetables is another strategy to modify the gut microbiota and improve human metabolic health¹⁸³. Some of their benefits could be attributed to the fiber contents and prebiotic-like compounds, but also to the high content in bioactive substances, like polyphenolic or sulphur-containing

compounds that can improve some of the obesity features according to the literature ^{184–186}. For example, the administration of polyphenols (e.g., curcumin, quercetin, resveratrol) to obese individuals have been associated to insulinopenia and reduction of waist circumference and inflammatory markers ¹⁸⁷. Yet, some challenges are required to be overcome for their applicability, like the bioavailability in the food matrix, the susceptibility to degradation and the safety. In the last years, technical and scientific advances are helping to override some of these limitations, for example, through the overproduction of these compounds biotechnologically or the use of nano-encapsulation ^{188,189}.

There are also human studies evaluating the effects of probiotic bacteria on obesity and metabolism ¹⁹⁰. A randomized clinical trial in overweight, obese and IR patients confirmed some benefits of *A. muciniphila*. More specifically, after a three-month intervention with viable and pasteurized bacteria, the subjects who received the bacteria presented improved insulinemia and plasma cholesterol, and decreased markers of hepatic and renal dysfunction ¹⁴⁰. Similarly, one strain of *Bifidobacterium longum* restored fasting glucose levels, but not BMI in obese subjects ¹⁹¹. Additionally, another intervention study performed by our team in obese children have proven the beneficial effects of *Bifidobacterium pseudocatenulatum* CECT 7765 in inflammatory and IR markers ¹⁹².

Last, synbiotics represents another strategy to modulate the gut microbiota and potentially achieve metabolic benefits. Trials in obese humans have confirmed the ability of certain synbiotics to improve metabolic and immune parameters, such inflammation, glucose imbalance, body weight and waist circumference along with the improvements in the microbiota composition ^{193,194}. A double-blind randomized clinical trial in obese women revealed that the administration of *Bifidobacterium lactis* with FOS alleviated inflammation and reduced body weight linked led to metabolite changes (increase in pyruvate and alanine, and decrease in citrate and branched-chain amino acids) ¹⁹⁴. Another randomized trial in obese adults investigated the effects of the administration of FOS plus a mix of probiotic strains (*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum* and *L. bulgaricus*) which led to a reduction in the BMI, fat mass and blood pressure ¹⁹⁵.

Herein, these examples evidence the potential of microbiome-modulating strategies to combat obesity and metabolic dysfunction. However, the understanding of the key microbiome components contributing to a healthy metabolic phenotype and the molecular mechanism by which these strategies exert their action is very limited. Besides, the effectiveness is, in most cases modest and variable, which could be partly due to the high inter-individual variability of the gut microbiota and the response to these dietary strategies. Therefore, future research is still needed to harness the microbiome potential and translate the findings into practical and effective solutions to better combat obesity.

OBJECTIVES

OBJECTIVES

The overall objective is to progress in the knowledge of how the gut microbiota influences obesity and associated metabolic disorders and evaluate new microbiome-based interventions that through immunomodulatory mechanisms could improve or prevent obesity development.

To this end, we divided the Thesis in three specific objectives:

SO1. Evaluate the potential of dietary and microbiome-based strategies with immunomodulatory properties to prevent metabolic alterations in obesity.

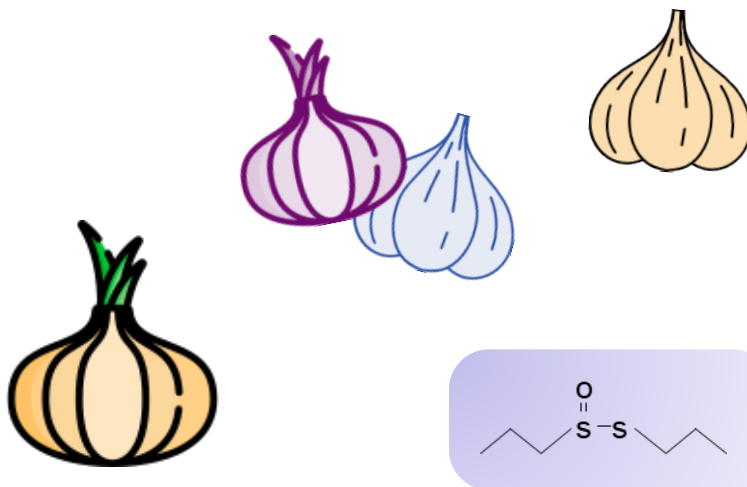
SO2. Identify whether the effect of the diet or the selected microbiome-based strategy on metabolism depend on components of the immune system.

SO3. To unravel the mechanisms underlying the communication between immune cells and the intestinal bacteria mediating their effects on obesity.

RESULTS

CHAPTER 1

STRATEGIES BASED ON BIOACTIVE COMPOUNDS TO TACKLE OBESITY



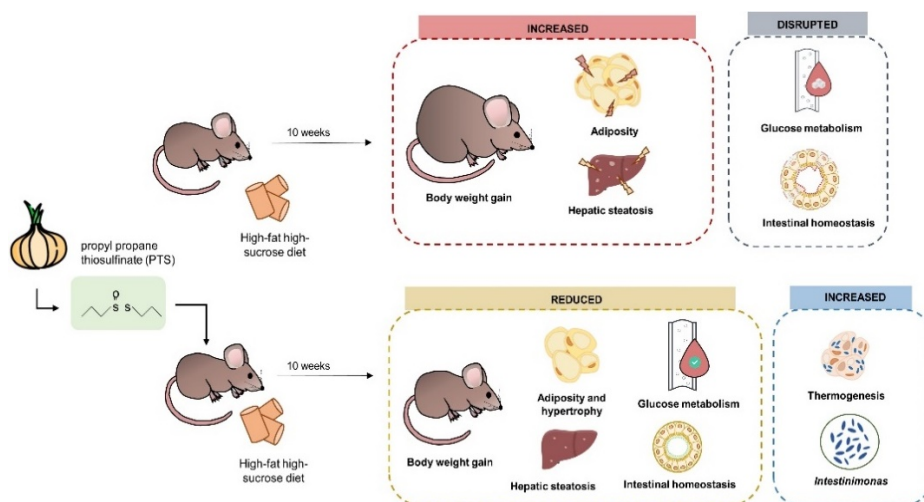
Rebeca Liébana-García, Marta Olivares, Sonia Rodríguez-Ruano, Verónica Tolosa-Enguís, Isabel Chulia, Lidia Gil-Martínez, Enrique Guillamón, Alberto Baños, Yolanda Sanz (2022). The Allium derivate propyl propane thiosulfinate exerts anti-obesogenic effects in a murine model of diet-induced obesity.

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My contribution to this research article comprehended:

Conceive and design the experiments. Perform the animal experiments. Data acquisition, analysis and interpretation. Figure design and manuscript writing.

THE ALLIUM DERIVATE PROPYL PROPANE THIOSULFINATE EXERTS ANTI-OBESOGENIC EFFECTS IN A MURINE MODEL OF DIET-INDUCED OBESITY



ABSTRACT

Allium species and their organosulfur-derived compounds could prevent obesity and metabolic dysfunction, as they exhibit immunomodulatory and antimicrobial properties. Here, we report the anti-obesogenic potential and dose-dependent effects (0.1 or 1 mg/kg/day) of propyl propane thiosulfinate (PTS) in a murine model of diet-induced obesity. The obesogenic diet increased body weight gain and adipocyte size, and boosted inflammatory marker (*Cd11c*) expression in the adipose tissue. Conversely, PTS prevented these effects in a dose-dependent manner. Moreover, the higher dose of PTS improved glucose and hepatic homeostasis, modulated lipid metabolism, and raised markers of the thermogenic capacity of brown adipose tissue. In the colon, the obesogenic diet reduced IL-22 levels and increased gut barrier function markers (*Cldn3*, *Muc2*, *Reg3g*, *DefA*); however, the highest PTS dose normalized all of these markers to the levels of mice fed a standard diet. Gut microbiota analyses revealed no differences in diversity indexes and only minor taxonomic changes, such as an increase in butyrate producers, *Intestinimonas* and *Alistipes*, and a decrease in *Bifidobacterium* in mice receiving the highest PTS dose. In summary, our study provides preclinical evidence for the protective effects of PTS against obesity, which if confirmed in humans, might provide a novel plant-based dietary product to counteract this condition.

INTRODUCTION

Obesity is characterized by excessive body fat accumulation, which increases the risk of suffering from chronic diseases such as heart failure, cancer, and type 2 diabetes¹. Many single-nucleotide polymorphisms are associated with the development of obesity; however, these differences in the genetic background alone do not explain the incidence of obesity^{2,3}. Indeed, the constant rise in obesity prevalence can only be explained by broad environmental changes, given the stasis of human biology within the same time frame⁴. Changes in food systems and dietary patterns undoubtedly influenced the epidemiology of obesity. In this respect, hypercaloric diets disrupt the energy balance, immune system, and gut microbiome, whereas a balanced, plant-based diet protects from fat deposition and metabolic impairment⁵.

Some of the health benefits associated with the intake of vegetables are due to their content in bioactive compounds. Accordingly, the administration of extracts rich in certain bioactive compounds to obese human subjects^{6–8} or rodents^{9,10} is proven to reduce some of the features associated with obesity and its comorbidities (e.g., decreases in body weight and plasma cholesterol levels or improvements in waist/hip and LDL/HDL ratios). Among the bioactive compounds currently stirring interest are the organosulfur compounds obtained from vegetables of the genus *Allium*—such as onions, garlic, or leeks—synthesized by these plants as a defense mechanism against tissue damage. The fact that these organosulfur compounds have functional properties exerting antibacterial, antifungal, anti-inflammatory, or antioxidant activities might underlie the century-old folk wisdom that vouches for their beneficial properties^{11,12}.

The most widely studied organosulfur compound is the so-called allicin (diallyl thiosulfinate). In the context of obesity, allicin is shown to curb obesity in animal models, an effect that could be, at least partly, mediated by the gut microbiota^{13,14}. However, one limitation of allicin is that it is precariously unstable, and its bioavailability raises doubts¹⁵. To a lesser extent, other organosulfur compounds from the *Allium* genus were also investigated, such as propyl-propane-thiosulfonate (PTSO) or propyl propane thiosulfinate (PTS). In this respect, the compound PTSO displayed beneficial effects on two murine models of colitis-induced by the dextran sodium sulfate (DSS) and the 2,4-dinitrobenzene sulfonic acid (DNBS) by reducing different pro-inflammatory mediators and improving the integrity of the intestinal epithelial barrier¹⁶. These two mechanisms are also pinpointed by Vezza et al. to explain the anti-obesogenic effects of this compound in a very recent in vivo study¹⁷. Regarding PTS, there is evidence of its potential use as an additive for animal nutrition¹⁸ and its antimicrobial and anticoccidial properties in vitro^{19,20}. Moreover, PTS was demonstrated to be toxicologically safe in rats²¹. However,

to our knowledge, research so far has not investigated PTS in the context of its potential protective effects on metabolic health.

In the present study, we describe the anti-obesogenic potential of the *Allium* derivate PTS, tested at two different doses in a murine model of diet-induced obesity. We describe how PTS administration prevents body weight gain and the metabolic impairment associated with a hypercaloric diet in a dose-dependent manner. We also shed light on its mode of action through the effects exerted on immune mediators and energy homeostasis.

MATERIALS AND METHODS

Mice and treatments

The experiments were performed with a total of 32 C57BL/6J mice (7-week-old males, Charles River Laboratories, Écully, France). Mice were housed in groups of four per individually ventilated cage under a 12 h light/dark cycle (lights on at 8:00) in a temperature-controlled room (23 ± 2 °C). Mice were acclimatized for 10 days with a standard diet. During the experiment, mice had ad libitum access to water and food. The experiment was approved and performed following European Union 2010/63/UE and Spanish RD53/201 guidelines, approved by the ethics committee of the University of Valencia (Animal Production Section, SCSIE, University of Valencia) and authorized by the competent authority (Generalitat Valenciana). Furthermore, all efforts were made to guarantee the three Rs before performing the experiment. Accordingly, we considered alternatives to animal experimentation (replacement), but given its unavoidability we paid attention to reducing the number (reduction) and minimizing the suffering of, or harmful effects (refinement) on, the experimental animals. The Generalitat Valenciana authorized the procedure under the title "*Evaluation of bioactive compounds obtained from plant sources*" and assigned the code 2020/VSC/PEA/0088 on 26 May 2020.

Randomization of mice into four experimental groups ($n = 8$) was conducted based on body weight to minimize baseline differences. For 10 weeks, mice were treated with: (1) control diet (D12450K Ssniff; 10% of energy from fat and no sucrose), (2) high-fat-high-sugar diet (HFHSD, D12451 Ssniff; 45% of energy from lard, and 17% from sucrose), (3) HFHSD plus low-dose PTS (0.1 mg/kg/day), and (4) HFHSD plus high-dose PTS (1 mg/kg/day). **Table S1** shows the detailed composition of the diets. PTS from *Allium cepa*. (95% purity) was developed and provided by DOMCA SAU (Granada, Spain). The compound was administrated daily by oral gavage, whereas the control

groups received PBS. Trends in body weight and food and water intake were monitored twice per week.

In the 10th week, animals were fasted, anesthetized with isoflurane, and sacrificed by cervical dislocation. Blood samples were collected in EDTA tubes, and plasma was immediately transferred after centrifugation (12,000 g, 3 min). One aliquot of plasma was kept on ice to assess intestinal permeability, and one aliquot was stored at 80 °C for biochemical and immune analyses. A section of white adipose tissue (WAT) was immersed in a solution of 4% paraformaldehyde for histological analysis. Liver, brown adipose tissue (BAT), WAT (subcutaneous (inguinal) and visceral (epididymal)), two sections of the intestine (ileum and colon), and the cecal content were snap-frozen in liquid nitrogen and stored at -80 °C until use.

Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was conducted at week 9 after 4 h fasting. Blood from the saphenous vein was collected at 0, 15, 30, 60, and 120 min after an oral glucose challenge (2 g/Kg). Glucose was measured using glucose test strips (CONTOUR®-Next meter, Bayer, Leverkusen, Germany).

Intestinal permeability

Fluorescein isothiocyanine (FITC)-dextran 4 kDa (Sigma-Aldrich, St. Louis, MO, USA, 600 mg/Kg) was administered by oral gavage one hour before the sacrifice. Plasma was diluted in an equal volume of PBS, and fluorescence was measured at the excitation wavelength of 485 nm and 535 nm emissions (CLARIOstar® Plus Multi-Mode Microplate Reader, Ortenberg, Germany). Standard curves were obtained by diluting FITC-dextran in the plasma of non-treated mice, as previously described ²².

Biochemical analyses

Plasma levels of insulin, glucose, triglycerides, cholesterol, and non-esterified fatty acid (NEFA) were measured with the following commercial kits: ultrasensitive insulin ELISA kit (Mercodia, Uppsala, Sweden), glucose liquid (Química Analítica Aplicada SA, Tarragona, Spain), triglyceride colorimetric assay kit (Elabscience, Houston, TX, USA), cholesterol liquid kit (Química Analítica Aplicada SA, Spain), and NEFA colorimetric assay kit (Elabscience, USA), respectively. The HOMA-IR index was calculated as fasting plasma insulin (mU/L) x fasting plasma glucose (mmol/L)/22.5.

The citrate synthase activity was measured in the BAT using a colorimetric assay kit (BioVision, Milpitas, CA, USA). Hepatic glycogen was quantified according to the manufacturer's instruction of a commercial kit (Sigma-Aldrich, USA). In addition, the lipid content was quantified in the liver after extraction with chloroform, as previously described ²³. Briefly, the tissues were homogenized in chloroform/methanol (2:1) solution. After 3 h of shaking, Milli-Q

water was added, and the organic layer was separated by centrifugation (16,000 xg, 20 min) and dried overnight. Triglyceride concentration was measured as described above.

Immune parameters

One section of the colon and one section of the liver were homogenized in RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was centrifuged (12,000 rpms, 10 min). In the supernatant from the colon and the liver, the levels IL-22 or IL-6 were, respectively, quantified with an ELISA kit (ELISA MAXTM) Deluxe Set, Biolegend, San Diego, CA, USA). The values were normalized with the amount of protein quantified using the Bradford method. Plasma levels of IL-6 were measured using the LuminexTM IL-6 Mouse Bead kit (Invitrogen, Waltham, MA, USA). In suspensions of the cecal content, secretory immunoglobulin A (sIgA) was measured using another commercial kit (Invitrogen, Waltham, MA, USA).

Gene expression analyses

Total RNA was isolated from different sections of the intestine (ileum and colon) using the commercial kit Nucleo Spin RNA (Macherey-Nagel, Nordrhein-Westfalen, Germany) and from the WAT and liver using the TRIsureTM reagent (Bioline, London, UK). Complementary DNA was prepared by the reverse transcription of 1 µg of total RNA using the kit High-Capacity cDNA Reverse Transcription (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. The RT-qPCR was performed with the LightCycler[®] 480 Instrument (Roche, Boulogne-Billancourt, France). The reaction consisted of LightCycler 480 SYBR Green I Master mix (Roche) and 300 nM of gene-specific primer pairs. Samples were run in duplicate, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method. The qPCR program was previously described ²³. Targeted genes were normalized with the expression of ribosomal protein L19 (*Rpl19*), the housekeeping gene. Primer sequences are detailed in [Table S2](#).

Histological analysis

The visceral WAT was stained with hematoxylin/eosin to quantify the size of the adipocytes. The bright-field digital images were taken using an E90I Nikon microscope (Nikon Corporation, Tokyo, Japan) with an 8x objective, equipped with a digital camera (Nikon DS-5Mc). A combined analysis was carried using Fiji (ImageJ 1.49q Software, National Institutes of Health, Bethesda, MD, USA) and NIS Elements BR 3.2 software (Nikon Corporation, Japan). The cross-sectional area of each adipocyte was automatically recognized and calculated by the NIS-elements software. Artifacts were manually discarded. Three independent measurements from different sections were performed on each mouse. At least 350 adipocytes were quantified per measurement.

Gut microbiota composition

Genomic DNA was extracted from the cecal content using a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany), and gut microbiota composition was analyzed by sequencing the V3-V4 hypervariable region of the 16S rRNA gene in an Illumina platform (MiSeq). The complete bioinformatics analysis is described in [Supp. Materials](#).

Statistical analyses

G*Power 3.1.9.2 was used to calculate sample size and GraphPad software (version 8, San Diego, CA, USA) for statistical analyses and plots. Shapiro–Wilk test was employed to assess data normality. For normally distributed data, differences were determined with one-way analysis of variance (ANOVA) or two-way ANOVA (as suitable) followed by *post hoc* Tukey's multiple comparison tests. Welch's correction was applied when variances were not equally distributed, followed by a Dunnett's T3 multiple comparisons test. Non-normally distributed data were analyzed with the Kruskal–Wallis test followed by Dunn's multiple comparisons test. For gut microbiota analyses, Benjamini–Hochberg method was used to adjust *p*-values and Permutational Multivariate Analysis of Variance (adonis) followed by a pairwise *post-hoc* test (pairwiseAdonis). The results were considered statistically significant at $p < 0.05$. For all analyses, the Grubbs test was used for outlier detection.

RESULTS

PTS curbed obesity by influencing the white and brown adipose tissue metabolism in adose-dependent manner

The HFHSD significantly increased body weight ([Figure 1A](#)) due to the expansion of the epididymal and inguinal WAT ([Figure 1B](#)). The administration of PTS curbed obesity in a dose-dependent manner; however, this effect only reached the statistical cut-off point at the higher dose. Accordingly, the histological analysis of the white fat pads showed that, at this dose, PTS prevented the increase in the adipocyte size induced by the HFHSD ([Figure 1C, D](#)). The morphological changes caused by the obesogenic diet were accompanied by clear increases in the expression of *Itgax*, coding for CD11c, a marker of M1 macrophages that are infiltrated in the adipose tissue in obesity²⁴ ([Figure 1E](#)). Only PTS at the higher dose reduced the expression of *Itgax* and *Ccl2* (coding for the chemokine MCP-1), which mediates the recruitment of macrophages.

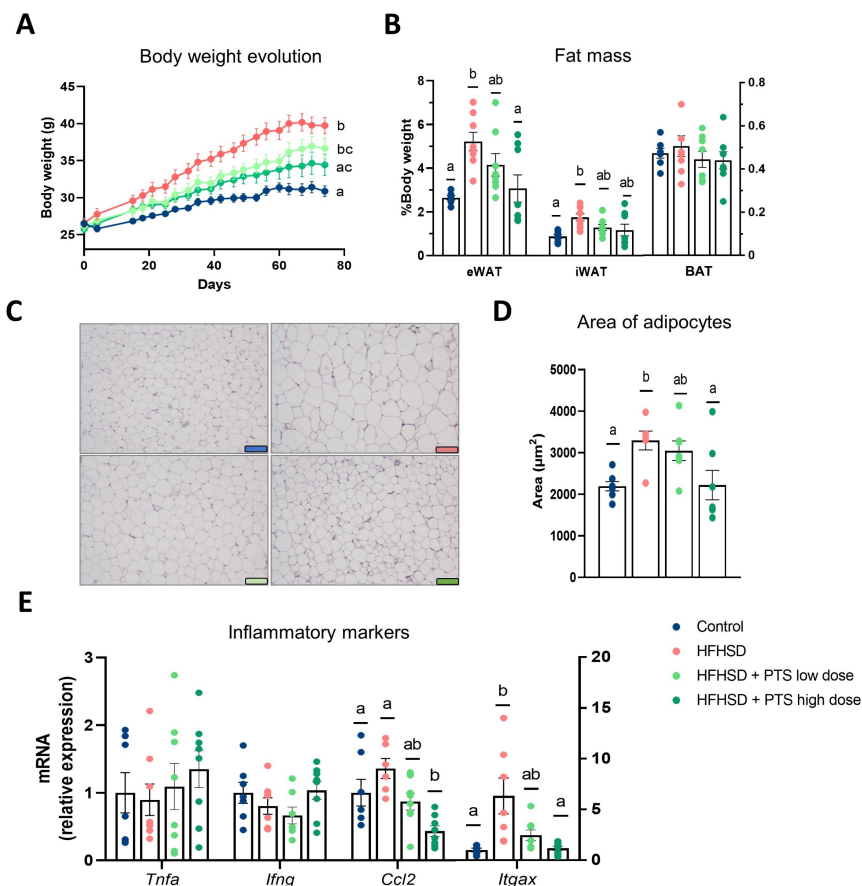


Figure 1. (A) Body weight development, (B) weight of the fat mass: eWAT and iWAT, (C) histology of the visceral adipose tissue (8x), (D) area of the adipocytes and (E) expression of markers of inflammation: *Itgax* and *Ccl2*. Mice were fed a control diet ($n = 6-7$); a high-fat, high-sucrose diet (HFHSD) ($n = 6-8$); and HFHSD with propyl propane thiosulfinate (PTS) at a low dose (0.1 mg/kg day) ($n = 7-8$) and a high dose (1 mg/kg day) ($n = 7-8$). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests for normally distributed data, except for body weight development, which was analyzed with a two-way ANOVA followed by Tukey's *post hoc* test. Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$. *Ccl2* gene codes for MCP-1; the *Itgax* gene codes for CD11c.

We also analyzed the expression of molecular markers involved in fat mobilization (**Figure 2A**) and storage (**Figure 2B**). In the WAT, the HFHSD did not modify fat mobilization, but it significantly upregulated pathways related to fat uptake and storage, such as *Lpl*, *Cd36*, and *Dgat2*. In contrast, the effect of PTS on lipid metabolism was balanced, with reductions in either fat mobilization and storage (**Figure 2A, B**). Specifically, the higher dose of PTS reduced the expression of markers involved in catabolic processes such as lipolysis (*Atgl*, *Hsl*), and beta-oxidation (*Cpt1a*); and in anabolic processes such as fatty acid uptake (*Lpl*, *Cd36*), fatty acid synthesis (*Acc1*, *Fas*), and adipogenesis (*Cebpb*, *Pparg*). However, PTS did not counteract the decline in markers that were caused by the obesogenic diet (**Figure S1**). All the changes attributed to PTS administration occurred without any differences in the water or food intake, or in the plasmatic levels of triglycerides, NEFA, or cholesterol (**Table S3**).

Finally, we investigated whether the anti-obesogenic effect of PTS could be attributed to a higher thermogenic activity of the BAT. In agreement with our hypothesis, PTS increased the expression *Cpt1a* (the low and high dose) as well as *Ppara* and *Prdm16* (at the higher dose) (**Figure 2C**). Hence, to confirm the capacity of PTS to increase the thermogenic activity of the BAT, we analyzed the mitochondrial activity by quantifying the citrate synthase activity. Accordingly, we found that PTS boosted this activity in a dose-dependent manner, which was only statistically different between the group treated with the highest dose of PTS versus the obese group (**Figure 2D**).

PTS improved systemic glucose homeostasis, hepatic metabolism and inflammatory response

High-calorie diets are well-known to impair glucose metabolism. Here, we confirmed that mice fed an HFHSD had a worse response to the glucose tolerance test and exhibited uncontrolled insulinemia (**Figure 3A, B**). The administration of PTS tended to attenuate both effects in a dose-dependent manner. Moreover, even if fasting glycaemia and the hepatic glycogen levels remained unchanged between groups (**Figures 3C and S2A**), the metabolic disruption in response to the HFHSD led to a pre-diabetic state, as estimated by the HOMA-IR index, which was not observed in PTS-treated animals (**Figure S2B**). Moreover, the HFHSD triggered hepatic steatosis, as evidenced by an increase in the inflammatory tone (assessed by IL-6 quantification) (**Figure 3D**) and higher hepatic weight due to triglycerides accumulation (**Figure 3E, F**). No changes were detected in the plasmatic levels of IL-6 (**Figure S2C**). Meanwhile PTS at the highest dose significantly prevented both effects. Furthermore, the molecular analysis of the liver showed that the HFHSD upregulated lipid storage markers (*Cd36*, *Dgat2*, and *Fas*), which all remained at the same level as the control group in mice treated with the highest dose of PTS (**Figure 3G**).

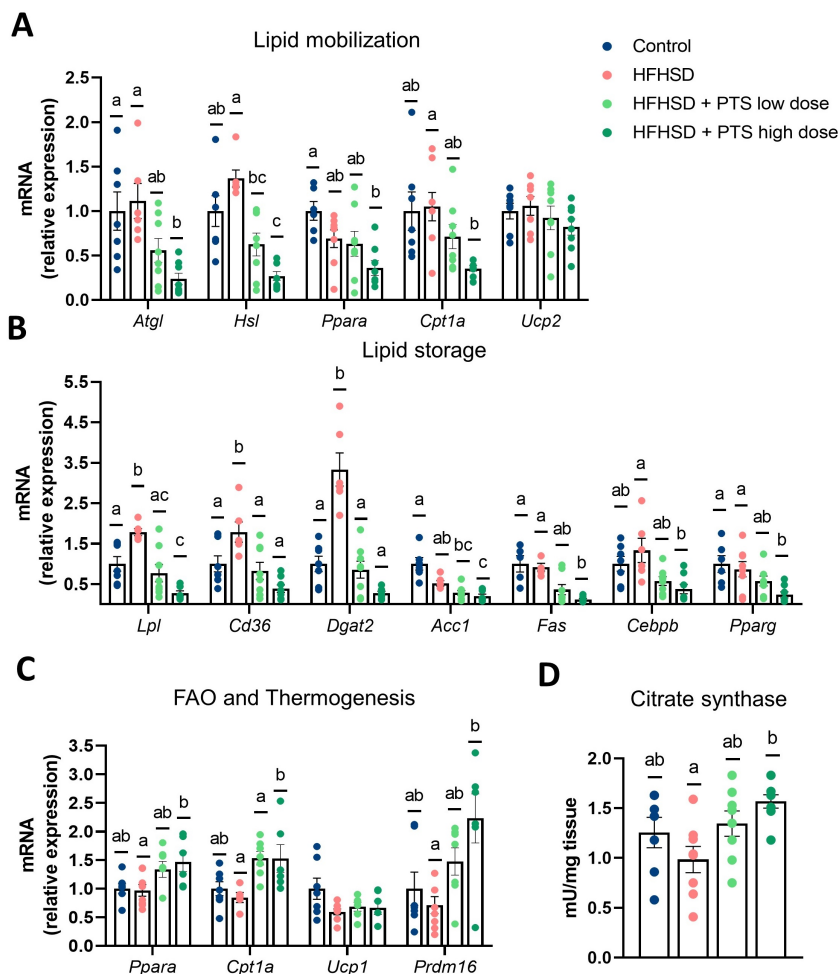


Figure 2. In the white adipose tissue: (A) expression of markers of lipid mobilization (B) lipid storage. In the brown adipose tissue: (C) expression of fatty acid oxidation (FAO) and thermogenesis markers (D) citrate synthase activity. Mice were fed a control diet (n = 6–7); a high-fat, high-sucrose diet (HFHSD) (n = 6–8); and HFHSD with propyl propane thiosulfinate (PTS) at a low dose (0.1 mg/kg day) (n = 6–8) and a high dose (1 mg/kg day) (n = 6–8). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests for normally distributed data, and Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the post hoc test when $p < 0.05$.

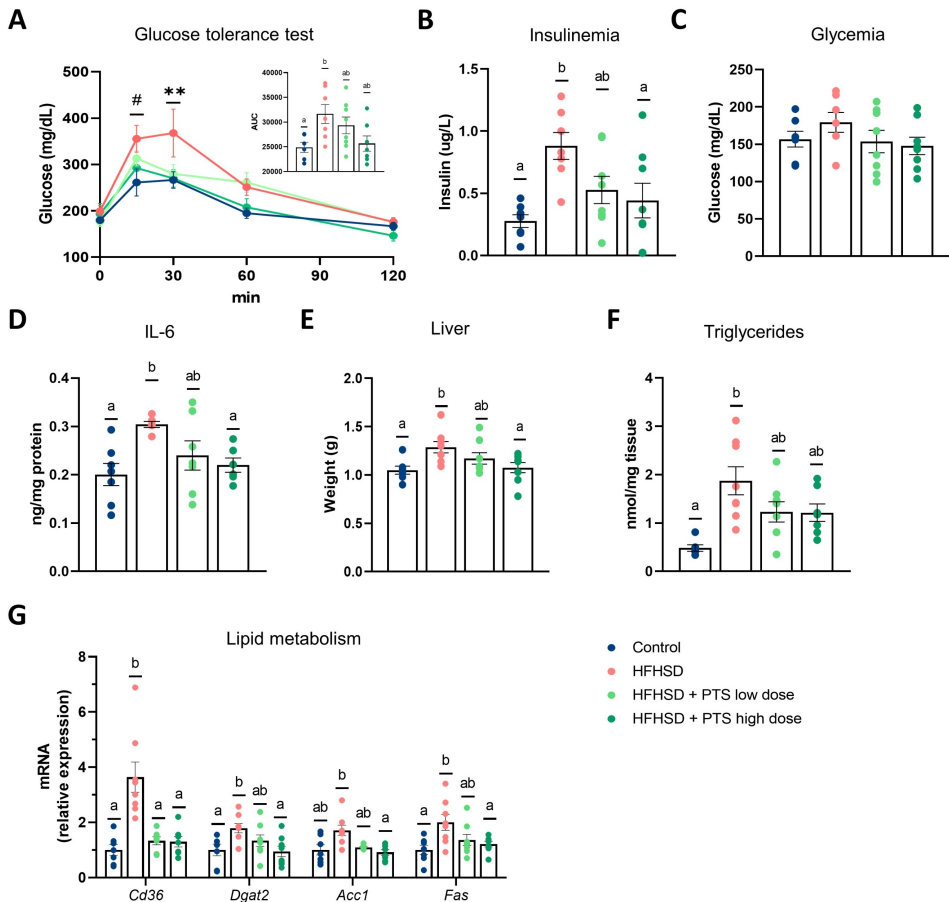


Figure 3. Systemic circulation: **(A)** Glucose tolerance test; AUC, **(B)** Insulinemia, and **(C)** glycemia. In the liver: **(D)** hepatic IL6 levels, **(E)** weight of the liver, **(F)** triglycerides, and **(G)** expression of markers of lipid metabolism. Mice were fed a control diet ($n = 6-7$); a high-fat, high-sucrose diet (HFHSD) ($n = 6-8$); and HFHSD with propyl propane thiosulfinate (PTS) at a low dose (0.1 mg/kg day) ($n = 7-8$) and at a high dose (1 mg/kg day) ($n = 7-8$). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's multiple comparison tests for normally distributed data, and Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$. “**” means significantly different within all groups, “#” means significantly different compared with the control group. ** $p < 0.01$; and # $p < 0.05$.

PTS had minor effects on the intestinal permeability and the gut barrier function

The in vivo assessment of intestinal permeability showed no differences caused either by diet or by PTS (**Figure 4A**). The HFHSD significantly altered the expression of genes involved in barrier function in the colon (*Cldn3*, *Zo1*, and *Muc2*) (**Figure 4B**), and ileum (*Cldn3* and *Ocln*) (**Figure S3A**). In contrast, PTS at the highest dose restored the expression of *Zo1* and *Muc2* to the level of the control group (**Figure 4B**). Based on the role attributed to IL-22 in the gut barrier function²⁵, we measured this cytokine in the colon. The group fed an HFHSD presented a sharp drop in IL-22 levels, which the highest dose of PTS completely counteracted (**Figure 4C**). Additionally, in the colon, the HFHSD impaired the expression of the antimicrobial peptides (AMPs), known as *Reg3g* and *DefA*, both restored by the highest dose of PTS (**Figure 4D**). There were no changes in the expression of cell renewal markers in the colon (**Figure 4D**) or of AMPs in the ileum (**Figure S3B**). Lastly, in terms of the cecal content, we quantified the sIgA and observed an increase linked to the obesogenic diet, which was not prevented by PTS at either of the doses tested (**Figure 4E**).

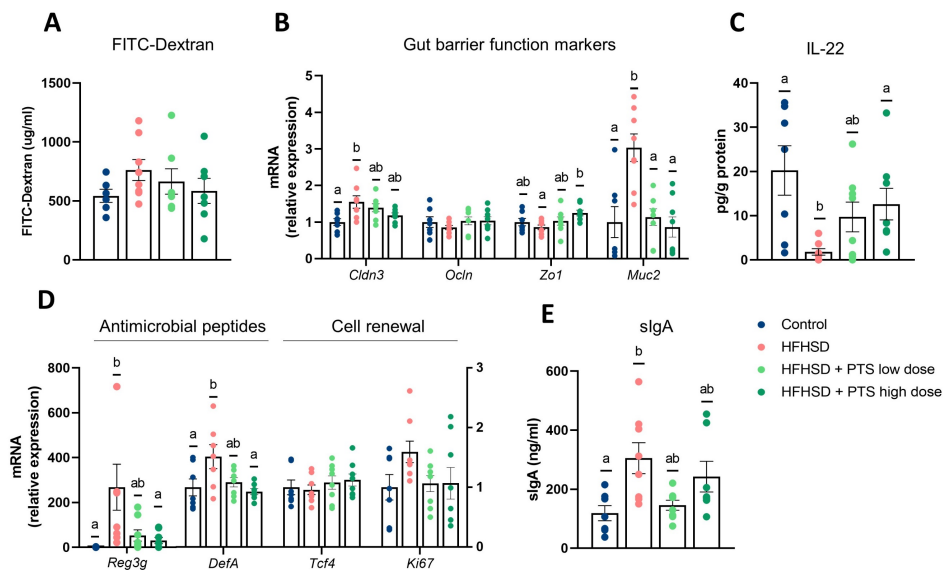


Figure 4. Gut barrier function assessed (A) by FITC translocation in the systemic circulation, and (B) expression of tight junction markers and mucin in the colon, (C) levels of IL-22 in the colon, and (D) colonic expression of antimicrobial peptides and cell renewal markers. In the cecal content, (E) levels of secretory immunoglobulin A (sIgA). Mice were fed a control diet (n = 6–7); a high-fat, high-sucrose diet (HFHSD) (n = 7–8); and HFHSD with propyl propane thiosulfinate (PTS) at a low dose (0.1 mg/kg day) (n = 7–8) and at a high dose (1 mg/kg day) (n = 7–8). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests. Non-normally distributed data were analyzed with the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the post hoc test when $p < 0.05$.

PTS caused no differences in gut microbiota diversity and only minor taxonomic changes

Non-metric multidimensional scaling (NMDS) showing the beta-diversity based on the Bray–Curtis dissimilarity indicated that the samples clustered based on the diet (control versus HFHSD) (**Figure 5A**). Accordingly, the three experimental groups that received the HFHSD presented a significant reduction in the Shannon index (**Figure 5B**) but no changes in the Chao1 diversity index (**Figure 5C**). When considering the changes in those OTUs identified at the genus level, the intake of HFHSD, independently of whether it was accompanied with the PTS treatment or not, caused decreases in *Parabacteroides* (**Figure 5D**) and increases in *Bacteroides* (**Figure 5E**) and *Blautia* (except at the high dose) (**Figure 5F**). Conversely, the administration of PTS at both doses was only associated with increases in the bacterial genus *Intestinimonas* (**Figure 5G**). Additionally, the highest dose of PTS increased *Alistipes* (**Figure 5H**) and reduced the relative abundance of *Bifidobacterium* (**Figure 5I**).

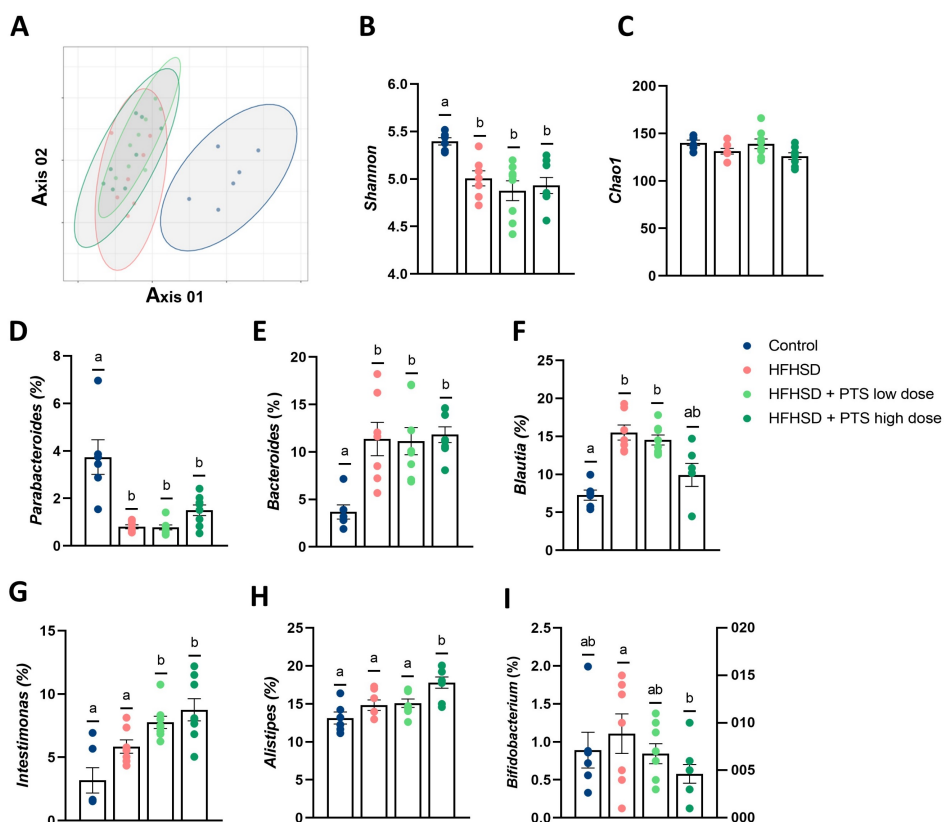


Figure 5. (A) NMDS showing the beta-diversity based on Bray–Curtis dissimilarity, (B) Shannon and (C) Chao1 diversity indexes, genera modified by the obesogenic diet. (D) *Parabacteroides*, (E) *Bacteroides*, (F) *Blautia*, genera modified by the compound propyl propane thiosulfinate

(PTS), (G) *Intestinimonas*, (H) *Alistipes*, (I) *Bifidobacterium*. Mice were fed a control diet ($n = 6-7$); a high-fat, high-sucrose diet (HFHSD) ($n = 7-8$); HFHSD with PTS at low dose (0.1 mg/kg/day) ($n = 8$); and HFHSD + PTS high dose (1 mg/kg/day) ($n = 8$). Taxonomic composition differences were statistically tested by Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$ or $q < 0.05$.

DISCUSSION

Plants belonging to the genus *Allium* were used in ancient medicine for the management of several health conditions. Nowadays, we have scientific evidence that supports this empirical knowledge. For instance, a recent meta-analysis including sixteen randomized clinical trials concluded that garlic supplementation reduces the level of circulating inflammatory markers (C-reactive protein, TNF α , and IL-6)²⁶. The perspective of obesity as an inflammatory condition justifies the search for compounds that counteract inflammation and their subsequent use as new therapeutic tools in this context²⁷. Based on the anti-inflammatory properties recently attributed to some *Allium*-derived compounds^{16,17}, we evaluated the potential effects of PTS in an animal model of diet-induced obesity for the first time. Our results show that PTS prevented adiposity and improved several metabolic parameters through a network of interconnected mechanisms, including anti-inflammatory effects, thermogenesis induction, and gut homeostasis protection.

High-calorie diets lead to gradual body weight gain due to fat deposition. This expansion of adipose tissue is accompanied by adipose macrophage recruitment and inflammation, which are described in other studies and confirmed here^{10,24,28}. Specifically, we describe the potential of PTS to reduce the expansion of the adipose tissue via the downregulation of inflammatory immune cells and chemokines (*Ccl2*, *Itgax*) and of genes involved in lipid storage (*Cd36*, *Lpl* and *Dgat2*). The HFHSD also caused ectopic fat accumulation in the liver that, similarly to what is observed in the adipose tissue, can respond to the overexpression of genes involved in lipid storage and lipogenesis. Hepatic steatosis and pro-inflammatory responses, such as those mediated by IL-6, are frequently accompanied by impaired glucose homeostasis²⁹⁻³³. Accordingly, HFHSD-fed mice displayed an increase in fasting insulinemia and had a worse response to the glucose tolerance test. Conversely, the prevention of fatty liver, together with the down-regulation of pro-inflammatory mediators caused by PTS in the adipose tissue (*Ccl2*, *Itgax*) and liver (IL-6), might have buffered the dietary effects and protected metabolic health, as shown by the better response of PTS-fed mice in the OGTT. In line with our observations, another study reported similar results with the related compound PTSO, which reduced the expression of IL-6 in the liver and inhibited the infiltration of macrophages in the fat and liver¹⁷. As in our case, the authors of the

aforementioned study attribute the anti-obesogenic effect of their compound to its action on these pathways.

Together with the prevention of inflammatory responses, PTS administration caused a sharp increase in the thermogenic activity of BAT. Similarly, the anti-obesogenic properties of other plant extracts, such as rhubarb or camu camu, were linked to the potential of promoting energy expenditure^{10,34}. Moreover, and more specifically, in obese mice treated with allicin, Zhang et al. observed an increase in the thermogenesis of BAT and an inhibition of the decrease in body temperature after cold stimulation¹³. In humans, the activity of brown fat is negatively correlated to body mass index and positively correlated to glucose tolerance and insulin sensitivity³⁵. For this reason, the induction of BAT activity is one of the strategies used to increase energy expenditure and combat obesity^{36,37}, and in the specific case of our study, might have contributed to the lower body weight gain displayed by the mice treated with PTS.

The third mechanism explaining the protective effects of the PTS compound reported herein is related to the preservation of intestinal homeostasis. Although we found no differences in the FITC-dextran test, which measures intestinal permeability, we did find changes in markers of intestinal immune homeostasis. The most notable change concerns the levels of IL-22. In line with our observations, in previous studies, a reduction in IL-22 was associated with hypercaloric diets³⁸. Moreover, this cytokine was closely associated with the regulation of antimicrobial peptides²⁵; however, in our study, the obesogenic diet caused an increase in the AMPs expression of *Reg3g*, and *DefA*. This paradoxical behavior can be explained by the fact that AMPs production does not depend solely on the cytokine IL-22 as demonstrated, for instance, by the absence of differences in the expression of AMPs between IL-22 KO and wild-type mice³⁹. Our study suggests that PTS administration could reverse the changes caused by an obesogenic diet in both IL-22 and AMPs, as one of the most plausible mechanisms explaining its protective effect against obesity.

Gut microbiota changes were proposed to explain the beneficial effects of other organosulfur compounds from the genus *Allium* in the context of obesity. For instance, in the case of allicin, a study transferring feces from mice treated with allicin to new recipients showed that the preventive effects exerted by the organosulphur compound in the former were mimicked in the latter¹³. In the present study, PTS did not substantially change the microbial ecosystem, as concluded by the results of the diversity indexes and the limited taxonomical modifications, suggesting that its protective effects are not primarily mediated by the microbiota. One of the changes consisted of an increase in *Intestinimonas*, a butyrate producer, due to PTS administration at the highest dose. The impact of this genus on metabolic health has received little attention, with one of the few studies describing reductions in obese subjects suffering from binge-eating disorders⁴⁰. Our observation that *Bifidobacterium* was reduced by PTS seems to contrast with

the findings of other authors, who describe increases in this genus with allicin¹³ and PTSO¹⁷ in animal models of obesity. However, an evaluation of the antimicrobial activity exerted by PTS in vitro showed its ability to affect the growth of bifidobacteria⁴¹. Thus, we can speculate that the reductions in the genus *Bifidobacterium* could be due to a direct and specific effect of PTS on this bacterial group, rather than being the consequence of other indirect mechanisms.

Recently, the genotoxicity of PTS in male and female rats was assessed and 55 mg/kg was established as the maximum safe dose²¹. In the present study, we evaluated two dosages (0.1 or 1 mg/kg), which were far below this maximum threshold, and were thus considered safe. Safety concerns aside, these two doses displayed differences in their potential to prevent metabolic alterations in response to an obesogenic diet in this pre-clinical trial.

CONCLUSIONS

Overall, our study provides pre-clinical evidence for the protective effects and mode of action of PTS in an animal model of diet-induced obesity in a dose-dependent manner. Specifically, we demonstrate that the administration of PTS at the highest dose evaluated can prevent body weight gain, adiposity, and alterations in glucose metabolism. The observed effects were mainly attributed to the potential of the compound to reduce inflammation in peripheral tissues, maintain hepatic and intestinal homeostasis and increase thermogenic activity in BAT. In the future, these protective effects of PTS against obesity should also be confirmed in humans.

Author Contributions: R.L.-G., M.O. and Y.S. conceived and designed the experiments. R.L.-G. and M.O. performed the animal experiments. L.G.-M., E.G. and A.B. were responsible for providing the compound. R.L.-G., M.O., S.M.R.-R., V.T.-E. and I.C. were responsible for data acquisition, or data analysis and interpretation. R.L.-G., M.O. and Y.S. wrote the paper. S.M.R.-R., V.T.-E., L.G.-M., E.G., A.B. revised the article and provided important intellectual content. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experiment was approved and performed following European Union 2010/63/UE and Spanish RD53/201 guidelines, approved by the ethics committee of the University of Valencia (Animal Production Section, SCSIE, University of Valencia) and authorized by the competent authority (Generalitat Valenciana). The Generalitat Valenciana

authorized the procedure under the title “Evaluation of bioactive compounds obtained from plant sources” and assigned the code 2020/VSC/PEA/0088 on 26 May 2020.

Data Availability Statement: Amplicon sequences of the 16S rRNA gene of study microbiotas were uploaded to ENA database under project accession number PRJEB50178. Raw data of the parameters analyzed in the study have also been made available as **Supp. Material**.

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Conflicts of Interest: The authors declare that there is no duality of interest associated with this manuscript.

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SUPPLEMENTAL MATERIAL

Gut microbiota composition

Samples were sequenced at IPBLN-CSIC in an Illumina MiSeq run (PE 2x300 output mode) using the 16S ProV3V4 primer pair (amplicon size about 600 bp)¹ and the Nextera XT v2 Index kit for multiplexing. The mean sequencing depth was 140.000 raw reads per sample. Demultiplexed reads were processed using USEARCH v9.2.64². Briefly, R1 and R2 reads were merged (with the parameters minmergelen set to 400 and maxmergelen set to 460) and sequences were trimmed to an equal length of 424 bp after primer removal and prior to quality filtering (maxee parameter set to 2). Chimeras were removed and de novo OTU picking performed (global alignment option set to 97% identity). Taxonomy was assigned using BLAST+ algorithm v.2.12 against the SILVA 132 database³. Very low abundant OTUs (as recommended in⁴), and all nonbacterial, chloroplast, and mitochondrial OTUs were filtered out using QIIME 1.9⁵. Data was rarefied at 10000 sequences per sample. No samples were discarded up to this step, so a total of 31 samples were used in the subsequent analyses. Alpha-diversity was estimated by the Chao1 index (richness) and the Shannon index (abundance and evenness) as calculated in QIIME 1.9⁵. Statistical analyses and plots were performed in R⁶ using the following packages: phyloseq⁷ microbiome⁸, vegan⁹, FSA¹⁰, pairwiseAdonis¹¹, ANCOMBC¹², and ggplot2¹³.

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Table S1: Composition of the diets used in this study

	Control diet (g/100g)	HSFHD (g/100g)
Composition (%)		
Casein	20.7	25
Lard	1.6	20.8
Soybean oil	2.4	2.8
Corn starch	48.85	7
Maltodextrin	14	11
Sucrose	-	20.17
Cellulose powder	5	5.7
L-cysteine	0.25	0.3
Vitamin mixture	1	1
Mineral and trace elements mixture	6	6
Energy content (% kJ)		
Fat	10	45
Protein	20	20
Carbohydrates	70	35

High-fat high-sucrose diet, HSHSD

Table S2: Primer sequences for gene expression analyses by RT-PCR

Targeted gene	Sequence S (5'-3')
<i>Cldn3</i>	F: TCATCGGCAGCAGCATCATCAC R: ACGATGGTGATCTTGGCCTTGG
<i>Zo1</i>	F: TTTTGTACAGGGGGAGTGG R: TGCTGCAGAGGTCAAAGTTCAAG
<i>Occludin</i>	F: ATGTCCGGCCGATGCTCTC R: TTTGGCTGCTCTTGGGTCTGTAT
<i>Tnfa</i>	F: TGTCTCAGCCTCTTCTCATTCC R: TGAGGGTCTGGGCCATAGAAC
<i>Reg3g</i>	F: TTCCTGTCCTCCATGATCAAA R: CATCCACCTCTGTTGGGTTT
<i>DefA</i>	F: GGTGATCATCAGACCCAGCATCAGT R: AAGAGACTAAACTGAGGAGCAGC
<i>Tcf4</i>	F: ATGGCAAACAGAGGAAGTGG R: GCCTGCTGAGAGTGAAGGAG
<i>Ki67</i>	F: CAGACTTGCTCTGGCCTACC R: GGTTGGCGTTTCTCCTCTTT
<i>Ifng</i>	F: TTCTTCAGCAACAGCAAGGC R: ACTCCTTTTCCGCTTCCTGA
<i>Ccl2</i>	F: GCAGTTAACGCCCCACTCA R: CCCAGCCTACTCATTGGGATCA
<i>Itgax</i>	F: FACGTCAGTACAAGGAGATGTTGGA R: ATCCTATTGCAGAATGCTTCTTTACC
<i>Cpt1a</i>	F: TTTGAATCGGCTCCTAATGG R: CCCAAGTATCCACAGGGTCA
<i>Acc1</i>	F: TAATGGGCTGCTTCTGTGACTC R: CTCAATATCGCCATCAGTCTTG
<i>Fas</i>	F: GGAGGTGGTGATAGCCGGTAT R: TGGGTAATCCATAGAGCCAG
<i>Cd36</i>	F: GCCAAGCTATTGCGACATGA R: ATCTCAATGTCCGAGACTTTTCAAC
<i>Dgat2</i>	F: GCGCTACTTCCGAGACTACTT R: GGGCCTTATGCCAGGAACT
<i>Atgl</i>	F: CAACGCCACTCACATCTACGG R: GGACACCTCAATAATGTTGGCAC
<i>Hsl</i>	F: ATGCCACTCACCTCTGATCC R: CTGTCCTGTCCTTCCCGTAG
<i>Pla2ga</i>	F: AAGGATCCCCAAGGATGCCAC R: CAGCCGTTTCTGACAGTTCTGG
<i>Pparg</i>	F: GCATGGTGCCTTCGCTGA R: TGGCATCTCTGTGTCAACCATG
<i>Prdm16</i>	F: CAGCACGGTGAAGCCATTG R: GCGTGCATCCGCTTGTG
<i>Ucp1</i>	F: ACTGCCACACCTCCAGTCATT R: CTTTGCCTCACTCAGGATTGG
<i>Ucp2</i>	F: ACCTTTAGAGAAGCTTGACC R: TTCTGATTTCTGCTACCTC
<i>Cidea</i>	F: TGCTCTTCTGTATCGCCAGT R: GCCGTGTTAAGGAATCTGCTG
<i>Ppara</i>	F: GATGTCACACAATGCAATTC R: CAGTTTCCGAATCTTTCAGG
<i>Pgc1a</i>	F: AGCCGTGACCACTGACAACGAG R: GCTGCATGGTTCTGAGTGCTAAG
<i>Muc2</i>	F: CCCAGAAGGGACTGTGTATG R: TGCAGACACACTGCTCACA

Table S3: Total food and water intakes and plasmatic parameters. Mean and standard error. Mice were fed a control diet, a high-fat high-sucrose diet (HFHSD), HFHSD with propyl propane thiosulfinate (PTS) at low dose (0.1 mg/kg/day) and high dose (1 mg/kg/day). Statistical analyses were performed by Kruskal-Wallis for non-normally distributed data and one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests. Different superscript letters represent statistical differences. Non-esterified fatty acid, NEFA.

	Control diet	HFHSD	HFHSD + PTS low dose	HFHSD + PTS high dose
Total food intake (g/mouse)	81.7 ± 7.4	138.9 ± 12.5	149.3 ± 16.9	135.2 ± 13.1
Total water intake (ml/mouse)	155.6 ± 11.3	163.1 ± 10.1	165.7 ± 8.9	169.1 ± 16.7
Triglycerides	75.6 ± 5.9	60.2 ± 6.4	55.1 ± 4.6	56.5 ± 6.7
Cholesterol	162.1 ± 5.4 ^a	208.2 ± 14.5 ^b	197.3 ± 12.3 ^{ab}	190.5 ± 8.3 ^{ab}
NEFA	81.3 ± 6.5 ^a	67.1 ± 5.2 ^{ab}	64.1 ± 4.6 ^{ab}	59.0 ± 5.4 ^b

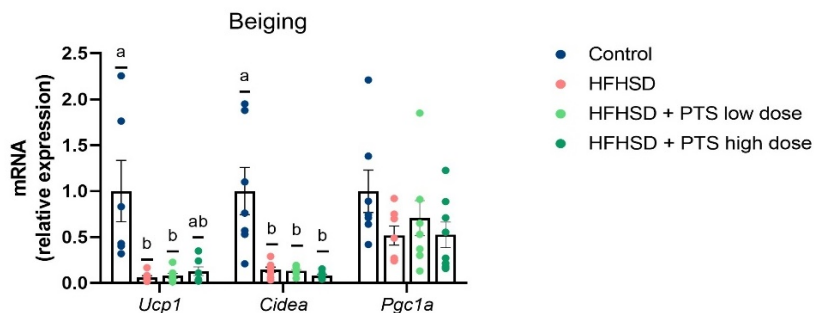


Figure S1: Markers of beiging in the white adipose tissue. Mice were fed a control diet (n=6-7), a high-fat highsucrose diet (HFHSD) (n=7-8), HFHSD with propyl propane thiosulfinate (PTS) at low dose (0.1 mg/kg day) (n=7-8) and high dose (1 mg/kg day) (n=7-8). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests. Non-normally distributed data were analyzed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$.

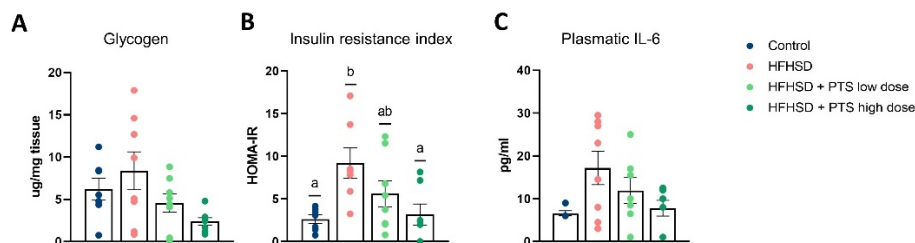


Figure S2. **A)** hepatic levels of glycogen, **B)** index of insulin resistance addressed by the HOMA-IR, **C)** plasmatic levels of IL-6. Mice were fed a control diet (n=5-7), a high-fat high-sucrose diet (HFHSD) (n=7-8), HFHSD with propyl propane thiosulfinate (PTS) at low dose (0.1 mg/kg day) (n=7-8) and high dose (1 mg/kg day) (n=7-8). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests. Nonnormally distributed data were analyzed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$.

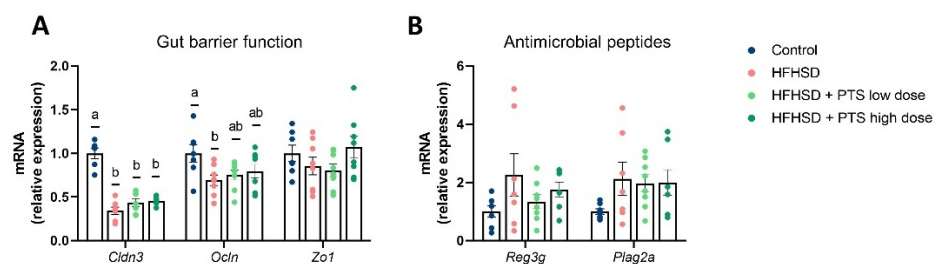
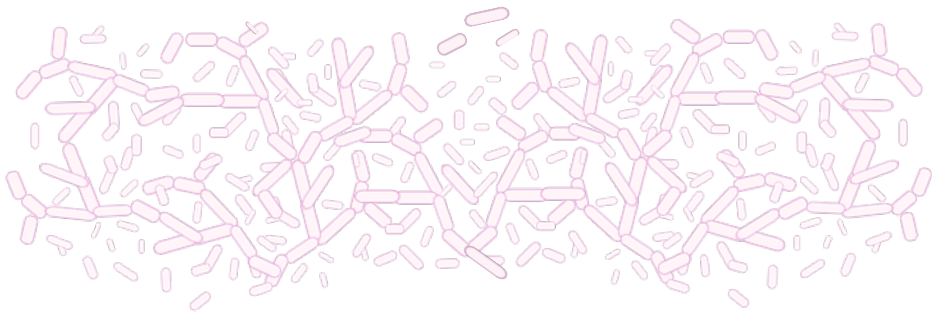


Figure S3: In the ileum, gene expression of **A)** markers of gut barrier function, **B)** antimicrobial peptides. Mice were fed a control diet (n=6-7), a high-fat high-sucrose diet (HFHSD) (n=7-8), HFHSD with propyl propane thiosulfinate (PTS) at low dose (0.1 mg/kg day) (n=7-8) and high dose (1 mg/kg day) (n=7-8). Statistical analyses were performed by oneway analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests for normally distributed data, and Welch's correction was applied when variances were not equally distributed. Different superscript letters show statistical differences in the *post hoc* test when $p < 0.05$.

CHAPTER 2

STRATEGIES BASED ON BACTERIA OF THE HUMAN GUT MICROBIOTA TO TACKLE OBESITY AND MECHANISM OF ACTION

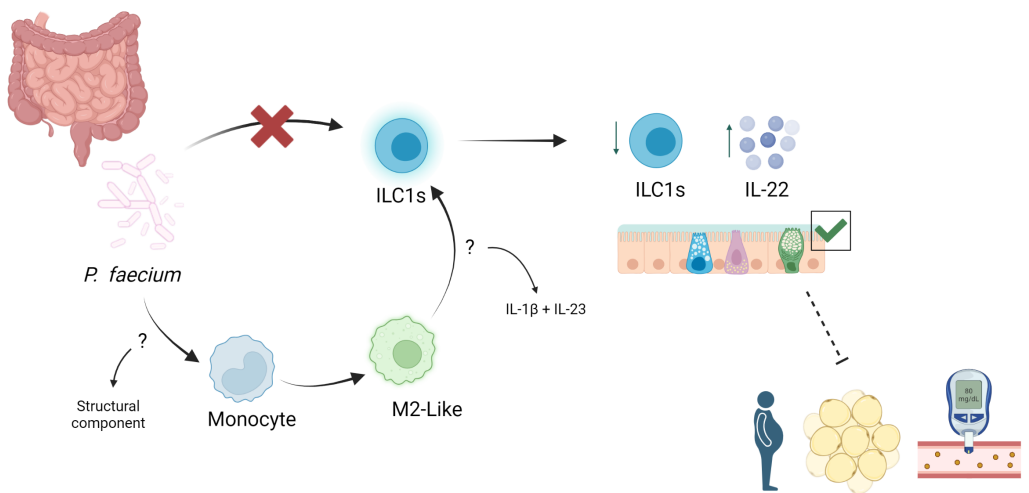


Rebeca Liébana-García*, Inmaculada López-Almela*, Marta Olivares, Paolo Manghi, Marina Romani-Pérez, Verónica Tolosa, Alejandra Flor Duro, Clara Bullich-Vilarrubias, Teresa Rubio, Valerio Rossini, Nicola Segata, Yolanda Sanz. *Phascolarctobacterium faecium* resets the macrophages and innate lymphoid cells dialogue conferring resistance to obesity in mice.
(submitted to Gut)

My contribution to this research article comprehended:

Conceive, design and perform the in vitro and the 3th and 4th animal experiments. Data acquisition, analysis and interpretation. Figure design and manuscript writing.

***PHASCOLARCTOBACTERIUM FAECIUM* RESETS THE MACROPHAGES AND INNATE LYMPHOID CELLS DIALOGUE CONFERRING RESISTANCE TO OBESITY IN MICE**



ABSTRACT

Objective: We have evaluated the mode of action of *Phascolarctobacterium faecium* in obesity to deepen our understanding of how intestinal bacteria suppress diet-induced inflammation and ameliorate metabolic dysfunction.

Design: The anti-obesity properties of live *P. faecium* were evaluated in male wild-type (WT) C57BL/6J, and *Rag1*^{-/-} mice fed a high-fat high-sucrose diet (HFHSD) to induce obesity. The interactions between *P. faecium* and the intestinal immune circuitry were elucidated using cultures of bone marrow-derived macrophages and group 1 innate lymphoid cells (ILC1s). A large metagenomic survey including 6,361 human subjects was performed to confirm the link between *P. faecium* and a non-obese phenotype.

Results: Treatment of HFHSD-fed mice with *P. faecium*, regardless of its viability, shifted the macrophage phenotype towards an M2-type, which counteracted the obesity-induced increase in gut-resident ILC1s and ultimately mitigated glucose intolerance and body weight gain. Moreover, *P. faecium* treatment prevented inflammation, boosted secretory immunoglobulin A production and induced antimicrobial peptide and interleukin 22 expression. These metabolic benefits were maintained in the absence of an adaptive immune system but were lost when the bacterium was co-administered with an inhibitor (GW2580) of M2 macrophage polarization. We confirmed that *P. faecium* was more prevalent in the gut metagenomes of non-obese adults regardless of nationality, sex or age, suggesting that it might contribute to safeguard metabolic health in humans.

Conclusion: *P. faecium* acts as a checkpoint inhibitor of the ILC1-induced inflammatory cascade through the enhancement of gut M2 macrophages, and is associated with normal weight in humans, overall suggesting its potential to protect against obesity and metabolic comorbidities.

INTRODUCTION

Obesity is a complex disease with an upward prevalence mainly due to the adoption of Westernized lifestyle habits worldwide. Obesity greatly contributes to the burden of noncommunicable diseases and causes substantial reductions in quality of life and well-being, accounting for considerable costs that place a strain on healthcare systems, economic productivity, and social resources ¹. The continued exposure to inflammatory dietary insults, such as refined sugars and saturated fats, profoundly impacts on the gut microbiota and impairs the gut barrier function and intestinal immunity ²⁻⁴. The alteration of these systems secondarily influences adiposity, insulin resistance, and other hallmarks of obesity and the metabolic syndrome. Indeed, growing evidence indicates that a skewed gut immunity towards an inflammatory state is an early trigger of obesity and its associated disorders, such as type 2 diabetes (T2D), fatty liver disease or cardiovascular problems ^{5,6}.

In obese individuals, increased recruitment of pro-inflammatory macrophages appears in the stomach, duodenum, and colon that, interestingly, correlates to body weight and unhealthy lifestyle behaviors, like low vegetable or high processed meat intakes ⁷. Similarly, an increase in other leukocytes in the intestinal mucosa, like group 1 of innate lymphoid cells (ILC1s) and pro-inflammatory T helper (Th)1 cells is described in people with obesity ⁸⁻¹⁰. The inflammatory response to energy-dense diets in the gut is thought to prime inflammation downstream in metabolic organs, such as the visceral adipose tissue, a significant driver of obesity-related insulin resistance ^{11,12}. In line with this, the treatment of obese mice with an anti-inflammatory drug used for colitis led to improvements in glucose and insulin tolerance, confirming the contribution of gut inflammation to obesity complications ⁸. It can be hypothesized that obesogenic diets act as pro-inflammatory signals that break the tuning between immune cells and intestinal epithelial cells (IECs) instigating metabolic derangements ¹³. These diet-immune interactions may also affect the gut microbiota and, in turn, the gut microbiota contributes regulating immunity and metabolism ¹⁴. For instance, We and others have found that obesogenic diets reduce intestinal interleukin (IL)-22 ^{14,15}, which is a cytokine that induces antimicrobial peptide (AMPs) secretion by IECs and IgA production, thus regulating the gut microbiota composition ¹⁶. However, supplementation of the obesogenic diet with inulin restores IL-22 production in a microbiota-dependent fashion and mitigates the main obesity alterations ¹⁷.

Epidemiologic studies describe that obesity and obesity risk are associated with gut microbiota aberrancies that, theoretically, could be restored through interventions, constituting a promising tool for nutrition and medicine ^{18,19}. In the last years, efforts have focused on evaluating the role of newly isolated intestinal bacteria that could aid in obesity prevention in preclinical models ¹⁹. For example,

Holdemanella biformis improves glucose homeostasis through modulation of the enteroendocrine systems in diet-induced obesity (DIO) models ²⁰. And *Akkermansia muciniphila* mitigates obesity-associated alterations through its ability to protect the gut barrier potentially via mucus production and Toll-like-receptor (TLR)2 signaling ²¹ and to increase the glucagon-like peptides 1 (GLP-1) secretion ²², in DIO models. It has been also demonstrated that *A. muciniphila* effectively improves insulin sensitivity, reduces insulinemia, and total plasma cholesterol in obese adults ²³. Here, we have evaluated the potential of *Phascolarctobacterium faecium* to mitigate obesity motivated by previous observational studies that associated this genus to a lower risk of developing excessive weight gain in children ¹⁸ and greater weight loss under a lifestyle intervention in overweight adults ²⁴. Specifically, we deciphered the mechanisms by which the intestinal symbiont *P. faecium* DSM 32890 interacts with the host immune system to mitigate obesity using a DIO murine model. We also confirmed the relevance of this bacterial species as a biomarker of obesity in humans by performing a large meta-analysis.

MATERIALS AND METHODS

Animal experimental design

Four animal experiments were performed. Seven-week-old C57BL/6J wild-type (WT) mice were purchased from Charles River Laboratories (Écully, France) and *Rag1*^{-/-} mice with a C57BL/6J background were supplied by The Jacksons laboratory (Bar Harbor, Maine, USA). In all experiments, mice were randomly housed in groups of 4-5 animals per cage in a ventilated rack under controlled temperature (23 ± 2°C), relative humidity (40-50%), and a 12-hour-light/dark cycle. Mice were acclimatized for 10 days and had *ad libitum* access to water and food. Mice were randomized based on body weight to minimize baseline differences. Body weight evolution and food and water intake were monitored once or twice per week.

Experiment 1. 30 WT males C57BL/6J mice were divided into three experimental groups (n=10): (i) fed a control diet (D12450K Ssniff; 10% of energy from fat and no sucrose), (ii) fed a high-fat high-sugar diet (HFHSD, D12451 Ssniff; 45% of energy from lard fat, and 17% from sucrose), and (iii) fed HFHSD and orally gavage with a dose of *P. faecium* of ~2x10⁹ colony forming units (CFUs)/day (HFHSD + *P. faecium*) for 14 weeks. PBS supplemented with 0.05% L-cysteine and 10% glycerol was used as vehicle for the control groups. No adverse effects due to any of the treatments were observed.

Experiment 2. 30 males *Rag1*^{-/-} mice were divided in three experimental groups to receive for 14 weeks: (i) control diet, (ii) HFHSD, and (iii) HFHSD + *P. faecium* (~2x10⁹ CFUs/day). Control groups received the corresponding vehicle.

Experiment 3. 32 males C57BL/6J mice were divided into four groups treated for 14 weeks with: (i) control diet, (ii) HFHSD, (iii) HFHSD + *P. faecium* and, (iv) HFHSD and a daily dose of *P. faecium* pasteurized (HFHSD + *P. faecium* pasteurized). Viable and pasteurized bacteria were administered orally ($\sim 2 \times 10^9$ CFUs/day). Control groups received the corresponding vehicle.

Experiment 4. 40 males C57BL/6J mice were treated with (i) control diet, (ii) HFHSD, (iii) HFHSD + *P. faecium* ($\sim 2 \times 10^9$ CFUs/day), (iv) and HFHSD + *P. faecium* + GW2580 (inhibits colony stimulating factor 1 receptor phosphorylation) to avoid M2 macrophages polarization, starting at week 4 (HFHSD + *P. faecium* + GW2580). For validation purposes, a fifth group was treated with HFHSD and the inhibitor (HFHSD + GW2580). The inhibitor was dissolved in PBS 0.1% Tween 80 and 0.5% hydroxyl methyl cellulose and orally administered at 160 mg/kg/day as previously described²⁵. Control groups received containing PBS with 0.1% Tween 80, 0.5% hydroxyl methyl cellulose, 0.05% L-cysteine and 10% glycerol. Additionally, group (iii) received a vehicle containing PBS with 0.1% Tween 80 and 0.5% hydroxyl methyl cellulose. All groups received the same final volume of the respective treatments.

At the end of the experiment animals were fasted, anesthetised with isoflurane, and sacrificed one animal from each group intercalary by cervical dislocation. Blood was collected by cardiac puncture in EDTA-containing tubes. Plasma was immediately collected after centrifugation (1000xg, 5 min, 4°C). Plasma, intestinal tissue, adipose tissue (AT), liver, and cecal content were snap-frozen in liquid nitrogen or keep on ice for flow cytometry analysis. All samples were stored at -80°C until further use.

Statistical analysis

G*Power 3.1.9.2 was used to calculate sample size allowing the primary outcomes (adiposity and glucose metabolism) and GraphPad software (v.9 San Diego, CA, USA) for data analysis. Grubbs test was used for outlier detection and Shapiro–Wilk test was employed to assess data normality. One-way analysis of variance (ANOVA) or two-way ANOVA (as suitable) followed by *post hoc* Tukey's multiple comparison tests was apply to determine differences for normally distributed data. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to analyse non-normally distributed data. The results are shown as mean $\pm$ SEM and n represent the number of biological replicates shown as individual dots. Differences with p-values <0.05 were considered significant and are represented using different superscript letters unless otherwise stated. Data analysis was not blinded.

Please see supplemental material for Supplementary Methods.

RESULTS

P. faecium curbs body weight gain and restores glucose homeostasis.

The HFHSD increased body weight gain and epididymal white adipose tissue (eWAT), while *P. faecium* reduced body weight gain and fat depots in HFHSD-fed mice (**Fig. 1A-C**). Additionally, *P. faecium* fully normalized the plasmatic levels of triglycerides increased by the HFHSD but had no effect on cholesterol levels (**Fig. 1D, Supp fig.1A**). The HFHSD also disrupted the glucose metabolism but *P. faecium* improved whole-body glucose clearance and restored the basal glucose levels and, consequently, the index of the homeostatic model assessment for insulin resistance (HOMA-IR) (**Fig.1E-G, Supp fig.1B**), but not insulinemia (**Fig.1H**). *P. faecium* reduced circulating levels of leptin and glucose-dependent insulintropic polypeptide (GIP) that were dramatically increased by the HFHSD (**Fig. 1I, J**). Together, these results demonstrated the potential of *P. faecium* to mitigate the metabolic disturbances triggered by an obesogenic diet.

P. faecium restores the HFHSD-induced intestinal and systemic inflammation

HFHSD increased the proportions of intestinal ILC1s and induced intra-epithelial lymphocytes (IELs), but both were normalized by *P. faecium* (**Fig. 2A, B**). The bacterium *per se* enhanced the percentage of natural IELs reducing the induced/natural IELs ratio increased by the HFHSD (**Fig. 2C, D**). The HFHSD shifted the phenotype of intestinal macrophages increasing the dominance of a pro-inflammatory phenotype as showed the increased M1/M2 macrophage ratio attributed to the reduction of M2 population (**Fig. 2E-G**). In contrast, *P. faecium* caused a reduction in the M1/M2 ratio by boosting the M2 and slightly reducing the M1 population ($p=0.052$) (**Fig. 2E-G**). *P. faecium* substantially increased gut T regulatory (Treg) cells compared to control and HFHSD-fed mice (**Fig. 2H**).

We analyzed the expression of proteins related to lymphocyte anchoring and cytolytic activity in the gut, such as $\alpha E\beta 7$ integrin (known as CD103) and the granzyme B (*GrB*), respectively. *P. faecium* increased the expression of both genes (**Fig. 2I**). The same response was observed in secretory immunoglobulin A (sIgA) and the expression of the AMPs *Pla2g2a* and *Reg3 γ* which were increased by *P. faecium* (**Fig. 2J, K**). The bacterium did not change the gut barrier markers claudin-3 (*Cldn3*) or occludin (*Ocln*) compared to HFHSD-fed mice (**Fig. 2L**) or plasmatic lipopolysaccharide-binding protein (LBP) levels (**Fig. 2M**). HFHSD increased most of the plasmatic cytokines quantified (7 out of 8) with the exception of IL-1 β , but *P. faecium* normalized all cytokines concentrations to control diet fed-mice level (**Fig. 2N-O**). Our results suggest that *P. faecium* buffers the HFHSD-induced intestinal immune cell dysregulation and improves the gut defense mechanisms associated with a reduction of the systemic inflammation.

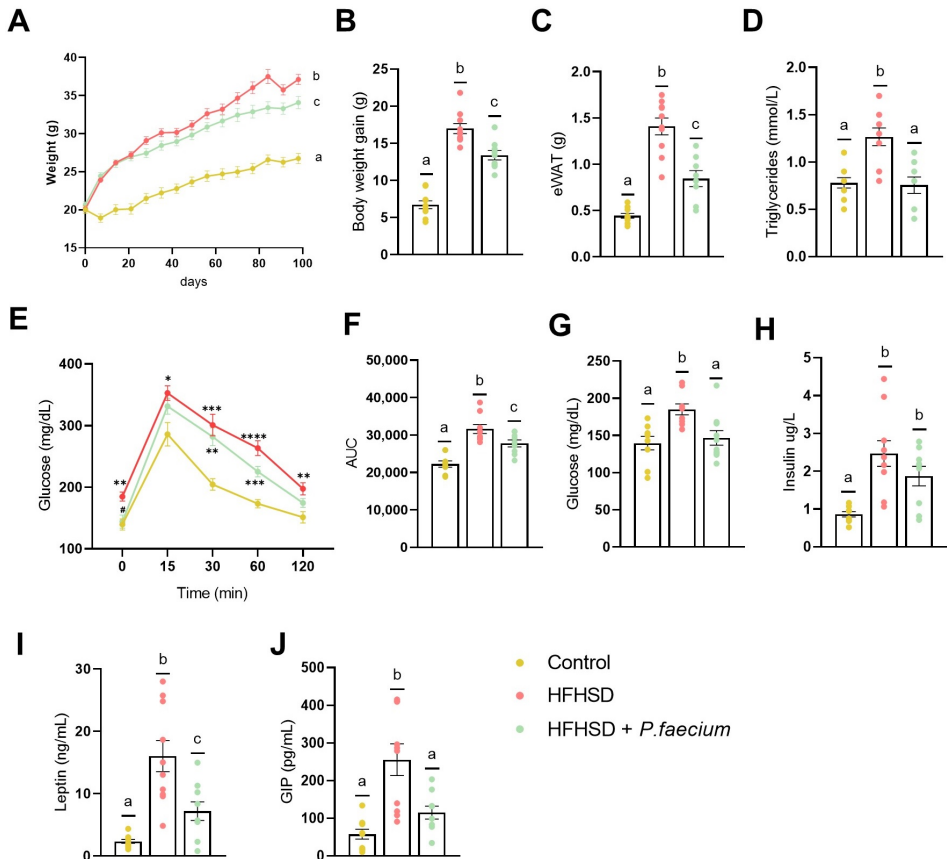


Figure 1. (A) Body weight evolution, (B) body weight gain (g), (C) weight (g) of epididymal white adipose tissue (eWAT), (D) triglycerides concentration in plasma, (E) blood glucose levels after an oral load of glucose (2 g/kg) and (F) area under the curve (AUC) at week 10 of intervention, (G) fasting glucose (mg/dL), (H) insulin (ug/L), (I) leptin (ng/mL) and glucose-dependent insulinotropic polypeptide (GIP) (pg/mL) levels in plasma. Values are presented as mean (n=8-10) $\pm$ SEM. Significant differences were assessed by two-way ANOVA or one-way ANOVA. Different superscript letters, asterisk (*) or pound sign (#) represent statistical significance (at $p < 0.05$). “**” is used when compared to control diet, and “#” when compared to HFHSD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and # $p < 0.05$.

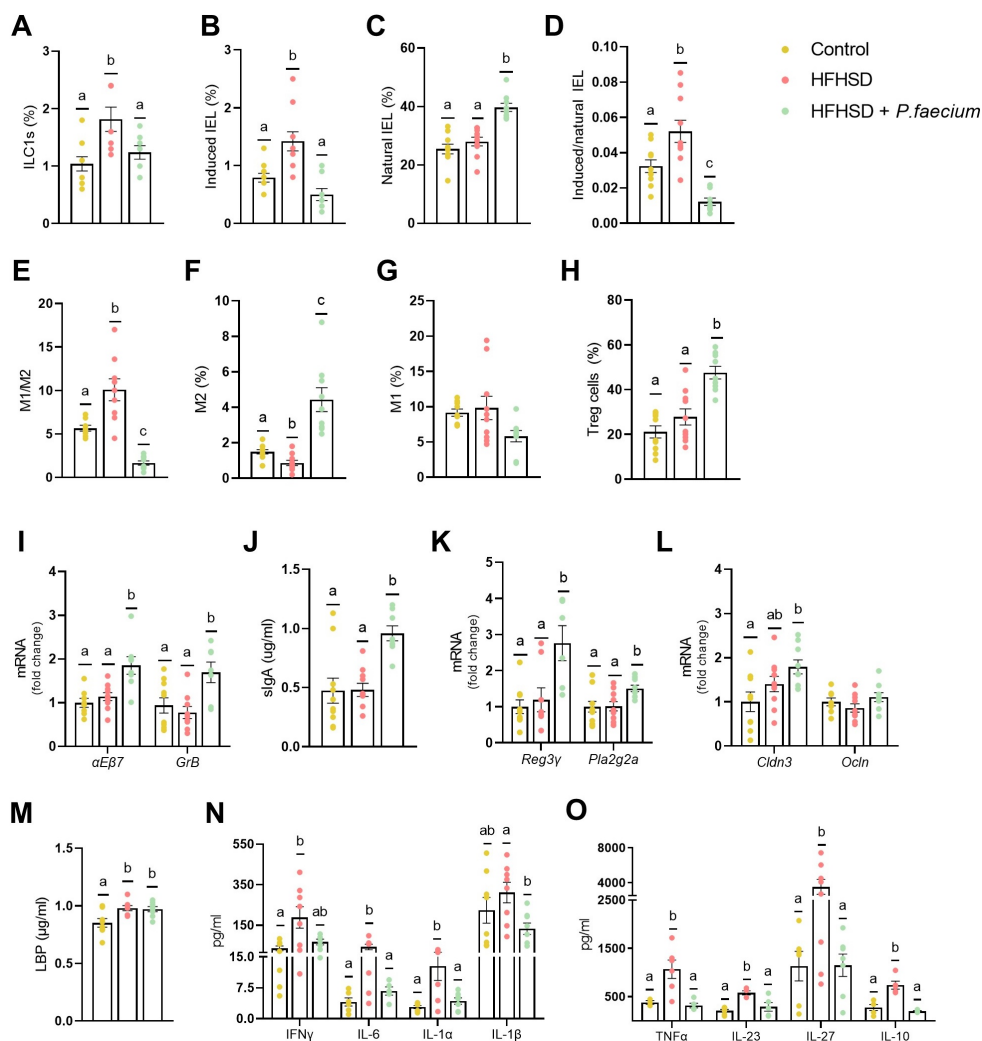


Figure 2. In the small intestine, (A) ILC1 cells of total intestinal epithelial cells), (B) Induced intraepithelial lymphocytes (IEL) (percentage of CD45⁺ cells of the total intestinal epithelial cells), (C) natural IEL (percentage of CD45⁺ cells of the total intestinal epithelial cells), (D) induced and natural IEL ratio, (E-G) pro-inflammatory (M1) and anti-inflammatory (M2) macrophages (percentage of F4/80⁺ cells of the total lamina propria cells) and ratio of M1 to M2, (H) regulatory T (Treg) cells (percentage of CD4⁺ cell of the total lamina propria cells), (I) mRNA levels of $\alpha E\beta 7$ integrin and *granzyme B* in the small intestine, (G) secretory immunoglobulin A (sIgA) in the cecal content (ug/mL), (K) mRNA levels of *Reg3 γ* and *Pla2g2a* and (L) mRNA levels of claudin (*Cldn*) 3 and occluding (*Ocln*) in the small intestine, (M) plasmatic lipid binding protein (LBP) levels (ug/mL), (N-O) plasmatic levels of the indicated cytokines (pg/mL). Values are presented as mean (n=8-10) ± SEM. Significant differences were assessed by one-way ANOVA or Kruskal-Wallis test. Different superscript letters represent statistically significance (at p < 0.05).

P. faecium* improves metabolic health in absence of adaptive immunity in obesity and directly promotes M2 macrophage polarization *in vitro

To test the role of adaptive immunity in the metabolic effects of *P. faecium*, we used utilized *Rag1*^{-/-} mice, which lack mature B and T cells. Results showed that *Rag1*^{-/-} mice also gained weight and fat mass under HFHSD-feeding accompanied by glucose intolerance (Fig. 3A-D). *P. faecium* treatment prevented these metabolic hallmarks despite the lack of adaptive immunity (Fig. 3A-D). Interestingly, *P. faecium* boosted the proportions of M2 macrophages and prevented the HFHSD-induced increase in ILC1s (Fig. 3E, F), as in WT mice (Fig. 2A, F). Whereas, M1 macrophages were unaffected either by the diet or the bacterium (Fig. 3G).

Given these findings, we next investigated the nature of the interactions between *P. faecium*, macrophages and ILC1s *in vitro*. Flow cytometry analysis revealed that exposure of bone marrow-derived macrophages (BMDMØ) to *P. faecium* (1:10 cell/bacteria) triggered a marked increase in the expression of M2 differentiation markers (Arg1, CD206 and CD163) (Fig. 3H), which was accompanied by increases in IL-10 and IL-1β concentrations in the supernatant of *P. faecium*-stimulated BMDMØ cultures (Fig. 3I). Interferon (IFN)γ, IL-22, IL-23, IL-12p70, GM-CSF and IL-4 remained below the detection threshold. We next questioned whether the changes in ILC1s were macrophage-mediated by exposing intestinal ILC1s cultures to the supernatants of *P. faecium*-stimulated BMDMØ cultures. Results showed increased gene expression of *Ifng*, *Tbx21*, *Il22*, and *Il23r*, decreased *Rorc* and caused no changes in *Tnfa* in ILC1s cultures (Fig. 3J). We also detected higher IL-22 concentrations in ILC1s cultures and a slight IFNγ reduction ($p=0.087$) (Fig. 3K). None of the effects were reproduced in ILC1s cultures directly exposed to the bacterium (1:10 cell/bacteria) (Fig. 3L). Therefore, the protective effects of *P. faecium* are not mediated by the adaptive immune system; instead, the bacterium primes macrophages towards the M2 phenotype which, in turn, control the inflammatory cascade patrolled by ILC1s.

***P. faecium* exerts anti-obesogenic effects and boost M2 macrophages *in vivo* regardless bacterial ability**

We next evaluated whether the immunomodulatory properties of *P. faecium* on M2 cells required the viability of the bacterium by treating HFHSD-fed mice with live or pasteurized bacteria. Pasteurized *P. faecium* succeeded in triggering the intestinal M2 increase to the same extent as live bacteria (Fig.4A). Both treatments equally reduced the M1/M2 ratio increased by the HFHSD (Fig. 4B), although differences were detected in M1 (Fig 4C). Similarly, pasteurized *P. faecium* reduced ILC1s percentages as did the live bacteria (Fig. 4D). Pasteurized *P. faecium* exerted the same metabolic benefits as live bacteria in terms of body weight gain reduction and oral glucose tolerance (Fig.4E-G). Therefore, the anti-obesogenic and M2 immune effects of *P. faecium* do not depend on the bacterium viability.

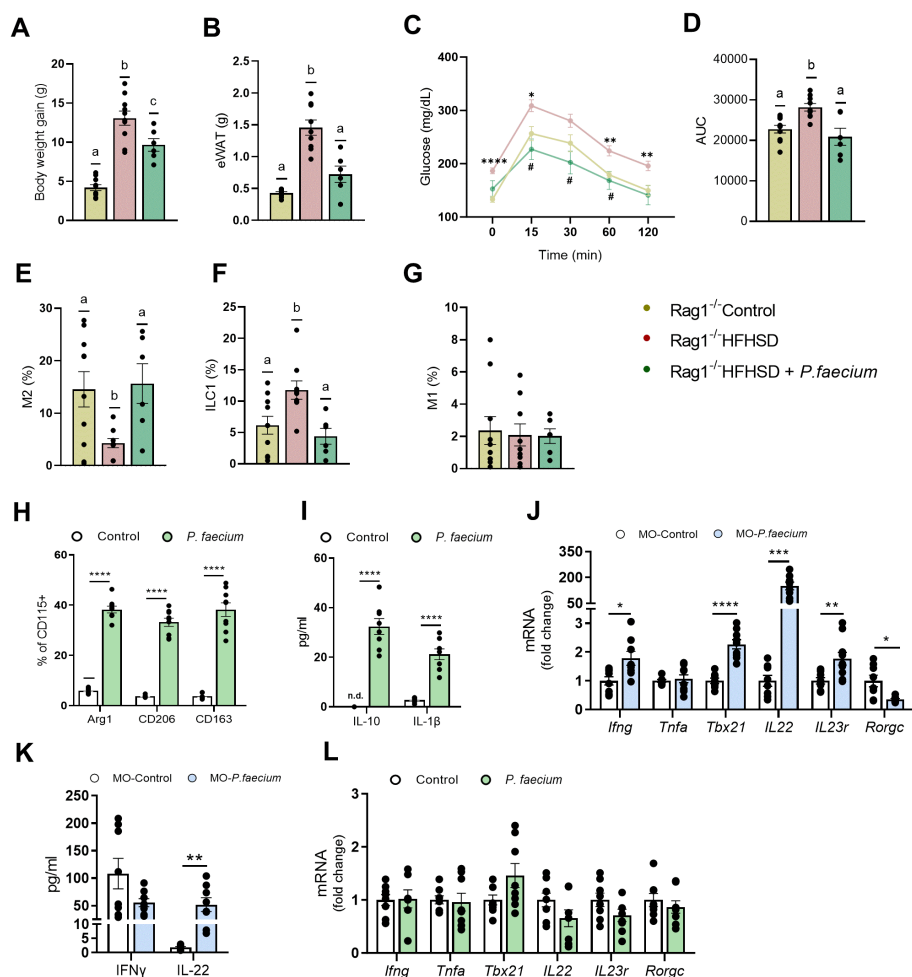


Figure 3. (A) Body weight gain (g), (B) weight (g) of epididymal white adipose tissue (eWAT), (C) blood glucose levels after an oral load of glucose (2 g/kg) and (D) area under the curve (AUC), (E) anti-inflammatory (M2) macrophages (percentage of F4/80+ cells of the total lamina propria cells) in the small intestine, (F) ILC1 (percentage of LIN- cells of total intestinal epithelial cells) in the small intestine, (G) pro-inflammatory (M1) macrophages, (percentage of F4/80+ cells of the total lamina propria cells), (H) Arg1, CD206 and CD163 (percentages of CD115+ in total bone marrow-derived macrophages (BMDM0)) and (I) cytokine levels in the BMDM0 culture supernatant (pg/mL), (J) mRNA levels of intestinal ILC1s cultures exposed to control BMDM0-supernatants (MO-control) or supernatants of *P. faecium* primed BMDM0 (MO-*P. faecium*) and (K) cytokine levels in the ILC1 culture supernatant (pg/mL), (L) mRNA levels of pro-inflammatory genes of intestinal ILC1s cultures challenged with *P. faecium*. Values are presented as mean (n=8-10) ± SEM. Significant differences were assessed by two-way ANOVA, one-way ANOVA or by unpaired t-test. Different superscript letters, asterisk (*) or pound sign (#) represent statistically significance at p < 0.05. “**” is used when compared to control diet, and “#” when compared to HFHSD. *p<0.05, **p<0.01, ****p<0.0001 and #p<0.05.

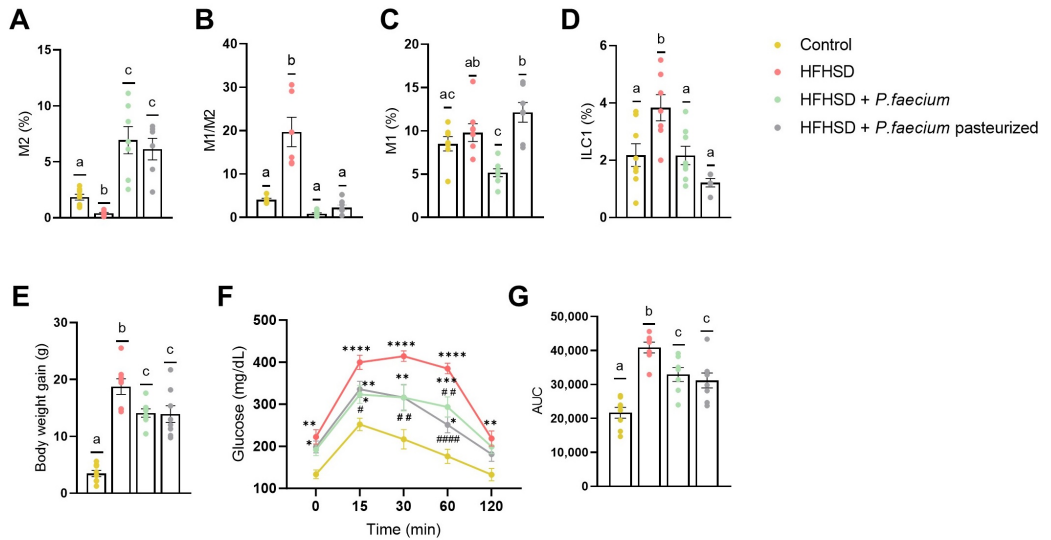
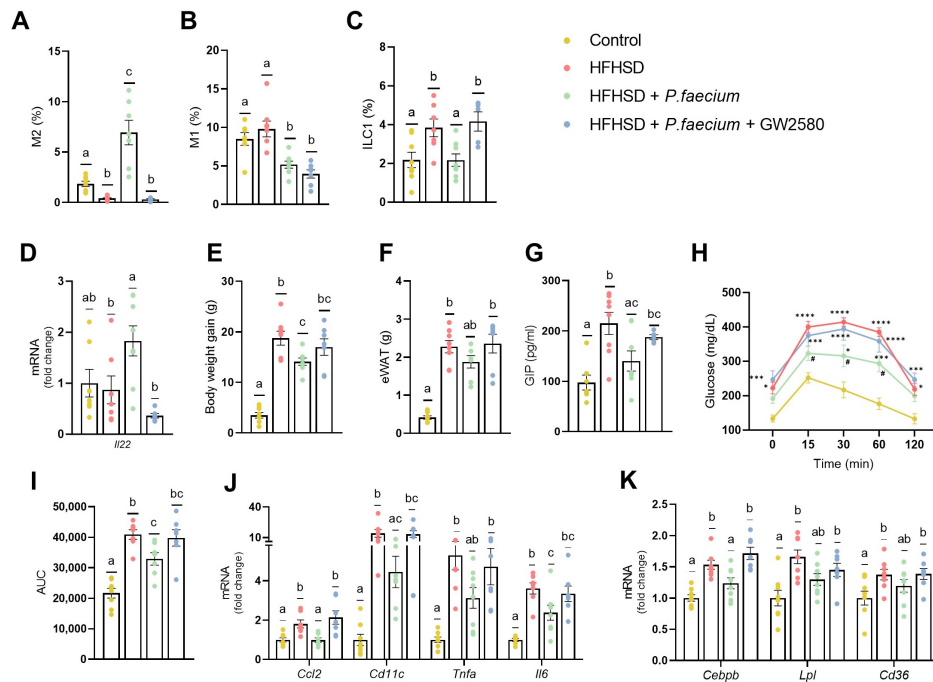


Figure 4. In the small intestine, (A) anti-inflammatory (M2) macrophages (percentage of F4/80+ cells of the total lamina propria cells), (B) ratio of M1 to M2, (C) pro-inflammatory (M1) macrophages (percentage of F4/80+ cells of the total lamina propria cells), (D) ILC1 (percentage of LIN- cells of total intestinal epithelial cells), (E) body weight gain (g), (F) blood glucose levels after an oral load of glucose (2 g/kg) and (G) area under the curve (AUC) at week 10 of intervention. Values are presented as mean (n=7-8) $\pm$ SEM. Significant differences were assessed by two-way ANOVA or one-way ANOVA. Different superscript letters, asterisk (*) or pound sign (#) represent statistically significance at $p < 0.05$. “**” is used when compared to control diet, and “#” when compared to HFHSD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, # $p < 0.05$, ## $p < 0.01$ and #### $p < 0.0001$

P. faecium display metabolic benefits through M2 macrophages.

We evaluated whether the induction of M2 macrophages is the mechanism through which *P. faecium* exerts metabolic benefits in obesity. With this aim, we administered GW2580 to specifically inhibit M2 polarization as previously described^{26,27}. First, we proved that the GW2580 administration did not affect body weight gain, adiposity, or glucose tolerance in HFHSD-fed mice (Sup fig 2A-D). Then, we confirmed, that when *P. faecium* was administered together with GW2580, the increase in intestinal M2 macrophages was blocked (Fig. 5A) without detectable changes in the M1 levels (Fig. 5B). Remarkably the inhibition of the M2 macrophage increase also blunted some of the beneficial effects of *P. faecium*, such as the reductions of ILC1s (Fig. 5C) and the increases of *Il22* expression in the small intestine (Fig. 5D). However, it had no impact on *Tfna* or *Il10* expressions (Sup fig 2E). Moreover, *P. faecium* failed to prevent successfully weight gain, adipose expansion, and GIP increases when the M2 increase was inhibited (Fig. 5E-G). Similarly, the beneficial effects on glucose tolerance and basal glycemia elicited by *P. faecium* were partly lost in mice treated with GW2580 (Fig. 5H-I; Supp fig. 2 F). Lastly, we explored the consequences of M2 inhibition in metabolically active organs. In the eWAT, the M2 blockade impeded

P. faecium-induced reduction of the *Ccl2* expression (coding for the monocyte chemoattractant protein 1 [MCP1]), which is involved in immune cell recruitment (Fig. 5J). Similarly, the M2 inhibitor blunted *P. faecium*-induced reductions on *Cd11c*, *Tnfa* and *Il6* expression (Fig. 5J). These changes in the inflammatory milieu also impacted metabolic functioning of the WAT. When the M2 polarization was inhibited, *P. faecium* failed to reduce adipogenesis, as reflected in the *Cebpb* expression (Fig. 5K), which was not modified compared to the obese group. In the liver, *P. faecium* reduced IL-6, TNF α and increased IL-22 concentrations, but these effects tended to be less accused in the presence of the M2 inhibitor (Supp fig. 2G-I). Our results indicated that the M2 macrophages are mediators of the metabolic benefits of *P. faecium* in the DIO obesity model.



***P. faecium* partially restores the HFHSD-induced changes in the gut microbiota.**

Analysis of the microbiota structure revealed that *P. faecium* partially normalized the HFHSD-induced alterations, but not when co-administered with the M2 inhibitor (Fig. 6A; Supp. Fig 2J). All HFHSD-fed groups showed decreased microbial richness (Fig. 6B). Only the group treated with the M2 inhibitor showed reduced Shannon's alpha-diversity index and normalized population evenness (Fig. 6C, D). Regarding the taxonomy, 9 ASVs identified at genus (2 out of 9) or specie (7 out of 9) levels were modified by *P. faecium* compared to non-treated HFHSD-fed mice (Supp. Table 3). *P. faecium* buffered the HFHSD-induced reduction of the two mucus-dwelling bacteria *A. muciniphila* and *Mucispirillum* spp. and that these effects disappeared when the M2 inhibitor was administered together with the bacterium (Fig. 6E, F). *P. faecium* also increased the abundance of butyrate producers like *Ruminococcaceae*_UBA1819 spp., but this effect was not blunted by the M2 inhibitor (Fig. 6G). Therefore, *P. faecium* avoided the loss of functionally relevant bacterial taxa caused by the HFHSD and the M2 blockade blunted some of the benefits of *P. faecium* in the intestinal ecosystem.

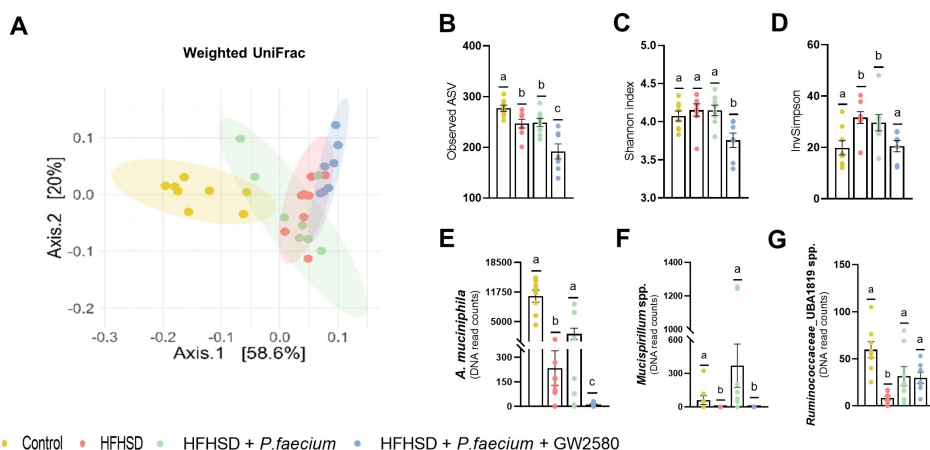


Figure 6. In the cecal content, (A) beta-diversity based on weighted UniFrac distances, (B) observed amplicon sequence variants (ASVs), (C) Shannon diversity index, (D) Inverse Simpson index, (E) and normalized abundance of (E) *Akkermansia muciniphila*, (F) *Mucispirillum* spp. and (G) *Rumunococcaceae* UBA1819 spp. Values presented are mean (n=8) $\pm$ SEM. Non-parametric methods were applied for statistical analysis of alpha diversity and differential abundance analysis was performed using DESeq2 v.1.36 R package. The resulting p-values were corrected using the Benjamini-Hochberg false discovery rate (BH FDR) procedure. Different superscript letters represent statistically significance at $p < 0.05$.

P. faecium is linked to normal-weight in a large-human multi-cohort study

We finally assessed whether the presence of *P. faecium* is associated with body weight in a multi-cohort study including 6,361 stool metagenomes from adult subjects (20 nationalities) from curatedMetagenomicData 3 (unpublished data). In total, we compared the metagenomes of 1,295 obese individuals with those of 5,066 normal-weight subjects (Fig. 7A). The detection of *P. faecium* was 17% less likely to happen in obese subjects (log odd-ratio = -0.188, $p = 0.016$) (Fig. 7B). The detection of *P. faecium* was also 15% less likely to happen in obese individuals when also sex and age were considered as confounding factors in the analysis ($p = 0.037$). Therefore, *P. faecium* is associated with leanness in the healthy, adult, human population regardless of the nationality, and remains such when sex and age are also taken into consideration.

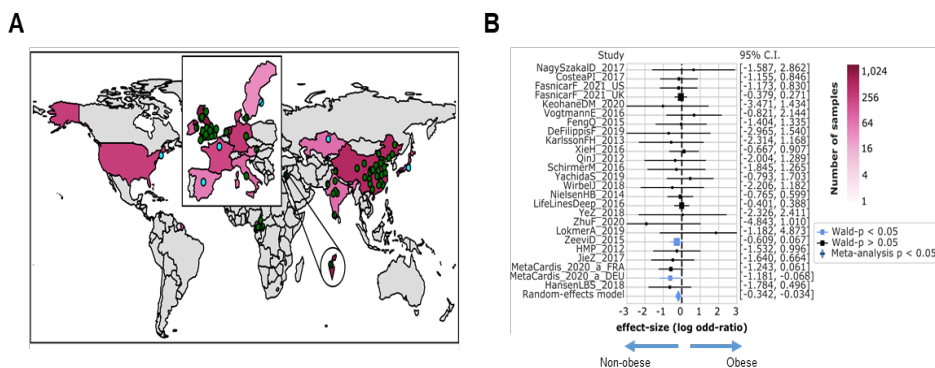


Figure 7. (A) Gut microbiome from obese and normo-weight adult participants from 16 countries in curatedMetagenomicData3, and (B) meta-analysis result regarding the prevalence of *P. faecium*. To assess differential detection of *P. faecium*, wald-test of the logistic regression coefficients relative to obesity yes/no were used to assess significance of the log odd-ratio with respect to the detection of *P. faecium* while the meta-analysis coefficient was computed as a Z-score of the tendency of the mean effect to be greater than zero. $p < 0.05$ was used as the significance threshold.

DISCUSSION

Herein, we demonstrate the anti-obesogenic properties of *P. faecium* DSM 32890, a new symbiotic intestinal bacterial strain, and its mode of action on specific intestinal immune circuits. Taking together, our results showed that *P. faecium* reduces around 25% the body-weight gain, 35% the adiposity, and improves glucose homeostasis, independently of the bacterium viability. These protective effects are mediated by the ability of the bacterium to boost the M2 macrophages polarization which, in turn, blunted the expansion of proinflammatory ILC1, as proven by blocking M2 cells *in vivo*. Moreover, the meta-analysis of metagenomic data from a large multi-cohort study in humans

showed that *P. faecium* may safeguard metabolic health as its prevalence is associated with human leanness independently of sex, age and nationality.

The gut immune system is constantly challenged by the consumption of hypercaloric diets²⁸. Certainly, chronic exposure to energy-dense foods triggers chronic inflammation in the gut, which plays an important role in long-term metabolic complications of obesity⁵. Here we show that the HFHSD intake prompted the accumulation of pro-inflammatory cell subsets, such as induced IELs (TCR $\alpha\beta^+$) and ILC1s and an increase of M1/M2 ratio in the small intestine, partly in line with previous studies^{29,30}. In the context obesity, this represents an early pathogenic gut immune signature, that if sustained becomes systemic and causes metabolic dysfunction^{29,31}. Despite the obesogenic diet is the primary trigger, diet-induced microbiota alterations are also causally involved in the dysregulation of gut immunity and inflammation, as demonstrated via fecal microbial transplants¹². Interestingly, alterations in the gut immune system can be reverted and, thus, it could become a rising therapeutic target for obesity control⁸. In this sense, the inhibition of intestinal M1 macrophages infiltration in genetic engineering animals (e.g., Ccr2 KO mice and tamoxifen-inducible models) or by the oral administration of gut anti-inflammatory molecules (e.g., 5-aminosalicylic acid) effectively pale obese-derived inflammation and metabolic alterations^{8,11}. Similarly, here we noticed that controlling the intestinal immune response to energy-dense diets with the administration of the intestinal bacteria especially prevalent in lean human subjects, such as *P. faecium*, could alleviate systemic HFHSD-triggered inflammation and confer resistance to obesity development. In fact, *P. faecium* prevented the increases in pro-inflammatory immune cells in the gut and drastically reduce the plasmatic levels of inflammatory cytokines in the different DIO models and studies here presented.

Besides, in WT HFHSD-fed mice, *P. faecium* elicited an intestinal pro-tolerogenic and anti-inflammatory tone evidenced by the increases in Treg cells, natural (TCR $\gamma\delta^+$) IELs, and M2 macrophages, that are potential underlying protective immune mediators. Furthermore, our findings in *Rag1^{-/-}* mice (lacking T and B cells) revealed that the adaptive immunity is no essential for *P. faecium* to prevent body weight gain and metabolic dysfunction, proving that IELs and Tregs are not the most critical cell subsets for controlling obesity-associated inflammation and the metabolic dysfunction. Instead, changes in intestinal M2 macrophages and ILC1s could be the primary drivers. The role of macrophages in obesity has been thoroughly studied^{6,11,31}, as well as the benefits of inducing a M2 polarization for reversing obesity-associated inflammation³². Notwithstanding, evidence of the ability of *P. faecium* to reduce the ILC1 by priming macrophages towards a M2 phenotype, expands our previous knowledge. ILCs are known to be involved in tissue homeostasis, morphogenesis, and regeneration³³, but particularly the role of gut resident ILC1 in the regulation of metabolism and obesity is still largely unknown. Okamura *et al.*, Okamura *et al.*, related the increased gut ILC1 (and T-bet⁺ ILC3 cells) with the reduction of IL-22, and the exacerbation of metabolic

disease in a DIO murine model ¹⁴. We have recently published that the blockage of the intestinal ILC1s increase, induced by the HFHSD in mice, prevented the M1 macrophages skewing and reinforced the intestinal barrier, thus alleviating the metabolic deleterious effects ³⁰. Here, we have shown that *P. faecium* administration reduces intestinal ILC1s along with improvements in the gut defensive mechanisms (reflected in increased sIgA production and over-expression of AMPs and IL-22) ³⁰. Collectively, our results reveal a chain of immune events induced by *P. faecium* in obesity. The bacterium directly primes M2 macrophages polarization which, in turn, reduces the obesity-induced ILC1s numbers, and induces IL-22 (expression in vivo and production in vitro). It is tempting to speculate that *P. faecium*-primed macrophages prompts the conversion of ILC1 into ILC3-like, which show high plasticity, as reported by other authors ³⁴. Indeed, we observed that *P. faecium* caused a reduction of ILC1 in vivo and increased IL-1 β levels in BMDM $\emptyset$ cultures, which participates in the ILC1 to ILC3 differentiation. However, this was not accompanied by an increment of *Rorc* expression or a decrease in *Tbx21* in ILC1 cultures stimulated with those *P. faecium*-stimulated BMDM $\emptyset$ supernatants, which classically are described to be characteristic of the switch between ILC1 and ILC3 ^{34,35}. Nonetheless, some authors have described that during pulmonary infection of mice, natural killer cells (members of the ILC1s group) increase a IL-22 production to promote host defense independently of ROR γ t levels ^{36,37}, suggesting that IL-22 production is not completely tied to the presence of this transcription factor. So, the different transient states acquired by ILCs in response to environmental stimuli awaits to be better characterized.

We consistently proved that *P. faecium*-induced M2 are critical to intercept the expansion of the pro-inflammatory ILC1. The administration of the M2 inhibitor not only canceled the M2 increase, but also the ILC1 reductions, ultimately leading to relevant loss of *P. faecium* anti-obesogenic effects (canceling 62% of body weight reduction and 64% of the improvements in the glycemic control). The fact that effects were not completely annulled by M2-depletion point to additional mechanism controlling energy balance that deserves further investigation. The co-administration of the M2 inhibitor GW2580 averts *P. faecium*-driven GIP reduction in HFHSD-fed mice. This incretin contributes to obesity development since it exerts anabolic effects leading to fat accumulation, and WAT inflammation characterized by increases of pro-inflammatory chemokines and cytokines, such CCL-2 and IL-6 ³⁸. When GIP is neutralized by antibodies or by GIP receptor antagonists, obesity development is attenuated ^{39,40}. Additionally, GIP is known to impair neuronal leptin sensitivity, an important controller of energy homeostasis, in response to excess caloric intake ^{41,42}, but its levels were restored by *P. faecium*. Furthermore, metabolically active organs (WAT and liver) showed lower inflammation and fat expansion in animals treated with *P. faecium*, an effect that was canceled when it was co-administered with GW2580. The findings support that the M2 induction by *P. faecium* is crucial for the control not

only of inflammation but also adiposity. More precisely, mice treated with *P. faecium* along with the GW2580 inhibitor maintained the HFHSD-driven overexpression of *Ccl2*, *Cd11c*, *Tnfa* and *Il6* in the WAT. In the fat pads, the chemokine CCL2 mediates monocyte recruitment, M1 polarization and greater TNF α and IL-6 concentrations, thereby contributing to an inflammatory milieu well connected with a pathological WAT-functioning and obesity-associated comorbidities ^{43,44}. Accordingly, the conditional ablation of CD11c⁺ cells decreased inflammatory markers in the WAT and rapidly improved glucose intolerance in DIO mice ⁴⁵.

Our findings also revealed that *P. faecium* buffered some of the microbiota alterations caused by the HFHSD, that were not observed in the animals treated simultaneously with M2 inhibitor. More specifically, the bacterium enhanced *A. muciniphila* abundance, which could potentiate the metabolic benefits exerted by *P. faecium* in obesity, given the extensive bibliography reporting the role of this species in obese-associated metabolic dysfunction ^{21,23}. At the same time, *P. faecium*, which is a propionate and potential butyrate producer ⁴⁶, favors the presence of butyrate and mucus producers (such *Ruminococaceae*_UBA1819 spp. and *Mucispirillum* spp) which could play additional beneficial roles in strengthening the gut barrier. The ability of *P. faecium* to downregulate the inflammatory environment may have contributed to the maintenance or recovery of these cluster of protective bacterial species, similarly to the effects observed with the supplementation of butyrate ⁴⁷, or with the administration of an antibody to deplete ILC1s ³⁰. Taking together, our findings suggest that *P. faecium* eases the restoration of a beneficial ecological niche under HFHSD-feeding. In turn, the ecological changes observed might help *P. faecium* colonization through cross-feeding mechanisms, since *A. muciniphila* is a succinate-producer and *P. faecium* a succinate consumer species, which could also partly explain the metabolic benefits on the host. Nonetheless, comparisons between live and inactivated *P. faecium* indicates that this bacterium per se displays immunoregulatory and anti-obesogenic properties. Furthermore, our study proves that the species *P. faecium* is more prevalent in normal-weight individuals than in obese subjects through a large meta-analysis of human metagenomes, supporting the previous hypothesis that this bacterial genus reduces obesity risk.

In conclusion, our study provides novel insights into the role of *P. faecium* in obesity in models and humans. By conducting a series of *in vivo* and *in vitro* experiments, we came out with an immune mechanism through which this bacterium is causally involved in obesity prevention. *P. faecium* resets the communication between macrophages and gut resident ILCs, priming the M2 macrophages polarization and, thereby, buffering the hypercaloric diet-induced ILC1s increases and, ultimately, resetting systemic inflammation and metabolic dysfunction. These results, together with the large meta-analysis confirming the associations between *P. faecium* and a lean phenotype in humans, contributes

to identify keystone bacteria of potential relevance to understand the origin of obesity and the breaking points of circular causalities.

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Competing interest: YS, MR-P and IL-A are authors of a patent on *Phascolarctobacterium faecium* DSM 32890. The rest of the authors declares no competing interests.

Patient and public involvement: Volunteers were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Data availability: The sequencing data used in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI, under accession number PRJEB59864.

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SUPPLEMENTAL MATERIAL

Bacteria growth conditions

P. faecium DSM 32890 was isolated from the stools of a healthy volunteer ¹. *P. faecium* was grown in a modified PYG medium, replacing glucose by succinate (8 g/L), and incubated at 37°C under anaerobic conditions (80% N₂, 10% CO₂ y 10% H₂) using a Bactron300-2 anaerobic chamber (Shel Lab, Cornelius, OR, USA). For animal experiments, the bacterial biomass was collected by centrifugation (10,000 xg, 10 min, 4°C), washed twice with PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) supplemented with 0.05% L-cysteine (Sigma-Aldrich, San Louise, CA, USA) and resuspended in PBS with 0.05% L-cysteine and 10% glycerol. The pasteurization of *P. faecium* consisted of heat 70°C for 30 min and freeze avoiding important temperature changes. Bacterial stocks were stored at -80°C until use. After freezing and thawing, the number of viable bacteria was calculated using BD Trucount™ Tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and propidium iodide staining (Sigma-Aldrich) in the flow cytometer BD LSRFortessa (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The absence of contaminations and bacterium identity was routinely confirmed respectively. by Gram staining and 16S rRNA gene sequencing by SANGER technology using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1401R (5'-CGGTGTGTACAAGACCC-3'). To confirm no viability, pasteurized *P. faecium* was plated in the modify PYG agar plates and incubated for 72 h at 37°C in anaerobic conditions.

Ethics

Animal procedures were evaluated and approved by the ethics committee of the University of Valencia (Animal Production Section, SCSIE, University of Valencia) and authorized by the competent authority (Generalitat Valenciana) who assigned the following approval IDs: 2017/VSC/PEA/00015, 2018/VSC/PEA/0171 and 2021/VSC/PEA/0177. The procedures conformed to EU directive 2010/63/UE and the Spanish RD53/2013 regulation, regarding the protection of animals used for experimental and other scientific purposes.

Oral glucose tolerance test

Two to three weeks before the sacrifice, an oral glucose tolerance test (OGTT) was conducted. Mice were maintained 4 h in fasting before collecting blood from the saphenous vein at 0, 15, 30, 60, and 120 minutes in response to an oral glucose challenge (2 g/Kg of body weight). Glycemia was determined by a Contour® XT glucometer (Bayer, Leverkusen, Germany).

Intestinal immune cells isolation and flow cytometry analysis

Isolation and staining of intestinal immune cells were conducted as previously described by our group ^{2,3}. Briefly, the small intestine was washed with ice cold PBS, opened longitudinally, and cut into small pieces. Intestinal tissue was

incubated for 30 minutes at 37°C in Hansk's balanced salt solution (HBSS) (ThermoFisher Scientific, MA, USA), supplemented with 5 mM EDTA (Scharlab, Barcelona, Spain), 1 mM DTT (Sigma-Aldrich), 100 µg/mL streptomycin and 100 U/mL penicillin (P/S) (Merck, Darmstadt, Germany). Cells were passed through 100 µm nylon cell strainers (Biologix, Shandon, China), washed in PBS supplemented with 5% of fetal bovine serum (FBS) (Mercodia, Uppsala Sweden), and centrifuged (300 xg, 5min, 4°C) to harvest the intra-epithelial cell fraction. The remaining tissue in the cell strainer was incubated twice at 37°C for 30 minutes in HBSS supplemented with 0.5 mg/mL collagenase D (Roche Diagnostics GmbH, Mannheim, Germany), 3 mg/mL dispase II (Sigma-Aldrich), 50 U/mL DNase I (Roche Diagnostics GmbH, Mannheim, Germany), and P/S. Cells from the lamina propria (LP) were collected by centrifugation after filtering the supernatant with 70 µm nylon cell strainers (Biologix) and washed in PBS supplemented with 5% FBS. In the intestinal epithelium, we characterized three immune cell subsets as follows: Innate lymphoid cells (ILC)1s: Lin⁻T-bet⁺ IFN-γ⁺; induced intra-epithelial lymphocytes (IELs): CD45⁺CD3⁺CD2⁺CD5⁺TCRαβ⁺; and natural IELs: CD45⁺CD3⁺CD2⁻CD5⁻TCRγδ⁺. In the LP, we analyzed pro-inflammatory macrophages (M1): F4/80⁺ CD80⁺ iNOS⁺; anti-inflammatory macrophages (M2): F4/80⁺ CD206⁺ Arg1⁺; and regulatory T (Treg) cells: CD19⁻CD3⁺CD4⁺CD25⁺Foxp3⁺. For the lineage, the commercial, BD PerCP-Cy™5.5 Mouse Lineage Antibody Cocktail was used. All the antibodies used for the flow cytometry analyses are detailed in [Supp. Table 1](#) In all cases, for the detection of intracellular markers, cells were previously permeabilized and fixed (fixation/permeabilization solution kit, BD Bioscience). Data was acquired with the flow cytometer BD LSRFortessa (Becton Dickinson Biosciences) operated by FACS Diva software v.7.0 (BD Biosciences). Data was analyzed using FCS express version 5 flow cytometry software or by FACS Diva software v.7.0. *In vitro* exposure of immune cell culture to *P. faecium*

GM-CSF bone marrow-derived cells, generation and stimulation

The isolation and differentiation of murine bone marrow-derived cells was conducted based on Hickey *et al.*⁴, with slight modifications. Briefly, bone marrow cells were extracted from the femurs and tibias of 4-7 weeks-old mice C57BL/6 mice by flushing with RPMI-1640 medium. Cells were filtered using a 70 µm strainer (Biologix) and collected (300 xg, 5 min, 4°C). First, cells were cultured on Petri dishes in RPMI containing 10% FBS, 1% P/S and 20 ng/mL GM-CSF (PeproTech London, UK) under microaerobic conditions (5% CO₂) at 37°C. On day 3, the media was refreshed and on day 6, the media was removed and the bone marrow-derived macrophages (BMDMØ) were detached using acutase solution (Sigma-Aldrich). Cells were pelleted and resuspended in RPMI with 10% FBS and plated in 6-well plates (1x10⁶ cells/well). The next day, BMDMØ were exposed for 6h to *P. faecium* in a ratio of 1:10 cell/bacteria. Supernatant was recollected for cytokines quantification and ILC1 stimulation, and the BMDMØ cells for flow cytometry as described hereafter. Additionally, 1 ml of the

supernatant was plate in modified PYG agar to certify the viability of the bacteria. Two independent experiments were performed with 6 and 9 replicates per condition each time. Challenged BMDMØ cells were incubated with anti-mouse FcγRIII/II CD16/CD32 before staining. Macrophages were characterized as: CD11c⁺CD11b^{high}MHC-II⁺CD115⁺ cells, the polarization to M2 was determined by the expression of: Arg-1, CD206 and/or CD163 as previously described ^{5,6}. All the antibodies used for the flow cytometry analyses are detailed in **Supp. Table 1**.

ILC1 isolation, purification, and co-culture

ILC1 were isolated from the lamina propria of the small intestine of 9-12 weeks-old C57BL/6 mice and purified using the FACs Aria Cell sorter (Becton Dickinson Biosciences) as previously describe ⁷. ILC1s were incubated with anti-mouse FcγRIII/II CD16/CD32 before staining and ILC1 population was gated in live cells defined as: CD45.2⁺Lin⁻CD127⁺CD90.2⁺NK1.1⁺NKp46⁺. Linage cocktail included: CD3ε, CD8a, CD19, Ter119, Cd11c, TCRb, TCRgd, Gr1, Cd11b as previously described ^{7,8}. Antibody details can be found in **Supp. Table 1**. After sorting, cells were plated in 96-well plates (2.5x10³ cells/well) and incubated at 5% CO₂ and 37°C with murine IL-2, IL-7 and IL-12 (all at the final concentration of 10 ng/ml) in complete RPMI media containing RPMI-1640 (L-Glutamine), 10% of FBS, 0.1% 2-mercaptoethanol (50 mM), 2 mM L-glutamine, 100 U penicillin, 0.1 mg/mL streptomycin, 2.5 mM HEPES, 1 mM sodium pyruvate, and 1 mM MEM non-essential amino acids. Next day, ILC1s were exposed to *P. faecium* in a ratio cell/bacteria of 1:10 or to *P. faecium*-stimulated BMDMØ supernatants for 6h. Culture supernatant was used for cytokine quantification and cells were homogenized in a lysis buffer (Macherey-Nagel, Nordrhein-Westfalen, Germany) for RNA extraction. Two independent experiments were performed with 4-5 biological replicates per condition each time.

Biochemical and immune parameters

In the plasma, fasting glycemia was determined in blood by a Contour® XT glucometer before sacrificing the animal. Plasmatic triglycerides and cholesterol were quantified with the triglyceride colorimetric assay kit (Elabscience, Houston, TX, USA), and cholesterol liquid kit (Química Analítica Aplicada SA, Madrid, Spain). Glucose-dependent insulintropic polypeptide (GIP), insulin, and leptin were quantified using the Luminex™ Mouse Metabolic Hormone Expanded kit (Merck & Co., Inc. Kenilworth, NJ, USA). Insulin resistance (IR) was calculated by the homeostatic model assessment for insulin resistance (HOMA-IR) index as fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5. Plasmatic levels of IFN-γ, IL-6, IL-1α, IL-1β, TNFα, IL-23, IL-27 and IL-10 were determined using the LEGENDplex mouse inflammation panel (Biolegend, San Diego, CA, USA) and circulating levels of lypopolysaccharide binding protein (LBP) where determined with the corresponding enzyme-linked immunosorbent assay kit (Cloud-clone Corp, Houston, TX, USA In BMDMØ supernatants, IL-1β, IL-10, IFN-γ, IL-22, IL-23, IL-12p70, GM-CSF and IL-4 were quantified. In the

supernatants of the ILC1 cultures, IFN- γ and IL-22 levels were measured. In both cases we used a Milliplex mouse Th17 magnetic bead panel (Merck & Co).

Hepatic levels of TNF α , IL-6 and IL-22 were quantified using commercial ELISA kit (Biolegend). 100 mg of the liver were suspended in 0.5 mL of RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (EASYPack, Roche) and homogenized using a TissueLyser II (Qiagen, Hilden, Germany). The homogenized tissue was centrifuged (21,000 xg 10 min, 4°C) and the supernatant was used for the interleukin quantification. The values were normalized with the amount of protein quantified using the Bradford method (Bio Rad). Prior to measure the soluble immunoglobulin A (sIgA), the cecal content was disaggregated in PBS (100 mg/ml), homogenized using the Lysing Matrix D tubes (MP biomedical, Santa Ana, CA, USA), and centrifuged (1,000 xg, 5 min, 4°C). The supernatant was diluted (1:1,000) with assay buffer and sIgA was quantified using an ELISA commercial kit (Invitrogen, Waltham, MA, USA).

Gene expression

RNA isolation from small intestine was performed using the TRIsureTM reagent (Bioline, London, UK) as previously described ⁹. RNA concentration was determined using Nanodrop TM 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). In each reaction, 1-2 μ g of total RNA was reverse transcribed to cDNA using the kit High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The RT-qPCR was performed with the LightCycler® 480 Instrument (Roche, Basel, Switzerland). The reaction consisted of LightCycler 480 SYBR Green I Master mix (Roche) and 300 nM of gene-specific primer pairs. For cultured ILC1, RNA was extracted using NucleoSpin RNA XS (Macherey-Nagel). RNA was retrotranscribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA; USA), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). The RT-qPCR was performed with TaqMan Gene Expression Master mix using a QuantStudio 5 Real-Time PCR system (Applied Biosystems). Primers and probes sequences can be found in **Supp. Table 2**. In all cases, samples were run in duplicate and the data were analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$). Target genes were normalized with *Rpl19*, *Gapdh*, or *Hprt* housekeeping genes and expressed as mRNA fold-change compared to the mice of the control diet group.

Gut microbiota analysis

DNA from the cecal content of mice was extracted using the QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Library preparation was performed using Nextera XT v2 Index (Illumina, San Diego, CA) targeting the V3-V4 region of the 16S rRNA gene, and sequenced on an Illumina® MiSeq platform (2×300 bp paired-end reads). The amplicon sequence variants (ASVs) quantification was obtained with the DADA2 v.1.24 R package¹⁰. Raw reads were truncated after 250 bp for forward and reverse reads and 30 nucleotides were additionally

removed at the start of both paired-end reads. Reads were filtered for quality assurance; clean pairs of reads were merged into contig sequences and chimeric sequences were discarded. Taxonomy was assigned by crossing sequences with SILVA v.138 database ¹¹. Taxa with a prevalence below 5% were removed from subsequent analyses. Data was rarefied according to the minimum number of lectures in the matrix 33935. Alpha diversity was calculated through the estimation of the observed ASVs, Shannon index and Inverse Simpson index using the Phyloseq v.1.40 R package¹². Non-parametric methods such as Kruskal–Wallis and post hoc Wilcoxon Rank Sum tests were applied for statistical analysis of alpha diversity. The evaluation of the community structure across groups was performed through principal coordinate analysis (PCoA) (phyloseq::ordinate()) function and “weighted UniFrac” distance). The analysis of the differential abundance of microbial taxa was performed using DESeq2 v.1.36 R package ¹³. The DESeq2 implemented function normalized our data and performed hypothesis testing using Wald test. The resulting p-values were corrected using the Benjamini-Hochberg false discovery rate (BH FDR) procedure.

Meta-analysis of human metagenomes

To quantify differences in the probability of detecting *P. faecium* at a larger scale, we used curatedMetagenomicData (cMD) 3 (Manghi et al, in revision). We queried cMD 3 for all samples from healthy (not presenting alternative pathologies to obesity) adults (age > 16 years), obtaining a total of 6,361 samples from 32 different datasets from 4 different continents. Obesity was defined at BMI ≥ 30 in all countries but China, India, and Japan in which it was defined as BMI ≥ 28 . Based on these thresholds, 1,295 samples were considered as derived from obese people, and 5,066 from non-obese people. To assess differential detection of *P. faecium*, we run the tool available at ¹⁴, which implements a random-effects meta-analysis of logistic regression coefficients. The analysis was run first on all samples, then on the same subjects adjusting by sex, and by age and sex, and then excluding underweight individuals (defined as BMI ≤ 18). Wald-test of the logistic regression coefficients relative to obesity yes/no were used to assess significance of the log odd-ratio with respect to the detection of *P. faecium*, while the meta-analysis coefficient was computed as a Z-score of the tendency of the mean effect to be greater than zero. In both cases $p < 0.05$ was used as a significance threshold.

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Supplemental Table 1. Immune anti-mouse and cell viability markers' information used for flow cytometry

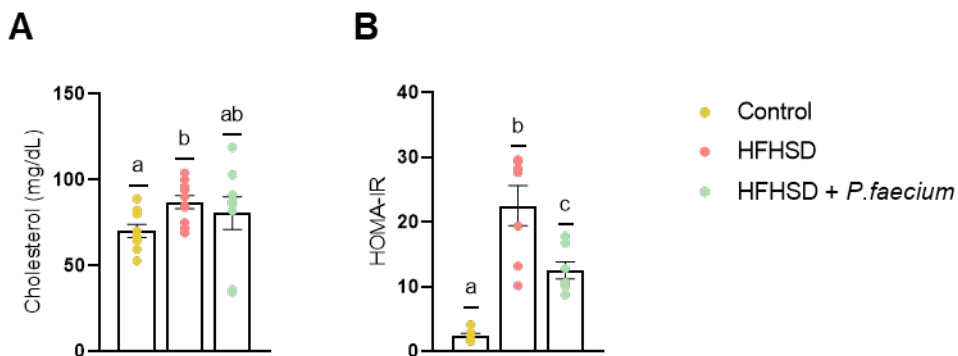
Marker	Clone	Fluorophore	Manufacturer
CD16/CD32	2.4G2	-	BD Bioscience
CD25	PC61	APCCy7	BD Bioscience
CD3ε	145-2C11	PerCPCy5.5	BD Bioscience
CD4	GK1.5	BV711	BD Bioscience
TCR β	H57-597	BUV395	BD Bioscience
CD115 (CSF-1R)	T38-320	APC	BD Bioscience
I-A/I-E (MHC-II)	2G9	BV421	BD Bioscience
CD11c	HL3	PE-Cys7	BD Bioscience
LIN	145-2C11; RB6-8c5; RA3-6B2; Ter-119; M1/70	PerCP-Cy™5.5	BD Bioscience
CD206	C068C2	PerCPCy5.5	Biolegend
CD45.2	104	APC/Cy7	Biolegend
CD127 (IL7ra)	A7R34	BV421	Biolegend
Thy1.2 (CD90.2)	53-2.1	BV605	Biolegend
iNOS	CXNFT	APC	eBioscience
NK1.1	PK136	Pe/Cy7	eBioscience
Nkp46	29A1.4	PerCP EF710	eBioscience
CD11b	M1/70	APC-eFluor 780	Invitrogen
CD163	TNKUPJ	FITC	Invitrogen
LIVE/DEAD™ Fixable Aqua	-	BV510	Invitrogen
CD19	REA749	APC	Miltenyi biotec
CD45	REA737	FITC	Miltenyi biotec
F4/80	REA126	FITC	Miltenyi biotec
Foxp3	REA788	PE	Miltenyi biotec
Tbet	REA102	PE-Vio615	Miltenyi biotec
TCRγδ	REA633	PE	Miltenyi biotec
CD2	RM2-5	Pe-Vio770	Miltenyi biotec
CD5	REA421	APC	Miltenyi biotec
CD80	16-10A1	Pe-Vio770	Miltenyi biotec
IFN _γ	REA638	APC	Miltenyi biotec
Arg1	Met1-Lys322	PE	R&dSystems
CD3ε	145-2C11	APC	Biolegend
CD8a	53-6.7	APC	Biolegend
CD19	6D5	APC	Biolegend
Ly-76 (Ter119)	TER-119	APC	Biolegend
Cd11c	N418	APC	Biolegend
TCR β	H57-597	APC	eBioscience
TCRγδ	GL3	APC	Biolegend
Ly-6G/Ly-6C(Gr1)	RB6-8C5	APC	Biolegend
CD11b	M1/70	APC	eBioscience

Supplemental Table 2. Gene names, abbreviations and primer sequences or *TaqMan* references use for gene expression analysis.

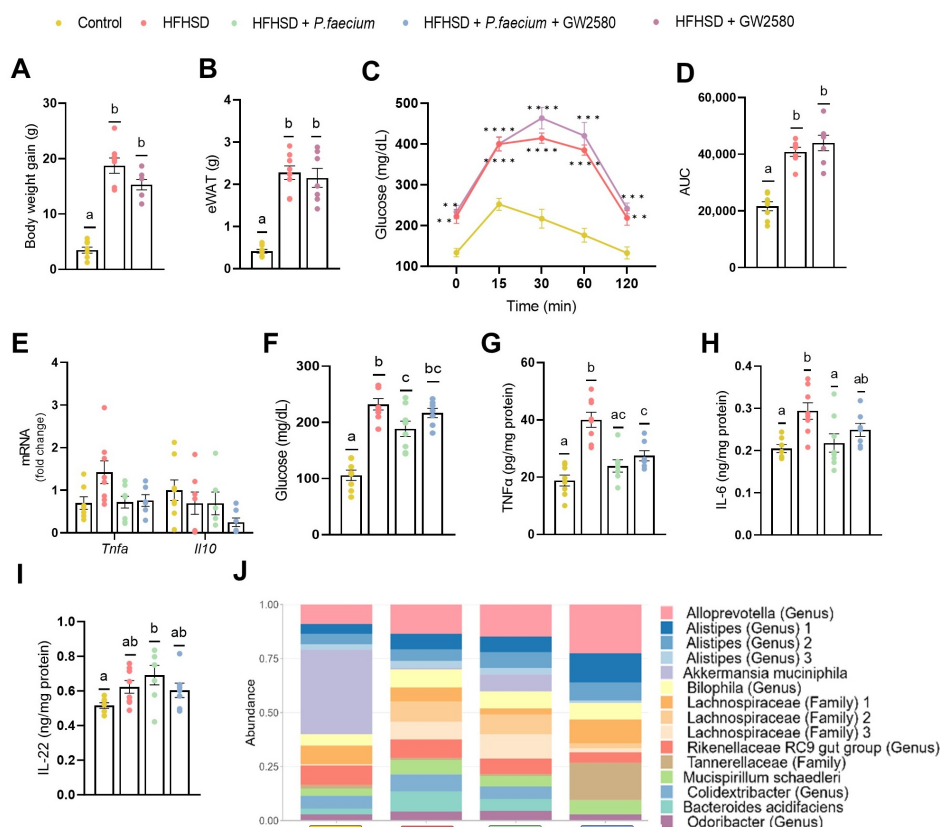
Gene name (abbreviation)	Sequence 5'-3'/ <i>TaqMan</i> Gene reference
CCAAT/enhancer binding protein beta (<i>Cebpb</i>)	Forward: TCGGGACTTGATGCAATCC Reverse: AAACATCAACAACCCCGC
Chemokine (C-C motif) ligand 2 (<i>Ccl2</i>)	Forward: GCAGTTAACGCCCCACTCA Reverse: CCCAGCCTACTCATTGGGATCA
Claudin 3 (<i>Cldn3</i>)	Forward: TCATCGGCAGCAGCATCATCAC Reverse: ACGATGGTGATCTTGGCCTTGG
Cluster of differentiation 36 (<i>Cd36</i>)	Forward: GCCAAGCTATTGCGACATGA Reverse: ATCTCAATGTCCGAGACTTTTCAAC
Granzyme B (<i>GrB</i>)	Forward: GCCCACAACATCAAAGAACAG Reverse: AACCAGCCACATAGCACACAT
Integrin $\alpha E\beta 7$ (<i>$\alpha E\beta 7$</i>)	Forward: CCTGTGCAGCATGTAAAAGAATG Reverse: CAAGGATCGGCAGTTCAGATAC
Integrin, alpha X (<i>Itgax</i> or <i>cd11c</i>)	Forward: ACGTCAGTACAAGGAGATGTTGGA Reverse: ATCCTATTGAGAATGCTTCTTTACC
Interleukin 10 (<i>Il10</i>)	Forward: GCTCTTACTGACTGGCATGAG Reverse: CGCAGCTCTAGGAGCATGTG
Interleukin 22 (<i>Il22</i>)	Forward: GACATAAACAGCAGGTCCAGTT Reverse: AGAAGGCTGAAGGAGACAGT
Interleukin 6 (<i>Il6</i>)	Forward: ACAAGTCGGAGGCTTAATTACACAT Reverse: TTGCCATTGCACAACCTCTTTC
Lipo-protein lipase (<i>Lpl</i>)	Forward: TGAAAGCCGGAGAGACTCAG Reverse: AGTGTCAGCCAGACTTCTTCAG
Occludin (<i>Ocln</i>)	Forward: ATGTCCGGCCGATGCTCTC Reverse: TTTGGCTGCTCTTGGGTCTGTAT
Phospholipase A2 group IIA (<i>Pla2g2a</i>)	Forward: AAGGATCCCCCAAGGATGCCAC Reverse: CAGCCGTTTCTGACAGTTCTGG
Regenerating islet-derived protein 3 gamma (<i>Reg3γ</i>)	Forward: TTCCTGTCTCCATGATCAAA Reverse: CATCCACCTCTGTTGGGTTTC
Ribosomal protein 19 (<i>RpL19</i>)	Forward: CCTTGTCTGCCTTCAGCTTGT Reverse: GAAGGTCAAAGGGAATGTGTTCA
Tumor necrosis factor alpha (<i>Tnfa</i>)	Forward: TGTCTCAGCCTCTTCTCATTCC Reverse: TGAAATCTGGGCCATAGAAC
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	Mm99999915_g1
Hypoxanthine-guanine phosphoribosyltransferase (<i>Hprt</i>)	Mm00446968_m1
Interferon gamma (<i>Ifng</i>)	Mm01168134_m1
Interleukin 22 (<i>Il22</i>)	Mm01226722_g1
Interleukin 23 receptor (<i>IL23r</i>)	Mm00519943_m1
RAR-related orphan receptor c (<i>Rorgc</i>)	Mm01261022_m1
T-box transcription factor 21 (<i>Tbx21</i>)	Mm00450960_m1
Tumor necrosis factor alpha (<i>Tnfa</i>)	Mm00443260_g1

Supplemental Table 3. ASVs identified at the genus or specie levels modified by *P. faecium* compared to non-treated HFHSD-fed mice.

ASV	ASV3	ASV279	ASV344	ASV180	ASV251	ASV108	ASV290	ASV188	ASV157
Family	Akkermansiaceae	Blifidbacteriaceae	Lachnospiraceae	Ruminococcaceae	Lachnospiraceae	Deferribacteraceae	Oscillospiraceae	Sutterellaceae	Sutterellaceae
Genus	Akkermansia	Blifidobacterium	Roseburia	UBA1819	NK4A136 group	Mucispirillum	Oscillibacter	Parasutterella	Parasutterella
Species	<i>muciniphila</i>	<i>animalis</i>							
Control	10805,50	66,75	12,50	59,63	24,00	61,38	0,00	123,00	12,50
HFHSD	233,25	4,88	17,00	8,25	13,63	0,00	0,00	29,38	17,00
HFHSD + <i>P. faecium</i>	2190,00	0,00	0,00	31,50	0,00	369,75	32,00	0,00	0,00
HFHSD + <i>P. faecium</i> + GW2580	11,14	0,00	0,00	29,71	22,57	0,00	31,86	0,00	0,00
HFHSD vs Control	2,1E-05	8,9E-03	ns	1,1E-06	ns	1,1E-17	ns	ns	ns
HFHSD + <i>P. faecium</i> vs Control	ns	4,7E-09	1,4E-06	ns	1,5E-03	ns	2,5E-07	6,4E-13	1,0E-08
HFHSD + <i>P. faecium</i> + GW2580 vs Control	9,0E-16	7,3E-09	2,0E-06	ns	ns	1,0E-17	3,5E-07	2,0E-12	7,2E-13
HFHSD + <i>P. faecium</i> vs HFHSD	5,0E-02	1,2E-02	3,8E-07	1,2E-02	2,4E-02	7,0E-22	1,0E-06	1,8E-07	2,5E-04
HFHSD + <i>P. faecium</i> + GW2580 vs HFHSD	1,2E-03	3,6E-03	2,1E-07	2,8E-03	ns	ns	4,9E-07	8,4E-08	1,5E-08
HFHSD + <i>P. faecium</i> + GW2580 vs HFHSD + <i>P. faecium</i>	2,5E-10	ns	ns	ns	1,7E-03	4,8E-22	ns	ns	ns



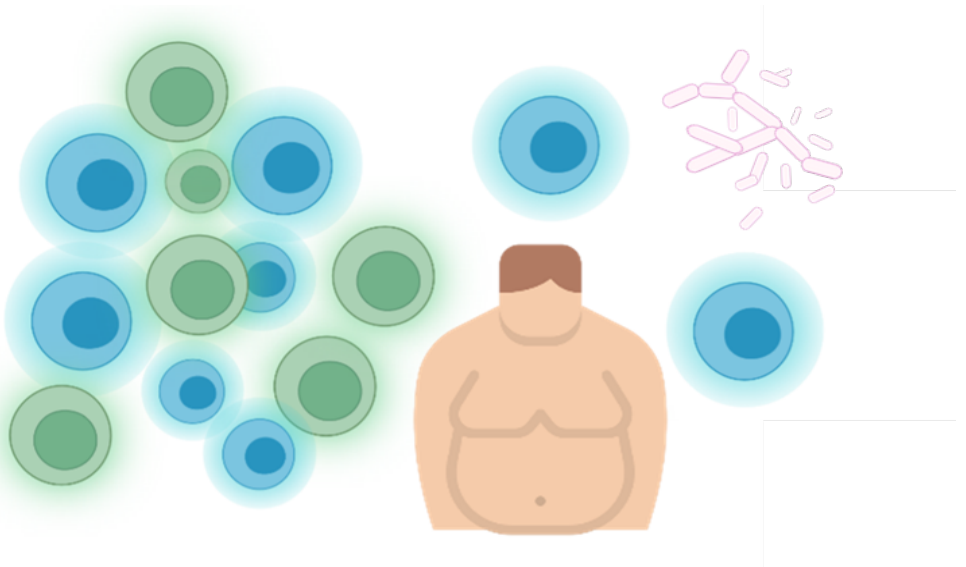
Supplemental Figure 1. (A) Cholesterol concentration in plasma (mg/dL) and (B) the homeostatic model assessment for insulin resistance (HOMA-IR) index. Values presented are mean ($n=8-10$) $\pm$ SEM. Significant differences were assessed by one-way ANOVA. Data with different superscript letters are statistically significant at $p < 0.05$.



Supplemental Figure 2. (A) Body weight gain (g), (B) Weight (g) of epididymal white adipose tissue (eWAT), (C) Blood glucose levels after an oral load of glucose (2 g/kg) and (D) area under the curve (AUC) at week 10 of intervention, (E) mRNA levels of indicated genes in the small intestine (F) fasting glucemia (mg/dL), (G-I) hepatic levels of IL-6 (ng/mg protein), TNFα (pg/mg protein) and IL-22 (ng/mg protein). and (J) mean relative abundance by group of the 15 most abundant genera/families. Values are presented as mean (n=7-8) ± SEM. Significant differences were assessed by two-way ANOVA or one-way ANOVA. Differential abundance analysis was performed using DESeq2 v.1.36 R package. Different superscript letters or asterisk (*) represent statistical differences (at least $p < 0.05$). ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. “*” when compare with control diet

CHAPTER 3

UNDERSTANDING THE INTERACTIONS BETWEEN IMMUNE CELLS MODULATED BY *P.* *faecium* AND THEIR ROLES IN OBESITY

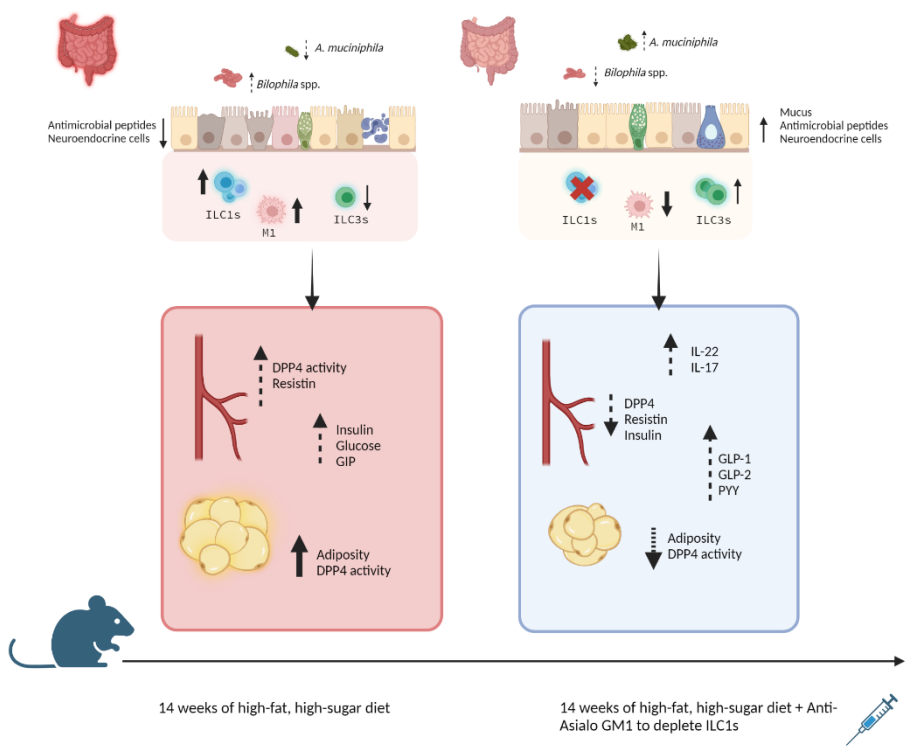


Rebeca Liébana-García, Marta Olivares, Carlos Francés-Cuesta, Teresa Rubio, Valerio Rossini, Guillermo Quintas & Yolanda Sanz (2023). Intestinal group 1 innate lymphoid cells drive macrophage-induced inflammation and endocrine defects in obesity and promote insulinemia, **Gut Microbes**, 15:1, 2181928, DOI: [10.1080/19490976.2023.2181928](https://doi.org/10.1080/19490976.2023.2181928).

My contribution to this research article comprehended:

Conceive and design the experiments. Performance of the animal experiments. Data acquisition, analysis and interpretation. Figure design and manuscript writing.

INTESTINAL GROUP 1 INNATE LYMPHOID CELLS DRIVE MACROPHAGE-INDUCED INFLAMMATION AND ENDOCRINE DEFECTS IN OBESITY AND PROMOTE INSULINEMIA



ABSTRACT

Hypercaloric diets overactivate the intestinal immune system and disrupt the microbiome and epithelial cell functions, impairing glucose metabolism. The origins of this inflammatory cascade are poorly characterized. We investigated the involvement of intestinal proinflammatory group 1 innate lymphoid cells (ILC1s) in obesity progression and metabolic disruption. In obese mice, we studied longitudinally the ILC1s response to the diet and ILC1s depletion to address its role in obesity. ILC1s are required for the expansion of pro-inflammatory macrophages and ILC2s. ILC1s depletion induced the ILC3-IL-22 pathway, increasing mucin production, antimicrobial peptides, and neuroendocrine cells. These changes were translated into higher gut hormones and reduced insulinemia and adiposity. ILC1s depletion was also associated with a bloom in *Akkermansia muciniphila* and decreases in *Bilophila* spp. Intestinal-ILC1s are upstream activators of inflammatory signals, connecting immunity with the microbiome, the enteroendocrine system, and the intestinal barrier in the control of glucose metabolism and adiposity.

INTRODUCTION

Obesity is characterized by excessive fat storage that represents the main metabolic risk factor for the development of complications, such as type 2 diabetes (T2D), cardiovascular disease, or cancer. This fact, together with its global relentless advance in the last decades, makes urgent the intensification of the research aim to understand the precise mechanisms by which unbalanced diets trigger obesity. It is well-proven that obesity is associated with chronic inflammation that drives changes in microbiota, glucose metabolism, and insulin resistance.¹ A change in the microbial configuration *per se* can also modify the equilibrium between different immune cell types and their activation, thereby, increasing the risk of inflammatory pathology driven by a biased-immune response.² In obesity, this loss of intestinal immune homeostasis is an early event that precedes and contributes to systemic inflammation.³ While the large excess of energy provided by the diet is ultimately stored as fat in metabolic organs, the intestine is the first compartment affected by obesogenic diets, which can alter the composition and function of intestinal immune cells and the microbiota and their metabolome, in turn, influencing metabolic responses to diet.^{4,5} In fact, this crosstalk between intestinal immune cells and microbiota is essential in shaping immune function.⁶ For example, a fat-enriched diet results in specific changes in the gut microbiota that impair the intestinal immune system and, ultimately, leads to systemic low-grade inflammation.⁵

Innate lymphoid cells (ILCs) are a recently discovered family of immune cells that are mainly localized at epithelial surfaces where they sense intrinsic and extrinsic signals (such as microbes and dietary stimuli) and function to preserve immune homeostasis.⁷ The ILC family comprises natural killer (NK) cells, ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer (LTi) cells.⁸ Although, immunologists commonly gather them in three groups: ILC1 (comprising ILC1s and NKs), ILC2, and ILC3 (comprising ILC3 and LTi cells), mainly based on the type of immune response they drive.² Emerging evidence links ILCs to obesity through a mechanism that is not fully understood. Obesogenic diets diminish the proportion of intestinal interleukin (IL)-22-producing ILC3s, which might contribute to the gut barrier dysfunction seen in obesity,^{9,10} although uncertainties exist.¹¹ In the context of inflammatory bowel diseases, dysregulation of ILC3s has been implicated in their pathogenesis.¹² Colonic ILC3 mediates a protective response in acute colitis, but sustained intestinal damage leads to a proinflammatory intestinal response mediated by ILC3.¹³ A protective role for ILC2s in adipose tissue has been reported in mice and humans,¹⁴ and group 1 ILCs have been described to regulate and polarize macrophages to drive inflammation, which promotes insulin resistance.^{15–17} Also, another study reported that intestinal ILC2s, but not ILC2s from adipose tissue, are involved in obesity development in mice fed hypercaloric diets.¹⁸ This unequivocally points to the tissue-specific functions played by this predominantly tissue-resident family of immune cells. So

far, the implications of adipose ILC1s in obesity and metabolic complications have been widely evidenced, but the potential function of the intestinal-group 1 ILCs awaits further investigations.

We hypothesized that gut-resident ILC1s increase in abundance as a consequence of hypercaloric diets, and initiate a cascade of pathogenic events that stimulate inflammation and trigger intestinal barrier and enteroendocrine cell dysfunction. Given the links between the microbiota and immunity, we further postulated that ILC1- triggered inflammation suppresses host-microbiota symbiosis, which would account for the systemic dysregulation of glucose homeostasis. Here, we present intestinal ILC1s implication in the cascade of events promoting inflammation in obesity and its links to intestinal and endocrine homeostasis. As well, we have evidence of the impact of the depletion of intestinal ILC1s on the microbiota and the metabolic profile.

MATERIALS AND METHODS

Mice and treatments

Experiments were performed using a total of 44 male C57BL/6 J mice (7 weeks of age; Charles River Laboratories, Écully, France). Mice were housed in groups of 4/5 per individually-ventilated cage under a 12-h light/dark cycle, in temperature-controlled conditions ($23 \pm 2^{\circ}\text{C}$). Mice were acclimatized for 10 days and had *ad libitum* access to water and food. Mice were randomized based on body weight to minimize baseline differences and were exposed for 4, 10 or 14 weeks to: (i) control diet (D12450K Ssniff Spezialdiäten GmbH, Soest, Germany; 10% of energy from fat and no sucrose), or (ii) HFHSD (D12451 Ssniff; 45% of energy from lard, and 17% from sucrose). From week 10 to 14, a subgroup of mice on the HFHSD ($n = 8$) was treated by intraperitoneal injection with 50 μl of the AGM1 antibody (Biolegend, San Diego, CA) following manufacturer's instruction and twice weekly according to Lee et al.¹⁵ Control groups received PBS instead. Body weight evolution and food intake were monitored twice weekly.

After 4 and 10 weeks of treatment, 5 animals of each experimental group were anesthetized with isoflurane and sacrificed by cervical dislocation under fasted conditions. The remaining 24 animals were sacrificed at week 14. To minimize potential confounders, the sacrifice order was designed so mice of different experimental groups were interspersed. Blood was collected in EDTA-containing tubes with or without a DPP4 inhibitor (Millipore, Burlington, MA). The plasma was recollected after centrifugation ($12,000 \times g$, 3 min) and stored at -80°C . Samples of WAT, ileum and colon were fixed in 4% paraformaldehyde for histological analyses. Liver, WAT (subcutaneous [inguinal] and visceral

[epididymal]), ileum, colon, and cecal content were snap-frozen in liquid nitrogen and stored at -80°C until use.

The experiment was approved and performed following European Union 2010/63/UE and Spanish RD53/201 guidelines, was approved by the ethics committee of the University of Valencia (Animal Production Section, SCSIE, University of Valencia), and was authorized by the competent authority (Generalitat Valenciana). The procedure was entitled “*Influence of innate lymphoid cells in obesity development*” and assigned the code 2020/VSC/PEA/0114.

Biochemical analysis

In the plasma containing the DPP4 inhibitor, the levels of PYY, active and total GLP-1, GIP, insulin, resistin and leptin were measured using the Luminex™ Mouse Metabolic Hormone Expanded kit (Merck Chemicals and Life Science, Madrid, Spain). Total GLP-2 was quantified using a commercial kit (Abyntek Biopharma, Bizcaia, Spain). We also quantified the levels of glucose (Química Analítica Aplicada SA, Spain). The homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated as fasting plasma insulin (mU/L) $\times$ fasting plasma glucose (mmol/L)/22.5.

DPP4 activity

The DPP4 activity was measured in different samples (liver, adipose tissue and, plasma). The inhibitory DPP4 activity of the AGM1 antibody was also investigated using a pool of plasma as a source of DPP4 activity. The DPP-4 was measured as previously described⁴¹ and detailed in [Supp. Material](#).

Immune parameters

Plasma levels of IL-22 and IL-17A were measured using the Luminex™ Mouse Th17 Bead Panel kit (Merck Chemicals and Life Science, Madrid, Spain).

Isolation of intestinal immune cells and flow cytometry analysis

Cell isolation was performed as described.⁴² Briefly, the small intestine was washed with cold PBS, longitudinally opened, and cut into small pieces. The epithelium and the lamina propria were isolated, digested, and stained with the corresponding antibodies as described in [Supp. Material](#). Data acquisition and analysis were performed using a BD LSRFortessa flow cytometer operated with FACS Diva software v.7.0 (BD Biosciences). Data were analyzed using FCS express version 5.

Gene expression analyses

Total RNA was isolated from different sections of the intestine (ileum and colon) using a commercial kit (Nucleo Spin RNA, Nordrhein-Westfalen, Germany) and from the WAT using the TRIsure Reagent (Bioline, London, UK). Complementary DNA was prepared by the reverse transcription of 1 μg of total RNA using a High-

Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Reactions contained the LightCycler 480 SYBR Green I Master mix (Roche, Boulogne-Billancourt, France) and 300 nM of gene-specific primer pairs. RT-qPCR was performed with the LightCycler® 480 Instrument (Roche)⁴³ Data were analyzed using the 2- $\Delta\Delta$ CT method. Targeted genes were normalized against ribosomal protein L19 (Rpl19) as the housekeeping gene; primer sequences are detailed in **Supp Table S3**.

Histological and immunohistochemical analysis

Adipocyte size was quantified in visceral WAT after staining with hematoxylin/eosin. Bright-field digital images were taken using an Eclipse 90I (E90I) Nikon microscope (Nikon Corp., Tokyo, Japan) and analyzed as previously described⁴³ NECs in the ileum were detected in paraffin sections (5 μ m) immunostained with an antibody to synaptophysin, using the Autostainer link 48 (DAKO, Glostrup, Denmark). The results were expressed as the average NEC number per mucosal area (number/mm²). Mucin production in the colon was calculated after staining with Alcian blue, using eosin as a contrast dye. Mucin production was expressed as the total area of mucin-producer cells relative to the whole mucosa area. Histological and immunohistochemical analyses involved a specialist-blinded researcher. All detailed protocols can be found in **Supp. Material**.

Metabolomic analysis

The cecal content was resuspended in methanol (750 mg/ml) and centrifuged at full speed (15,000x g, 10 min), and filtered (0.22 μ m) . Metabolites were studied by a non-targeted approach using a UPLC chromatograph (Agilent Technologies, Santa Clara, CA). Full scan MS data from 100 to 1700 *m/z* was collected on an iFunnel quadrupole time-of-flight (TOF) Agilent 6550 spectrometer (Agilent Technologies). Detailed information and experimental settings for every step of the analysis are in **Supp. Material**.

DNA extraction and sequencing

DNA from the cecal content of the 14-week-old group (n = 24) was extracted using the QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Library preparation was performed using Nextera XT v2 Index (Illumina, San Diego, CA) targeting the V3-V4 region of the 16S rRNA gene, and sequenced on an Illumina® MiSeq platform (2 × 300 bp paired-end reads).

Intestinal bacterial diversity and taxonomic analysis

Raw reads were filtered for quality assurance and clean pairs of reads were merged into contig sequences. An ASV table was constructed, and chimeric sequences were removed. Taxonomy was assigned by checking sequences with the SILVA v.138 database. All steps were performed using DADA2 v.1.24 package in R v.4.2. Taxa with a prevalence below 5% were removed from

subsequent analyses. The alpha diversity within samples was computed through the estimation of the richness (observed potential species), Simpson, and the inverse of Simpson indices using Phyloseq v.1.40. A maximum-likelihood phylogenetic tree of ASVs was constructed using the general time reversible substitution model with four gamma categories using Phangorn v.2.8.1. The phylogenetic tree was used to calculate the weighted UniFrac distance for computing differences between microbial communities using Phyloseq. Microbiome differential abundance analysis was performed using DESeq2 v.1.36 R package. The DESeq2 implemented function was used to normalize the data and hypothesis testing was performed using the Wald test. The resulting p-values were corrected using the BH-FDR procedure.

Statistical analysis

G*Power 3.1.9.2 was used to calculate the sample size allowing the primary outcomes (adiposity and glucose metabolism). Data from biochemical, histological, and gene expression studies were analyzed using GraphPad software (v.9 San Diego, CA). The Shapiro-Wilk test was employed to assess data normality. The differences for normally distributed data were determined using one-way analysis of variance (ANOVA) or two-way ANOVA followed by post hoc Tukey's multiple comparison tests or t-test (as suitable). Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed using the Mann-Whitney or Kruskal-Wallis test (as suitable) followed by Dunn's multiple comparisons test. A χ^2 test was used for categorical data (active GLP-1). Correlations between resistin and eWAT were calculated with the Pearson test. The Grubbs test was used for outlier detection. For all analyses, results were expressed as mean $\pm$ SEM and considered statistically significant at $p < .05$. Data analysis was not blinded.

RESULTS AND DISCUSSION

Obesogenic diet boosted ILC1s in the gut closely linked to the expansion of the adipose tissue

We first investigated the effect of a high-fat, high sugar-diet (HFHSD 45% of energy from lard, and 17% from sucrose) on intestinal ILC1s, expecting that, similar to adipose tissue ILC1s,¹⁹ gut ILC1s will dynamically respond to dietary changes. Additionally, in order to test our hypothesis, we used a neutralizing antibody to deplete ILC1s in male C57BL/6 mice fed an HFHSD (**Figure 1A**). Specifically, we chose the anti-Asialo GM1 (AGM1) that targets the asialo-GM1, a cell-surface glycolipid present in ILC1 group (referred to as ILC1s hereinafter). This is a well-described strategy for blocking ILC1s function, but not exempt from limitations, such as the possible off-target interactions with other cells and the lack of tissue specificity.

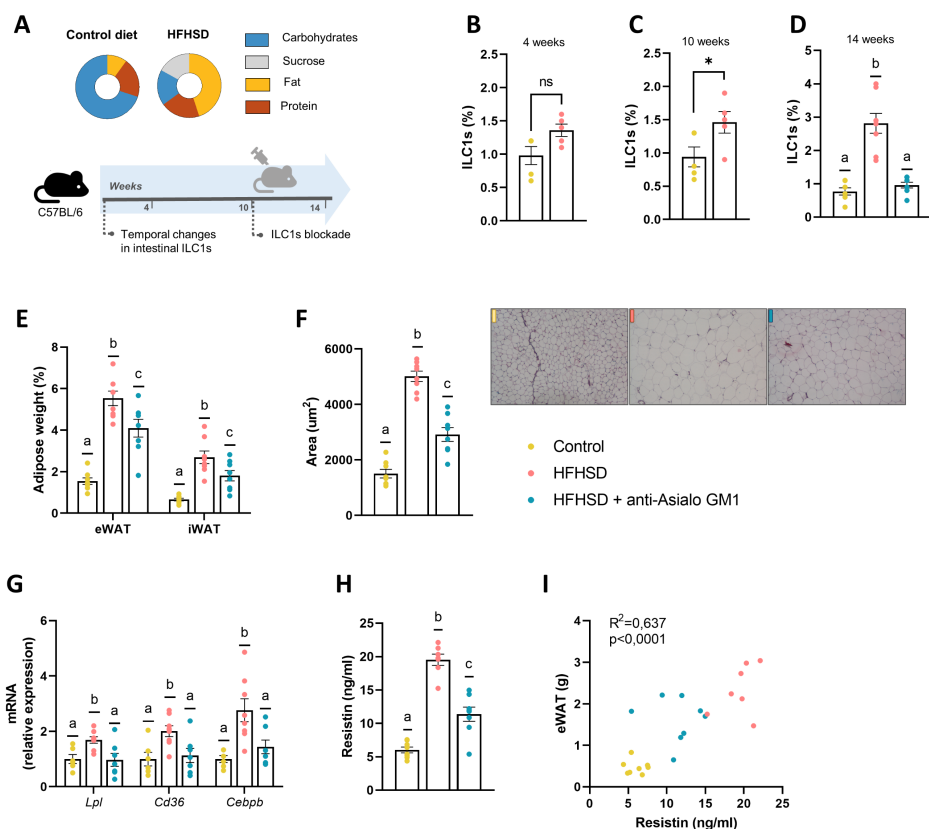


Figure 1. Obesogenic diet boosted ILC1s in the gut and adipose tissue expansion countered by ILC1s depletion (a) Schematic representation of the experimental design. (b–d) ILC1s (percentage of LIN⁺ cells of total intestinal epithelial cells) in the small intestine at 4 weeks, 10 weeks, and 14 weeks, respectively (n = 5–8). (e) Weight of epididymal and inguinal white adipose tissue (eWAT and iWAT, respectively) (n = 8). (f) adipocyte area (n = 8) and representative images of the histological staining of visceral adipose tissue (8×). (g) Expression of markers of fatty acid uptake and adipogenesis, *Lpl*, *Cd36* and *Cebpb* (n = 7–8). (h,i) Plasma resistin levels and correlation between resistin levels and eWAT content (n = 7–8). Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. For normally distributed data statistical analyses were performed by t-test or ANOVA, as appropriate, followed by *post hoc* Tukey's multiple comparison test. Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Mann-Whitney or Kruskal-Wallis test (as appropriate) followed by Dunn's multiple comparisons test. Correlations were calculated with Pearson's test. “*” p < .05. Data with different superscript letters are significantly different at p < .05.

We found that fat mass and body weight were greater in mice on HFHSD than on a control diet after 4 weeks, whereas no significant changes were observed small intestine ILC1s abundance (**Figure 1B, Supp Figure S1A, B**). By contrast, ten weeks of HFHSD increased gut ILC1s abundance, body weight, and fat mass (**Figure 1C, Supp Figure S1C, D**), which is consistent with a previous study reporting an increase in intestinal ILC1s abundance in mice fed an obesogenic diet for 12 weeks.²⁰ Similar to the observations with obesogenic diets, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis course with elevated intestinal ILC1s abundance.^{21,22} Interestingly, no increase in ILC1s was observed in non-inflamed tissue in patients without inflammatory bowel disease, unequivocally linking ILC1s with an inflammatory milieu.²² To study the role of ILC1s in obesity, we treated mice with an AGM1 antibody, which depletes ILC1s,^{15,19} from weeks 10 to 14 of HFHSD. No changes in food intake were observed in mice treated or not with AGM1 during this period when compared with mice on a control diet (**Supp Figure 1E**). As expected, the abundance of ILC1s was significantly lower in AGM1-treated mice than in non-treated mice (**Figure 1D**). Additionally, adiposity in two fat depots (epididymal [eWAT] and inguinal white adipose tissue) was significantly lower in AGM1-treated mice than in non-treated mice (**Figure 1E**), which was accompanied by a tendency for reduced body weight gain (**Supp Figure 1F**). Likewise, adipocyte size was significantly smaller in AGM1-treated mice (**Figure 1F**), consistent with a downregulation in the expression of genes involved in fatty acid uptake (*Lpl* and *Cd36*) and adipogenesis (*Cebps*), which were all increased by HFHSD (**Figure 1G**). In agreement with these data, previous studies point to ILC1s as major promoters of adipose tissue hypertrophy and inflammation.^{17,23} Specifically, ILC1s have been shown to promote CD11c+ macrophage activation resulting in adipose fibrogenesis, an indicator of advanced obesity;¹⁷ other authors, however, failed to find a link between ILC1s and adiposity in animal models.^{15,16} We also found that ILC1s depletion decreased significantly the plasma levels of resistin and tended to reduce those of leptin, both well-recognized adipokine markers of adiposity^{24,25} (**Figure 1H**). Resistin was positively correlated to eWAT mass (**Figure 1I**). Overall, our results indicate that gut-resident ILC1s increase in response to HFHSD and likely contribute to several features of obesity.

ILC1s depletion reduced intestinal proinflammatory response and protects from the gut homeostasis breakdown

Given these findings, we next investigated the dialog between ILC1s and macrophages in the gut. This immune-cell interaction has been studied mainly in obese adipose tissue.^{16,19} As anticipated, HFHSD feeding for 14 weeks created a pro-inflammatory milieu that boosted the expansion of type 1 (M1) macrophages and the M1/M2 ratio together with ILCs in the gut, as revealed by flow cytometry analysis (**Figure 2A, B**). Additionally, ILC1s depletion blunted the expansion of M1 macrophages and reduced the M1/M2 ratio in the gut (**Figure 2A, B**). No diet or antibody-induced changes were observed for M2 macrophages

(**Supp Figure S2A**). These results indicate that activation of ILC1s is obligatory for the subsequent expansion of pro-inflammatory M1 macrophages in the gut.^{19,23} The changes in the inflammatory tone due to ILC1s depletion secondary impacted other ILC subsets. Recent studies have reported an increase in the proportion of ILC2s in high-fat diet-induced obesity.¹⁸ We found that ILC1s depletion suppressed the HFHSD-induced increase in ILC2 abundance, with levels equal to those in the control diet group (**Figure 2C**). Contrastingly, we observed a reduction in ILC3s in HFHSD-fed mice and an increase in the obese group treated with AGM1 (**Figure 2D**). ILC3s are involved in tissue-protective responses to damage,⁸ and an increase in ILC1s in inflamed tissue has been linked to a reduction in ILC3s as a consequence of a shift from the latter to the former.²¹ We noted the same relationship between ILC1s and ILC3s in the obesity model; however, our experimental approach does not allow us to assess this phenomenon directly. To support these findings, we measured the plasma levels of IL-22 and IL-17A, two effector cytokines of ILC3s. At 10 weeks of HFHSD, IL-22 levels were significantly lower than those in the control diet group (**Supp Figure S2B**). No differences were observed at 14 weeks between the control diet and the HFHSD-fed groups; however, ILC1s depletion led to an increase in the plasma levels of both IL-22 and IL-17A (**Figure 2E, F**). Furthermore, IL-22 and IL-17 showed increased expression in the ileum, reflecting how intestinal ILC1s depletion affects those cytokines also in the intestine (**Supp Figure S2C**).

The ILC3/IL-22 pathway bolsters gut barrier function by inducing the production of antimicrobial peptides (AMPs) and mucin.¹⁰ Accordingly, ILC1s depletion led to a significant increase in the ileal gene expression of the AMP regenerating islet-derived 3-gamma (*Reg3g*) in HFHSD-fed mice, which was accompanied by a trend for an increase in the expression of phospholipase A2g2 (*Pla2g2a*). This response occurred without changes to the expression of *Tcf4*, a transcription factor involved in the differentiation of AMP-producing Paneth cells (Figure 2g). In the colon, no differences were found (**Supp Figure S2D**). The lack of an immune response mediated by ILC1s also influenced the expression of two of the three tight junction protein genes analyzed (*Zo1* and *Ocln*) in the ileum (**Figure 2H**), whereas, in the colon, only *Ocln* showed a trend to be increased in mice treated with the antibody AGM1 compared to untreated obese mice (**Supp Figure S2D**). Moreover, AGM1 treated mice presented an increase in mucin (*Muc2*) expression and mucus production (**Figure 2I, J**), which could be secondary to the increase in ILC3 abundance.¹⁰ Collectively, these findings indicate that the pro-inflammatory microenvironment driven by ILC1s impairs different mechanisms involved in strengthening the gut barrier. Interestingly, some of the effects of ILC1s depletion have been, similarly, attributed to anti-obesogenic treatments including prebiotics or vegetable-rich extracts.^{26,27}

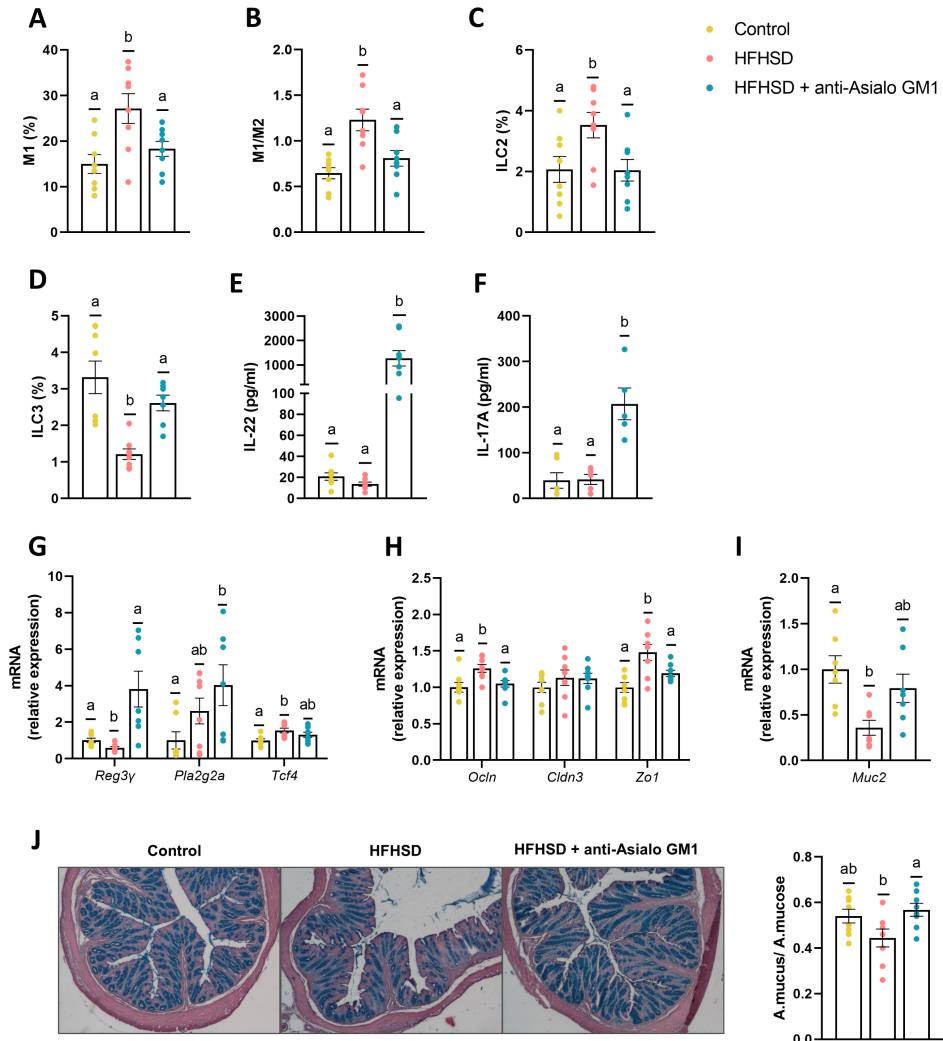


Figure 2. Depletion of intestinal ILC1s improved gut homeostasis. (a) Pro-inflammatory macrophages (M1) (b) ratio of M1/M2 macrophages (n = 8) in the small intestine. (c,d) ILC2s and ILC3s (percentage of LIN⁺ cells of the total lamina propria cells) in the small intestine (n = 7–8). (e,f) Plasma levels of IL-22 and IL-17A (n = 5–8). (g) Ileal gene expression of antimicrobial peptides (*Reg3g*, and *Pla2g2a*), the Paneth differentiation marker *Tcf4* and, (h) tight junction markers (*Ocln*, *Cldn3* and *Zo1*). (i) Colonic expression of *Muc2*, and (j) representative images of the histological staining of the colonic tissue with alcian-blue and quantification of mucus production as the area of mucus relative to the area of the total mucosa (x8). Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. Statistical analyses were performed by ANOVA followed by *post hoc* Tukey's multiple comparison test for normally distributed data. Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data with different superscript letters are significantly different at $p < .05$.

ILC1s depletion alleviated gut hormones dysregulation which could improve glucose metabolism

We further investigated the consequences of ILC1s depletion on the enteroendocrine system to explore possible links to obesity. Guided by previous evidence, we found a relationship between endocrine hormones and intraepithelial lymphocytes. Immunohistochemical analysis of the ileum revealed that neuroendocrine cells (NECs) were less abundant in the HFHSD-fed group than in the control diet group, whereas the opposite was seen in AGM1-treated mice on HFHSD (**Figure 3A**). The expression of neuronal differential 1 (*NeuroD1*), but not neurogenin 3 (*Ngn3*) (factors related to NEC development and differentiation) negatively correlated with NEC abundance, likely as a compensatory response (**Supp Figure S3A**). We then questioned whether changes in NECs led to differences in circulating gut hormones involved in glycemic regulation. Although we found no differences in response to the diet, ILC1s depletion caused a sharp rise in peptide YY (PYY) and in glucagon-like peptide 1 (GLP-1) (total and active) and total GLP-2 (**Figure 3B–E**). In addition to the canonical role of GLP-1 in glucose homeostasis, its role in buttressing the gut barrier has been recently established,²⁸ similar to the role attributed to GLP-2.²⁹ Depletion of ILC1s also tended to restore the levels of the gastric inhibitory polypeptide (GIP) to those in the control diet group (**Supp Figure S3B**). Incretins are inactivated by dipeptidyl-peptidase 4 (DPP4), a ubiquitous protease closely linked to obesity.³⁰ In line with previous observations, we found an increase in plasma DPP4 activity in HFHSD-fed mice, but this effect was not observed in AGM1-treated mice (**Figure 3F**) and was not a direct effect of the AGM1 antibody (**Supp Figure S3C**). We next investigated the possible origin of the higher circulatory DPP4 activity. Adipose tissue is a major source of DPP4, where it modulates key metabolic features of obesity including inflammatory tone, glucose homeostasis, and adiposity.³¹ The obesogenic diet increased DPP4 activity in eWAT, and this was prevented by AGM1 treatment (**Figure 3G**). No differences were found in liver, another important source of DPP4 activity (**Supp Figure S3E**). Then, the observed increase in active GLP-1 can be explained by the greater abundance of NECs in the intestine and the diminished adipose and plasma DPP4 activity. In agreement with previous studies,^{15,16,23} ILC1s depletion rescued the hyperinsulinemia caused by the HFHSD and the homeostatic model assessment for insulin resistance (HOMA-IR) (**Figure 3H, I**). Overall, these studies allow us to hypothesize that the increased intestinal hormone concentrations secondary to ILC1s depletion in the intestine explain the observed improvement in gut homeostasis and insulinemia.

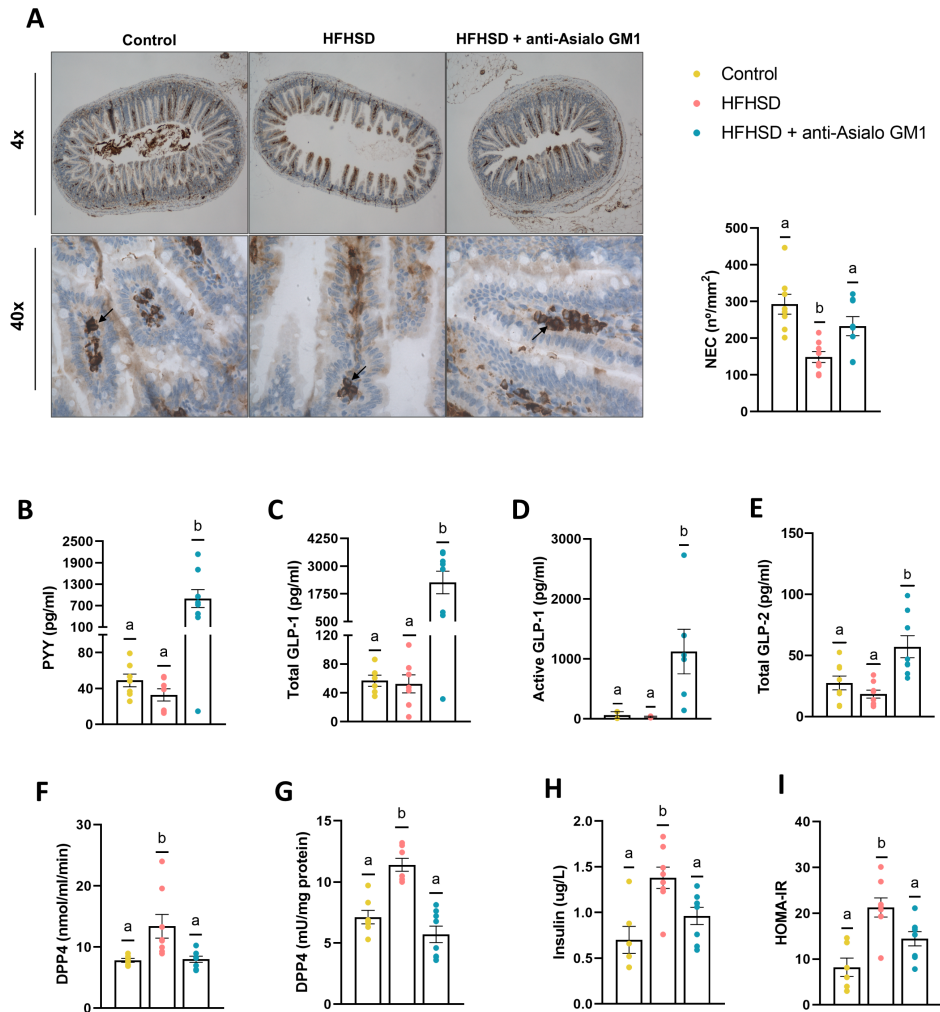


Figure 3. Depletion of ILC1s influenced enteroendocrine control. (a) Immunolabeling of ileal sections and the number of immune-positive neuroendocrine cells per mucosa area ($n = 8$). (b) Plasma levels of the hormones PYY ($n = 7-8$), (c) total GLP-1 (d) and active GLP-1 ($n = 6-8$), and (e) total GLP-2, (f) DPP4 activity in plasma and in (g) visceral adipose tissue ($n = 6-8$). (h) Insulinemia ($n = 6-8$) and (i) HOMA-IR index ($n = 6-8$). Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. Statistical analyses were performed by ANOVA followed by post hoc Tukey's multiple comparison test for normally distributed data. Welch's correction was applied when variances were not equally distributed. Non-normally distributed data were analyzed with the Kruskal- Wallis test followed by Dunn's multiple comparisons test. Active GLP-1 was analyzed by Chi-square contingency tests. Data with different superscript letters or the symbol "*" show significant differences at $p < .05$.

ILC1s depletion imprinted microbiota and metabolic changes toward a healthier intestinal microenvironment

Finally, we investigated whether the gut microbiota composition and function are affected by ILC1s depletion. According to principal coordinates analysis (PCoA) of the weighted UniFrac distances, 14 weeks of HFHSD caused an evident structural change in the microbial community, explained by the PC1 (56.3% of the variability). The PC2 (16.1%) can be attributed to ILC1s depletion (**Figure 4A**). The AGM1 antibody also reduced the observed amplicon sequence variants (ASVs) as compared with mice fed a control diet or HFHSD (**Figure 4B**), and normalized the Simpson's diversity and evenness indices (**Figure 4C, D**). Overall, we observed that the families that best discriminated between the three experimental groups were *Muribaculaceae* and *Lachnospiraceae* (**Figure 4E**). Both groups of mice fed an HFHSD present reductions in ASV of the *Muribaculaceae* family. In the *Lachnospiraceae* family, two clusters can be distinguished. The ASVs of the first cluster were increased by the obesogenic diet and restored by the ILC1s depletion, while in the other cluster we observed an increase in several ASV associated with the ILC1s. Among the ASVs (identified at genus or species levels) that were significantly modified by ILC1s depletion *versus* HFHSD only, two ASVs were prominent: *Bilophila* spp. and *Akkermansia muciniphila*. *Bilophila* spp. is known to synergize with an obese diet, promoting intestinal inflammation and gut barrier dysfunction.³² Increases in *Bilophila* spp. were counteracted by ILC1s depletion (**Figure 4F**). By contrast, *A. muciniphila* abundance was blunted by the HFHSD and recovered after ILC1s depletion (**Figure 4G**). *A. muciniphila* plays a key role in metabolic health and is generally associated with a lean phenotype; indeed, its administration neutralizes several obesity hallmarks.³³ Other genera affected by the antibody treatment are shown in **Supp Figure S4A–H**.

We also conducted a non-targeted metabolomic analysis of the cecal content. In total, 6984 different metabolic features were detected, after peak detection and clean-up, among which the mean value of 2387 features was significantly altered (**Supp Table S1**). An exploratory PCA revealed that most of the changes were caused by ILC1s depletion (24.9% of explained variance), although, as expected, the HFHSD also triggered a shift in the metabolic profile (12.9%) (**Figure 4H**). From the total detected features, only 263 (~4%) were adequately annotated, with several notable examples. Imidazole propionate is a novel microbial-derived metabolite associated with T2D and is elevated in patients with gut inflammation.³⁴ We found that imidazole propionate was significantly decreased by ILC1s blockade as compared with the control diet group but not with the HFHSD group (**Supp Figure S4I**). Moreover, a significant increase in the dietary flavonoid rutin was evident in the AGM1-treated group (**Supp Figure S4J**). It has been shown that rutin supplementation in obese mice increases serum GLP-1 levels and dose-dependently inhibits DPP4 activity.³⁵ A deeper metabolic network analysis using the *mummichog* algorithm revealed 9 altered pathways (containing

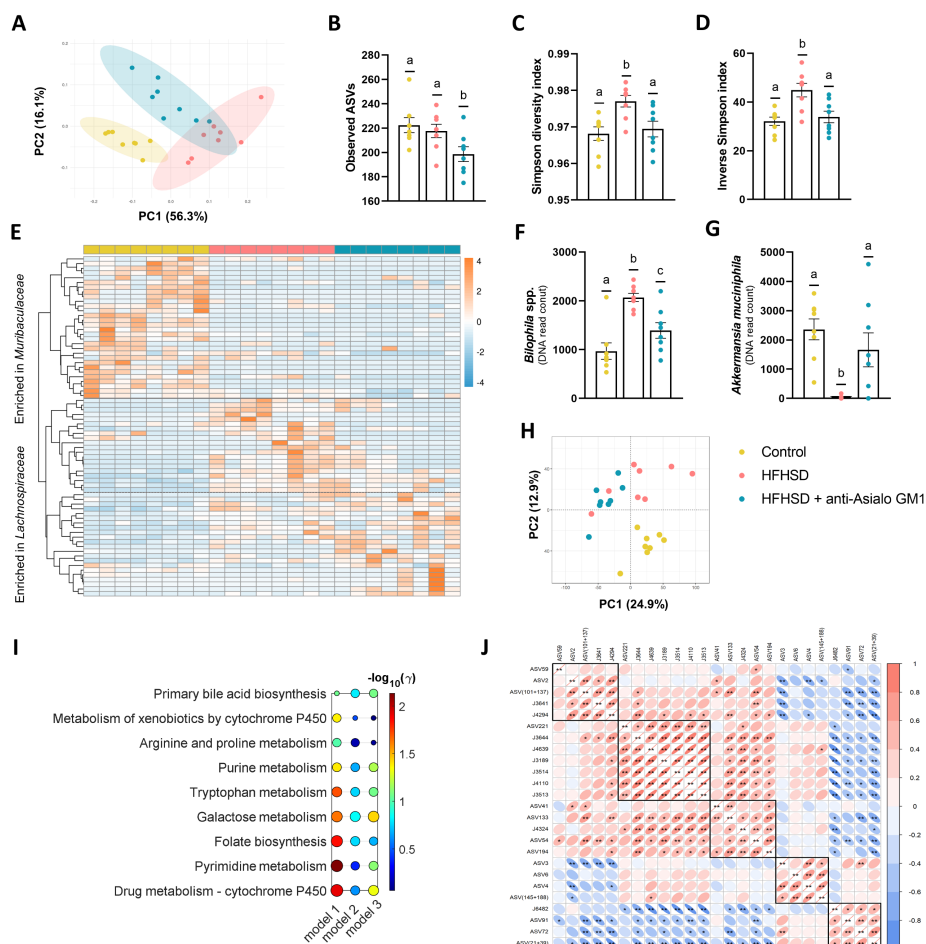


Figure 4. Depletion of intestinal ILC1s shaped the gut microbiome and the metabolic signature. (a) Beta-diversity based on weighted UniFrac distances. (b) Observed ASVs. (c, d) Simpson's diversity and evenness indexes. (e) Heat-map representing the clustering of the two more abundant families (*Muribaculaceae* and *Lachnospiraceae*). (f) *Bilophila* spp. and (g) *Akkermansia muciniphila* abundance. (h) Principal component analysis based on metabolomic data. (i) Scheme of the altered pathways between HFHSD-fed animals and HFHSD-fed mice depleted for ILC1s (model 1: HFHSD vs HFHSD anti-Asialo GM1; model 2: control diet vs HFHSD anti-Asialo GM1; model 3: control diet vs HFHSD), and (j) correlations between the ASVs and the metabolites significantly different between HFHSD vs HFHSD anti-Asialo GM1. ASV59: *Lachnospiraceae* NK4A136 group; ASV2: *Bilophila*; ASV (101 + 137): *Colidextribacter*; J3641: uridine; J4294: glucosiduronic acid; ASV221: *Lachnospiraceae* GCA-900066575; J3644: Fatty acid; J4639: D-maltose; J3189: suberic acid; J3514: sedanonic acid; J4110: qesitol; J3513: traumatic acid; ASV41: *Oscillospiraceae*; ASV133: *Oscillospiraceae* UCG-003; J4324: Fatty acyls; ASV54: *Lachnospiraceae* NK4A136 group bacterium; ASV194: *Lachnospiraceae* A2; ASV3: *Akkermansia muciniphila*; ASV6: *Rikenellaceae* RC9 gut group; ASV4: *Lachnospiraceae* NK4A136 group bacterium; ASV (145 + 188): *Ruminococcaceae*; J6482: rutin; ASV91: *Coriobacteriaceae* UCG-002; ASV72: *Parasutterella*; ASV (21 + 39): *Bacteroides*. In all cases, n = 8. Bars represent the mean ± SEM and biological replicates are shown as individual dots. Pathway analysis was carried out using the mummichog algorithm with a p-value cutoff of 0.05 and the human KEGG pathway database.

at least 3 tentatively assigned metabolites and more than 3 significantly altered) between HFHSD-fed animals treated or not with AGM1 (model 1) (**Figure 4I**). In total, 7 pathways were significantly modified (γ -value < 0.05), of which 2 also appeared significantly altered in model 3 (control vs HFHSD) (**Figure 4I, Supp Table S2**). No pathways were significantly altered in model 2 (control vs HFHSD+AGM1) (**Figure 4I, Supp Table S2**). The greatest metabolic change in model 1 was found in the pathway of pyrimidine metabolism. Alterations in pyrimidine metabolism have been associated with lipid accumulation and gestational diabetes.^{36,37} Among the 34 metabolites tentatively assigned to the pyrimidine pathway, uridine was one of those identified (**Supp Table S2**). The role of uridine in obesity remains controversial. Uridine administration can ameliorate diet-induced obesity,³⁸ but high uridine plasma levels are related to obesity and T2D.³⁹ We found that uridine levels were reduced by ILC1s depletion (**Supp Figure S4K**). Additionally, the analysis revealed that uridine was positively correlated with *Bilophila* spp., *Colidextribacter* spp., and *Lachnospiraceae* NK4A136 group bacterium (a member of the Lachnospiraceae family), which we associated with the obese phenotype, and negatively correlated with *A. muciniphila* (**Figure 4J**).

Overall, there is an improvement in the microbial ecosystem secondary to the depletion of ILC1s and the pro-inflammatory intestinal milieu in obesity. Given that non-targeted metabolic analysis lacks an initial hypothesis, some of our conclusions remain speculative; however, our approach allowed us to characterize metabolite changes associated with the microbiota and ILC1s depletion that warrant further investigation.

CONCLUSIONS

We establish, for the first time to our knowledge, the contribution of intestinal ILC1s to the activation of macrophage-induced inflammation, with downstream consequences for endocrine peptides responsible for metabolic dysregulation in obesity. We cannot, however, exclude the possibility that AGM1 can reach other tissues (like the adipose and the liver^{19,40}) and some of our data (such as the lower adiposity or improved insulin resistance index) might also be an effect of ILC1s depletion in other locations. Nonetheless, we confirmed that the depletion of intestinal ILC1s coincided with the restoration of intestinal immune and endocrine markers compromised by the obesogenic diet. Moreover, ILC1s depletion prevented changes to the integrity of the mucus barrier through activation of the ILC3/IL-22 pathway and reset the microbiota and metabolome profiles to a healthier symbiotic state. It remains to be established whether intestinal ILC1s might be a primary target to alleviate unhealthy diets effects and improve or prevent the metabolic complications in obesity.

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Author contributions: RL-G, MO and YS conceived and designed the study, RL-G and MO performed the *in vivo* experiment and analyzed the samples, CF-C analyzed the 16S rRNA data; TR and GQ analyzed the metabolic data; VR: contributed to the immune analysis and critically reviewed the manuscript. RL-G, MO, and YS wrote the manuscript. All authors reviewed and agreed with the final version.

Data availability statement: The sequences corresponding to the analyses of the gut microbiota composition are uploaded in the ENA (project number PRJEB53615; <https://www.ebi.ac.uk/ena/browser/view/PRJEB53615>). The non-targeted metabolic data used in this study is available in the Zenodo repository (https://zenodo.org/record/7104987#.Y_OwfR_MJdg, doi:10.5281/zenodo.7104987).

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SUPPLEMENTAL MATERIAL

DPP4 activity

The DPP-4 activity was determined by the cleavage of para-nitroanilide (PNA) from the substrate Gly-Pro-PNA (Sigma, Madrid, Spain). Briefly, 20–50 mg of tissue was homogenized in Tris buffer (50 mM, 1% n-octyl-glucoside, pH 8.3). A volume of the supernatant (20 μ L) was incubated with Gly-Pro-PNA and enzymatic activity was measured kinetically for 30 minutes at 37°C (380 nm). DPP4 activity was quantified with a standard curve of free PNA. In the adipose tissue and liver samples, the values were normalized by the amount of protein, quantified by the Bradford method (Bio-Rad, Hercules, CA).

Immune staining

The epithelium and the lamina propria were isolated, digested, and stained with the following antibodies: ILC1s (CD90⁺ CD127⁺ LIN⁻ Tbet⁺ IFN γ ⁺) in the intraepithelial fraction were labeled with a PerCP-CyTM5.5-conjugated anti-Lineage antibody cocktail (LIN) (BD Biosciences, Franklin Lakes, NJ), phycoerythrin (PE)-Vio615-conjugated anti-Tbet and allophycocyanin (APC)-conjugated anti-IFN γ (Miltenyi Biotec, Bergisch Gladbach, Germany). Lamina propria ILC2s (CD90⁺ CD127⁺ LIN⁻ GATA3⁺ IL-4⁺) and ILC3s (CD90⁺ CD127⁺ LIN⁻ ROR γ t⁺ IL-22⁺) were determined using PerCP-CyTM5.5-conjugated anti-LIN, Brilliant Ultraviolet 395 (BUV395)-conjugated anti-GATA 3 (BD Biosciences), PE-Cy7-conjugated anti-IL-4 (Biolegend, San Diego, CA), VioB515-conjugated anti-ROR γ t (Miltenyi Biotec) and PE-conjugated anti-IL-22 (Biolegend) antibodies. Pro-inflammatory (M1: F4/80⁺ CD80⁺ iNOS⁺) and anti-inflammatory (M2: F4/80⁺ CD206⁺ Arg1⁺) macrophages were labeled with FITC-conjugated anti-F4/80⁺, PE-Vio770-conjugated anti-CD80 (Miltenyi Biotec), APC-conjugated anti-iNOS2 (ThermoFisher Scientific), PerCPCy5.5-conjugated anti-CD206 (Biolegend) and PE-conjugated anti-Arg1 (R&D Systems, Minneapolis, MN) antibodies. For all intracellular marker staining, cells were permeabilized and fixed (fixation/permeabilization solution kit, BD Biosciences). Data acquisition and analysis were performed using a BD LSRFortessa flow cytometer operated with FACS Diva software v.7.0 (BD Biosciences). Data were analyzed using FCS express version 5.

Histological and immunohistochemical analysis

Adipocyte size was quantified in visceral WAT after staining with hematoxylin/eosin. Bright-field digital images were taken using an Eclipse 90I (E90I) Nikon microscope (Nikon Corp., Tokyo, Japan) equipped with a 5-megapixel cooled digital color camera (DS-5Mc; Nikon Corp.) under a CFI Plan Fluor 4X (MRH00040) Nikon objective with an optical zoom factor of 2 $\times$. A combined analysis was carried out using Fiji (ImageJ 1.49q Software, NIH) and NIS-elements BR 3.2 software (Nikon Corp.). The cross-sectional area of each adipocyte was automatically recognized and calculated by the NIS-elements

software. Artifacts were manually discarded. Three independent measurements from different sections were performed on each mouse, and 80–350 adipocytes were quantified on each measurement.

Neuroendocrine cells (NECs) in the ileum were detected in paraffin sections (5 μm) immunostained with an antibody to synaptophysin, using the Autostainer link 48 (DAKO, Glostrup, Denmark). The number of immune-positive cells in two non-serial sections was counted by a blind specialist researcher using an E90I Nikon microscope. The mucosa area of the sections was then manually delineated using NIS-elements software and the results were expressed as the average of NEC number per mucosal area (number/ mm^2).

Mucin production in the colon was calculated after staining with Alcian blue using eosin as a contrast dye. Bright-field digital images were taken using an E90I Nikon wide-field microscope, as above. The main individual image properties were as follows: RGB 24 bits, frame size of 2560*1920 pixels, image dimensions of the field of view of 1.08*0.815 mm (0.43 microns/pixel). To analyze the complete colon section, several overlapping images of each intestinal section were stitched automatically with Fiji software (ImageJ 1.49q software). The area positive for Alcian blue and the total mucosal area were selected using a macro with fixed binary segmentation parameters. Both areas were calculated using NIS-elements BR 3.2 software. Mucin production was expressed as the total area of mucin-producer cells relative to the whole mucosa area.

Metabolic analyses

Reversed-phase chromatography analysis was performed using an Acquity BEH C18 (100 $\times$ 2.1 mm, 1.7 μm) column (Waters Corp., Wilmslow, UK) on an Agilent 1290 Infinity UPLC chromatograph (Agilent Technologies, Santa Clara, CA). Full scan MS data from 100 to 1700 m/z was collected on an iFunnel quadrupole time-of-flight (TOF) Agilent 6550 spectrometer (Agilent Technologies). Electrospray ionization was used for sample volatilization. ESI+ and ESI- analysis were carried out in independent batches. Peak table generation was carried out for each batch separately using XCMS in RStudio (RStudio Inc., Boston, MA). Metabolite annotation was performed using an iterative data-dependent acquisition (DDA) method previously developed¹, and matching m/z values, isotopic profiles, and MS/MS data against the Human Metabolome Database (www.hmdb.ca), METLIN and LipidBlast (LipidEx) databases. Data acquisition and manual integration were carried out employing the MassHunter Workstation (version B.07.00) from Agilent. Raw data were converted into mzXML format using ProteoWizard (<http://proteowizard.sourceforge.net/>). Peak detection, integration, deconvolution, alignment, and pseudospectral identification were carried out using XCMS and CAMERA in R v.4.2.

For reversed-phase chromatography, autosampler and column temperatures were set to 4°C and 55°C, respectively, and the injection volume used was 4 μl .

Gradient elution was performed at a flow rate of 400 $\mu\text{l}/\text{min}$ as follows: initial conditions of 98% of mobile phase A (H_2O , 0.1% v/v HCOOH) followed by a linear gradient, 2–20%, of mobile phase B (CH_3CN , 0.1% v/v HCOOH) in 3.5 min and 20–95% B in 4 min. The 95% elution of phase B was held for 1 min and then a gradient of 0.1 min was used to return to the initial conditions, which were held for an additional 2.4 min. Standard needle washes were carried out between injections using the mobile phase to reduce autosampler carryover between injections. The following electrospray ionization parameters were selected: gas temperature, 200°C; drying gas, 14 l/min; nebulizer, 37 psig; sheath gas temperature, 350°C; sheath gas flow, 11 l/min. Automatic MS spectra calibration during the analysis was carried out by introducing a reference standard into the source via a reference sprayer valve and using the following ions (m/z) as references: 149.02332, 121.050873 and 922.009798 (positive mode), and 119.036 and 980.0163 (negative mode). Data obtained during initial column conditioning were excluded from data analysis. For peak detection, the centWave algorithm was used with the following parameters: mass accuracy, 15 ppm; peak width, (5,20); snthresh, 6; prefilter, (3,1000). Intensity weighted m/z values of each feature were calculated using the mean function. Peak limits used for integration were found through descent on the Mexican hat-filtered data. Grouping before and after retention time correction was carried out using the density method. Missing data points were filled by reintegrating the raw data files in the regions of the missing peaks using the fill Peaks method. Accuracy of the peak integration and alignment were assessed by comparing peak area values obtained by manual and automated (i.e., XCMS) integration results for deuterated injection standards and known metabolites, obtaining linear correlation coefficients > 0.99. A set of 11 QC replicates were injected at the beginning of each batch to ensure system conditioning, and a QC was analyzed every 6 samples. Three consecutive blanks were analyzed at the end of each batch for background elimination. QC replicates were used for the correction of within-batch effects using the quality control samples and support vector regression correction algorithm ^{2,3} and a radial basis function kernel. Selection of the tolerance threshold (ϵ), the penalty term applied to margin slack values (C), and the kernel width (γ) was carried out using a pre-selection of C and optimization of ϵ and γ using a grid search, leave-one-out cross-validation, and the root mean squared error of cross-validation (RMSECV) as target function. C was selected for each UPLC-MS feature as the median value of the intensities observed in QC replicates. The ϵ search range was selected based on the expected instrument precision (4–10% of the median value of the intensities observed for the whole set of QC replicates). The γ search interval selected was $[1, 10^5]$. UPLC-MS features were removed from further analysis if the relative standard deviation of QCs >20% after batch effect correction. A second data clean-up was carried out after within-batch effect correction using the set of blank samples independently for each batch. For metabolite annotation, an aliquot of the QC was repeatedly analyzed using the auto DDA mode with the following inclusion m/z precursor

ranges, 100–200, 200–400, 400–600, 600–800 and 800–1200, using in all replicates centroid mode at a rate of six spectra/s in the extended dynamic range mode (2 GHz). The collision energy was set to 20 V, MS/MS fragmentation with an automated selection of five precursor ions per cycle and an exclusion window of 0.15 min after two consecutive fragmentations. The MS/MS spectral match was carried out using the geometric mean value of the dot and the reverse dot products. Fragment ions present in the reference spectra included in the same pseudospectrum of an annotated metabolite were annotated as fragments.

The differential concentration analysis of metabolomics was performed with the Wilcoxon Rank Sum test corrected by the Benjamini-Horchberg false discovery rate (BH-FDR) procedure. Principal component analysis (PCA) was performed with the FactoMineR v.2.4 R package, centering and scaling all the detected metabolites based on differences in concentration. Pathway analysis was performed with the mummichog algorithm⁴ with a p-value cut-off of 0.05 and the human KEGG pathway database. Data analysis was carried out in MATLAB 2021a (Mathworks Inc., Natick, MA) using in-house written scripts, and the PLS Toolbox 8.9 (Eigenvector Research Inc., Wenatchee, WA). For the integration analysis, significant features in the comparisons of ILC1-targeted and non-targeted obese mice from the differential abundance analysis were selected in both metabolomic and 16S datasets. Regarding repeated features, non-molecular ions were removed from the list of selected metabolites. Spearman's correlation between features was calculated with the cor.test function in R and p-values were adjusted by BH-FDR. The correlation matrix was graphically displayed with the corrplot v0.92 R package with hierarchical clustering order using the ward.D agglomeration method.

References

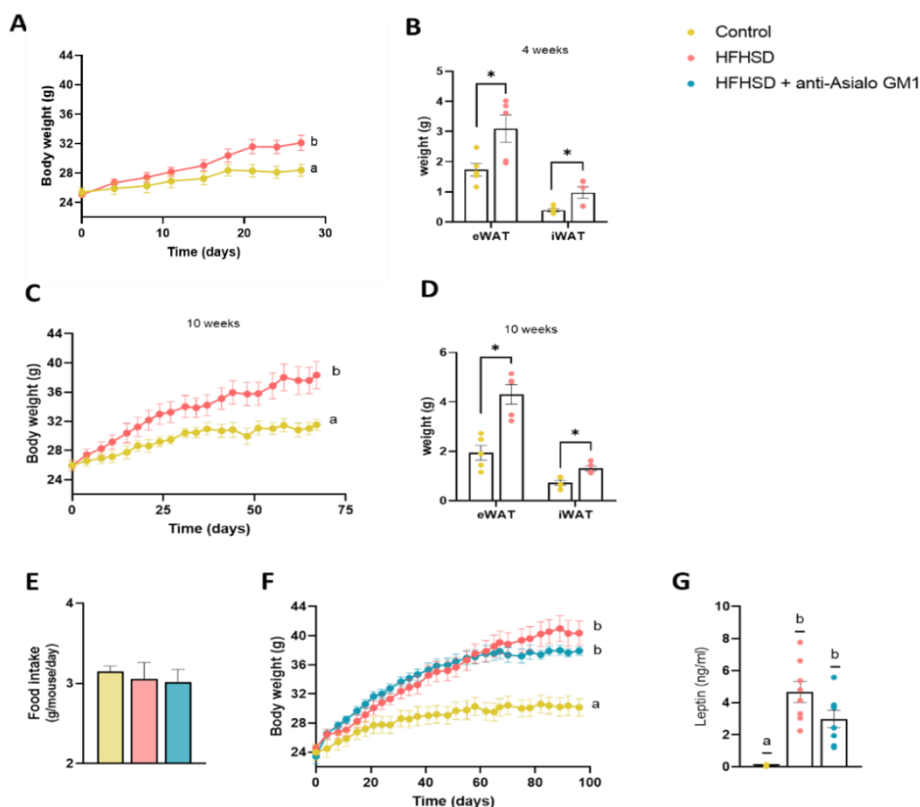
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Supp. Table 1: Metabolic features detected in the cecal-content by a non-targeted analysis. → <https://doi.org/10.1080/19490976.2023.2181928>

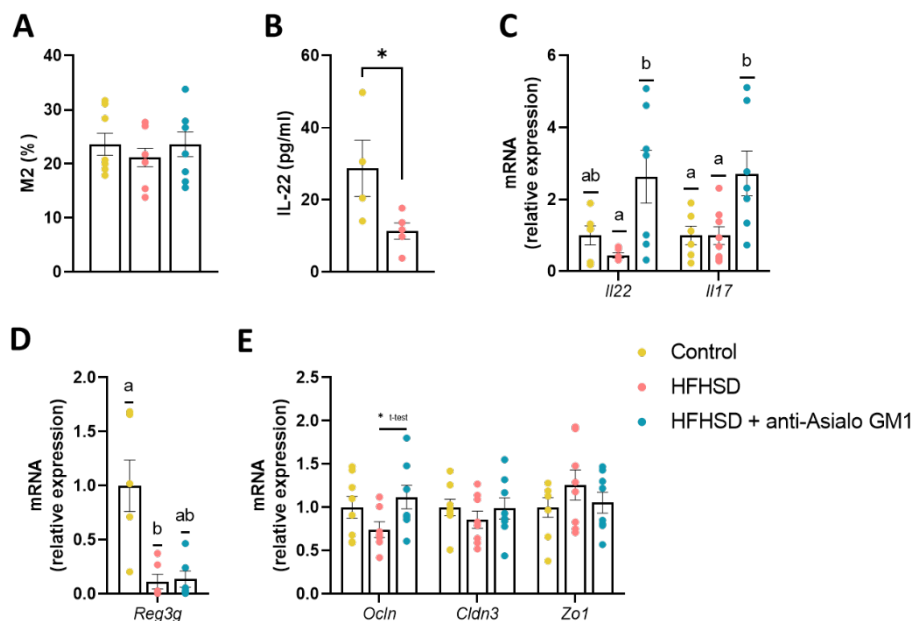
Supp. Table 2: Metabolic network analysis by *mummichog* algorithm. → <https://doi.org/10.1080/19490976.2023.2181928>

Supp. Table 3: Primers used in this study

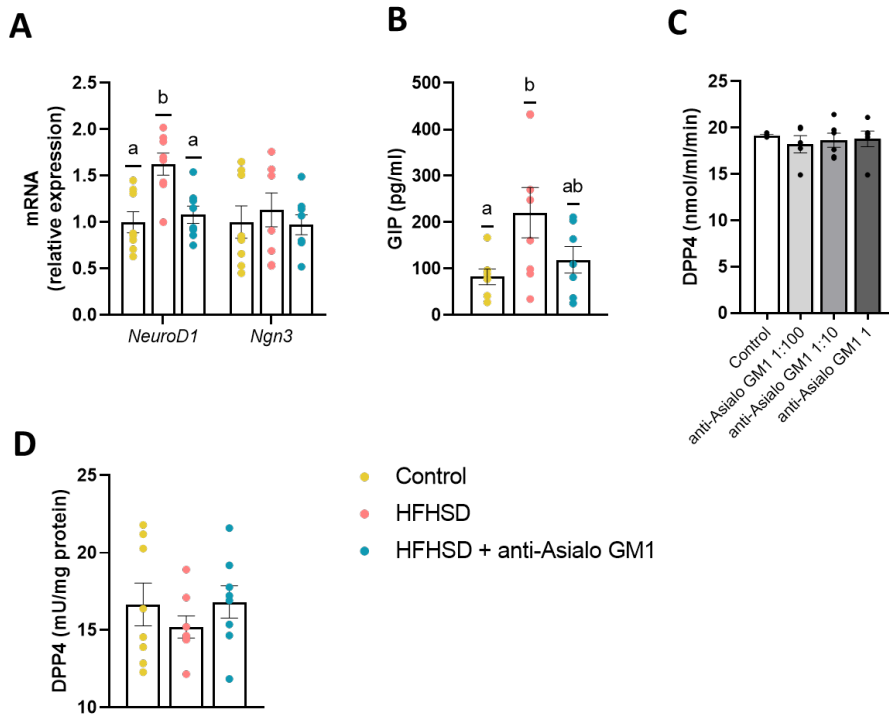
Targeted gene	Sequences (5'-3')
<i>Cd36</i>	F: GCCAAGCTATTGCGACATGA R: ATCTCAATGTCCGAGACTTTTCAAC
<i>Cebpb</i>	F: TCGGGACTTGATGCAATCC R: AAACATCAACAACCCCGC
<i>Cldn3</i>	F: TCATCGGCAGCAGCATCATCAC R: ACGATGGTGATCTTGGCCTTGG
<i>Il17</i>	F: CTCCAGAAGGCCCTCAGACTAC R: GGGTCTTCATTGCGGTGG
<i>Il22</i>	F: GACATAAACAGCAGGTCCAGTT R: AGAAGGCTGAAGGAGACAGT
<i>Lpl</i>	F: TGAAAGCCGGAGAGACTCAG R: AGTGTCAGCCAGACTTCTTCAG
<i>Muc2</i>	F: CCCAGAAGGGACTGTGTATG R: TGCAGACACACTGCTCACA
<i>NeuroD1</i>	F: AGGAATTCGCCACGCAGAAAG R: CTCCTCTGCATTTCATGGCTTCAAG
<i>Ngn3</i>	F: ACTCAGCAAACAGCGAAGAAG R: CAGTGCCCAGATGTAGTTGTG
<i>Ocln</i>	F: ATGTCCGGCCGATGCTCTC R: TTTGGCTGCTCTTGGGTCTGTAT
<i>Pla2ga</i>	F: AAGGATCCCCCAAGGATGCCAC R: CAGCCGTTTCTGACAGTTCTGG
<i>Reg3g</i>	F: TTCCTGTCCTCCATGATCAAA R: CATCCACCTCTGTTGGGTTC
<i>Tcf 4</i>	F: ATGGCAAACAGAGGAACTGG R: GCCTGCTGAGAGTGAAGGAG
<i>Zo1</i>	F: TTTTGTGACAGGGGGAGTGG R: TGCTGCAGAGGTCAAAGTTCAAG



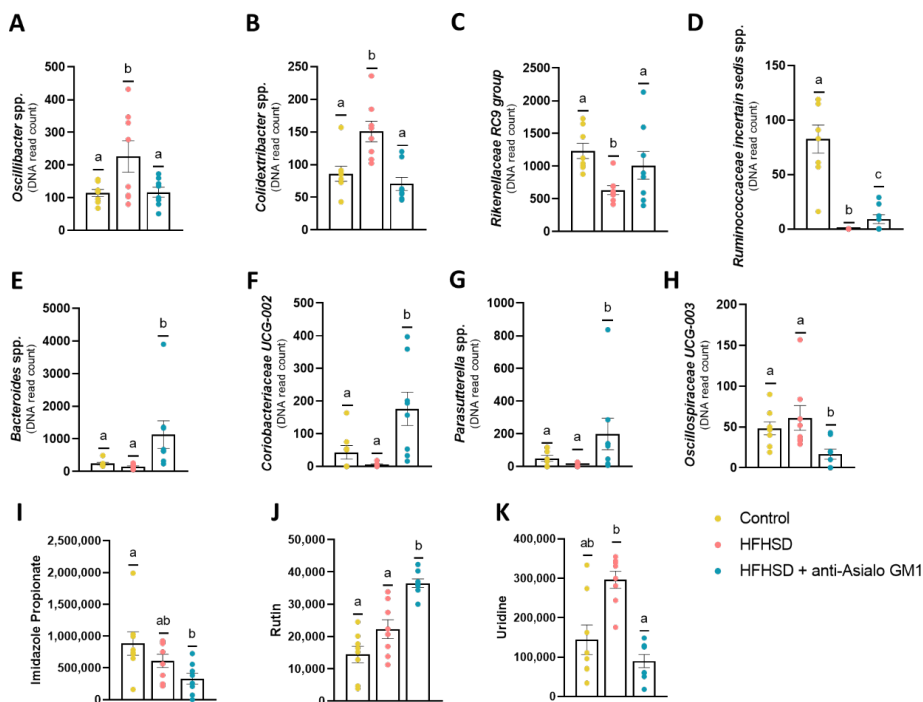
Supp. Fig 1. Changes in body weight and adiposity after exposure to an obesogenic diet. **(A)** Body weight evolution and **(B)** percentage of epididymal (eWAT) and inguinal (iWAT) white adipose tissue after 4 weeks of HFHSD (n=5), **(C)** body weight evolution and, **(D)** percentage of eWAT and iWAT after 10 weeks of HFHSD (n=5), **(E)** food intake from week 10 to week 14 in HFHSD-fed mice and **(F)** body weight evolution after 14 weeks of HFHSD (n=8) **(G)** Plasma leptin levels. Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. Changes in body weight were analyzed by two-way ANOVA followed by Tukey's post hoc test. Changes in adiposity were assessed by using a t-test. Food intake and leptin levels were analyzed by one-way ANOVA. Different superscript letters or the symbol “*” show statistical differences when $p < 0.05$.



Supp. Fig 2. Changes in immune mediators. **(A)** Anti-inflammatory M2 macrophages (percentage of F4/80⁺ cells of total lamina propria cells) in mice fed a HFHSD for 14 weeks (n=8) **(B)** Plasma levels of IL-22 after 10 weeks of an HFHSD (n=5) **(C)** Gene expression of IL-22 and IL-17 in the ileum (n=6-8), **(D)** Gene expression of regenerating islet-derived 3-gamma (*Reg3g*) in colon (n=6-8) and **(E)** Gene expression of tight junction proteins in the colon (n=7-8). Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. The M2 (A) and *Reg3g* expression changes (D) were analyzed with the Kruskal–Wallis test, changes in IL-22 by an unpaired t-test and ileal (C) and colonic (E) relative expression changes analyzed by One-Way ANOVA. Different superscript letters or the symbol “*” shows significant differences at $p < 0.05$.



Supp. Fig 3. Changes in endocrine markers in mice. **(A)** Gene expression of the indicated factors of enteroendocrine cell differentiation in the ileum (n=8). **(B)** Plasma GIP levels (n=7–8). **(C)** DPP4 activity in a pool of plasma with or without different dilutions of anti-Asialo GM1 (n=4–6). **(D)** DPP4 activity in the liver (n=8). Bars represent the mean $\pm$ SEM and biological replicates are shown as individual dots. Data were analyzed by one-way ANOVA. Data with different superscript letters are significantly different at $p < 0.05$.



Supp. Fig 4. Bacterial abundance and metabolites in the cecal content. **(A)** *Oscillibacter* spp., **(B)** *Colidextribacter* spp., **(C)** *Rikenellaceae* RC9 group, **(D)** *Ruminococcaceae incertain sedis* spp., **(E)** *Bacteroides* spp., **(F)** *Coriobacteriaceae* UCG-002, **(G)** *Parasutterella* spp., **(H)** *Oscillospiraceae* UCG-003, **(I)** imidazole propionate (arbitrary units), **(J)** rutin (arbitrary units) and **(K)** uridine (arbitrary units). n=8 in all cases. Bars represent the mean ± SEM and biological replicates are shown as individual dots. Differential metabolite abundance between groups were compared with a Wilcoxon rank sum test and the p-value was corrected with the Benjamini-Hochberg procedure. Data with different superscript letters are significantly different at $p < 0.05$.

GENERAL DISCUSSION

GENERAL DISCUSSION

Obesity is a major risk factor for morbidity, disability and death worldwide and, therefore, a major public health challenge. According to the WHO, overweight and obesity have reached epidemic proportions in the European region, affecting almost 60% of adults and one in three school-aged children ¹⁹⁶. Obesity substantially reduces health and well-being and, consequently, generates considerable costs, which place a strain on national healthcare systems, economic productivity, and social resources. Accordingly, addressing obesity is a priority for achieving the Sustainable Development Goals (SDG). However, despite policy efforts, there have been a steady increase in the prevalence of overweight and obesity in the European Region, and no Member State is on track to reach the target of halting the expected rise in obesity by 2025. There is thus an urgent need to better understand the interplay between environmental obesity drivers (e.g., unhealthy diets) and individual response to such drivers (e.g., the response mediated by gut microbiota), and to develop more effective interventions to combat obesity at population and individual levels.

In this Doctoral Thesis we have evaluated different dietary approaches that could help ameliorate obesity and its comorbidities. In the First Chapter, we have proven the thermogenic and anti-inflammatory properties of a new plant-based extract namely propyl propane thiosulfinate (PTS) in an animal model of obesity. In a Second Chapter, we have deciphered the immune-mediated mechanisms of action of the newly isolated bacterial strain of the species *Phascolarctobacterium faecium* to prevent obesity. In the last study, we have unraveled, for the first time, the involvement of intestinal ILC1s in the cascade of events triggered by an obesogenic diet that causes local and systemic inflammation and provided insights on the ILC1 links with intestinal and endocrine homeostasis (**Figure 4**).

The progress on the identification of novel approaches to combat obesity, as the ones presented in this Doctoral Thesis, aligns with the third SDG, which aims to promote good health and wellbeing ⁶. Besides, the re-valorization of end-products from onions as raw material to generate PTS fits well with the 12 SDG and the European Green Deal of the European Commission towards the promotion of a circular and resource-efficient food system ^{6,197}.

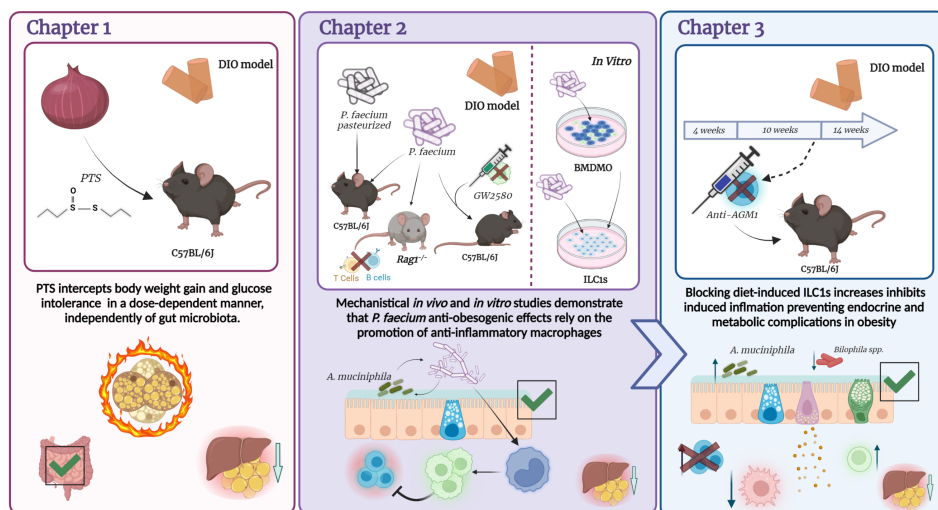


Figure 4. Graphical summary of the studies and main results that comprise this Doctoral Thesis. Created with BioRender.com (Agreement number: CT25OA9LZ9).

THE ROLE OF INTESTINAL ILC1s IN OBESITY

In this Doctoral Thesis we have used DIO mice as a model of obesity instead of other alternatives like genetically modified mice. Genetically modified obesity models, as the *Lep^{ob/ob}* or *Lep^{db/db}* mice (both defective in leptin signaling) have been widely used and provided a valuable information of the pathophysiology of obesity and T2D ^{198,199}. However, these animal models have important limitations. For example, *Lep^{ob/ob}* mice present an impaired immune function and lower physical activity ¹⁹⁹. Besides, knock out (KO) models represent an oversimplification of obesity causes to a monogenic one (for instance, leptin). However, the most common form of obesity is multifactorial and requires the involvement of several genetic and environmental factors ²⁰⁰. As a result, genetically modified mice generally provide less translational information than do wild-type mice.

Feeding animals with a high energy diet is a common alternative to reproduce obesity and its metabolic complications ²⁰¹. The C57BL/6J strain is susceptible to develop obesity when feeding hypercaloric diets in view of the increased adiposity, and the onset of IR and glucose intolerance ²⁰¹. Accordingly, C57BL/6J mice fed an obesogenic diet are widely used to model obesity ²⁰². Specifically, here we have fed C57BL/6J mice with a high-fat high-sugar diet (HFHSD), where the 45% of the Kcal are provided by saturated fat and the 17% of the Kcal by sucrose. The composition of this diet aims to resemble the Western diet that is rich in fats and sugars. Alternatively, some authors have used another obesogenic diet that is based on increasing only the fat content (≥60% of the total Kcal) ²⁰³. Feeding mice with this diet versus the HFHSD could explain, at least in part, some discrepancies across studies. For instance, when using diets with the 60% to 80% of Kcal from fat, the increase in the weight gain and the

glucose tolerance worsening and IR can be appreciated after one or two weeks of dietary exposure ^{203,204}. In contrast, when feeding a HFHSD, there is a slower development of obesity, glucose intolerance and IR (noticed at least after 4-5 weeks of exposure). Despite of diets with a fat content greater than 60% lead to a rapid obesity development, this composition significantly deviates from the normal fat content. Note that, in a human diet, fat provides ~25-30% of daily Kcal and then, the use of HFD $\geq$ 60% brings results less comparable to human physiology that does a diet of 45% of Kcal coming from fat ¹⁹⁸.

This Doctoral Thesis shows the relevance of the inflammatory state in obesity and its metabolic complications. In particular, we have focused on the pro-inflammatory tone caused by obesogenic diets in the intestine and the tie with the microbiota and the impairment of metabolic organs ^{24,205}. The intestine is the first organ exposed to the effects of the diet, which modulate intestinal immunity and the gut microbiota ²⁴. Indeed, an impairment of the intestinal homeostasis has been proposed as one of the causal mechanisms underlying the pathophysiology of obesity. The microbiota interacts with the host regulating immunological, endocrine, and neural pathways that mediate communication between the gut and peripheral organs, ultimately influencing metabolic health ²⁹. In the study presented in Chapter 3, we have investigated longitudinally the changes that the HFHSD causes in the intestinal ecosystem and immune cell populations. Our final goal was to establish more precisely the sequence of events by which obesogenic diets compromise the metabolic health. Specifically, we have analyzed the changes occurring after 4, 10 and 14 weeks of HFHSD exposure. The first significant insight of the dietary impact was observed in the AT and the body weight gain. Four weeks of HFHSD led to increases in the fat depots, that were associated with the over expression of genes related to FA uptake and adipogenesis (*Pparg*, *Cd36*, and *Lpl*; not shown) contributing to higher fat deposition. Moreover, also in the AT, we observed an increased in the expression of pro-inflammatory markers (*Cd11c* and *Mcp1*; not shown) predicting the adipose hypertrophy observed in the AT after 14 weeks of obesogenic-diet feeding. Other authors have similarly reported a macrophage increased infiltration in the AT already at four weeks of feeding with an obesogenic diet (60% HFD), as well as increases in *Cd11c* and *Mcp1* in liver and AT ^{160,206}. In fact, increased levels of CD11c⁺ cells in AT have been related to immune cell recruitment, mainly inducing pro-inflammatory cell accumulation, AT inflammation, and insulin resistance, both in mice and human ^{207,208}.

We also analyzed the effect of the HFHSD on the intestinal immune cell populations at different time points of exposure (4, 10 and 14 weeks). Specifically, we have focused on two subsets of the innate arm: the ILCs and macrophages. In agreement with other authors ⁸², some results obtained in this Doctoral Thesis (mainly in the Chapters 2 and 3) have proven the importance of ILC subsets for obesity development. The longitudinal study presented in the Chapter 3 showed that the obesogenic diet boosts intestinal ILC1s and M1 percentages after 10 and

14 weeks of feeding with a HFHSD. Indeed, these increases were observed already after 4 weeks of dietary exposure, although the changes did not reach statistical significance until later stages of exposition. Part of our findings are consistent with previous research that observed greater ILC1s abundance in the gut, after treating mice with a HFD for 12 weeks ²⁰⁹. Interestingly, in our study, these increases in ILC1s and M1 macrophages occurring at the intestinal level were simultaneously to the significant disturbances in the systemic circulation (higher insulinemia, cholesterol, and IL-22 decrease).

Furthermore, we looked into the impact of HFHSD in some of the obesity complications, such hepatic steatosis, IR and glucose dysregulation. All them were worsening progressively under the exposure to the obesogenic diet. Additionally, from the beginning (forth week) there was a significant increase in GIP levels (data not shown) that continued to rise along the 14 weeks that lasted the experiment. As we discussed in Chapter 2, the increased circulating levels of GIP might be contributing to the overexpression of genes related to FA uptake, adipogenesis and inflammation in the AT, given its well-known anabolic function and its contribution to inflammation ³⁴. However, the role of GIP remains paradoxical. Some studies have indicated that GIP might act as an obesogenic peptide since higher GIP secretion worsen the development of IR and obesity in DIO models ²¹⁰. Similarly, KO mice in the GIP receptor showed protection against the development of obesity in either WT and *Lep^{ob/ob}* mice ²¹¹. In contrast, some authors have evidenced beneficial effects of GIP receptor agonists (e.g., tirzepatide) in the treatment of obesity ²¹².

Finally, in the study presented in Chapter 3 we also cancelled the HFHSD-induced ILC1s increase through the administration of an antibody (AGM1). The ILC1s depletion resulted, on one hand, in a reduction of the diet-induced inflammatory response, including a decrease in M1 macrophages, and on the other hand, in a better preservation of mucus barrier integrity via the activation of the ILC3/IL-22 pathway and NECs, with subsequent improvements in metabolic parameters such as reduced adiposity and insulinemia. Taken together, these findings emphasize the importance of ILC1s in the development of metabolic complications associated with the obesogenic diet ²⁴. Additionally, the ILC1s depletion significantly impacts on the microbiome, likely as a consequence of the inflammatory tone reduction, leading to a favorable reset of the microbial communities and the metabolome profile. For example, we found an increase in *A. muciniphila*, associated with protection against obesity ¹⁶⁹, and a decrease in *Bilophila* spp., related to the promotion metabolic dysfunction ²¹³. Our findings confirm the relationship between intestinal immunity, particularly the ILC1s, and the gut microbiota, and its critical role in the development and progression of metabolic disease ^{29,214}.

NEW DIETARY AND MICROBIOME-BASED STRATEGIES TO COMBAT OBESITY

Within the framework of this Doctoral Thesis, we have also provided pre-clinical evidenced of the preventive effects of two potential dietary alternatives for the treatment of obesity. In particular, we have proven the efficacy of a sulfur-derived extract (Chapter 1) and a new potential probiotic bacterium (Chapter 2) to attenuate the adverse effects of the obesogenic diet on the metabolic phenotype, specially reducing the body weight gain and improving glucose homeostasis.

The First Chapter is focused on the role of a bioactive compound named PTS that is extracted from *Allium cepa*, commonly known as onion. We justify the evaluation of PTS in an animal model of DIO given the proven anti-inflammatory properties of this and other *Allium*-derivate compounds ^{160,215,216}. Besides, initially we also hypothesized that PTS could exert a prebiotic-like effect, according to previous observations in *in vitro* fermentations ²¹⁷, and with other related compounds such as allicin or PTSO *in vivo* ^{160,218}. In our study, we showed that PTS can prevent body weight gain, adiposity, and alterations in glucose metabolism in a dose-dependent manner. However, microbiota analysis revealed limited effects over the microbial communities with only minor taxonomic changes, such as an increase in butyrate producers, *Intestinimonas* and *Alistipes*, and a decrease in *Bifidobacterium* in mice receiving the highest PTS dose. Our results pointed to three interconnected mechanisms throughout which PTS could exert anti-obesogenic effects: 1) the promotion of the thermogenic activity, 2) the decrease of inflammation in peripheric organs and 3) the preservation of intestinal homeostasis (normalization of the expression of gut barrier function markers, AMPs and IL-22 levels). Adipocyte browning and thermogenic potentiation have been recognized as crucial mechanisms underlying the anti-obesogenic properties of several plants and fruits-based extracts ^{158,219}. To what concerns *Allium* derivatives, this thermogenic effect has not been reported in PTSO (a degraded form of PTS), but in allicin (analog compound) ^{160,220}. However, in contrast to our results, the thermogenic potential of allicin is, at least in part, attributed to the changes induced in the gut microbiota ²²⁰. In accordance, BAT activation was reproduced after allicin-induced gut microbiota transplant to 60% HFD fed mice ²¹⁸. Interestingly, while allicin directly promoted *Ucp1* and FAO genes expression, our results show that PTS promoted FAO genes expression and citrate activity increase, but not *Ucp1* ^{218,220}. Recently, the anti-obesogenic properties of *Bifidobacterium bifidum*, *B. longum*, *Pediococcus pentosaceus*, *Parabacteroides goldsteinii*, and *Lactobacillus plantarum* have been related to their ability to increase thermogenesis, mainly by upregulation of UCP1 and UCP2 levels in DIO animal models ^{221–224}. However, none of this bacterial species were increased by PTS and, thus, in contrast to allicin, we cannot consider that microbiota changes act as key mediators of the observed increase in the thermogenic activity.

PTS can be from *in situ* from onions, and is known to reach the intestine (personal communication from the partner company), where it might be absorbed. PTS formation occurs when the onion is cut or chopped. In industrial conditions, as a broad estimation, each onion can generate around one to five milligrams of PTS. Then, an individual with an average weight of 60-70 kg will have to consume approximately 15 onions per day to be exposed to the higher dose used in our mouse study (1 mg/kg/day). This means that the effects we report here will only be achieved by the consumption of the PTS as a supplement, but not as part of foods in the context of a normal healthy diet. Also, considering the physiological differences between mice and humans, further research will be needed to confirm whether the anti-obesogenic effects we observed are replicated in humans. In this sense, recent *in vitro* studies have proven the anti-inflammatory properties of PTS in several human tumor cell lines ²²⁵. Some related beneficial properties (e.g., anti-inflammatory, anti-oxidant and cardioprotective properties) of garlic and other species of the *Allium* genus have also been described in human studies and mainly attributed to their content in organosulfur compounds ²²⁶. Indeed, supportive studies have already described the potential of allicin to promote browning in humans constituting an alternative for ameliorating obesity and related diseases ²²⁷. Therefore, forecasting a promising transference of observed PTS pre-clinical benefits to humans.

In Chapter 2, we have evaluated the interest of *P. faecium* as a potential next-generation probiotic to counteract the effects of an obesogenic diet. *P. faecium* is a Gram-negative, non-motile, non-sporulating, highly pleomorphic and anaerobic bacterium that belongs to the Firmicutes phylum ²²⁸. The species was firstly isolated from koala's feces ²²⁸, while the strain of *P. faecium* DSM 32890 here evaluated was isolated in our team from the feces of a healthy and lean human donor. Indeed, *P. faecium* is an abundant gastrointestinal symbiont in humans, representing the 0.004% to 1.479% of the total human bacterial population ²²⁹. Besides, our team have recently published its whole genome sequence ²³⁰. A previous observational study in which we were involved found a link between this genus and a reduced risk of excessive weight gain in children ²³¹. Furthermore, the study presented in Chapter 2 included a broad metagenomic analysis (>6,000 subject) performed in collaboration with the team of Prof. N. Segata (Trento University, Italy) that has confirmed the specific association between the specie *P. faecium* and normal weight in humans. Moreover, we have also demonstrated, the anti-obesogenic properties of *P. faecium* using different murine models of obesity. Taken together, our results proved the ability of the bacterium to reduce body weight gain and adiposity and to restore glucose homeostasis in a reproducible manner. We have attributed these metabolic benefits to the immunomodulatory capacity of *P. faecium* and deciphered its mode of action by using animal models and *in vitro* studies. First, in WT mice fed a HFHSD, we observed that *P. faecium* improved the M1/M2 ratio (by promoting M2 increases), increased T reg cells and reduced ILC1s in the intestine. Besides,

in the circulation, its administration reduced the release of cytokines induced by the HFHSD. Similar outcomes, including prevention of obesity development together with an up regulation of M2 macrophages and downregulation of ILC1s were observed in the second experiment performed in a DIO *Rag1*^{-/-} mice model (devoid of adaptive immunity) with *P. faecium*. This additional experiment evidenced that the ability of *P. faecium* to modulate adaptive immunity, for example Treg cells are not essential for retaining its metabolic benefits. In contrast, when the bacterium together with the M2 inhibitor (GW2580) were administered in the next experiment, the ILC1s reduction and metabolic benefits were cancelled. These findings evidenced that the ability of *P. faecium* to boost the M2 polarization is a key mechanism through which it exerts beneficial metabolic effects. Similarly, very recently it has been reported that *A. muciniphila* protects against obesity-associated inflammation and helps maintain immune homeostasis through the induction of M2 polarization via TLR2 signaling that ends in IL-10 production ²³². Also anti-obesogenic properties of *Parabacteroides goldsteinii* and *Hirsutella sinensis* have been attributed, at least partially, to their ability to promote IL-10 expression, but in this case by Treg cells ²³³. In another study, the benefits of the administration of *Limosilactobacillus fermentum* CRL1446 and *Lactococcus lactis* CRL1434 consisted in plasmatic reductions of TNF α and IL-6 levels ²³⁴. In this sense, *P. faecium* did stimulate IL-10 production *in vitro* by M2-primed macrophages and reduced plasmatic levels of pro-inflammatory cytokines, such as TNF α and IL-6 (among others). Besides, *P. faecium* reduced *in vivo* the ILC1s numbers and increased IL-22 secondary to the *P. faecium*-driven M2 macrophages promotion, an effect that we highlight as the most important proof for its anti-obesogenic properties. Indeed, as presented in Chapter 3, counteracting the HFHSD-driven ILC1s increases through the administration of an antibody (AGM1) restored the immune and endocrine alterations in the intestine, re-equilibrated the gut microbiota, improved the glucose homeostasis and reduced the adiposity. In other words, we observed that the ILC1 depletion partially mirrored the beneficial effects exerted by *P. faecium* in obesity.

The anti-inflammatory properties of *P. faecium* may also be due to its ability to generate metabolites such as propionate (resulting from the consumption of succinate) or butyrate ²³⁰. Propionate is known to down-regulate the secretion of pro-inflammatory cytokines such as TNF α in macrophages *in vitro* ²³⁵. Similarly, butyrate supplementation reduced IL-6 and TNF α , and induced IL-10 *ex vivo* responses in peripheral blood mononuclear cells from obese patients ²³⁶. Indeed, SCFAs (including propionate and butyrate) have shown to decrease systemic inflammation markers in several human interventions ²³⁷. However, *P. faecium* retained the immunomodulatory (and anti-obesogenic) effects when pasteurized, suggesting that a structural bacterial component may be the main trigger of its protective effects. Similarly, the pasteurized form of *A. muciniphila* has been shown to retain its beneficial effects and that the administration of Amuc_1100

(protein isolated from its outer membrane) recapitulated many of the beneficial effects exert by the live bacterium ¹²⁵.

Additionally, this Doctoral Thesis provides evidence on the interaction occurring between ILC1s and macrophages. Our findings support that ILC1 have the ability to promote M1 polarization in the intestine, which is consistent with previous observations in AT ^{78,79,238}. Moreover, as pointed above, we have observed that M2 macrophages possess the ability to reduce the ILC1 cells and shift their cytokine secreting profile (as described in the following section) similarly to DCs ⁵⁸. These novel findings contribute to the understanding of the intricate regulation of immune responses in the obese intestine and the role played by the gut microbiota.

ROLE OF IL-22 IN THE IMMUNE-METABOLIC AXIS IN OBESITY

The three intervention studies conducted in the present Doctoral (PTS, *P. faecium* or the AGM1 antibody to deplete ILC1) have shown to increase IL-22 expression and/or IL-22 concentrations at the intestinal level in obese treated mouse groups. Similarly, the anti-obesogenic mechanism of action of some prebiotic fibers, such inulin, has been attributed to the IL-22 induction ⁴¹. IL-22 plays a crucial role in host innate immunity, the regulation of commensal bacteria and the maintenance of intestinal barrier integrity ^{239,240}. Indeed, IL-22 induction is impaired in DIO models, *Lep^{ob/ob}* and *Lep^{db/db}* obese mice, and IL-22 KO mice fed HFD develop more severe metabolic impairments ²⁴⁰. Moreover, the administration of IL-22 to obese mice have proven to reinforce mucosal barrier, reverse microbial changes and ease oxidative stress, ultimately counteracting metabolic damages exerted by HFD ²⁴¹, which is in line with our observations.

In our study, the mechanism by which PTS induces an elevation in colonic IL-22 levels, either through direct stimulation of IL-22-producing cells or indirect suppression of type 1 immune responses, remains to be elucidated. In this sense, Vezza *et al.* attributed the anti-inflammatory effects shown by the compound PTSO to the reduction of macrophage/DC cell infiltration in the AT ¹⁶⁰. Therefore, we could hypothesize that the immunomodulatory properties of PTS may not only result from a reduction in the expression of pro-inflammatory markers and cytokine levels in metabolic active organs (Chapter 1), but also from the modulation of immune cell numbers in the intestine, ultimately contributing to the IL-22 increase. In our *in vitro* experiments with *P. faecium* (Chapter 2), we confirmed a direct interaction of the bacterium with macrophages by priming them towards an M2-like phenotype. Moreover, the exposition of intestinal ILC1s cultures to the *P. faecium*-primed M2 promoted IL-22 production by the ILC1s. The ability of ILC1s to produce IL-22 has been already described ^{58,242–244}. Some authors attributed this outcome to ILC1s plasticity and potential differentiation into ILC3-like cells driven by IL-23 and IL1 β ⁵⁸, while others observed direct *in vivo* and *ex vivo* IL-22 production by NK cells (members of ILC1s group) after bacterial

infection ^{242–244}. Interestingly, when we depleted ILC1s *in vivo* (Chapter 3), we observed that the inhibition of HFHSD-induced ILC1s increases itself is sufficient to rise IL-22 levels. This suggests that the reduction of ILC1s levels may be acting as a pro-inflammatory suppressive mechanism, favoring type 3 protective response instead (as the one carried by Treg and/or ILC3), driving the enhancement of IL-22 levels ⁶⁴. Overall, we could hypothesize that *P. faecium* may induce an increase of IL-22 through M2-mediated mechanisms including: 1) ILC1s downregulation favoring the type 3 responses, and 2) IL-22 production by the group ILC1, the later still to be confirmed in *in vivo* studies. Undeniably, the molecular interactions between *P. faecium*, M2 and ILC1s, as well as the different transient states acquired by ILCs in response to different environmental cues (dietary or microbial) deserve further investigations. This would allow a complete understanding of this intricate dialog which seems to be key to the bacterium's immune-mediated anti-obesogenic properties.

As already stated, our results support that the protection of intestinal immune homeostasis, commonly disrupted by the HFHSD, can help mitigate metabolic dysfunction associated with obesity. Indeed, our results suggest that the alleviation of intestinal inflammation (reduction of M1/M2 ratio and ILC1s increases) and the enhancement of gut barrier function (promotion of IL-22, slgA, AMPs and gut barrier markers increases), could be key for the reestablishment observed of intestinal hormone levels (like GIP or GLP-1) and the reduction of systemic inflammation, ultimately, contributing to the restoration of the metabolic alterations of peripheral tissues. In this sense, treated animals (either with PTS, *P. faecium* or even with the ILC1s inhibitor) presented lower adiposity and hypertrophy, as evidenced by reduced AT weight, adipose area, and down-regulation of pro-inflammatory markers (*Ccl2*, *Cd11c*, *Tnfa* and *Il-6*). In turn, this could lead to the restoration observed in adipokines levels, such as resistin or leptin, closely linked to adiposity, inflammation and glucose dysregulation ^{22,23}. Furthermore, enhance AT functioning can contribute to the improved hepatic status. Studies on the cross-talk between AT and liver have reported, for example, that persistent stress induces AT dysfunction associated with increased lipid influx to the liver, inflammation, IR, and the development of obesity complications, such as T2D and NAFLD ^{245,246}. In agreement, greater expression of genes related to FA uptake (*Lpl* and *Cd36*), inflammatory markers (IL-6), TG levels and hepatic weight were observed in the HFHSD-fed mice, although, these were total or partially reduced when treated with *P. faecium* and PTS. In this respect, it is worth noticing the reduction of IL-6 levels achieved by our treatments (mainly by *P. faecium*) at plasmatic, hepatic, and adipose level. Despite the role of this multifactorial interleukin still controversial, IL-6 is considered a predictor of IR and obesity derived problems in mice and humans ^{247–249}. Clinical trials have related bariatric surgery or sleeve gastrectomy with significant reductions in serum IL-6 levels among other pro-inflammatory markers, as a sign of improvement ^{250,251}. Moreover, similar studies focusing on the development of

novel anti-obesity treatments have consistently shown that the reduction of IL-6 levels contributes to improvements in obesity onset ^{160,175}. Therefore, we suggest that this decrease in IL-6 levels may contribute to the observed restoration of glucose metabolism, as a secondary outcome of the intestinal immune homeostasis protection in the treated HFHSD-fed mice of our studies.

FUTURE PERSPECTIVES

Overall, the administration of *P. faecium* and PTS have shown to be effective approaches to combat obesity in our experimental conditions and preclinical models of obesity. Interestingly, both strategies share part of the mechanisms of action. In the case of PTS, future research is needed to better characterize its immunomodulatory activity and effectiveness in humans. In this regard, the highest safe dose for PTS was recently determined to be 55 mg/kg in male and female rats, which is far higher than the two doses used in the study presented in this Doctoral Thesis ²⁵². Therefore, additional studies on dose-effects relationships should be performed with a view to translate results to humans.

In the case of *P. faecium*, further studies should be warranted to unravel the exact molecules through which this bacterium interacts with macrophages and shifts their phenotype reducing obesity-associated inflammation. The maintenance of the anti-obesogenic properties by pasteurized *P. faecium* provides additional information on the potential active molecules and suggests its possible use as a postbiotic ²⁵³, but additional studies would be required to progress in this direction. Lastly, the results obtained in the large human metagenomic analysis, where *P. faecium* prevalence was negatively associated with the obese phenotype, suggest that *P. faecium* abundance could be a promising obesity biomarker.

CONCLUSIONS

GENERAL CONCLUSIONS

1. The *Allium* derivate Propyl-propane-thiosulfinate (PTS) **intercepts body weight gain and glucose intolerance** induced by obesogenic diet in rodents in a dose-dependent manner and **independently of gut microbiota modulation**.
2. The anti-obesogenic effect of PTS can be attributed to the **down-regulation of pro-inflammatory mediators** in metabolically active tissues, the promotion of **thermogenesis** and the **maintenance of intestinal homeostasis**.
3. *Phascolarctobacterium faecium* associates with **normal weight**, independently of nationality, sex or age, suggesting a role in **metabolic health protection in humans**. In preclinical models of DIO, *P. faecium* **prevents obesity development** and its metabolic disturbances, **regardless of its viability**.
4. The anti-obesogenic effects of *P. faecium* are partly mediated by immunomodulatory mechanisms. Specifically, *P. faecium* directly **promotes polarization of M2 macrophages** that, in turns, reduces the **obesogenic diet-induced ILC1s increase** and, thereby, **local and systemic inflammation**.
5. **Gut-resident ILC1s act as promoters of intestinal inflammation** triggered by hypercaloric diets, and connect immunity with the microbiome, the enteroendocrine system and the intestinal barrier **in the context of obesity and associated metabolic disorders**.

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CONTRIBUTIONS

1. LIST OF PUBLICATIONS

The present Doctoral Thesis is composed of three original articles. Two have been published in highly ranked indexed journals and the third is under revision in the moment of the Thesis submission.

1. Liébana-García R.*, López-Almela I.*, Olivares M., Manghi P., Romaní-Pérez M., Tolosa V., Flor Duro A., Bullich-Vilarrubias C., Rubio T., Rossini V., Segata N. and Sanz Y. *Phascolarctobacterium faecium* resets the macrophages and innate lymphoid cells dialogue conferring resistance to obesity in mice. **(Submitted)** *These authors equally contributed

2. Liébana-García, R., Olivares, M, Francés-Cuesta C., Rubio T., Rossini V., Quintas G., and Sanz Y. Intestinal group 1 innate lymphoid cells drive macrophage-induced inflammation and endocrine defects in obesity and promote insulinemia, Gut Microbes **2023**, 15:1;2181928, Doi: 10.1080/19490976.2023.2181928.

3. Liébana-García, R., Olivares, M., Rodríguez-Ruano, S.M., Tolosa-Enguís, V., Chulia, I., Gil-Martínez, L., Guillamón, E., Baños, A., and Sanz, Y. The allium derivate propyl propane thiosulfinate exerts anti-obesogenic effects in a murine model of diet-induced obesity. Nutrients **2022**, 14;440. Doi: org/10.3390/nu14030440.

In addition to the original articles, I am coauthor of two bibliographic reviews.

1. Liébana-García R., Olivares M., Bullich-Vilarrubias C et al. The gut microbiota as a versatile immunomodulator in obesity and associated metabolic disorders, Best Practice & Research Clinical Endocrinology & Metabolism, **2021**.101542. Doi: org/10.1016/j.beem.

2. Romaní-Pérez, M. Bullich-Vilarrubias C, López-Almela I, **Liébana-García R**, Olivares M, Sanz Y. The Microbiota and the Gut–Brain Axis in Controlling Food Intake and Energy Homeostasis. Int. J. Mol. Sci. **2021**, 22, 5830. Doi: org/10.3390/ijms22115830.

2. CONGRES PARTICIPATION

I have disseminated some of my research results in different congresses, symposiums workshops and seminars:

1. **Liébana-García R.**, Olivares M, Francés-Cuesta C, Rubio T, Rossini V, Quintas G, Sanz Y. Intestinal group 1 innate lymphoid cells are key drivers of obesity-associated inflammation and insulinemia. NeuroGastro. Bucarest, Rumania. August, 2023. Poster presentation.
2. **Liébana-García R.**, Olivares, M., Francés-Cuesta, C., Rubio, T., Rossini, V., Quintas, G., Sanz Y. Intestinal group 1 innate lymphoid cells cause gut barrier defects and microbiota imbalances preceding obesity and metabolic dysfunction. 9th International Human Microbiome Consortium Congress (IHMC). Online. November 2022. Poster presentation.
3. **Liébana-García, R.**; Olivares, M.; Francés-Cuesta, C.; Rubio, T.; Sanz, Y. Intestinal group 1 innate lymphoid cells drive macrophage-induced inflammation and endocrine defects in obesity and promote insulinemia. IX PhD student symposium CSIC 2022. IATA-CSIC, October 2022. Poster presentation.
4. **Liébana-García R.**, Olivares, M., Francés-Cuesta, C., Rubio, T., Sanz Y. Intestinal group 1 innate lymphoid shape the intestinal homeostasis in a murine model of obesity. XIII Workshop of Spanish society of microbiota, probiotics and prebiotics (SEMIP). Valencia, Spain. June 2022. Poster presentation.
5. **Liébana-García, R.**; Olivares, M., Sanz Y. Obesogenic diet boosts ILC1 in the gut that directly influences and metabolic balance. Workshop Innovation-Research Exchange Wageningen University & IATA-CSIC. Paterna-Valencia, Spain. March 2022. Oral presentation.
6. **Liébana-García, R.**; Olivares, M., Chulia I., Sanz Y. The *Allium* derivate propyl propane thiosulfinate exerts anti-obesogenic effects in a mouse model of diet-induce obesity. VIII PhD student symposium. Paterna-Valencia, Spain. November 2021. Oral presentation.
7. **Liébana-García, R.**; Olivares, M., Chulia I., Sanz Y. The garlic derivate propyl propane thiosulfinate exerts anti-obesogenic effects in a mouse model of diet-induce obesity. 8th International Human Microbiome Consortium congress. Online. June 2021. Poster presentation.

By presenting my research, I have contributed to the dissemination of new knowledge, as well as receiving feedback and constructive criticism from fellow researchers. Additionally, I also attended several other congresses and student seminars with the purpose of enhancing my knowledge and understanding of the field and which have contributed to keep increasing my network:

1. International food data science Workshop (Paterna-Valencia, Spain) December 2022
2. MACSQuant user day symposium (Valencia, Spain) November 2022
3. IV CSIC PhD student symposium (online) October 2022
4. Champalimaud Research Symposium; (Lisbon, Portugal) October 2022.
5. II CSIC PhD student symposium (online) October 2020

3. ACHIVEMENTS, COURSES AND DIVULGATION ACTIVITIES

During my thesis, I have completed various courses that have enriched my knowledge and skills beyond the mandatory curriculum. Notably, some such as the ones dedicated to experimental animal handling or the cytometry specific courses have significantly contributed to my overall scientific training and have enhanced my ability to conduct research effectively. Additionally, the three-month internship at group of immunophysiology supervised by Prof. Henrique Veiga-Fernandes (Champalimaud foundation, Portugal) significantly enhanced my formative and educational journey. It provided me with invaluable hands-on experience, new theoretical knowledge and practical research scenarios. This internship offered a unique opportunity to collaborate with immunity experts, broaden my scientific skills, and deepen my understanding of the subject matter, ultimately contributing to my overall growth and development as a researcher.

Furthermore, I have participated in the creation of the content of a massive open online course (MOOC) entitled "The Human Microbiome" funded by European EIT-FOOD. Moreover, I have also been involved in teaching activities at the UPV (90 h in total), where I have supervised practical lessons related to topics such as microbiology, human physiology, and biotechnology. These experiences allowed me to develop effective teaching and communication skills, and enhance my knowledge in the field while contributing to the education of future generations. In this sense I have also collaborated in the supervision of a master's student's, which has provided me with valuable experience in mentoring and guiding students through the research process.

Finally, along my Doctoral Thesis I have been actively committed in sharing scientific knowledge with the general public through outreach activities such as ExpoCiencia, Women in Science events, and talks in schools. Also, I participated in coloring book entitled "Microbes, diet and beyond" in the context of the MicrobiomeSupport EU project (H2020). I believe that science is not only for scientists, but for everyone, and that it is important to promote scientific literacy and engage the public in discussions about science and its impact on society.

ABBREVIATIONS TABLE

AGM1	Anti-Asialo GM1
AhR	Aryl hydrocarbon receptor
AMPs	Antimicrobial peptides
ANOVA	One-way analysis of variance
ARG-1	Arginase-1
ASVs	Amplicon sequence variants
AT	Adipose tissue
AX	Arabinoxylans
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic
CCK	Cholecystokinin
CNS	Central nervous system
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
DIO	Diet-induced obesity
DNBS	Dinitrobenzene sulfonic acid
DPP4	Dipeptidyl-peptidase 4
DSS	Dextran sodium sulfate
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
FA	Fatty acids
FAO	Fatty acid oxidation
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanine
FOS	Fructo-oligosacchaides
GABA	Gamma-aminobutyric acid
GIP	Gastric inhibitory peptide
GLP	Glucagon-like peptide
GOS	Galacto-oligosacchaides
GPCRs	G protein-coupled receptors
HFD	High-fat-diet
HFHSD	High-fat-high-sugar-diet
HIF-1α	Hypoxia-inducible factor 1 alpha
HMOs	Human milk oligo-saccharides
HOMA-IR	Homeostatic model assessment for insulin resistance
HPA	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocytes

IFNγ	Interferon-gamma
IgA	Immunoglobulin A
IL	Interleukin
ILCs	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS	Insulin receptor substrate
ISAPP	International Scientific Association for Probiotics and Prebiotics
KO	knock out
LPS	Lipopolysaccharides
LTi	Lymphoid tissue-inducer
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory macrophages
MAMPs	Microbe-associated molecular patterns
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1 (<i>also referred as CCL-2</i>)
NAFLD	Non-alcoholic fatty liver disease
NEC	Neuroendocrine cell
NF-κB	Nuclear factor kappa-B
NK	Natural killer
NLRs	nucleotide-binding oligomerization domain-like receptors
NMDS	Non-metric multidimensional scaling
NOD	Nucleotide-binding oligomerization domain
PRRs	Pattern recognition receptors
PCoA	Principal coordinates analysis
PTS	Propyl propane thiosulfinate
PTSO	Propyl propane thiosulfonate
PYY	Peptide YY
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpl19	Ribosomal protein L19
SCFAs	Short-chain fatty acids
T2D	Type 2 diabetes
TG	Triglycerides
Th	T helper
TLRs	Toll-like receptors
TMAO	Trimethylamine N-oxide
TNFα	Tumor necrosis factor-alpha
Treg	T regulatory
WAT	White adipose tissue
WHO	World Health Organization

