ROLE OF D-FAGOMINE AND OMEGA-3 POLYUNSATURATED FATTY ACIDS ON GUT MICROBIOTA AND RELATED METABOLIC CHANGES IN HEALTHY RATS AND IN A MODEL OF FAT-INDUCED PRE-DIABETES

MERCÈ HEREU PLANELLAS

2019

UNIVERSITY OF BARCELONA

FACULTY OF BIOLOGY

DOCTORAL PROGRAM IN BIOMEDICINE

ROLE OF D-FAGOMINE AND OMEGA-3 POLYUNSATURATED FATTY ACIDS ON GUT MICROBIOTA AND RELATED METABOLIC CHANGES IN HEALTHY RATS AND IN A MODEL OF FAT-INDUCED PRE-DIABETES

Report presented by Mercè Hereu to obtain a doctorate degree from the University of Barcelona.

Thesis carried out at the Department of Nutraceuticals and Free Radicals,

Institute for Advanced Chemistry of Catalonia, Spanish Research Council (IQAC-CSIC).

MERCÈ HEREU PLANELLAS

PhD candidate

SARA RAMOS ROMERO

Supervisor and UB tutor

MARTA ROMEU FERRAN

Supervisor

This thesis was supported by the Spanish Ministry of Economy and Competitiveness

(Grant AGL2013-49079-C2-1,2-R and a graduate fellowship to Mercè Hereu: BES2014-068592).

Als meus pares

ACKNOWLEDGEMENTS

En primer lloc vull donar les gràcies a totes les persones que han fet possible aquesta tesi, sobretot a les meves directores; la Sara i la Marta. Moltes gràcies per tota la feina feta que no és poca.

Sara, gracias por acompañarme y compartir este gran viaje conmigo. Gracias por confiar en mi un 200% desde el primer momento y sobre todo por enseñarme tanto; enriqueciéndome intelectual y personalmente. Gracias por tus tranquilizadoras charlas, apreciados consejos y por entenderme tanto.

Josep Lluís, és un plaer formar part del teu grup. Gràcies per fer-me sentir com a casa des del primer dia. També agraeixo moltíssim la teva gran dedicació a aquesta tesi, els valuosos consells, les benvingudes correccions, i tots els suggeriments i ajuts rebuts durant aquests anys. Seguim.

Susana, gràcies per ensenyar-me amb tanta perfecció. Agraeixo moltíssim totes les hores dedicades a aquesta tesi i a mi, les ganes infinites de treballar i sobretot de treballar molt bé. Sense tu hagués sigut molt més difícil i alguna cosa gairebé impossible!

També m'agradaria donar les gràcies a totes les persones del centre que d'alguna manera formen part d'aquesta tesi, al laboratori Benjamí Piña per acollir-me com a una més del grup i ajudar-me sempre que m'ha fet falta, gràcies Marta! A tot l'equip de l'estabulari per cuidar a les rates amb el mateix amor que ho he fet jo i estar sempre disposats a ajudar-nos, gràcies Eva!

També agrair a tots els estudiants de grau i màster que m'han ajudat durant aquests anys, sobretot a la Roser i en Carles. A vosaltres dos, gràcies per escoltar-me sempre i ser com sou!

I d'una manera molt especial, vull donar gràcies infinites als meus companys de viatge, alegries i penes. Roser, Raquel, Ana, Mireia, Cristina, Pol, Carlos i Karel. No podia haver tingut millors companys de feina, de laboratori ni millors amics. Gràcies per ensenyar-me tanta química i sobretot gràcies per tants somriures! Sou la part més important d'aquesta tesi. També us la dedico a vosaltres!

I per últim, a la meva família per ser-hi sempre. Sobretot als meus pares, pels valors transmesos i per ensenyar-me des de ben petita que la paciència és la mare de la ciència. I als més importants; Rosa, Jonte, Ivar, Carla, Jordi, Júlia, Pol, Sònia, Xavi, Laia i Martí. Gràcies pel vostre amor i recolzament

incondicional que només vosaltres sou capaços de donar, sobretot quan més ho he necessitat. Us estimo!

ABBREVIATIONS

AKT	Protein kinase B
ALA	α-Linolenic acid
ANGPTL4	Angiopoietin-like protein 4
ARA	Arachidonic acid
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
BMI	Body mass index
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FA	Fatty acid
FSA	Fructose-6-phosphate aldolase
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GPCR	G-protein-coupled receptor
GSE	Grape seed extract
HDAC	Histone deacetylase
HETE	Hydroxyeicosatetraenoic acid
HIV	Human immunodeficiency virus
IGT	Impaired glucose tolerance
IL	Interleukine
IR	Insulin resistance
IRe	Insulin receptor
IRS	Insulin receptor substrate
JNK	Jun-N-terminal kinase
LA	Linoleic acid
LPS	Lipopolysaccharide
LT	Leukotriene

MAMP	Microbe-associated molecular pattern
MAPK	Mitogen activated protein kinase
MODY	Maturity onset diabetes of the young
MUFA	Monounsaturated fatty acid
NJ	Nojirimycin
OGTT	Oral glucose tolerance test
PG	Prostaglandin
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PRR	Pattern recognition receptors
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SD	Sprague Dawley
SFA	Saturated fatty acid
STD	Group fed with a standard diet
TAG	Triacylglycerol
TGR5	G-protein-coupled bile acid receptor
TLR	Toll-like receptor
TX	Thromboxane
T1D	Type-1 diabetes
T2D	Type-2 diabetes
UPR	Unfolded protein response
WHO	World Health Organization
WKY	Wistar Kyoto
ZO-1	Zonula occludens 1

TABLE OF CONTENTS

TABLE OF FIGURES

INTEREST

Modern lifestyles in an increasing number of human societies include consuming an excess of saturated fats and refined sugars as well as enjoying microbiologically aseptic environments. The current worldwide epidemics of obesity (650 million adults) and type-2 diabetes (T2D; 422 million adults) are probably the price that part of humanity is paying for such opulence and safety. Informed changes in dietary habits together with the introduction of new healthy and tasty foodstuffs may help to improve human wellbeing.

This thesis will explore the changes that take place in both the intestinal microbiota and the host organism (rats) after dietary supplementation with an iminosugar (D-fagomine) and ω-3 polyunsaturated fatty acids (ω-3 PUFAs): eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with the aim of evaluating the influence of these compounds on gut bacterial populations and defining their possible role in the prevention of fat-induced T2D.

In mammals, the intestines are colonized by trillions of microorganisms that have co-evolved with the host. This microbiota encodes a consortium of genes from bacteria that exceeds by far the number of genes in the human genome. It is becoming evident that gut microbiota contributes significantly to host homeostasis. Maintaining the appropriate distribution of microbial populations (eubiosis) is emerging as an attractive approach to avoiding ectopic fat accumulation and insulin resistance (IR), with the aim of averting the progression of diabetes. As intestinal microbiota is strongly influenced by environmental agents (e.g. food, probiotics), we thought that it might be possible to minimize the dysbiosis (unbalanced microbiota) associated to ageing or high-fat diets by supplementing the diet with a combination of functional food components of different nature probably acting at different levels.

The very first metabolic alteration eventually leading to T2D is IR a reduced capacity to internalize glucose from the bloodstream as result of insensitivity to insulin. An excess of dietary fat and subsequent body fat accumulation results in the generation of pro-inflammatory cytokines and chemokines and a state of low-grade inflammation and impairment of insulin signaling. Systemic inflammation, IR, and obesity have been linked to gut dysbiosis. Some recent research is even suggesting that gut dysbiosis is the first cause of fat-induced low-grade inflammation.

This thesis is based on previous results by our group showing that the iminosugar D-fagomine induces changes in the composition of gut microbiota probably by inhibiting the adhesion of some types of

bacteria to the intestinal mucosa and on the well-known anti-inflammatory activity of ω-3 PUFAs (EPA/DHA 1:1).

This thesis is intended to contribute to curbing the current growing incidence of diabetes by generating new knowledge to understand the interactions between gut microbiota and host, and by suggesting novel dietary approaches.

SUMMARY

Modern lifestyles in an increasing number of human societies include consuming an excess of saturated fats and refined sugars as well as enjoying microbiologically aseptic environments. Maintaining the appropriate distribution of gut microbial populations (eubiosis) is emerging as an attractive approach to prevent ectopic fat accumulation and insulin resistance (IR), and to avert the progression of diabetes. Functional food components are those that help maintain the normal bodily functions beyond providing energy or building blocks. They may bring about physiological health benefits for both healthy subjects following a healthy lifestyle and people belonging to risk groups such as obese or pre-diabetic populations. D-Fagomine is an iminosugar originally present in buckwheat with the capacity for selectively inhibiting bacterial adhesion to the intestinal mucosa. ω-3 Polyunsaturated fatty acids (PUFAs) reduce blood pressure as well as levels of insulin, triacylglycerols, cholesterol and total lipids. Iminosugars and ω-3 PUFAs may be used as functional food ingredients or dietary supplements to maintain a healthy status over time and to reduce risk factors for diabetes.

This thesis focuses on the effects of buckwheat D-fagomine, fish ω-3 PUFAs (EPA/DHA 1:1) and their combination on the gut microbiota and related metabolic variables in the host both in healthy rats and a rat model of fat-induced pre-diabetes.

In the first part of this thesis we explored the effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on rats given a standard diet as a model for healthy subjects. We found that D-fagomine had the capacity for promoting microbial functional diversity by increasing the Bacteroidetes/Firmicutes ratio and for mitigating the age-related reduction in populations of the putatively beneficial Lactobacilliales and Bifidobacteriales. Also, the populations of the genus *Prevotella* remained stable over time in animals supplemented with D-fagomine, independently of ω-3 PUFAs supplementation. The combination between D-fagomine and ω-3 PUFAs provided the functional benefits of each supplement. Notably, it helped stabilize populations of *Prevotella* in the rat intestinal tract while reducing weight gain and providing the anti-inflammatory and cardiovascular benefits of ω-3 PUFAs.

In the second part we explored the effects of the same supplements on rats with fat-induced pre-diabetes as a model for people at risk of suffering from diabetes and cardiovascular diseases. We found that D-fagomine delayed the development of a fat-induced pre-diabetic state by reducing low-grade inflammation. We suggest that the anti-inflammatory effect of D-fagomine may be linked to a reduction in fat-induced overpopulation of minor gut bacterial groups such as Enterobacteriales. The

combined supplements counteracted the high-fat induced incipient IR, and liver inflammation, while increasing the cecal content, the Bacteroidetes/Firmicutes ratio as well as the populations of putatively beneficial Bifidobacteriales and Lactobacilliales. The functional effects of the combination between D-fagomine and EPA/DHA 1:1 against the gut dysbiosis and the very early metabolic alterations induced by a high-fat westernized diet were mainly those of D-fagomine complemented by the anti-inflammatory action of ω-3 PUFAs.

The results of this thesis point clearly towards a functional role for D-fagomine in the maintenance of the intestinal health by preserving diversity and mitigating the age-related reduction of some beneficial bacteria and also in the prevention of risk factors for diet-induced pre-diabetes reinforced by the action of ω-3 PUFAs by complementary mechanisms.

1 INTRODUCTION

There is increasing and convincing evidence that part of the beneficial effects of dietary components such as soluble fiber (indigestible polysaccharides), polyphenols and PUFAs are connected with the maintenance of a balanced gut microbiota (eubiosis) while dysbiosis (unbalanced microbiota) would be in the root of alterations and diseases (e.g. obesity, T2D) associated with bad dietary habits (Portune, Benitez-Paez, Del Pulgar, Cerrudo, & Sanz, 2017).

1.1 GUT MICROBIOTA

The human gut microbiota consists of a wide variety of bacteria, viruses and fungi that live in the intestine. The human gut microbiota is formed by 10^{14} bacteria, more than 10 times the number of eukaryotic cells in a healthy person (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). Bacteria colonize human body from birth, probably already in the uterus (Walker, Clemente, Peter, & Loos, 2017), and persist until death. These bacteria and their products interact with the host in many ways, influencing gut homeostasis and health outcomes. The preservation of microbial diversity and balance is fundamental for host health (Nicholson et al., 2012).

1.1.1 COMPOSITION OF GUT MICROBIOTA

The human gut microbiota mainly consists of 9 bacterial phyla with over 1,000 species, and more than 15,000 strains. A healthy gut microbiota is predominantly constituted by the phyla Firmicutes (≈ 60% of the gut microbiota) and Bacteroidetes (≈ 40%) in both humans and rodents (Ley et al., 2005). Bacteroidales is the major order among Bacteroidetes while Clostridiales is the major order among Firmicutes. Other quantitatively minor yet important subgroups of the gut microbiota are the orders Enterobacteriales, Lactobacilliales, and Bifidobacteriales, which belong to the phyla Proteobacteria, Firmicutes and Actinobacteria, respectively (Figure 1). *Bacteroides* and *Prevotella* are the major genera among Bacteroidales order. Both are well-known as dietary fiber fermenters and they play an important role in the production of short-chain fatty acids (SCFAs).

Enterobacteriales order is composed of non-pathogenic and opportunistic bacteria such as *Escherichia coli*, which coexist inside the healthy host as commensal (neither beneficial nor pathogenic) bacteria that may cause enteric diseases and extra-intestinal infections in immunocompromised hosts or when the normal gastrointestinal barriers are breached (Kaper, Nataro, & Mobley, 2004).

Lactobacilliales and Bifidobacteriales are known to confer health benefits on the host, including resistance to infection, amelioration of allergic symptoms and protection against inflammatory

processes (Roberfroid et al., 2010). *Lactobacillus acidophilus* is one of the major species of its genus found in the human gut, and together with *Lactobacillus plantarum*, it contributes to the maintenance of the normal barrier function of the intestinal epithelium (Gareau, Sherman, & Walker, 2010).

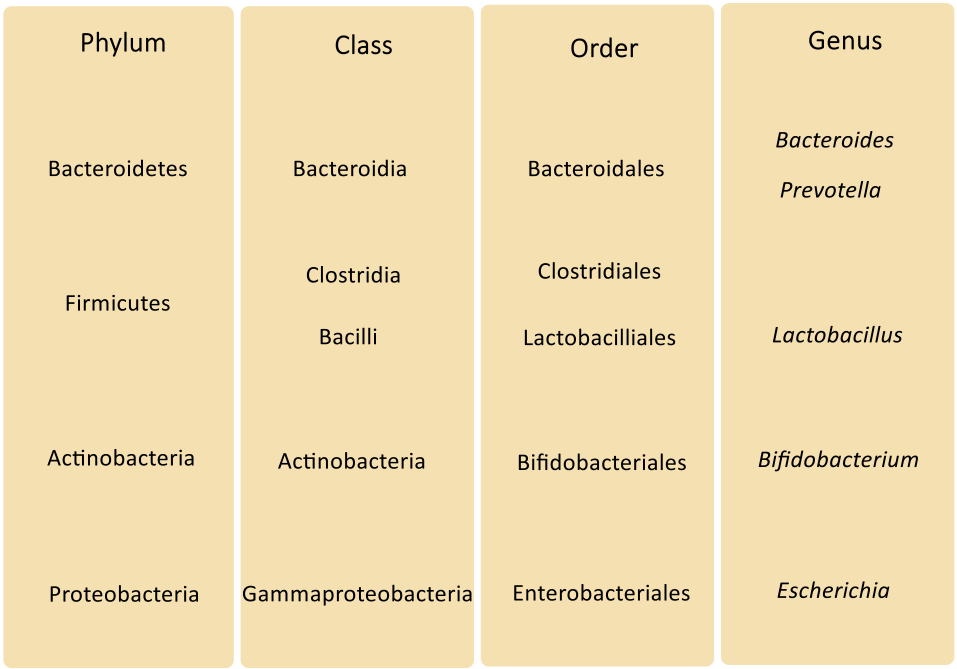


Figure 1. Main taxonomic composition of human gut microbiota

1.1.2 FUNCTIONS OF GUT MICROBIOTA IN THE HOST

The human intestinal microbiota plays a key role in several metabolic, nutritional, physiological, and immunological processes. The main functions of the intestinal microbiota are: i) nutrient acquisition; providing a range of essential nutrients for the host ii) contribution to immune system development, maturation and modulation, and iii) maintenance the epithelial barrier; protecting from colonization by exogenous pathogens.

1.1.2.1 Nutritional contribution

One of the most important functions of the gut microbiota is the optimization of energy harvesting. The gut microbiota makes an important contribution to the host metabolism by providing enzymes

that are not encoded by the host genome for the breakdown of non-digestible carbohydrates, which include large polysaccharides, such as resistant starch, cellulose, hemicellulose, pectin and some oligosaccharides that escape digestion (Cummings & Macfarlane, 1991). This functionality results in the recovery of energy and absorbable substrates for the host and supplies energy and nutrients for bacterial growth and proliferation. Thus, intestinal microbiota maximizes caloric availability of nutrients ingested by i) extracting additional calories from indigestible carbohydrates in the large intestine, in particular polysaccharides, and ii) modulating intestinal epithelium absorption capacity (Cummings & Macfarlane, 1991).

The capacity of the gut microbiota to process different carbohydrates as well as other macronutrients, such as lipids and proteins may, at least in part, explain how it may contribute to the development of obesity and other metabolic disorders.

1.1.2.2 Immune system modulation

Another important function of gut microbiota is to activate the immune system. The Paneth cells are one of the main cell types in the intestine epithelium that combat pathogens by signalling to the immune system through specific receptors (pattern recognition receptors; PRR), such as toll-like receptors (TLRs). TLRs recognize and bind to specific molecules associated with bacteria (microbe-associate molecular patterns; MAMPs), such as peptidoglycan, lipopolysaccharide (LPS) or lipid A, leading to the stimulation of a host immune response and the release of protective cytokines and white blood cells. The result is a tolerance response to commensal bacteria and an inflammatory response to pathogenic organisms (Salzman, Underwood, & Bevins, 2007).

1.1.2.3 Maintenance of the epithelial barrier

Gut microbiota also plays a central role in protecting the host from enteric bacterial infection by contributing to the maintenance and integrity of the normal barrier function through the maintenance of cell-cell junctions.

The host intestinal cells have attachment sites that can be recognized by both beneficial and pathogenic bacteria. Non-pathogenic bacteria, such as *Bifidobacterium* compete for these attachment sites in the brush border formed by intestinal epithelial cells, preventing the attachment and subsequent entry of pathogenic bacteria, such as some *E. coli* strains (Cani et al., 2009). The balance between the populations of beneficial, pathogenic and commensal bacteria is essential to maintain the normal gut barrier function through the maintenance of cell-cell junctions (**Figure 2**).

Modifications in the distribution and localization of tight junction proteins (mainly zonula occludens-1 (ZO-1) and occludin) are associated with changes in gut permeability, which results in increased plasma LPS levels (metabolic endotoxemia). This is a possible mechanism by which gut bacteria can initiate inflammatory processes leading to obesity and IR. Conversely, an improved gut barrier strongly correlated with reduced plasma LPS levels and low-grade inflammation (Cani & Delzenne, 2011).

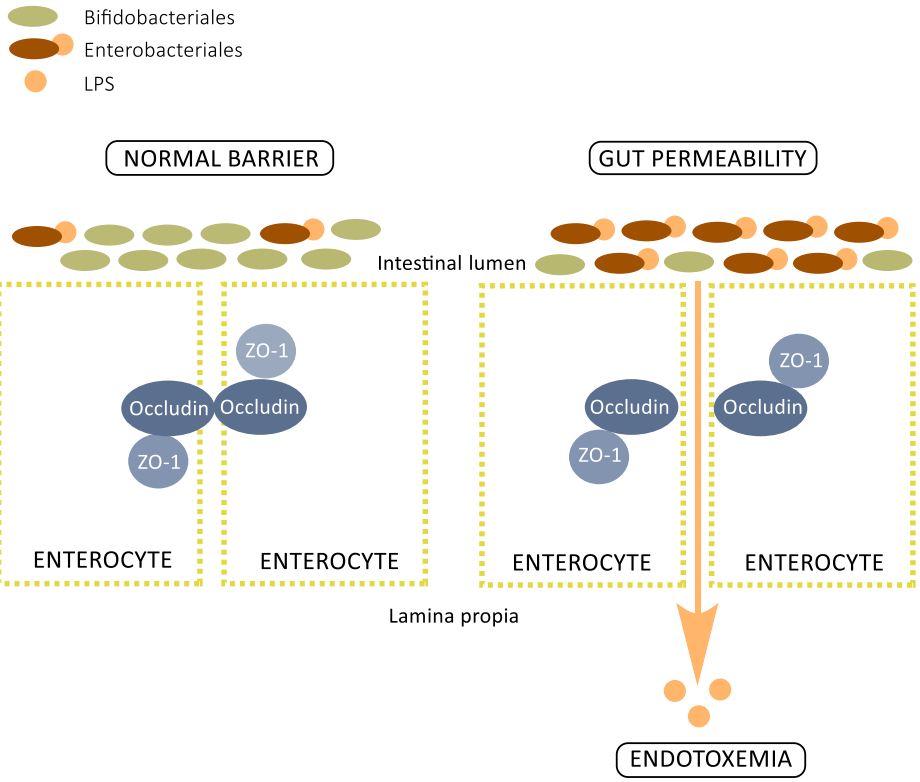


Figure 2. Gut barrier function

Another way of antimicrobial protection provided by microbiota-host interactions are lactic acid production by *Lactobacillus sp.*, because the lactic acid may increase the antimicrobial activity of host lysozyme by disrupting the gram-negative bacterial outer membrane (Alakomi et al., 2000).

1.1.3 MECHANISMS OF INTERACTION BETWEEN GUT MICROBIOTA AND HOST

The mechanisms by which gut microbiota interact with the host organism are still unclear. It has been suggested that SCFAs, LPS, angiopoietin-like protein 4 (ANGPTL4), and bile acids can be possible mediators of the host response (Janssen & Kersten, 2017). These molecules are direct or indirect mediators between gut microbiota and host that can be either protective or harmful, depending on their concentration and on the metabolic status of the host.

1.1.3.1 Short-chain fatty acids

SCFAs are fatty acids (FAs) with less than 6 carbon atoms. SCFAs are the main metabolites produced by the gut microbiota during the fermentation of non-digestible polysaccharides. Acetate, propionate and butyrate are the major SCFAs produced by bacteria. The highest levels of SCFAs are found in the proximal colon, where they are used locally by colonocytes or transported across the gut epithelium into the bloodstream. The capacity of SCFAs to modulate biological responses in the host are exerted by two mechanisms: i) regulation of gene expression by inhibiting histone deacetylases (HDACs) and ii) activation of G-protein-coupled receptors (GPCRs) (Tan et al., 2014).

Apart from being a major energy source for colonocytes, SCFAs in the gut have various physiological functions including: i) anti-inflammatory effect by modulating immune cell chemotaxis, reactive oxygen species (ROS) release and cytokine release, ii) antimicrobial activity by disrupting osmotic and pH balance and nutrient uptake and iii) preservation of gut integrity by maintaining mucosal homeostasis. SCFAs can fortify the epithelial barrier by affecting the mucus layer, epithelial cell survival, as well as tight junction proteins (Tan et al., 2014).

1.1.3.2 Lipopolysaccharide

LPS is a large molecule consisting of a lipid (lipid A) covalently linked to the O-antigen (a repetitive glycan polymer) through a core polysaccharide. LPS is the major component of the outer membrane of gram-negative bacteria. Bacterial LPS from intestinal microbiota could be a potential causal link between disruptions in the gut microbiota and obesity-related IR and adipose tissue (AT) inflammation (Cani et al., 2007a). LPS is continuously produced by gram-negative bacteria in the gut and can be translocated into intestinal capillaries triggering the secretion of pro-inflammatory cytokines when it binds to the complex TLR4 at the surface of innate immune cells (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Backhed, 2015; Cani et al., 2007a; Cani et al., 2008).

1.1.3.3 Angiopoietin-like protein 4

ANGPTL4 is a glycoprotein expressed in mice mainly in AT (followed by liver, kidney, muscle and intestine), and in humans mainly in liver. ANGPTL4 plays an important role in lipid metabolism by inhibiting the activity of the enzyme lipoprotein lipase and consequently the hydrolysis of circulating triglycerides. ANGPTL4 is a potential link between the gut microbiota and fat storage. Specific bacterial species, such as *Bacteroides thetaiotaomicron*, and SCFAs have the capacity to induce ANGPTL4 expression (Backhed et al., 2004; Backhed, Manchester, Semenkovich, & Gordon, 2007).

1.1.3.4 Bile Acids

Bile acids are steroid acids synthesized in the liver and then secreted into the small intestine. In the intestine, bile acids play an important role in the emulsification and absorption of dietary lipids as well as in restricting bacterial proliferation and overgrowth. Bacterial enzymes modify primary bile acids through deconjugation, dehydrogenation, dehydroxylation, and sulfation reactions to produce secondary bile acids, which are reabsorbed and returned to the liver for further processing. They also are known as important signalling molecules through the G protein-coupled bile acid receptor 1 (TGR5). Through TGR5, bile acids can influence a variety of biological processes as bile acid metabolism, intestinal hormone secretion, inflammation and lipid and glucose metabolism (Swann et al., 2011).

1.1.4 HOST CONDITIONS RELATED TO GUT MICROBIOTA DYSBIOSIS

Dysbiosis can lead to the development of a variety of alterations and diseases in the host including inflammatory bowel disease, colon cancer, irritable bowel syndrome, non-alcoholic fatty liver disease, asthma, atopy, hypertension, obesity and T2D, … (Nicholson et al., 2012).

1.1.4.1 Obesity

The ability to store energy would be a beneficial adaptation for ancient humans who had irregular access to food. However, nowadays, in developed societies where food is readily available, this benefit can become a problem.

Total energy intake refers to all energy consumed as food and drink that can be metabolized inside the organism. Energy density is defined as the amount of available dietary energy per unit of weight of foodstuff (expressed in kcal/g or kJ/g). Fat provides the highest energy per unit weight (9 kcal/g), and carbohydrate and protein the lowest (4 kcal/g). Fiber also contributes to energy density because it provides little energy and contributes to weight. Water accounts for most of the variability in

energy density because it provides weight but not energy. Therefore foods high in water and/or fiber are low-energy-dense. As fat provides the greatest amount of energy per gram, high-fat foods are high-energy-dense (Perez-Escamilla et al., 2012).

Body mass index (BMI) is an indicator of weight-for-height used to classify overweight and obesity in human adults. BMI is defined as a body weight in kilograms divided by the square of height in meters (kg/m^2). According to categories established by the World Health Organization (WHO) overweight adults have a BMI ≥ 25, class I obese have a BMI ≥ 30, class II ≥ 35 and class III ≥ 40 (WHO, 2000).

Obesity is defined as a condition of abnormal or excessive fat accumulation as a consequence of an energy imbalance that may be a threat to general health. Obesity is developed when energy intake exceeds energy expenditure over time. There are many complex and diverse factors that give rise to a positive energy balance. Nowadays physical activity has decreased due to the sedentary nature of many forms of work, modes of transportation, and urbanization. Moreover, dietary habits tend to shift towards an increased intake of energy-dense foods that are high in fat, salt and refined sugars, and low in vitamins, minerals and other micronutrients (WHO, 2000).

As mentioned before, gut microbiota is one of the factors that collaborate in the host energy intake. Obese and lean individuals have different proportions of bacteria subgroups in their gut microbiota, with differences in their ability to extract energy from host diet and to deposit that energy in fat (Turnbaugh & Gordon, 2009; Turnbaugh et al., 2006). Although much research is being focussed on the relationship between body weight and gut bacterial composition there is little information at levels below phyla. Body weight appears to be related to the Bacteroidetes/Firmicutes ratio as low Bacteroidetes/Firmicutes ratio has been associated with the obese phenotype in the host (Canfora, Jocken, & Blaak, 2015; Ley, Turnbaugh, Klein, & Gordon, 2006). At the order level, an increase in the population of Enterobacteriales has been connected to diet-induced obesity (de la Serre et al., 2010). At the genus level, the populations of *Prevotella* are high in non-industrialized populations whose diets include high levels of dietary fiber, while *Bacteroides* is more prevalent in Western populations who consume high-protein, low-fiber diets (Chen et al., 2017).

Obesity has been associated with many chronic diseases such as T2D, cardiovascular disease and cancer. As the prevention of T2D is one of the main goals of this thesis diabetes is described in more detail in the following section.

1.1.4.2 Diabetes

Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose. The maintenance of blood glucose levels within a very narrow range (70-100 mg/dL in both humans and rodents) is carried out by the opposing and balanced actions of pancreatic hormones insulin and glucagon produced in the islets of Langerhans. Insulin is a hormone produced by the β-pancreatic cells that signals the internalization of glucose into the hepatocytes, adipocytes and skeletal muscle cells where it is converted into energy via glycogenesis or lipogenesis. In contrast, glucagon is a hormone produced by α-pancreatic cells that signal the glucose release from the hepatocytes, adipocytes and skeletal muscle cells via glycogenolysis into the blood stream. Glucagon increases blood glucose levels while insulin decreases them. Between meals, when blood glucose levels are low, glucagon is released from α-pancreatic cells to promote hepatic glycogenolysis and increases blood glucose levels (**Figure 3**). In contrast, after a meal, insulin secretion from β-pancreatic cells, stimulated by elevated exogenous glucose levels, triggers glucose uptake into insulin-dependent muscle and AT as well as to promote glycogenesis (glucose storage as glycogen into the cells) in the liver (Berkowitz, 2007).

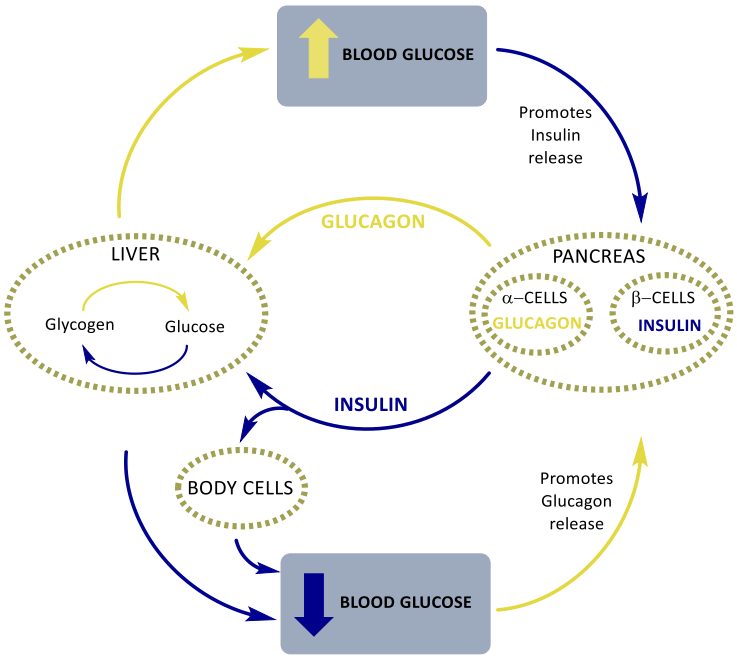


Figure 3. Glucose homeostasis

Diabetes mellitus refers to a heterogeneous group of chronic, catabolic and endocrine diseases characterized by hyperglycemia (Melmed, Polonsky, Larsen, & Kronenberg, 2017). Hyperglycemia has both short-term and long-term complications (Berkowitz, 2007; Melmed et al., 2017). When glucose cannot be internalized into the cells, it remains in the blood and then is excreted. Inefficient use of glucose can lead to weight disbalance and excessive appetite (polyphagia). Elevated blood glucose levels can predispose to recurrent infections because glucose may fuel bacterial growth as well as impair white blood cell function. Hyperglycemia renders blood hyperosmotic causing polydipsia (increased thirst) and polyuria (increased urine volume) due to the presence of glucose in the glomerular filtrate. Moreover, the loss of total body potassium from excessive urination and from catabolism of muscle protein (alternative energy source) produces a state of fatigue. Vision changes due to modifications in the water content within the eye lens are also caused by hyperglycemia. Chronic hyperglycemia is a risk factor for peripheral vascular disease, coronary artery disease and stroke. Hyperglycemia fosters the development and progression of cardiovascular disease by mechanisms that include derangements in the vessel wall through promotion of vascular inflammation and endothelial cell dysfunction; abnormalities in blood cells; and factors affecting homeostasis. These mechanisms likely contribute to increased plaque burden and plaque instability (Thiruvoipati, Kielhorn, & Armstrong, 2015).

The WHO estimates the global prevalence of diabetes in 422 million adults in 2014 (8.5% of adults). The greatest number of people with diabetes is found in the segment between 40 and 59 years of age, and 80% of them live in low and middle income countries. In 2016, an estimated 1.6 million deaths were directly caused by diabetes (WHO, 2016).

Diabetes is classified into 4 general categories depending on its etiology:

- Type-1 diabetes (T1D) (previously known as juvenile or insulin-dependent diabetes) results from the autoimmune progressive destruction of insulin-producing β-pancreatic cells by CD4+ and CD8+ T lymphocytes and macrophages infiltrating the islets of Langerhans (Foulis, McGill, & Farquharson, 1991). As a result, the pancreas cannot produce insulin and glycemia increases without control. T1D has a major genetic component and it is influenced by environmental factors. Patients with T1D constitute 5-10% of all people with diabetes. This type of diabetes can affect people of any age, but usually occurs in children or young adults (WHO, 2016).

- T2D is defined by the National Diabetes Data Group and the WHO as carbohydrate intolerance characterized by IR, relative insulin deficiency, excessive hepatic glucose production and hyperglycemia (Brashers, 2006).

- Gestational diabetes mellitus (GDM) occurs when the action of insulin is blocked by the hormones progesterone and cortisol produced by the placenta. Women experiencing IR and hyperglycemia during pregnancy have higher risk of developing T2D 5-10 years after pregnancy eventhough the alterations usually disappear after birth. GDM is basically addressed by controlling blood glucose, normally by dietary restriction of carbohydrate intake and moderate exercise (Kampmann et al., 2015).

- Other types of diabetes include monogenic diabetes syndromes such as neonatal diabetes and maturity-onset diabetes of the young (MODY), diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and chemically-induced diabetes by agents such as glucocorticoid, human immunodeficiency viruse (HIV) antiretroviral treatment, or immunosupressants after organ transplantation.

Type-2 diabetes

T2D is the most common type of diabetes worldwide and gut microbiota seems to be intimately linked to it. In T2D, the β-pancreatic cells produce insufficient amounts of insulin or its target tissues are unable to respond to its signal (IR), leading to hyperglycemia. It usually occurs in adults, but is increasingly observed in children and adolescents. In 2002, an estimated 18.2 million people in the United States (6.3% of the total population) were living with diabetes, of which approximately 17.2 million (95% of all cases) had T2D (WHO, 2016).

T2D is a multifactorial disease resulting from a combination of environmental and genetic risk factors. The risk factors for developing T2D include lifestyles such as sedentary behaviour, poor physical activity and a westernized diet with fat and refined sugars that can triggers to obesity (Melmed et al., 2017). The significance of T2D lies primarily in numerous associated troublesome symptoms and serious short- and long-term complications. Because of the very gradual way in which hyperglycemia develops over time, many patients can sustain extended periods of hyperglycemia without experiencing a loss of a sense of well-being.

Most of the T2D signs and symptoms are similar to those of other diabetes types. As mentioned before, classic symptoms of hyperglycemia include inappropriate levels of polydipsia, polyuria, fatigue, subtle losses of visual acuity and recurrent infections (Berkowitz, 2007). However, while T1D results in body weight loss, T2D has been connected to obesity. The link between obesity and T2D is the capacity of AT to trigger IR, which is a fundamental aspect of the etiology of T2D. Adipocytes in the AT have an endocrine function with wide-reaching effects on other organs. Particularly AT plays a major regulatory role in energy balance and glucose homeostasis by releasing a wide variety of molecules including hormones such as leptin, cytokines and substrates such as FAs (Kahn & Flier, 2000).

Five stages in the progression to T2D have been proposed, each one characterized by changes in several metabolic parameters and β-pancreatic cell function (Weir & Bonner-Weir, 2004). T2D is preceded by IR, a reduced capacity to internalize glucose from the bloodstream as consequence of insensitivity to insulin that may result from obesity, physical inactivity and/or genetic predisposition. To maintain normal glucose levels under IR, the pancreas responds by releasing more insulin by an increasing β-pancreatic cell mass. This phase is called compensation stage or stage 1. If IR proceeds further into diabetes, there is a drop in insulin secretion, with subsequent increased fasting plasma glucose (89-130 mg/dL) and impaired glucose tolerance (IGT; high glucose levels over a period of 2 h after ingestion) as a consequence of a loss and dedifferentiation of β-pancreatic cells. This phase is called adaptation stage or stage 2. Stage 3 is an unstable period of early decompensation in which glucose levels can rise rapidly to 130 mg/dL. Stage 4 is characterized by a stable decompensation with severe β-pancreatic cell dedifferentiation. Finally, stage 5 is characterized by even more severe decompensation representing a profound reduction in β-pancreatic cell mass and total dependence on insulin administration for survival. In stage 5 glucose levels are around 350 mg/dL (Weir & Bonner-Weir, 2004).

Physiopathology of insulin resistance

T2D develops from IR affecting to different tissues and organs, as AT, liver and muscle. The insulin receptor (IRe), is located in the membrane of hepatocytes, skeletal muscle and adipocytes, as well as other types of cells, such as neurons. Binding of insulin to IRe leads to a cascade of intracellular signalling events that regulate glucose uptake and metabolism. Activation of IRe leads to its auto-phosphorylation on tyrosine residues and phosphorylation of its substrate insulin receptor substrate

1 (IRS-1) on tyrosine residues. This initiates two major signalling cascades: i) the mitogen-activated protein kinase (MAPK) pathway that includes activation of extracellular-signal-regulated kinase (ERK) proteins leading to gene expression, protein translation, and cell growth, and ii) the phosphatidylinositol 3 kinase (PI3K) pathway that includes activation of protein kinase B (AKT) leading to translocation of glucose transporter 4 (GLUT4) vesicles from their intracellular pool to the cell membrane, where they allow uptake of glucose into the cell (**Figure 4**).

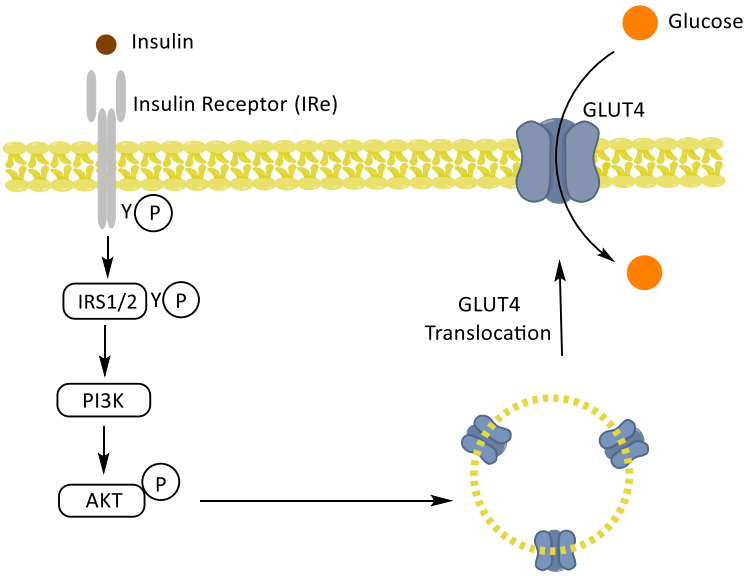


Figure 4. Regulation of glucose uptake by PI3K pathway

IR can be attributed to impaired insulin signalling and decreased insulin-stimulated glucose transport by agents acting at different steps in this pathway. As a consequence, GLUT4 is not translocated and glucose cannot be internalized into the cell.

There are three main mechanisms that explain the pathogenesis of IR in different organs: ectopic lipid accumulation, endoplasmic reticulum stress, and systemic inflammation (Samuel & Shulman, 2012).

- Ectopic Lipid Accumulation

Ectopic lipid accumulation is the displacement of lipid storage from the AT into other organs and tissues. Lipid droplets or adiposomes are considered the intracellular sites of synthesis and lipolysis of lipids. They possess a large number of enzymes that regulate the entry and exit of lipid species. Adipose triglyceride lipase (ATGL) is a lipase that catalyses the hydrolysis of triglycerides (TAGs) into diacylglycerols (DAGs). Intracellular lipid intermediates (DAGs and ceramides) are lipids that can activate protein kinase C (PKC) proteins. PKC proteins regulate numerous cellular responses including gene expression, protein secretion, cell proliferation, and the inflammatory response. The activation of PKC by DAGs or ceramides in muscle and liver leads to a phosphorylation of a serine residue, instead of tyrosine, of the IRe and IRS (**Figure 5**). Loss of PI3K activation from increased IRS serine phosphorylation by PKC reduces insulin-stimulated IRe and IRS tyrosine phosphorylation and leads to reduced translocation of GLUT4 to the cell membrane (Samuel & Shulman, 2012).

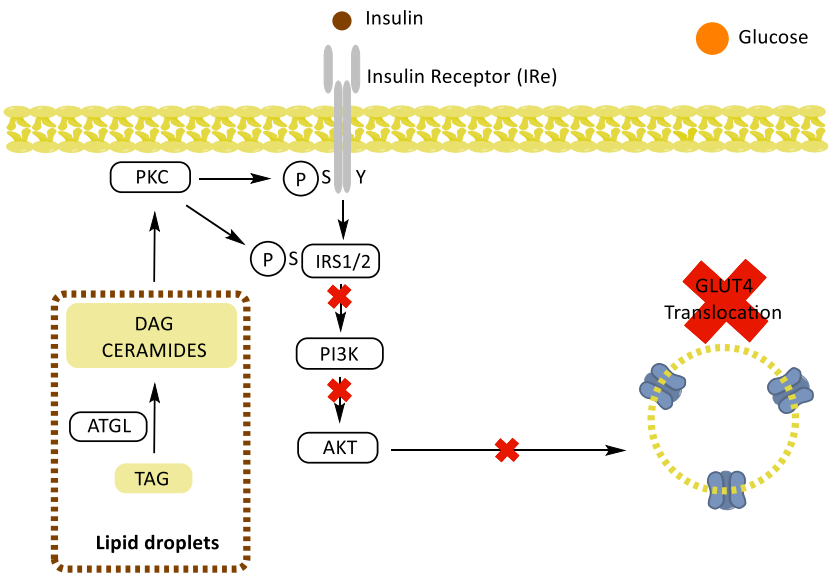


Figure 5. Ectopic lipid accumulation mechanism of insulin resistance

- Endoplasmic Reticulum Stress

When IR first develops, the increased requirements for insulin production could overwhelm the capacity of the endoplasmic reticulum (ER) to process and secrete insulin. The unfolded protein

response (UPR) is initiated with the accumulation of unfolded proteins in the ER lumen. The capacity of the UPR to cause insulin resistance may ultimately depend on whether UPR activation alters the balance of lipogenesis and lipid export to promote lipid accumulation. UPR regulates lipogenesis, lipid droplet formation, and lipid storage. Thus, activation of the UPR may primarily alter cellular lipid balance and, via lipid intermediates accumulation can alter insulin signalling (Samuel & Shulman, 2012).

- Systemic Inflammation

Despite the multiplicity of intracellular pathways that mediate the inflammatory responses, the explanation for the development of IR under inflammatory states often converges on the activation of Jun-N-terminal kinase 1 (JNK1) (**Figure 6**). The defect in insulin signalling can be attributed to serine, instead of tyrosine, phosphorylation of IRS, by activation of JNK1, providing a possible mechanistic link between inflammation and IR (Samuel & Shulman, 2012).

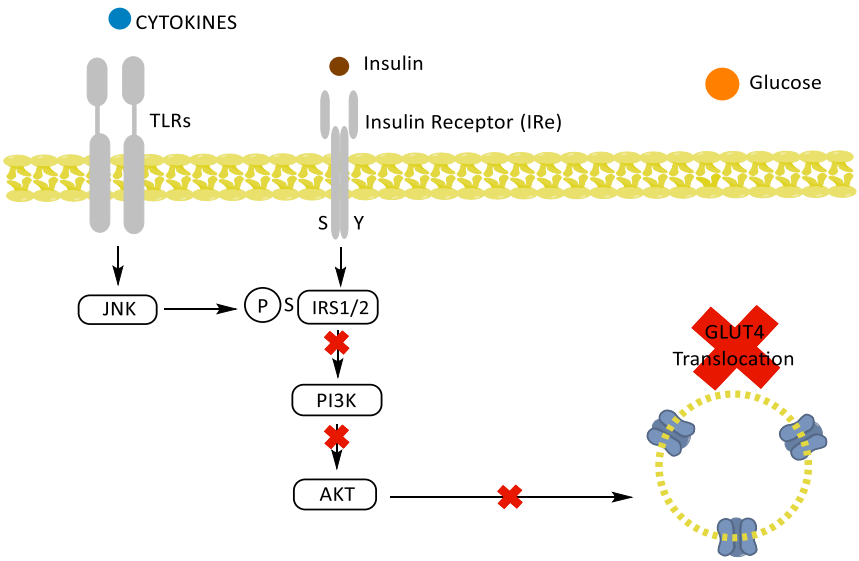


Figure 6. Systemic inflammation mechanism of insulin resistance

T2D and IR have been linked to shifts in the populations of gut microbiota. In humans, T2D is associated with higher levels of *Lactobacillus* spp. compared with non-diabetics. *Lactobacillus* spp. correlated positively with fasting glucose and glycated hemoglobin levels, while *Clostridium* spp.

correlated negatively with fasting glucose and plasma insulin and triglycerides (Blandino et al., 2016). As the modulation of the gut microbiota by dietary compounds is another main goal of this thesis; a more detailed explanation about modifications of the gut microbiota will be developed in the following section.

1.1.5 CHANGES IN GUT MICROBIOTA

Extrinsic factors such as age and diet continually influence the diversity and function of the gut microbiota with diverse implications for the host's health.

1.1.5.1 Age

The microbial populations in the human gut changes over time and these changes can influence health. The most dramatic changes in the composition of the intestinal microbiota take place in childhood (Favier, Vaughan, De Vos, & Akkermans, 2002). After this, only relatively small changes take place and a child has an adult-like microbiota by approximately two years of age. During adult life, the composition of the intestinal microbiota is relatively stable (Faith et al., 2013), however short-term disturbances can rapidly induce changes with possible effects on the host organism (David et al., 2014).

In humans, age-related differences in gut microbiota composition include an increase in the total number of facultative anaerobes, mainly Enterobacteriales (Hopkins & Macfarlane, 2002; Hopkins, Sharp, & Macfarlane, 2001; Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004), as well as a reduction in the populations of species belonging to the phylum Bacteroidetes (Mariat et al., 2009). Also the decline in the numbers of health-promoting *Lactobacillus* and *Bifidobacterium* is one of the most marked changes in the elderly gut (Hopkins & Macfarlane, 2002; Hopkins et al., 2001; Woodmansey et al., 2004). These changes, together with an overall reduction in microbial diversity as well as changes in digestive physiology such as intestinal transit time, may result an increased susceptibility to disease in the old age (Biagi et al., 2010).

1.1.5.2 Diet

There is mounting evidence of the role played by diet in modulating the composition and metabolic activity of the gut microbiota. Alterations in diet have been demonstrated to rapidly modify gut microbial composition (David et al., 2014; Hildebrandt et al., 2009).

Carbohydrates are the principal source of carbon and energy for colonic bacteria. Many of the health benefits ascribed to fiber are a consequence of their fermentation by the gut microbiota and the metabolites that are produced. Carbohydrates are fermented to organic acids, such as SCFAs which are the major end-products of carbohydrate fermentation that provide energy for other bacteria and peripheral tissues (Flint, Scott, Duncan, Louis, & Forano, 2012).

Dietary fat also influences the composition and metabolic activity of the gut microbiota (Hildebrandt et al., 2009). Bacteroidetes levels increase either by fat or carbohydrate restricted diets so that diets rich in fruits, vegetables and fibres are associated with a high diversity of gut microbiota. At the genus level, high intake of fat and protein is associated with increased levels of *Bacteroides*, whereas high fiber intake is associated with increased levels of *Prevotella* (De Filippo et al., 2010; Ley et al., 2006; Wu et al., 2011).

There is much interest in developing new preventive and therapeutic tools for manipulating the composition of the gut microbiota to benefit the host health. There are several nutritional strategies to avert dysbiosis or to restore a normobiotic/eubiotic state disrupted by age or diet. These strategies include the administration of probiotics (putatively beneficial microorganisms) and prebiotics (ingredients that promote the growth/activity of beneficial microorganisms) (Roberfroid et al., 2010). Other bioactive food compounds, such as iminocyclitols and ω-3 PUFAs may have the capacity to preserve gut microbial diversity through different mechanisms and finally promote health.

1.2 BIOACTIVE COMPOUNDS

Bioactive compounds are essential and non-essential compounds, such as vitamins or polyphenols, which are present as natural constituents in food and can provide health benefits in animals or humans that consume them. They exhibit different effects such as antioxidant activity, inhibition or activation of enzymes, regulation of gene expression and modulation of gut microbiota (Biesalski et al., 2009).

1.2.1 IMINOCYCLITOLS

1.2.1.1 Chemical structure, natural sources and synthesis

Iminocyclitols, also referred to as iminosugars or azasugars, are small monocyclic or bicyclic polyhydroxylated alkaloids (nitrogenated natural organic compounds, with biological activities at low

doses). They may be considered saccharide analogues in which the ring oxygen has been replaced by nitrogen (Watson, Fleet, Asano, Molyneux, & Nash, 2001). Iminocyclitols are present in many species of plants and bacteria. The first natural iminocyclitol discovered was nojirimycin (NJ), which was isolated from the bacterium *Streptomyces nojiriensis* (Inouye, Tsuruoka, & Nida, 1966). D-Fagomine was the first iminocyclitol isolated from a plant, buckwheat (*Fagopyrum esculentum*) (Koyama & Sakamura, 1974). Iminocyclitols may be obtained by extraction from plants or microorganisms, chemical synthesis and enzymochemical synthesis (Amézqueta & Torres, 2016).

1.2.1.2 Activity, functions and applications

As other secondary metabolites, iminocyclitols help plants in their defence against certain microorganisms and predators, particularly insects (Asano, 2003a; Lou, Zou, Yan, & Gui, 2011). The biological activities of iminocyclitols, when ingested, are due to their structural analogy to sugars (Watson et al., 2001). One of the biological activities of iminocyclitols is the inhibition of intestinal glycosidases such as intestinal brush border disaccharidases (Asano et al., 1995). Glycosidases are enzymes that catalyse the hydrolysis of the glycosidic bonds in complex carbohydates and glycoconjugates. They are involved in a wide range of important biological processes, such as intestinal digestion, posttranslational processing of the sugar chain of glycoproteins, quality-control systems in the ER and the lysosomal catabolism of glycoconjugates (Asano, 2003b). The inhibition of glycosidases may have profound effects on carbohydrate catabolism in the intestines, maturation, transport, and secretion of glycoproteins, and can alter cell-cell or cell-virus recognition processes. Another biological activity of iminocyclitols seems to be their interaction with gut microbiota (Gómez et al., 2012).

The applications of iminocyclitols range from inhibiting intestinal brush border disaccharidases to modifying the glycosylation of eukaryotic cells, the metabolism of carbohydrates and glycoconjugates, the carbohydrate-dependent properties of glycoproteins and the carbohydrate-mediated interaction of host cells with infective agents (Winchester, 2009). Thus, iminosugars could play a role in the prevention of disorders such as obesity, diabetes, metabolic syndrome, immune response imbalances, cancer, autoimmune diseases, transplant rejection or lysosomal storage diseases (such as Gaucher's disease) (Butters, Dwek, & Platt, 2005; Gómez et al., 2012).

1.2.1.3 D-Fagomine

Chemical Structure

D-Fagomine, (2R, 3R, 4R)-2-hydroxymethylpiperidine-3,4-diol (1,2-dideoxynojirimycin), is a polyhydroxylated piperidine (saturated six-atom ring formed by five carbon atoms and a nitrogen). The spatial configurations of the hydroxyl groups in D-fagomine are coincident with those of other simple sugars (**Figure 7**). D-Fagomine presents the same spatial configurations of D-glucose and D-mannose on carbons 3, 4, 5 and 6.

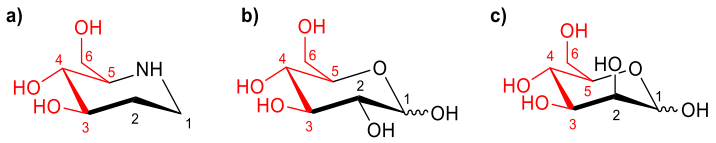


Figure 7. Chemical structures of a) D-fagomine, b) D-glucose and c) D-manose

Natural Sources and Synthesis

D-Fagomine was first isolated from seeds of buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) (Koyama & Sakamura, 1974) and later found in other plant sources such as mulberry (*Morus Alba*, Moraceae) leaves (Asano et al., 2001) and gogi (*Lycium chinense*, Solanaceae) roots (Asano et al., 1997).

Buckwheat flour is used to prepare a variety of foodstuffs including noodles (Japanese soba, Korean makguksu and Italian pizzoccheri), groats (Polish kasha), pancakes (French crêpes from Bretagne, Slavic blinis and North American ployes), boiled flour (Italian polenta, and Slovenian and Croatian zganci), fried dough (Spanish farinetes de fajol from Catalonia), beer, cookies or bread. D-Fagomine is stable during boiling, baking, frying and fermentation. The estimated total intake of D-fagomine resulting from a diet that includes such foodstuffs would be between 3 and 17 mg per day (Amézqueta et al., 2013).

D-Fagomine can be stereoselectively formed by chemo-enzymatic synthesis in three steps using fructose-6-phosphate aldolase (FSA) from *Escherichia coli* as biocatalyst (Castillo et al., 2006). This method was used by Bioglane SLNE for the generation of the sample used in this thesis.

Activity

As D-fagomine is an intestinal glycosidase inhibitor, it has the potential to modulate postprandial blood glucose concentration after oral administration of either sucrose or starch to rats (Gómez et al., 2012). Recent observations have uncovered a second possible activity of D-fagomine and maybe other iminocyclitols as well: the selective inhibition of bacterial adhesion to the intestinal mucosa. Studies *in vitro* have shown that D-fagomine selectively agglutinates fimbriated Enterobacteriales, such as *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium and consequently it inhibits the adhesion of these bacteria to pig intestinal mucosa (Gómez et al., 2012). This activity may modify the composition of the gut microbiota. The first evidence supporting this hypothesis was the observation that D-fagomine reduced diet-induced weight gain while counteracting the diet induced increase in the populations of gut Enterobacteriales (Ramos-Romero et al., 2014).

1.2.2 POLYUNSATURATED FATTY ACIDS

1.2.2.1 Chemical structure, natural sources and synthesis

FAs are carboxylic acids with a long aliphatic chain (**Figure 8**).

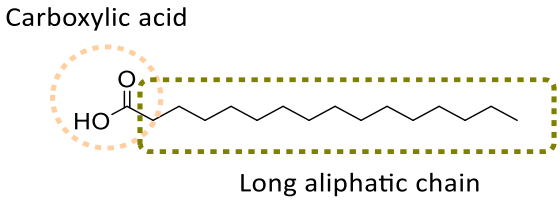


Figure 8. Chemical structure of a fatty acid

Naturally occurring FAs can be classified into three categories based on the number of double bonds present in their backbone (**Figure 9**): saturated FAs (SFAs, no double bonds), monounsaturated FAs (MUFAs, a single double bond), and polyunsaturated FAs (PUFAs, more than one double bond).

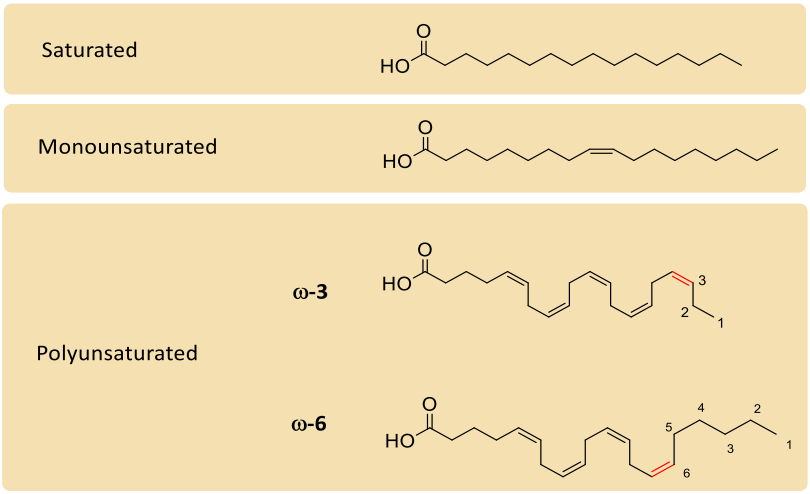


Figure 9. Classification of fatty acids

FAs can be further classified by their carbon chain length and the position of the first double bond from the terminal methyl group (ω-3 PUFAs or ω-6 PUFAs).

Linoleic acid (LA; ω-6 PUFA with 18-carbon chain and 2 unsaturations) and α-linolenic acid (ALA; ω-3 PUFA also with 18-carbon chain and 2 unsaturations) are the precursor molecules from which the rest of fatty acids belonging to the ω-6 or ω-3 PUFAs can be synthesized through a series of elongation and desaturation reactions (**Figure 10**). All the reactions are catalysed by an enzymatic system consisting in fatty acyl-CoA synthetases, Δ-6 and Δ-5 desaturases and elongases. These two fatty acid families share these enzymes and they compete for them, thus the levels of LA and ALA can influence the metabolic outcome of each other (for a review on the actions of ω-6 and ω-3 PUFAs see (Calder, 2013; Innes & Calder, 2018; Wiktorowska-Owczarek, Berezinska, & Nowak, 2015)).

LA and ALA are the two essential FAs for humans because the body is incapable of synthesizing them as a result of the limitation of the enzyme responsible for inserting *cis* double bonds. ω-6 PUFAs are found in crop seeds and vegetable oils, including canola, soybean, corn, and sunflower oils. ω-3 PUFAs are less abundant in food sources, and mainly found in marine algae and phytoplankton, fish and, to a lesser extent in plant seeds and oils (Calder & Yaqoob, 2009; Saini & Keum, 2018).

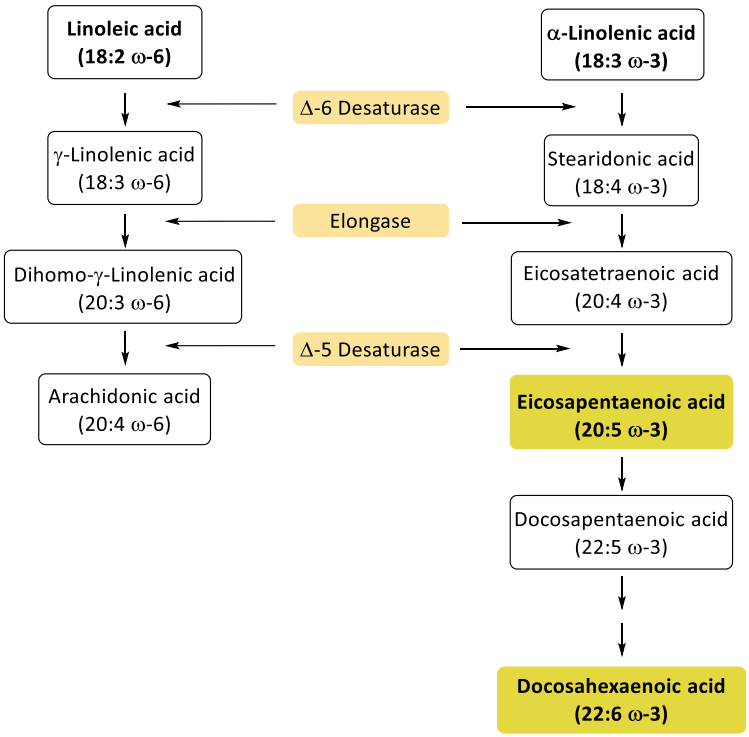


Figure 10. Metabolism of the essential linoleic and α-linolenic fatty acids to longer chain fatty acids. This figure shows the common enzymes required for these conversions

1.2.2.2 Activity, functions and applications

PUFAs are cell-membrane components. The presence of unsaturated fatty acids with numerous double bonds on cell membranes cause chain bending that forms free spaces and affects their physical properties: fluidity and elasticity (Stubbs & Smith, 1984).

To avoid chronic diseases and maintain good health, a balanced intake of ω-6 and ω-3 PUFAs is necessary because these two types of PUFAs have different and complementary effects on bodily metabolic functions. ω-3 PUFAs possess anti-inflammatory activity, predominantly through the displacement of lipid intermediates from the ω-6 pathway to the ω-3 pathway by competing for the same enzymes. Arachidonic acid (ARA) is a ω-6 PUFA precursor of prostaglandins (PG) and thromboxanes (TX) series 2 (PGE2, PGI2, TXA2) and leukotrienes (LT) series 4 (LTB4, LTC4, LTD4) with pro-inflammatory potential and the ability to induce platelet aggregation and vasoconstriction. The metabolism of ω-3 PUFAs generate series 3 PG and TX (PGE3, PGI3, TXA3) and series 5 LT (LTB5,

LTC5, LTD5); this group of ω-3 eicosanoids shows anti-inflammatory and antiplatelet properties (**Figure 11**). One of the most common pharmacological approaches to treat inflammation is to inhibit the biosynthesis of ω-6 eicosanoids. Therefore, ALA consumption and its derivatives EPA and DHA may be a good strategy to preclude the elongation of ω-6 fatty acids to yield ARA and its derivatives (for a review summarizing the metabolism of ω-6 and ω-3 PUFAs see (Naughton, Mathai, Hryciw, & McAinch, 2016; Wiktorowska-Owczarek et al., 2015)). ω-3 PUFAs exert a lowering effect on the synthesis of pro-inflammatory compounds while fueling the synthesis of cytokines with anti-inflammatory lipid mediators.

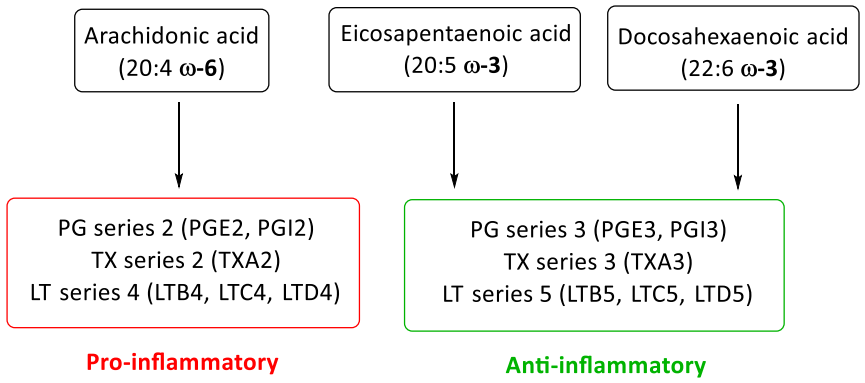


Figure 11. Eicosanoids derived from arachidonic, eicosapentaenoic and docosahexaenoic acids

1.2.2.3 EPA and DHA

Chemical Structure

EPA (20:5) and DHA (22:6) are ALA derived ω-3 PUFAs. They are carboxylic acids with 20 and 22 carbon atoms and, 5 and 6 double bonds respectively (**Figure 12**), with their first double bond between carbons 3 and 4 from the terminal methyl group.

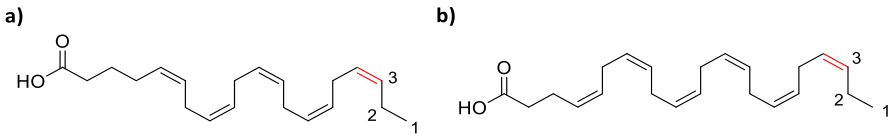


Figure 12. Chemical structures of a) eicosapentaenoic acid (EPA; 20:5) b) docosahexaenoic acid (DHA; 22:6)

Natural Sources and Synthesis

EPA and DHA are considered as almost essential PUFAs because their biosynthesis from ALA is too low to meet the needs of the organism under some circumstances such as disease or developmental problems. Therefore, they must be supplied with the diet. EPA and DHA are ω-3 PUFAs principally found in algae, marine food products and they are particularly concentrated in oily fish and fish oil. Some types of fish such as salmons, sardines, tunas, and halibuts contain EPA (15-19% of total FAs) and DHA (30% of total FAs) in slightly lower amounts. Numerous national and international health organizations recommend regular consumption at least 250-500 mg/day of EPA and DHA (Calder & Yaqoob, 2009). There also are small quantities of DHA in eggs, and EPA and DHA in milk.

Activity

EPA plays an important role in inflammation. The ω-6/ω-3 ratio in phospholipids determines the balance between prostaglandins of the 2 and 3 series derived from ARA and EPA, respectively (**Figure 11**). Eicosanoids of the 2 series promote inflammation and platelet aggregation, and they activate the immune response while series 3 prostaglandins tend to attenuate these effects. Thus, the inflammatory responses are modulated through the levels of ARA and EPA; when the proportions are favourable for ω-3 PUFAs, the response to inflammatory agents is weaker (Calder, 2006, 2013).

Apart from favouring the synthesis of anti-inflammatory mediators, DHA is an important cell-membrane component. Membrane rafts are especially rich in DHA, which provides proper fluidity, and proper functioning of membrane receptors, ion channels and transporting proteins. DHA is present in high proportions in brain tissue and retina (up to 50% and 60-80% membrane phospholipids, respectively) (Feller & Gawrisch, 2005; SanGiovanni & Chew, 2005).

EPA and DHA are associated with cardiovascular health as they reduce the triglyceride levels in plasma and normalize blood pressure through reducing the levels of TXA2, a strong vasoconstrictor. They also exert anti-thrombotic effect by reducing the platelet tendency towards adhesion and aggregation (for reviews, see (Calder, 2004; McEwen, Morel-Kopp, Chen, Tofler, & Ward, 2013)).

2 OBJECTIVES

The main objective of this thesis is to advance in understanding the interactions between gut microbiota and host metabolism, with the final aim of finding novel dietary approaches effective at preventing the current growing incidence of diabetes and obesity. This thesis focuses on the effects of buckwheat D-fagomine, fish ω-3 PUFAs (EPA/DHA 1:1) and their combination on the gut microbiota and related host metabolic variables in both healthy rats and a rat model of fat-induced pre-diabetes. This global objective can be split into the following specific goals:

In healthy animals:

- To elucidate the effect of D-fagomine on the gut microbiota in healthy rats with time.

- To explore the possible complementary or additive effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on the gut microbiota in healthy rats with time.

- To explore the possible complementary or additive effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on risk factors for type-2 diabetes in healthy rats with time.

In animals with pre-diabetes:

- To assess the possible use of D-fagomine as a functional food component using a rat model of fat-induced pre-diabetes.

- To explore possible molecular mechanisms behind the action of D-fagomine in a rat model of fat-induced pre-diabetes.

- To explore the possible complementary or additive effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota and related metabolic variables in a rat model of fat-induced pre-diabetes.

3 RESULTS

The results of this thesis have been published or submitted for publication by way of the following scientific papers:

PAPER 1: Eubiotic effect of buckwheat D-fagomine in healthy rats

PAPER 2: The influence of combined buckwheat D-fagomine and fish omega-3 PUFAs on beneficial gut bacteria in rats

PAPER 3: Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-induced pre-diabetes

PAPER 4: Effects of combined buckwheat D-fagomine and fish omega-3 PUFAs on the gut microbiota and risk factors for diabetes in Sprague Dawley rats fed a high-fat diet

3.1 PAPER 1

TITLE: Eubiotic effect of buckwheat D-fagomine in healthy rats

AUTHORS: **Mercè Hereu**, Sara Ramos-Romero, Natalia García, Susana Amézqueta and Josep Lluís Torres

JOURNAL: Journal of Functional Foods, 2018, 50, 120-126

IMPACT FACTOR (2017): 3.47

CLASSIFICATION AND CATEGORY: FOOD SCIENCE & TECHNOLOGY 16/133 (Q1)

ORAL COMMUNICATION: International Scientific Conference Probiotics and Prebiotics IPC2016. Budapest, Hungary. June 21-23, 2016.

REFERENCE: Hereu, M., Ramos-Romero, S., García-González, N., Amézqueta, S., & Torres, J. L. (2018). Eubiotic effect of buckwheat D-fagomine in healthy rats. *Journal of Functional Foods*, 50, 120-126.

PARTICIPATION OF MERCÈ HEREU IN PAPER 1:

Mercè Hereu performed the animal intervention, the biometric determinations, the qRT-PCR experiments, and the gas chromatography determinations. Mercè Hereu also analyzed the data and wrote the paper.

Sara Ramos Romero

Thesis Supervisor

Journal of Functional Foods 50 (2018) 120–126

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Eubiotic effect of buckwheat D-fagomine in healthy rats

Mercè Hereu[a], Sara Ramos-Romero[a,b,*], Natalia García-González[a], Susana Amézqueta[c], Josep Lluís Torres[a]

[a] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain
[b] Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain
[c] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Faculty of Chemistry, University of Barcelona, Barcelona, Spain

ARTICLE INFO

Keywords:
Gut microbiota
Lactobacillus
Bifidobacteria
Iminosugar
Iminocyclitol
Buckwheat

ABSTRACT

Diversity and balance of gut microorganisms is fundamental for health throughout life. The aim of this study is to explore the possible eubiotic effect of the buckwheat iminosugar D-fagomine (0.096% w/w in standard feed) in growing healthy Wistar Kyoto rats. Feed and energy intake, residual energy in feces, and body weight gain were independent of D-fagomine supplementation throughout the intervention (24 weeks). The populations of significant bacterial subgroups and species were determined in fecal and cecal DNA by quantitative real-time PCR. D-Fagomine increased the Bacteroidetes:Firmicutes ratio and partially counteracted the loss of Lactobacilliales and Bifidobacteriales over time. The supplementation reduced the levels of excreted short-chain fatty acids (SCFAs) as determined by gas chromatography. This paper provides preliminary evidence that D-fagomine has the capacity to promote microbial functional diversity by increasing the Bacteroidetes:Firmicutes ratio and to mitigate the age-related reduction in populations of the putatively beneficial Lactobacilliales and Bifidobacteriales.

1. Introduction

Human gut microbiota is formed of some 10^{14} bacteria: more than 10 times the number of eukaryotic cells in a healthy person. It mainly consists of 9 bacterial phyla encompassing over 1000 species, and more than 15,000 strains. Most of these bacteria belong to the two most abundant phyla in the gut: Bacteroidetes (40% of the gut microbiota) and Firmicutes (60%) (Ley et al., 2005). Their main biological function in the host is the optimization of energy harvesting through the degradation of indigestible biopolymers (e.g. polysaccharides) in the large intestine, and their conversion into smaller species that can be internalized and used as building blocks for lyposynthesis (Thomas, Hehemann, Rebuffet, Czjzek, & Michel, 2011). Bacteroidales is the major order among Bacteroidetes while Clostridiales is the major order among Firmicutes. Other quantitatively minor yet important subgroups of the gut microbiota are the orders: Lactobacillales, Bifidobacteriales and Enterobacteriales, which belong to the phyla Firmicutes, Actinobacteria and Proteobacteria, respectively. Lactobacillales and Bifidobacteriales may confer health benefits on their host, including resistance to infection, amelioration of allergic symptoms and protection against inflammatory processes (Roberfroid et al., 2010). *Lactobacillus acidophilus* is one of the major species of its genus found in the gut, and together with *Lactobacillus plantarum*, it contributes to the maintenance of the normal barrier function of the intestinal epithelium (Gareau, Sherman, & Walker, 2010). Enterobacteriales is composed of non-pathogenic and opportunistic bacteria such as *Escherichia coli*, a facultative anaerobic microorganism. Most *E. coli* strains can coexist inside a healthy host; but they may cause enteric diseases and extra-intestinal infections in immunocompromised hosts or when the normal gastrointestinal barriers are breached (Kaper, Nataro, & Mobley, 2004).

Microbiota products can be either protective or harmful, depending on their concentration and on the metabolic status of the host. These products include lipopolysaccharides (LPS: a component of the bacterial cell wall), angiopoietin-like protein 4 (a protein involved in lipid metabolism), bile acids and short-chain fatty acids (SCFAs) (Janssen & Kersten, 2017). SCFAs are the end products of the fermentation of dietary fiber by anaerobic intestinal bacteria (den Besten et al., 2013; Tan et al., 2014). Bacteroidetes and Actinobacteria are known to produce acetate and propionate; whereas butyrate is mainly generated by bacterial groups in the Firmicutes phylum (e.g. Clostridiales) (Mackie & White, 2012).

SCFAs are building blocks for *de novo* lyposynthesis as well as mediators of biological responses in the host. They interact with signaling pathways through activities such as inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs) (Tan et al., 2014).

* Corresponding author at: IQAC-CSIC, Jordi Girona 18-26, 08034-Barcelona, Spain.
E-mail address: sara.ramos@iqac.csic.es (S. Ramos-Romero).

https://doi.org/10.1016/j.jff.2018.09.018
Received 5 June 2018; Received in revised form 10 September 2018; Accepted 16 September 2018

The preservation of microbial diversity and balance is fundamental for host health (Nicholson et al., 2012). Many factors can produce disruptions in gut microbiota and lead to dysbiosis, which consequently increases the susceptibility of the host to contract diseases (Iebba et al., 2016). Physiological changes in the gastrointestinal tract, modifications in lifestyle, and functional alterations of the host immune system over time ultimately affect the bacterial ecosystem (Biagi et al., 2010). In humans, age-related differences in gut microbiota composition include an increase in the total number of facultative anaerobes, mainly Enterobacteriales; and a reduction in the populations of species belonging to the phylum Bacteroidetes, as well as of the health-promoting Lactobacillales and Bifidobacteriales (Woodmansey, 2007). *E. coli* and other opportunistic bacteria tightly adhere to mucosal surfaces (Svanborg, Agace, Hedges, Lindstedt, & Svensson, 1994) and may prevent gut colonization by the more loosely bound species belonging to the Lactobacillales and Bifidobacteriales orders.

Nutritional strategies to avert dysbiosis or to restore a normobiotic/eubiotic state include the administration of probiotics (putatively beneficial microorganisms) and prebiotics (ingredients that promote the growth/activity of beneficial microorganisms) (Roberfroid et al., 2010). Other food components may have the capacity to preserve gut microbial diversity through different mechanisms; together with probiotics and prebiotics these may generally be called eubiotics. Iminocyclitols, also called iminosugars, are carbohydrate analogues with a nitrogen atom in place of the endocyclic oxygen. D-Fagomine (1,2-dideoxynojirimycin) is a six-ring iminocyclitol first isolated from seeds of buckwheat (*Fagopyrum esculentum*) and also present in other plant sources such as mulberry (*Morus alba)* leaves, and gogi (*Lycium chinense)* roots (Amézqueta et al., 2012). D-Fagomine is partially absorbed and then rapidly (8 h) excreted in urine. It is partially metabolized into methyl-D-fagomine (about 10% in urine and 3% in feces) (Amezqueta et al., 2017). D-Fagomine inhibits intestinal disaccharidases *in vitro*, reduces the post-prandial blood glucose concentration in healthy rats and inhibits the adhesion of *E. coli* and *Salmonella enterica* serovar *Typhimurium* to pig intestinal mucosa (Gómez et al., 2012). D-Fagomine also maintains the glycemic status in pre-diabetic animals (Molinar-Toribio et al., 2015), it reduces fat-induced weigh gain (Ramos-Romero et al., 2014) and there is preliminary evidence that it may elicit these effects through an action on gut microbiota, particularly on Enterobacteriales (Ramos-Romero et al., 2014).

To evaluate the possible use of D-fagomine as a functional food component for the maintenance of balanced gut microbiota, here we explore the changes it induces in the populations of major microbial phyla and selected putatively beneficial minor orders in healthy rats over time.

2. Materials and methods

2.1. Animals

A total of 18 male Wistar-Kyoto rats from Envigo (Indianapolis, IN, USA), aged 8–9 weeks, were used. All animal handling was carried out in the morning, to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals (directive 2010/63/EU) under license from the regional Catalan authorities (reference no. DAAM7921), and were approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2. Experimental design and sample collection

The rats were housed under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h light-12 h dark cycle. To reduce the variation in microbiota between rats, the animals were accommodated in their cages (n = 3 per cage) for 4 weeks before the nutritional intervention. Then, they were randomly divided into 2 groups (n = 9/group): control group (STD), fed a standard diet of 2014 Teklad Global 14% Protein chow from Envigo; and a group fed the standard diet supplemented with 0.96 g D-fagomine/kg feed (> 98% from Bioglane SLNE, Barcelona, Spain) per kg feed (FG). The composition of the diets is provided in Table 1. The proportion of D-fagomine in the feed (2 mg/g carbohydrates) was defined in accordance with the results of previous studies *in vitro* (Gómez et al., 2012) and *in vivo* (Ramos-Romero et al., 2014). The mean daily dose of D-fagomine was 3.9 mg per 100 g body weight, calculated from a mean feed consumption of 4.1 g feed per day per 100 g body weight. The animals were fed *ad libitum* with free access to water (Ribes, Barcelona, Spain).

Table 1
Composition of the experimental diets.

	Standard [a]	Standard plus D-fagomine
Composition (g/kg)		
Protein	143.00	143.00
L-cystine	3.00	3.00
Available carbohydrate	480.00	480.00
Crude fiber	41.00	41.00
Fat	40.00	40.00
Mineral	28.37	28.37
Vitamins	1.20	1.20
Ash	47.00	47.00
Choline bitartrate	1.00	1.00
D-Fagomine [b]	–	0.96
Total energy (ks °C/g) [c]	704.3	627.0

[a] Teklad Global 14% protein rodent maintenance diet (2014) from Harlan.
[b] D-Fagomine (Batch: FG1008E) from Bioglane (Barcelona, Spain).
[c] Integrated SDTA signal proportional to energy in diets.

Feed consumption was monitored daily and body weight was measured three times per week throughout the experiment. Energy intake was calculated as estimates of metabolizable energy, based on the Atwater factors, assigning: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

Fecal samples were collected by abdominal massage after weeks 0, 1, 3, 9 and 24. The energy content in the feces collected after week 20 was determined by differential scanning calorimetry (25–600 °C in an O_2 atmosphere, 10 °C/min) by means of a TGA/SDTA851e thermogravimetric analyzer (Mettler-Toledo, Columbus, OH) with integrated SDTA signal.

After 24 weeks of supplementation, the rats were fasted overnight and anesthetized intraperitoneally with ketamine from Merial Laboratorios (Barcelona, Spain) and xylacine from Quimica Farmaceutica (Barcelona, Spain) (80 and 10 mg/kg body weight, respectively). The cecal content was collected, weighed and immediately frozen in liquid N_2. All the samples were stored at –80 °C until analysis.

2.3. Measurement of microbial populations

The relative populations of selected bacterial phyla, orders and species were estimated in fecal and cecal DNA by quantitative real-time PCR (qRT-PCR). Total DNA was extracted from both feces and cecal content using a QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany) and quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). All DNA samples were diluted to 20 ng/μL. The qRT-PCR experiments were carried out using a LightCycler® 480 II (Roche, Basel, Switzerland) in 96-well plates. Each qRT-PCR well was run in triplicate and contained DNA (2 μL) and a master mix (18 μL) consisting of 2X SYBR (10 μL), the corresponding forward and reverse primer (1 μL each), and water (6 μL). All the reactions were paralleled by a non-template control (water) and a positive control (Table 2) from DSMZ (Braunschweig, Germany). Water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA, USA). The qRT-PCR cycling conditions were as follows: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at the primer-specific annealing temperature (Table 2), and 30 s at 72 °C (extension). Following amplification, to determine the specificity of the qRT-PCR, melting curve analysis was carried out by heating for 2 s at 95 °C, then cooling for 30 s

Table 2
Quantitative real-time PCR primers and conditions.

Target bacteria	Annealing temperature (°C)	Sequences (5′-3′)	Positive control[a]	Reference
Total Bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	[b]	Hartman et al. (2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	*Bacteroides fragilis*	Abdallah Ismail et al. (2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	*Ruminococcus productus*	Haakensen, Dobson, Deneer, & Ziola (2008)
Bacteroidales	61	F: GGT GTC GGC TTA AGT GCC AT R: CGG AYG TAA GGG CCG TGC	*Bacteroides fragilis*	Hartman et al. (2009)
Clostridiales	60	F: CGG TAC CTG ACT AAG AAG C R: AGT TTY ATT CTT GCG AAC G	*Ruminococcus productus*	Hartman et al. (2009)
Lactobacilliales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus acidophylus*	Walter et al. (2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	*Bifidobacterium longum*	Queipo-Ortuno et al. (2013)
Enterobacteriales	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	*Escherichia coli* M15	Hartman et al. (2009)
Lactobacillus acidophylus	64	F: AGC TGA ACC AAC AGA TTC AC R: ACT ACC AGG GTA TCT AAT CC	*Lactobacillus acidophylus*	Walter et al. (2001)
Lactobacillus plantarum	55	F: GCC GCC TAA GGT GGG ACA GAT R: TTA CCT AAC GGT AAA TGC GA	*Lactobacillus plantarum*	Walter et al. (2001)
Escherichia coli	61	F: GTT AAT ACC TTT GCT CAT TGA R: ACC AGG GTA TCT AAT CCT GTT	*Escherichia coli* M15	Malinen, Kassinen, Rinttila, & Palva (2003)

[a] All strains of positive controls were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).
[b] Positive control for total bacteria was the same as that for each individual reaction.

at 60 °C, and a temperature gradient from 30 °C to 95 °C at a rate of 0.11 °C/s, with five fluorescence recordings per °C.

The relative DNA abundances for the different sequences were calculated from the second derivative maximum of their respective amplification curves (C*p*, calculated in triplicate) by considering C*p* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation: $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ (Pfaffl, 2001). Total bacteria was normalized as 16S rRNA gene copies per mg of wet feces (copies/mg).

2.4. Short-chain fatty acids

SCFAs were analyzed in feces after 12 weeks of supplementation and in the cecal content at the end of the study, by gas chromatography using a previously described method (Schwiertz et al., 2009) with some modifications. Briefly, the feces were freeze-dried and weighed (~50 mg dry matter) and a solution (1.5 mL) containing the internal standard 2-ethylbutiric acid (6.67 mg/L) and oxalic acid (2.97 g/L) in acetonitrile/water 3:7 was added. Then, SCFAs were extracted for 10 min using a rotating mixer. The suspension was centrifuged (5 min, 12,880*g*) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) and the supernatant passed through a 0.45 µm nylon filter. An aliquot of the supernatant (0.7 mL) was diluted to 1 mL with acetonitrile/water 3:7. SCFAs were analyzed using a Trace2000 gas chromatograph coupled to a flame ionization detector (ThermoFinnigan, Waltham, MA, USA) equipped with a Innowax 30 m × 530 µm × 1 µm capillary column (Agilent, Sta Clara, CA, USA). Chrom-Card software was used for data processing. This method has shown good selectivity for six different SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid), sensitivity, linearity in the working concentration range (acetic and butyric acids 3–750 ppm; propionic acid 1–250 ppm; isobutyric acid 0.3–75 ppm; isovaleric and valeric acids 0.2–40 ppm) and accuracy (trueness and precision). To check the method trueness and precision, a recovery study at three concentration levels and on three different days was performed. Precision (RSD < 15%) and recovery (> 70%) were adequate and intra-day reproducible.

2.5. Statistical analysis

The results are expressed as mean values with their standard errors (SEM). Normal distribution and heterogeneity of data were evaluated by Shapiro-Wilk test and F-tests, respectively. Intra-group statistical significance throughout the study was determined by repeated-measures ANOVA. Statistical significance between groups was determined by Student's *t*-test. Differences were considered significant when $P < 0.05$. All data calculations and statistical analysis were performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Body weight, and feed and energy intakes

Body weight was similar in the STD and FG groups, both before and after the nutritional intervention (Table 3). There were no differences between groups in either water, feed or energy intakes throughout the experiment (Table 3); nor were there in the energy excreted in feces at the end of the study (Table 3).

Table 3
Body weight, feed and energy intake, and energy in feces of rats supplemented (or not) with D-fagomine for 24 weeks.

	STD[a]		FG[b]	
	Mean	SEM	Mean	SEM
Initial body weight (g)	224.9	3.9	237.8	4.1
Final body weight (g)	416.4	12.9	435.7	11.15
Water intake (mL/day/100 g body weight)	7.4	0.2	7.3	0.2
Feed intake (g/day/100 g body weight)	4.8	0.7	4.1	0.3
Energy intake[c] (kcal/day/100 g body weight)	14.3	0.2	14.6	0.2
Excreted energy[d]	306.6	19.5	253.6	21.7

[a] STD (Control group): rats fed a standard diet (2014 Teklad Global 14% Protein chow from Envigo).
[b] FG (D-Fagomine group): rats fed the standard diet supplemented with 0.96 g D-fagomine/kg feed.
[c] Estimated as metabolizable energy based on Atwater factors, which assign: 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.
[d] Integrated STD signal (ks °C/g) proportional to energy in feces from week 20.

3.2. Major microbiota phyla and orders

The relative proportions of the two predominant bacterial phyla: Bacteroidetes and Firmicutes, and orders within these phyla: Bacteroidales and Clostridiales in the gut microbiome, were evaluated at time 0 and after 1, 3, 9 and 24 weeks of supplementation in feces, and also at the end of the study (24 weeks) in cecal content (Fig. 1).

Intragroup variations in the percentages of Bacteroidetes and Firmicutes over the entire experiment were not significant; while supplementation with D-fagomine clearly increased the populations of Bacteroidetes in feces, already after one week of supplementation, except at week 3 (Fig. 1A). This effect was also detected in the cecal content at the end of the study (Fig. 1A, B). The populations of Bacteroidales (the main order within Bacteroidetes) presented a similar pattern (Fig. 1D). No significant differences were observed in the populations of Firmicutes or its major order, Clostridiales, throughout the study (Fig. 1B, E).

3.3. Minor microbiota orders and species

The relative proportions of the orders Lactobacillales, Bifidobacteriales, and Enterobacteriales, as well as *L. acidophilus*, *L. plantarum*, and *E. coli* in the gut microbiota, were evaluated at time 0 and after 1, 3, 9 and 24 weeks of supplementation in feces, and at the end of the study (24 weeks) in cecal content (Fig. 2).

The relative populations of Lactobacillales at the end of the study (week 24) were significantly lower ($P < 0.001$) than those at time 0 in the STD group (Fig. 2A). D-Fagomine partially counteracted this age-

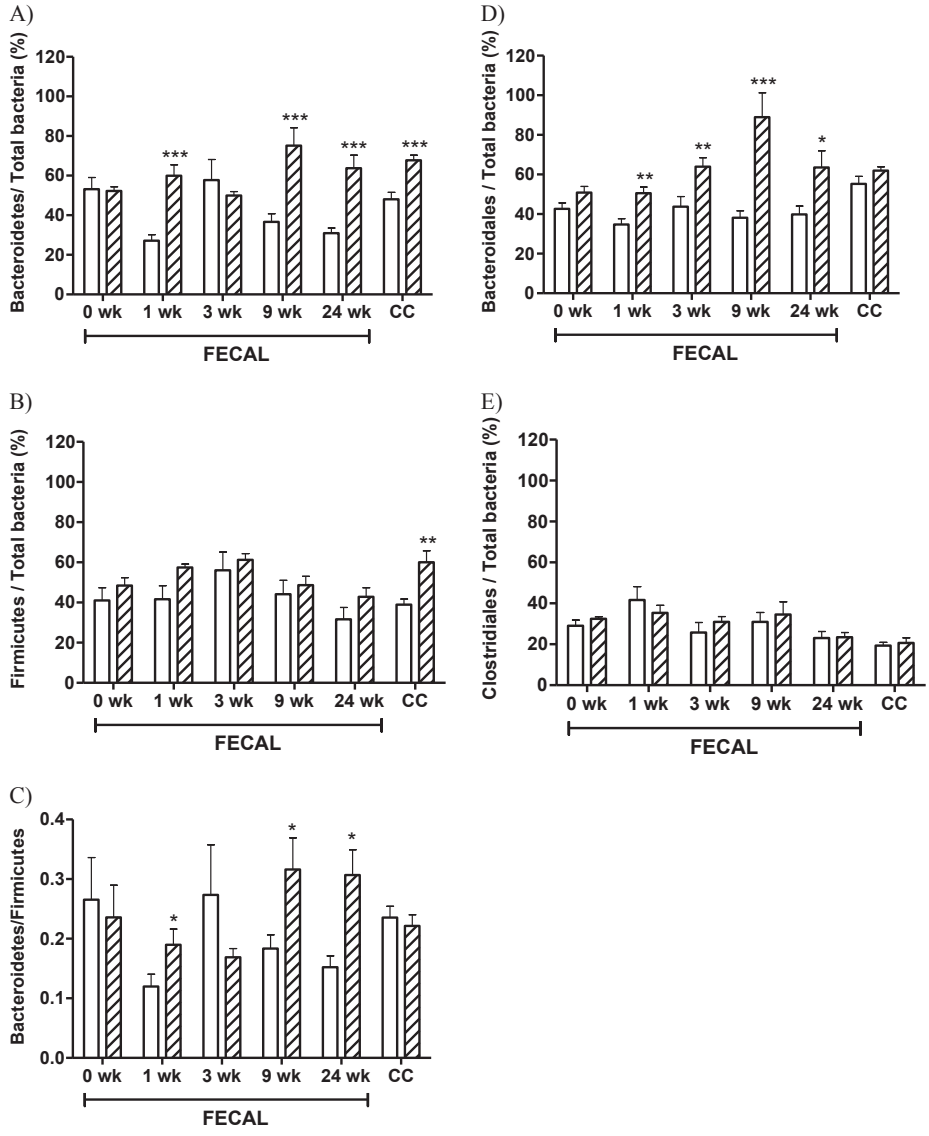


Fig. 1. Bacteroidetes (A), Firmicutes (B), Bacteroidetes:Firmicutes ratio (C) Bacteroidales (D) and Clostridiales (E) in fecal samples from rats fed a standard diet (STD, empty bars), or supplemented with D-fagomine (FG, striped bars) at different times, and in cecal content (CC) at the end of the study. Data are presented as means with their standard error. Comparisons were made using Student's *t*-test. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

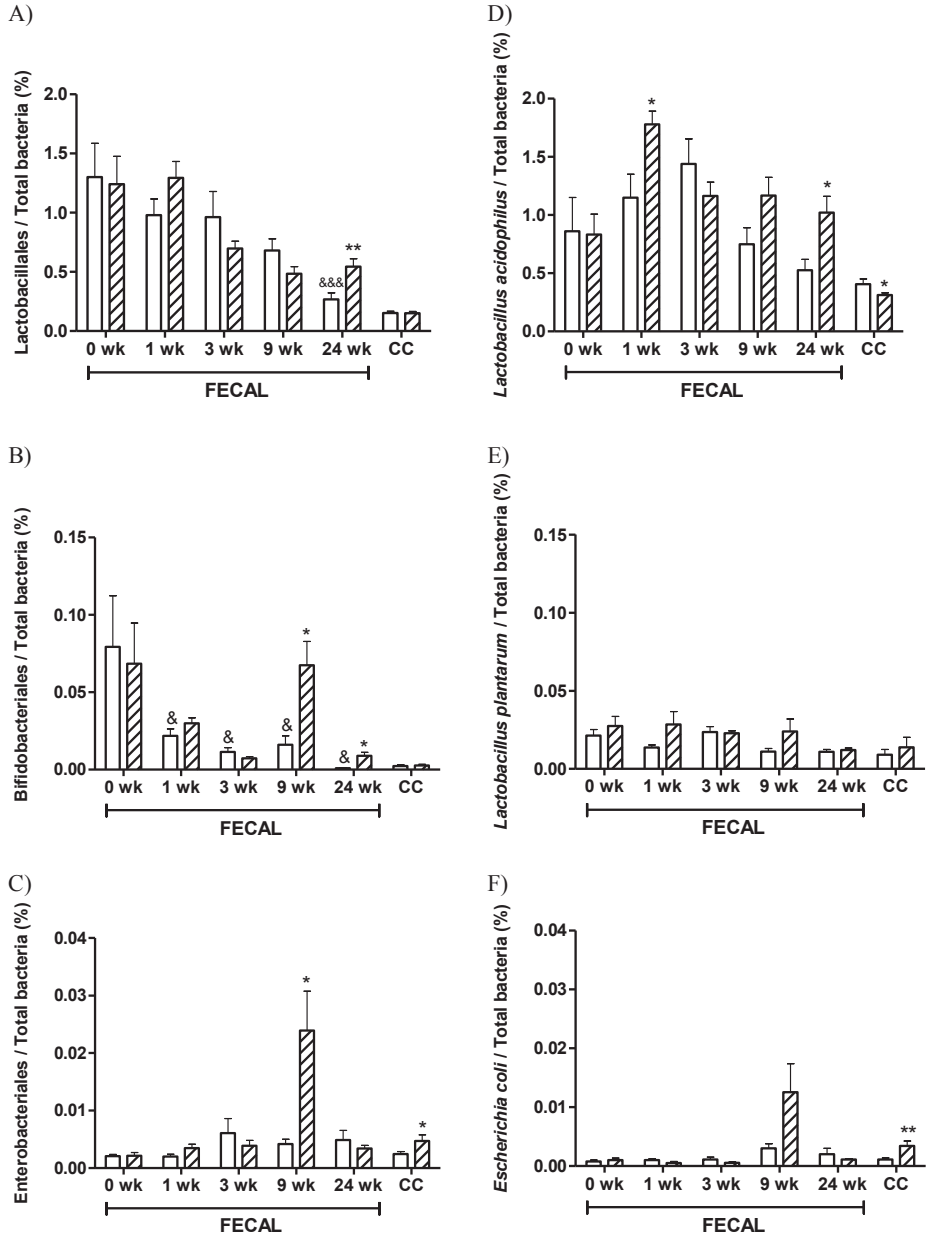


Fig. 2. Lactobacillales (A), Bifidobacteriales (B), Enterobacteriales (C), *Lactobacillus acidophilus* (D), *Lactobacillus plantarum* (E) and *E. coli* (F) in fecal samples from rats fed a standard diet (STD, empty bars), or supplemented with D-fagomine (FG, striped bars) at different times, and in cecal content (CC) at the end of the study. Data are presented as means with their standard error. Comparisons were made using Student's *t*-test or repeated-measures ANOVA. $^{*}P < 0.05$ *vs* STD. $^{**}P < 0.01$ *vs* STD; $^{\&}P < 0.05$ *vs* wk 0. $^{\&\&\&}P < 0.001$ *vs* wk 0.

related loss as after 24 weeks the population of Lactobacilliales in the supplemented group was significantly ($P < 0.01$) greater than that in the STD group (Fig. 2A). The same effect was detected for *L. acidophilus* (Fig. 2D). There were no differences between the groups in the percentage of *L. plantarum* (Fig. 2E).

The relative populations of Bifidobacteriales also significantly decreased ($P < 0.05$) over time in the feces of animals in the STD group (Fig. 2B); at the end of the experiment (week 24) the population was almost undetectable. Supplementation with D-fagomine also had an effect on these proportions of Bifidobacteria over time. Already after 9 weeks of intervention, the population of Bifidobacteriales was significantly higher ($P < 0.05$) in the supplemented group than in the STD group; and at the end of the study (week 24), the differences between the groups were still significant ($P < 0.05$).

The populations of Enterobacteriales and *E. coli* in the STD and FG groups were similar throughout the study except after week 9 of supplementation when a significant ($P < 0.05$) increase was recorded for Enterobacteriales in the FG group (Fig. 2C). At the end of the study, the

Table 4
Short-chain fatty acids determined in feces from rats supplemented (or not) with D-fagomine for 12 weeks and at the end of the study (24 weeks) in cecal content.

	FECES				CECAL CONTENT			
	STD[a]		FG[b]		STD[a]		FG[b]	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	310.94	61.1	142.07*	25.7	96.12	5.3	88.07	6.0
Propionic acid	27.42	6.2	16.70	2.7	25.41	2.0	19.95	1.3
Isobutyric acid	1.25	0.2	0.45*	0.1	3.66	0.3	3.00	0.1
Butyric acid	17.58	3.3	10.62	2.0	15.28	1.9	10.59	0.9
Isovaleric acid	1.00	0.3	0.44	0.1	3.92	0.3	3.02	0.2
Valeric acid	0.69	0.1	0.64	0.1	3.00	0.3	2.32	0.2
TOTAL SCFA	356.86	66.1	170.92*	28.5	136.71	10.8	125.77	7.2

Comparisons were made using Student's *t*-test.

* $P < 0.05$ *vs* STD group.

[a] STD (Control group): rats fed a standard diet (2014 Teklad Global 14% Protein chow from Envigo).

[b] FG (D-Fagomine group): rats fed the standard diet supplemented with 0.96 g D-fagomine/kg feed.

group supplemented with D-fagomine presented higher amounts of Enterobacteriales ($P < 0.05$) and *E. coli* ($P < 0.01$) in the cecal content (Fig. 2C, F).

3.4. Short-chain fatty acids

The concentrations of SCFAs were measured in feces after week 12 of the study and in the cecal content at the end (24 weeks) (Table 4).

D-Fagomine significantly ($P < 0.05$) reduced the concentration of acetic and isobutyric acids, and also the total content of SCFAs in feces (Table 4). There were no differences between groups in any SCFA determined in the cecal content (Table 4).

4. Discussion

The present study focuses on the effect of D-fagomine on gut microbiota of healthy WKY rats over a period of 24 weeks (from age 8–9 weeks to 32–33 weeks). The intragroup differences in the populations of Bacteroidetes, Firmicutes and their respective major orders, Bacteroidales and Clostridiales, were not significant. This result roughly agrees with a previous report of fecal microbiota variation in healthy Sprage-Dawley rats over a period of two years (Flemer et al., 2017). In that study, the populations of the two phyla and the Bacteroidetes:Firmicutes ratio showed a non-significant tendency to increase during the first year (Flemer et al., 2017). Now we have shown here that the feces of WKY rats supplemented with D-fagomine contains significantly higher populations of Bacteroidetes and Bacteroidales than those of rats given the STD diet, already after one weak and over the entire experiment, with the exception of Bacteroidetes at week 3 (Fig. 1A, D). As the level of functional diversity in the gut microbiome has been linked to the relative abundance of Bacteroidetes (Turnbaugh et al., 2009), D-fagomine may contribute to the maintenance of intestinal health in ageing rats by preserving diversity.

We have also recorded some intergroup differences in fecal SCFAs. The total SCFA content in the group supplemented with D-fagomine showed a tendency to be lower than in the STD group; this difference was only significant in the cases of acetate and isobutyrate (Table 4). This reduction in excreted SCFAs might be related to the increase in the Bacteroidetes:Firmicutes ratio (Fig. 1C), in agreement with studies that associate a reduced Bacteroidetes:Firmicutes ratio in obese *vs* lean mice with increased concentrations of acetate and butyrate (Turnbaugh et al., 2006) or acetate and propionate (Murphy et al., 2010). In humans, the transfer of intestinal microbiota from lean donors can improve insulin sensitivity of patients suffering from metabolic syndrome, while increasing the populations of butyrate-producing bacteria and reducing fecal SCFAs (acetate and butyrate) (Vrieze et al., 2012). This apparent contradiction may be explained by considering the host/microbiome ecosystem as a whole, in which the capacity to absorb bacterial metabolites by the host plays a determinant role and the fecal concentration of these metabolites may not be directly related to their generation rate. Fecal SCFAs may still be markers of the host's metabolic status. Hence, lower levels of excreted SCFAs together with higher Bacteroidetes:Firmicutes ratios are consistently associated in the literature with a lean healthy phenotype, compared to metabolically altered phenotypes (Canfora, Jocken, & Blaak, 2015).

The action of D-fagomine is also evident in the case of the putatively beneficial Lactobacillales and Bifidobacteriales, particularly *Lactobacillus acidophilus* (Fig. 2A, B, D). The fecal populations of these bacteria steadily and significantly decreased from week 3 until the end of the study in non-supplemented animals. There is little information in the literature about changes in the populations of putatively beneficial bacteria in healthy rats over time. In Wistar rats, Lactobacilliales show a slight tendency to increase during the first year of life, while species of the *Bifidobacterium* genus are detected only in the second year (Flemer et al., 2017). In humans, the populations of Bifidobacteriales remain relatively stable during adulthood and decrease considerably in old age (Arboleya, Watkins, Stanton, & Ross, 2016). This decline has been associated with the development of intestinal disorders, including diarrhea, irritable bowel syndrome, and inflammatory bowel disease (Gareau et al., 2010). In the present study, the supplemented group presented significantly higher populations of Lactobacilliales and Bifidobacteriales than those in the STD group at the end of the intervention (animals of 32–33 weeks of age). D-Fagomine might counteract the loss of beneficial bacteria by inhibiting the adhesion of opportunistic species such as *E. coli*, as previously reported (Gómez et al., 2012).

The feces of the Wistar-Kyoto rats in this study did not contain elevated percentages of Enterobacteriales, whether they were supplemented with D-fagomine or not. In the supplemented group, a significant increase of Enterobacteriales, and particularly *E. coli*, was recorded after 9 weeks of intervention (Fig. 2C, F). Yet these levels (up to 0.2%) fall within the normal range for healthy individuals and they are much lower than those triggered by an obesogenic diet (4%) (Ramos-Romero et al., 2014). The population of Bifidobacteriales also increased significantly at the same time point (Fig. 2B). At this particular time in the experiment, a singular event may have occurred. The combination of the standard diet and D-fagomine may have induced changes in the intestinal ecosystem when the rats were 17–18 weeks old. As commented before in the case of SCFAs, the increase in excreted Enterobacteriales and *E. coli* does not necessarily imply an increase of these populations in contact with the intestinal wall. In fact, the opposite might be the case in the supplemented group. The results at week 9 suggest that D-fagomine was eliminating Enterobacteriales and *E. coli* while favoring colonization by Bifidobacteriales. This assertion is supported by previous results which show that D-fagomine inhibits the adhesion of *E. coli*, but not of Bifidobacteria, to the intestinal mucosa (Gómez et al., 2012) and it reduces the populations of enterobacteria triggered by an obesogenic diet (Ramos-Romero et al., 2014). This explanation is also consistent with the recorded increased populations of *E. coli* in cecum content at the end of the study (Fig. 2F).

5. Conclusions

This paper provides preliminary evidence that the iminosugar D-fagomine has the capacity to promote diversity in gut microbiota and to mitigate the age-related reduction in the populations of some putatively beneficial bacteria in healthy rats. D-Fagomine increased the Bacteroidetes:Firmicutes ratio, reduced the loss of Lactobacilliales and Bifidobacteriales with aging and reduced the levels of excreted SCFAs. A comprehensive metagenomic study should shed more light on the

changes in gut microbiota induced by iminosugars such as D-fagomine and their functionality. D-Fagomine may have a eubiotic effect on the composition of intestinal microbiota that may be complementary to that of probiotics and prebiotics.

6. Ethics

I have read and adhere to the Publishing Ethics.

Conflict of interest

There are no conflicts of interest to declare

Acknowledgements

Language revision by Christopher Evans is appreciated.

This research was supported by the Spanish Ministry of Economy and Competitiveness (Grant AGL2013-49079-C2-1,2-R and a graduate fellowship to M. Hereu: BES2014-068592).

References

Abdallah Ismail, N., Ragab, S. H., Abd Elbaky, A., Shoeib, A. R., Alhosary, Y., & Fekry, D. (2011). Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Archives of Medical Science, 7*(3), 501–507.

Amézqueta, S., Galán, E., Fuguet, E., Carrascal, M., Abian, J., & Torres, J. L. (2012). Determination of d-fagomine in buckwheat and mulberry by cation exchange HPLC/ESI-Q-MS. *Analytical and Bioanalytical Chemistry, 402*(5), 1953–1960.

Amezqueta, S., Ramos-Romero, S., Martinez-Guimet, C., Moreno, A., Hereu, M., & Torres, J. L. (2017). Fate of d-Fagomine after oral administration to rats. *Journal of Agricultural and Food Chemistry, 65*(22), 4414–4420.

Arboleya, S., Watkins, C., Stanton, C., & Ross, R. P. (2016). Gut bifidobacteria populations in human health and aging. *Frontiers in Microbiology, 7*.

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., & Pini, E. (2010). Through ageing, and beyond: Gut microbiota and inflammatory status in seniors and centenarians. *PloS One, 5*.

Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews. Endocrinology, 11*(10), 577–591.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research, 54*(9), 2325–2340.

Flemer, B., Gaci, N., Borrel, G., Sanderson, I. R., Chaudhary, P. P., Tottey, W., ... Brugere, J. F. (2017). Fecal microbiota variation across the lifespan of the healthy laboratory rat. *Gut Microbes, 8*(5), 428–439.

Gareau, M. G., Sherman, P. M., & Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature Reviews.Gastroenterology & Hepatology, 7*(9), 503–514.

Gómez, L., Molinar-Toribio, E., Calvo-Torras, M.Á., Adelantado, C., Juan, M. E., Planas, J. M., ... Torres, J. L. (2012). D-Fagomine lowers postprandial blood glucose and modulates bacterial adhesion. *British Journal of Nutrition, 107*(12), 1739–1746.

Haakensen, M., Dobson, C. M., Deneer, H., & Ziola, B. (2008). Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *International Journal of Food Microbiology, 125*(3), 236–241.

Hartman, A. L., Lough, D. M., Barupal, D. K., Fiehn, O., Fishbein, T., Zasloff, M., & Eisen, J. A. (2009). Human gut microbiome adopts an alternative state following small bowel transplantation. *Proceedings of the National Academy of Sciences of the United States of America, 106*(40), 17187–17192.

Iebba, V., Totino, V., Gagliardi, A., Santangelo, F., Cacciotti, F., Trancassini, M., ... Schippa, S. (2016). Eubiosis and dysbiosis: The two sides of the microbiota. *The New Microbiologica, 39*(1), 1–12.

Janssen, A. W., & Kersten, S. (2017). Potential mediators linking gut bacteria to metabolic health: A critical view. *The Journal of Physiology, 595*(2), 477–487.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic Escherichia coli. *Nature Reviews. Microbiology, 2*(2), 123–140.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America, 102*(31), 11070–11075.

Mackie, R., & White, B. (2012). Gastrointestinal Microbiology: Volume 1 Gastrointestinal Ecosystems and Fermentations: Springer Science & Business Media.

Malinen, E., Kassinen, A., Rinttila, T., & Palva, A. (2003). Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology, 149*(Pt 1), 269–277.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Gómez, L., Taltavull, N., Nogués, M. R., ... Torres, J. L. (2015). D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food & Function, 6*(8), 2614–2619.

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., ... Shanahan, F. (2010). Composition and energy harvesting capacity of the gut microbiota: Relationship to diet, obesity and time in mouse models. *Gut, 59*(12), 1635–1642.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science, 336*(6086), 1262–1267.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), e45.

Queipo-Ortuno, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., ... Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One, 8*(5), e65465.

Ramos-Romero, S., Molinar-Toribio, E., Gómez, L., Pérez-Jiménez, J., Casado, M., Clapés, P., ... Torres, J. L. (2014). Effect of D-fagomine on excreted enterobacteria and weight gain in rats fed a high-fat high-sucrose diet. *Obesity, 22*(4), 976–979.

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., ... Meheust, A. (2010). Prebiotic effects: Metabolic and health benefits. *The British Journal of Nutrition, 104*(Suppl 2), S1–S63.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2009). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity, 18*(1), 190–195.

Svanborg, C., Agace, W., Hedges, S., Lindstedt, R., & Svensson, M. L. (1994). Bacterial adherence and mucosal cytokine production. *Annals of the New York Academy of Sciences, 730*, 162–181.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in Immunology, 121*, 91–119.

Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M., & Michel, G. (2011). Environmental and gut bacteroidetes: The food connection. *Frontiers in Microbiology, 2*, 93.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., ... Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature, 457*(7228), 480–484.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature, 444*(7122), 1027–1031.

Vrieze, A., Van Nood, E., Holleman, F., Salojarvi, J., Kootte, R. S., Bartelsman, J. F., ... Nieuwdorp, M. (2012). Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology, 143*(4), 913–916.

Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 67*(6), 2578–2585.

Woodmansey, E. J. (2007). Intestinal bacteria and ageing. *Journal of Applied Microbiology, 102*(5), 1178–1186.

3.2 PAPER 2

TITLE: The influence of combined buckwheat D-fagomine and fish omega-3 PUFAs on beneficial gut bacteria of rats

AUTHORS: Mercè Hereu, Sara Ramos-Romero, Roser Marín-Valls, Susana Amézqueta, Bernat Miralles-Pérez, Marta Romeu, Lucía Méndez, Isabel Medina and Josep Lluís Torres

JOURNAL: Submitted to Food Research International

IMPACT FACTOR (2017): 3.52

CLASSIFICATION AND CATEGORY: FOOD SCIENCE & TECHNOLOGY 14/133 (Q1)

PARTICIPATION OF MERCÈ HEREU IN PAPER 2:

Mercè Hereu supervised and performed the animal intervention, the biometric determinations, the evaluation of glycemic status, the qRT-PCR experiments and the gas chromatography determinations. Mercè Hereu also analyzed the data and wrote the paper.

Sara Ramos Romero

Thesis Supervisor

The influence of combined buckwheat D-fagomine and fish omega-3 PUFAs on beneficial gut bacteria in rats

Mercè Hereu[1], Sara Ramos-Romero[1,2*], Roser Marín-Valls[1], Susana Amézqueta[3], Bernat Miralles-Pérez[4], Marta Romeu[4], Lucía Méndez[5], Isabel Medina[5], Josep Lluís Torres[1]

[1] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

[2] Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

[3] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Spain

[4] Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la Salut, Reus, Spain

[5] Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

ABSTRACT

Some functional food components may help maintain homeostasis by promoting balanced gut microbiota. Here, we explore the possible complementary effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on putatively beneficial gut bacterial strains. Male Sprague Dawley rats were supplemented with D-fagomine, ω-3 PUFAs, or both, for 23 weeks. Bacterial subgroups were evaluated in fecal DNA by qRT-PCR and short-chain fatty acids were determined by gas chromatography. We found that the populations of the genus *Prevotella* remained stable over time in animals supplemented with D-fagomine, independently of ω-3 PUFA supplementation. D-Fagomine supplementation also maintained the relative populations of Bifidobacteriales, while ω-3 PUFAs mainly affected Lactobacilliales. ω-3 PUFAs reduced the amount of acetic acid in feces. Animals supplemented with D-fagomine gained less weight and tended to accumulate less fat than controls and rats given only ω-3 PUFAs. The plasma levels of pro-inflammatory ARA-derived metabolites, triglycerides and cholesterol were lower in groups supplemented with ω-3 PUFAs. The D-fagomine and ω-3 PUFAs combination provided the functional benefits of each supplement. Notably, it helped stabilize populations of *Prevotella* in the rat intestinal tract while reducing weight gain and providing the anti-inflammatory and cardiovascular benefits of ω-3 PUFAs.

KEYWORDS: Weight; *Prevotella*; lactobacillus; bifidobacteria; D-fagomine; ω-3 PUFAs.

INTRODUCTION

There is convincing evidence that dietary components such as soluble fiber, polyphenols and polyunsaturated fatty acids (PUFAs) are associated with functional effects that protect against metabolic disorders and cardiovascular diseases (Alkhatib et al., 2017). It is also becoming increasingly evident that some of these effects are mediated by changes in gut microbiota (Portune, Benitez-Paez, Del Pulgar, Cerrudo, & Sanz, 2017). Diet has been shown to influence microbiota in both animal models and humans (De Filippo et al., 2010; Hildebrandt et al., 2009; Turnbaugh et al., 2009). Although the gut microbiota is relatively stable in healthy adults (Faith et al., 2013), short-term disturbances can rapidly change its composition (David et al., 2014; Wu et al., 2011) with possible effects on the host organism.

D-Fagomine (1,2-dideoxynojirimycin) is a six-ring iminocyclitol: a carbohydrate analog with a nitrogen atom in place of the endocyclic oxygen. D-Fagomine was first isolated from seeds of buckwheat (*Fagopyrum esculentum*) and it is also present in other plant parts such as mulberry (*Morus alba*) leaves and gogi (*Lycium chinense*) roots (Amézqueta et al., 2012; Koyama & Sakamura, 1974). The functional effects of D-fagomine include a reduction of post-prandial blood glucose concentration, achieved through the inhibition of intestinal disaccharidases (Gómez et al., 2012); and reductions in high-fat-diet-induced weight gain, low-grade inflammation and impaired glucose tolerance, probably all achieved by counteracting adverse changes in gut microbiota (Ramos-Romero et al., 2018; Ramos-Romero et al., 2014). Moreover, D-fagomine promotes the diversity of gut microbiota by increasing populations of Bacteroidetes in healthy rats while mitigating the age-related reduction in the populations of putatively beneficial Lactobacillus and Bifidobacterium bacteria (Hereu, Ramos-Romero, García-González, Amézqueta, & Torres, 2018).

Eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are the major ω-3 PUFAs of marine origin. EPA and DHA are essential dietary components that reduce risk factors (plasma cholesterol and triglycerides, oxidative stress, and blood pressure) for cardiovascular diseases (Poudyal, Panchal, Diwan, & Brown, 2011), and other pathologies that involve inflammation (Calder, 2006). ω-3 PUFAs are believed to exert their anti-inflammatory effects by competing with arachidonic acid (ARA) metabolism and fostering the synthesis of anti-inflammatory mediators such as resolvins (Calder, 2006). However, the effects of ω-3 PUFAs on gut microbiota are poorly documented (Costantini

& Molinari, 2017). It has been reported that EPA and DHA significantly increase the populations of Firmicutes (Lactobacillus taxa) and Bifidobacteria in mice fed a high-fat diet (Mujico, Baccan, Gheorghe, Diaz, & Marcos, 2013; Robertson et al., 2017). In contrast, we found that the mixture EPA/DHA 1:1 reduced the population of Lactobacilliales in Wistar Kyoto (WKY) rats fed a standard diet (Ramos-Romero et al., 2017). As buckwheat D-fagomine and ω-3 PUFAs have different effects on Lactobacillus and Bifidobacterium, we decided to test whether their combination had any additive or complementary effects. We previously reported that a combination of EPA/DHA 1:1 and proanthocyanidins can be instrumental in promoting balanced gut microbiota (Ramos-Romero et al., 2017). In this paper, we now also focus on the populations of *Prevotella*, because this genus has been directly associated with improved glucose metabolism in humans (Kovatcheva-Datchary et al., 2015), which is ultimately one of our main interests. We measured different variables in test animals (fat accumulation, weight gain, lipid profile and inflammation markers) that are pertinent to the known effects of D-fagomine and ω-3 PUFAs. Other studies have examined the effects of supplements on animal models subjected to more or less severe dietary challenges such as high-fat or high-sugar loads. As functional food components are primarily supposed to maintain the normal functions of the body, here we chose to test the effects on normal rats fed a standard diet.

MATERIALS AND METHODS

Animals and diets

Male Sprague Dawley (SD) rats (n = 36) from Envigo (Indianapolis, IN, USA), aged 10-11 weeks were housed (n = 3 per cage) under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h light-12 h dark cycle. Prior to the nutritional intervention, the animals were fed a standard diet (2014 Teklad Global 14% Protein Diet from Envigo) *ad libitum* with free access to water (Ribes, Barcelona, Spain) for two weeks. Then they were divided into 4 groups (n = 9 per group): the control (CTL) group fed only the standard diet; a group supplemented with D-fagomine (FG); a group supplemented with ω-3 PUFAs (EPA/DHA 1:1) (ω-3); and a group supplemented with both D-fagomine and ω-3 PUFAs (FG + ω-3). D-Fagomine (> 98%) manufactured by Bioglane SLNE (Barcelona, Spain) was generously provided by Taihua Shouyue (HK) International Co. Ltd (Hong Kong, China). It was included in the feed at a proportion of 0.96 g/kg feed as defined in previous studies (Gómez et al., 2012; Ramos-Romero et al., 2018). The mixture EPA/DHA 1:1 was obtained by mixing the appropriate

quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and EnerZona Omega 3 RX (Milan, Italy). These ω-3 PUFAs were administered by oral gavage using a gastric probe once a week at a dose of 0.8 mL oil per kg of body weight. The dose and EPA/DHA proportions used were those reported previously (Molinar-Toribio et al., 2015). To compensate for the stress of probing and the excess calories from the fish oil in groups ω-3 and FG + ω-3, the animals in groups CTL and FG were administered soy bean oil at the same dose and at the same time.

All the procedures carried out strictly adhered to European Union Directive 2010/63/EU for the care and management of laboratory animals, and were licensed by the regional Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

Data and sample collection

Feed consumption was monitored daily and body weight was measured weekly throughout the experiment. Based on feed intake, the mean daily dose of D-fagomine was 4.5 mg/100 g body weight. Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

After week 21 of the experiment, fecal samples were collected by abdominal massage and blood samples were collected from the saphenous vein after overnight fasting. Plasma was separated by centrifugation and stored at −80 °C until analysis.

At the end of the experiment (week 23), rats were fasted overnight and anaesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg/kg body weight, respectively). Blood was collected by cardiac puncture, then plasma was immediately obtained by centrifugation and stored at −80 °C until analysis. Perigonadal fat was collected, weighed and immediately frozen in liquid N_2. All the samples were stored at −80 °C until analysis.

Glycemic status

An oral glucose tolerance test (OGTT) was performed at week 18 on fasted animals. A solution of glucose (1 g/kg body weight) was administered by oral gavage before the test, and blood glucose concentration was measured 15, 30, 45, 60, 90 and 120 min after the glucose intake. Blood glucose concentration was measured by the enzyme electrode method, using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care, Basel, Switzerland).

Fasting blood glucose and plasma insulin levels were also measured after week 21, in fasted animals. Plasma insulin levels were measured

using the rat/mouse insulin ELISA kit from Millipore Corporation (Billerica, MA, USA).

Plasma lipid profile

Plasma triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol were measured using a spectrophotometric method and the corresponding kits from Spinreact (Girona, Spain) as described elsewhere (Bucolo & David, 1973; Méndez et al., 2013).

Plasma lipid mediators of inflammation

Lipid mediators from the metabolism of ARA were determined in plasma by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a method modified from Dasilva et al. (Dasilva et al., 2014). Erythrocyte-free plasma samples (90 µL) were thawed, diluted in the presence of BHT, and spiked with the internal standard (12 hydroxyeicosatetraenoic acid-d8, 12HETE-d8, Cayman Chemicals, Ann Arbor, MI, USA). Then, the samples were centrifuged (800 g, 10 min), and the lipids in the supernatants were purified by solid-phase extraction (SPE). The LC-MS/MS analyzer consisted of an Agilent 1260 Series chromatograph (Agilent) coupled to a dual-pressure linear ion-trap mass spectrometer LTQ Velos Pro (Thermo Fisher, Rockford, IL, USA) operated in negative ESI mode. A C18-Symmetry 150 x 2.1 mm inner diameter, 3.5 µm column (Waters, Milford, MA, USA) with a C18 4 x 2 mm guard cartridge (Phenomenex, Torrance, CA, USA) were used in the separation step. Samples (10 µL) were eluted with a binary system consisting of 0.02% aqueous formic acid [A] and 0.02% formic acid in methanol [B] under gradient conditions of: 0 min, 60% B; 2 min, 60% B; 12 min, 80% B; 13 min, 80% B; 23 min, 100% B; 25 min, 100% B; and 30 min, 60% B, at a flow rate of 0.2 mL/min.

Fecal microbial populations

The relative populations of selected bacterial phyla, orders and genera were estimated in fecal DNA by quantitative real-time polymerase chain reaction (qRT-PCR). DNA was extracted from feces using a QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany) and its concentration was quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). qRT-PCR experiments were carried out in triplicate using a LightCycler® 480 II (Roche, Basel, Switzerland). Each qRT-PCR well contained DNA (2 µL of a 20 ng/µL solution) and a master mix (18 µL) made of 2X SYBR (10 µL), the corresponding forward and reverse primer (1 µL each), and water (6 µL). All the reactions were paralleled by analysis of both a non-template control (Milli Q water) and a positive control (Table 1) from DSMZ (Braunschweig, Germany). The qRT-PCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at the primer-specific

annealing temperature (Table 1), and 30 s at 72 °C (extension). The specificity of the qRT-PCR reactions was assessed by melting curve analysis which consisted of heating to 95 °C and maintaining this temperature for 2 s, then cooling to 65 °C and maintaining this temperature for 15 s, and running a temperature gradient from 65 °C to 95 °C at a rate of 0.11 °C/s, with five fluorescence recordings per °C. The relative DNA abundances for each bacterial subgroup were calculated from the second derivative maximum of their respective amplification curves (C*p*, calculated in triplicate) by considering C*p* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation: $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ (Pfaffl, 2001). Amounts of total bacteria were normalized as 16S rRNA gene copies per mg of wet feces (copies/mg).

Fecal short-chain fatty acids

Short-chain fatty acids (SCFAs) were analyzed in fecal samples after 21 weeks of supplementation by gas chromatography using a previously described method (Schwiertz et al., 2009) with some modifications. Briefly, the freeze-dried feces were weighed (~50 mg dry matter) and a solution (1.5 mL) containing the internal standard 2-ethylbutyric acid (6.67 mg/L) and oxalic acid (2.97 g/L) in acetonitrile/water 3:7 was added. Then, SCFAs were extracted for 10 min using a rotating mixer. The suspension was centrifuged (5 min, 12,880 g) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) and the supernatant filtered through a 0.45 µm nylon filter. Then an aliquot of the supernatant (0.7 mL) was diluted with acetonitrile/water 3:7 to a final volume of 1 mL. SCFAs were analyzed using a Trace2000 gas chromatograph coupled to a flame ionization detector (ThermoFinnigan, Waltham, MA, USA) equipped with an Innowax 30 m × 530 µm × 1 µm capillary column (Agilent, Santa Clara, CA, USA). Chrom-Card software was used for data processing. Helium was used as carrier gas with a linear velocity of 5 mL/min. GC oven temperature was programmed as follows: 80 °C (hold 1 min) to 120 °C at 15 °C/min (hold 4 min) to 130 °C at 5 °C/min (hold 4 min) to 235 °C at 8 °C/min (hold 4 min). FID detection was performed at a base temperature of 240°C. Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The precision (RSD < 15%) and recovery (> 70%) of the method were adequate and inter- and intra-day reproducible.

Statistical analysis

All data manipulation and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The results are expressed as means with their

standard errors (SEM). The normal distribution and heterogeneity of the data were evaluated, and statistical significance was determined by one-way ANOVA with each group as variable and the Tukey multiple-comparisons test, two-way ANOVA for repeated measures of body weight and glycemic response or Student's *t*-test to compare the populations of gut microbiota of CTL group at week 21 *vs* week 0. Differences were considered significant when $P < 0.05$ and were considered to indicate a tendency when $0.05 \leq P \leq 0.1$.

Table 1.- Quantitative real-time PCR primers and conditions

Target bacteria	Annealing temperature (°C)	Sequences (5'-3')	Positive Control DNA	Reference
Total Bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(a)	(Hartman et al., 2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	*Bacteroides fragilis*	(Abdallah Ismail et al., 2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	*Ruminococcus productus*	(Haakensen, Dobson, Deneer, & Ziola, 2008)
Lactobacillales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus acidophylus*	(Walter et al., 2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	*Bifidobacterium longum*	(Queipo-Ortuno et al., 2013)
Bacteroides	60	F: GGT TCT GAG AGG AGG TCC C R: GCT GCC TCC CGT AGG AGT	*Bacteroides fragilis*	(Schwiertz et al., 2009)
Prevotella	60	F: CAG CAG CCG CGG TAA TA R: GGC ATC CAT CGT TTA CCG T	*Prevotella copri*	(Schwiertz et al., 2009)

[a] The positive control DNA used for the evaluation of Total Bacteria for each subgroup was the one selected as positive control for that subgroup.

RESULTS

Feed and energy intake and body weight

Feed and energy intake were similar for all the groups throughout the experiment (Table 2). In contrast, the animals in the FG and FG + ω-3 groups gained less weight than those in the CTL and ω-3 groups (Figure 1A). At the end of the study (23 weeks) the animals supplemented with D-fagomine had significantly ($P < 0.05$) lower body weight than the controls or those supplement with only EPA/DHA 1:1 (Table 2).

Similarly, animals supplemented with D-fagomine showed a tendency (P = 0.06 FG *vs* CTL; P = 0.1 FG + ω-3 *vs* CTL) to store lower perigonadal fat than those not supplemented or supplemented only with ω-3 PUFAs (Figure 1 B).

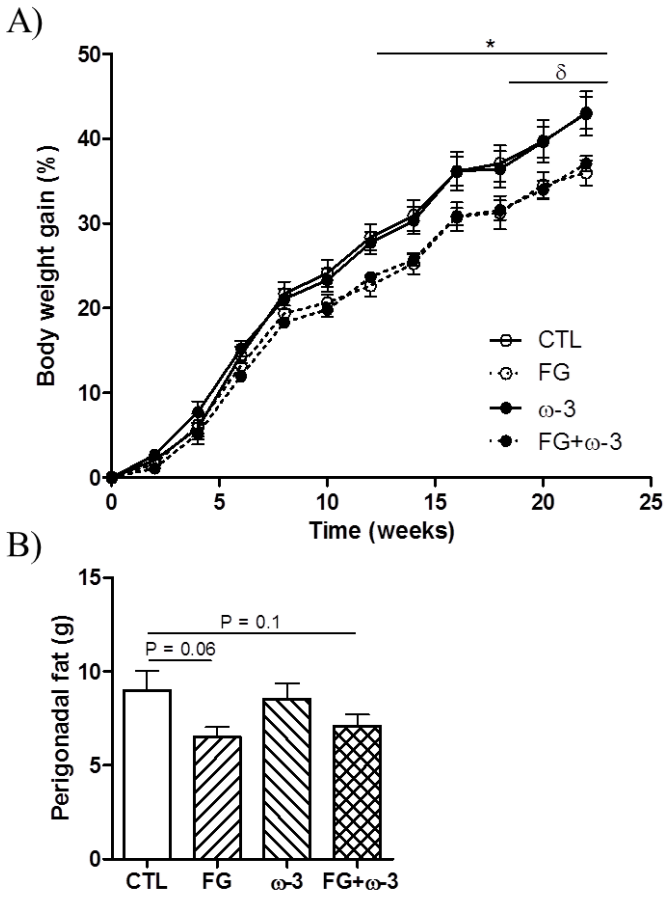


Figure 1.- Body weight gain (A) and perigonadal fat (B) of the different groups (CTL, FG, ω-3 and FG + ω-3) of Sprague Dawley rats fed a standard diet for 23 weeks. The data represent means with their standard errors. Comparisons were performed using two-way ANOVA for repeated measures (A), or one-way ANOVA followed by Tukey's post-hoc test (B). *P < 0.05 FG vs CTL group; δP < 0.05 FG + ω-3 vs CTL group.

Table 2.- Feed and energy intake, body weight, and plasma variables of rats supplemented with D-fagomine and/or ω-3 PUFAs for 23 weeks.

	CTL		FG		ω-3		FG + ω-3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/day/100 g body weight)	4.6	0.5	4.8	0.4	4.6	0.5	4.9	0.4
Energy intake [δ] (kcal/day/100 g body weight)	13.3	1.4	13.8	1.2	13.4	1.3	14.1	1.3
Initial body weight (g)	373	7	360	3	363	7	360	7
Final body weight (g)	540	16	493*	5	523	13	497*	11
Fasting glucose [&] (mg/dL)	65	2	62	1	67	2	63	2
Fasting insulin [&] (ng/mL)	0.56	0.10	0.34	0.03	0.65†	0.07	0.43	0.06
Triglycerides (mmol/L)	0.69	0.02	0.61*	0.02	0.56***†	0.01	0.53***	0.02
Cholesterol (mmol/L)	3.61	0.04	3.30**	0.03	3.23***	0.08	3.24***	0.06
HDL/LDL	2.82	0.08	2.13***	0.06	2.34***	0.03	2.11***ϕ	0.04

[δ] Energy intake is estimated as metabolizable energy based on Atwater factors: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrates.

[&] Samples from week 21.

Data are presented as means with their standard errors of the mean; n = 9 per group. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 vs CTL; †P < 0.05 vs FG; ϕ P < 0.05 vs ω-3

Glycemic Status

The areas under the curve from the OGTT performed at week 18 were statistically similar for all groups (Supplementary material, Figure 1). Fasting blood glucose and plasma insulin concentrations were measured after week 21 of the intervention. The levels of fasting glucose were statistically similar in all the groups and below 80 mg/dL (Table 2). The animals supplemented with D-fagomine showed a tendency (P = 0.1) towards lower fasting blood insulin concentrations than non-supplemented rats or those supplemented only with ω-3 PUFAs. The difference was statistically significant (P < 0.05) between the FG and ω-3 groups (Table 2).

Plasma lipid profile

The levels of total triglycerides, cholesterol and both HDL and LDL were measured in plasma

after 23 weeks of the intervention (Table 2). The concentrations of plasma triglycerides, cholesterol and the HDL/LDL ratio were significantly ($P < 0.05$) lower in the three supplemented groups than the control values.

Plasma lipid mediators of inflammation

The levels of ARA-derived pro-inflammatory eicosanoids were measured by LC-MS/MS in plasma samples collected at the end of the study (Figure 2). The plasma concentrations of 11HETE and 20HETE (Figure 2B, E) were significantly ($P < 0.05$) reduced in the two groups supplemented with ω-3 PUFAs with respect to the control group. The concentration of 12HETE (Figure 2C) was significantly ($P < 0.05$) lower in the three supplemented groups than the control values and the concentration of 5HETE and 15HETE (Figure 2A, D) were significantly ($P < 0.05$) reduced in the FG+ω-3 group compared to the FG group.

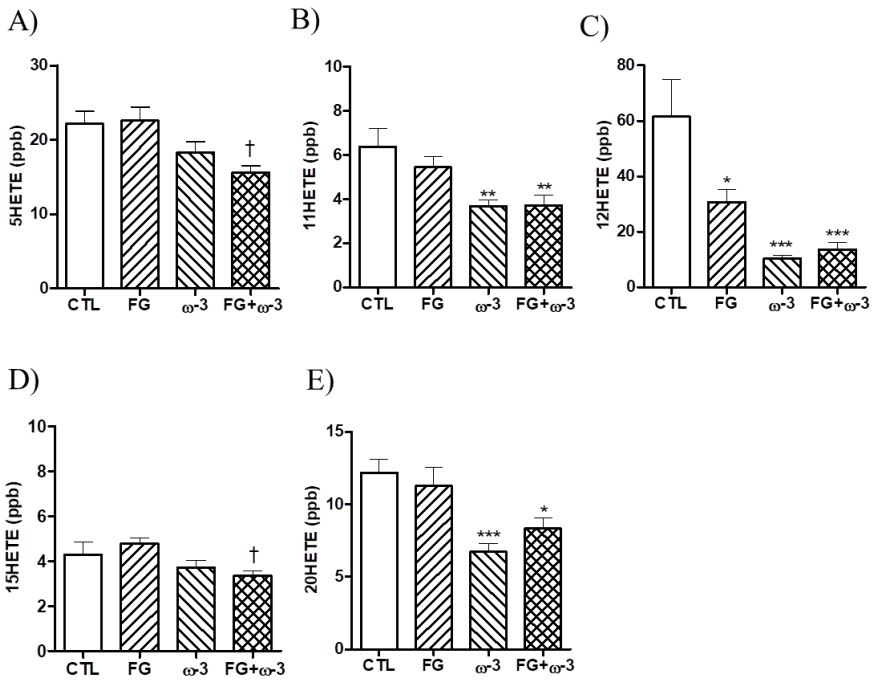


Figure 2.- Lipid mediators from ARA: 5HETE (A), 11HETE (B), 12HETE (C), 15HETE (D) and 20HETE (E) in plasma, at the end of the study of the different groups (CTL, FG, ω-3 and FG + ω-3) of Sprague Dawley rats fed a standard diet for 23 weeks. Data are presented as means with their standard error. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs CTL; †$P < 0.05$ vs FG.

Bacterial subgroups of gut microbiota

The proportions of the major bacterial phyla (Bacteroidetes and Firmicutes, Figure 3A, B), selected genera (*Prevotella* and *Bacteroides*, Figure 3D, E) and putatively beneficial orders (Lactobacilliales and Bifidobacteriales, Figure 3G, H) were estimated in fecal DNA at the end of the study. In the CTL group, the proportion of Bacteroidetes significantly ($P < 0.05$) decreased and the proportion of Firmicutes significantly ($P < 0.01$) increased after 21 weeks. The percentages of Bacteroidetes and Firmicutes were similar in all the groups except for a tendency ($P = 0.1$) for the population of Bacteroidetes to increase in the FG group at week 21.

Significant differences in the relative populations of the genera *Prevotella* and *Bacteroides* were detected when comparing the control group at different times and between groups at the end of the study (Figure 3D, E). The populations of both genera significantly decreased in the control group after 21 weeks. The proportion of *Prevotella* was significantly ($P < 0.05$) higher in animals supplemented with D-fagomine (FG and FG + ω-3 groups) than in the other groups (Figure 3D). The percentage of *Bacteroides* was higher ($P < 0.05$) in animals supplemented with the combination of D-fagomine and ω-3 PUFAs; while neither single supplementation significantly modified the proportions of this genus on its own (Figure 3E).

The percentage of Lactobacillales was significantly ($P < 0.05$) higher in the ω-3 group than in the FG group at the end of the intervention (Figure 3G), while the population of Bifidobacteriales was significantly ($P < 0.05$) higher in the FG group than in the ω-3 group (Figure 3H).

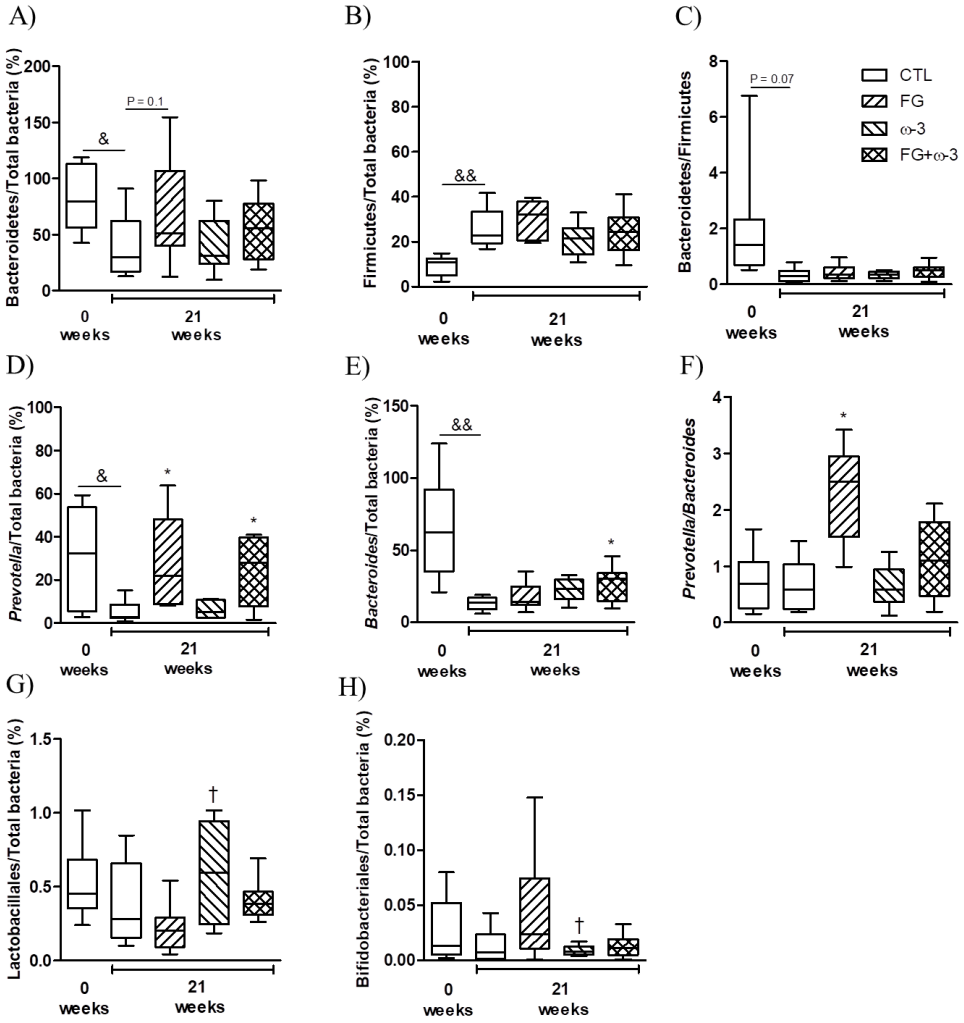


Figure 3.- Bacteroidetes (A), Firmicutes (B), Bacteroidetes/Firmicutes ratio (C), *Prevotella* (D), *Bacteroides* (E), *Prevotella*/*Bacteroides* ratio (F), Lactobacillales (G) and Bifidobacteriales (H) in fecal samples at the beginning of the study (week 0) and after 21 weeks, of the different groups (CTL, FG, ω-3 and FG + ω-3) of Sprague Dawley rats fed a standard diet. Data are presented as means with their standard error. Comparisons were made using Student's t-test or one-way ANOVA followed by Tukey's post-hoc test. &P < 0.05, &&P < 0.01 vs CTL week 0; *P < 0.05 vs CTL; †P < 0.05 vs FG.

Short-chain fatty acids

SCFAs were determined in feces at week 21 (Table 3). ω-3 PUFA supplementation (ω-3 and FG + ω-3 groups) reduced the fecal acetate content with respect to the control groups ($P < 0.001$). The concentration of isobutyric acid was significantly ($P < 0.001$) lower in the three supplemented groups than the control values.

Table 3.- Short-chain fatty acids in feces after 21 weeks.

	CTL		FG		ω-3		FG + ω-3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	115	13	125	14	23***	6	19***	4
Propionic acid	13.5	0.9	15.7	3.6	9.2	1.6	10	2
Isobutyric acid	2.7	0.2	0.9***	0.1	0.9***	0.2	0.9***	0.1
Butyric acid	17	2	27	9	12	3	16	3
Isovaleric acid	1.8	0.2	1.3	0.3	1.2	0.3	1.2	0.1
Valeric acid	1.4	0.1	1.7	0.5	1.3	0.3	1.6	0.2
Total SCFAs	152	9	158	19	48***	11	49***	7

Data are presented as means with their standard errors of the mean; n = 9 per group. Short-chain fatty acids (SCFAs) are given as millimoles per kilogram of feces. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. ***$P < 0.001$ vs CTL.

DISCUSSION

The present study focuses on the effect of the combination of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota of SD rats fed a standard diet. Our goal was to assess the capacity of these supplements to maintain a healthy status over time. Measuring and discussing biologically significant effects of food components in normal rats (or humans) is a particularly difficult task because the metabolic changes experienced by adequately fed animals are small. In the present study, the animals in all the groups were normoweight with normal growth curves (Figure 1A) and presented normal values of fasting blood glucose (Table 2) throughout the whole experimental intervention (≈ 5 months). Some statistically significant changes were recorded that may offer clues as to the putative protective effects of the supplementations and their combination. A weekly single dose of EPA/DHA (1:1) did not modify weight gain compared to the CTL group (SD rats) (Figure 1A), in agreement with our previous observations (Molinar-Toribio et al., 2015) in SHROB rats, which are a cross between

an SD male and a WKY female rat (Koletsky, 1975). Another study by our group showed that intensive daily supplementation with EPA/DHA (1:1) slightly increased both weight gain and perigonadal fat in female WKY rats compared to the group supplemented with the same dose of soybean oil (Ramos-Romero et al., 2017). These results highlight the differences between rat strains and doses in terms of response to putatively obesogenic components (Marques et al., 2016). D-Fagomine consistently reduced weight gain in the present study, as in previous reports. We have now shown here that this iminosugar reduced body weight gain by 15% over the 5 months of the intervention, when administered either alone or together with ω-3 PUFAs. These results are in line with previous studies which proved that D-fagomine was capable of reducing body weight gain in both SD and WKY rats fed energy-dense diets (Molinar-Toribio et al., 2015; Ramos-Romero et al., 2018; Ramos-Romero et al., 2014). Therefore, D-fagomine appears to be effective at reducing body weight gain in both SD and WKY rats fed either a standard or an obesogenic diet. The supplemented animals also showed reduced levels of plasma triglycerides and total cholesterol at the end of the intervention (Table 2). This reduction of plasma triglycerides as a result of D-fagomine supplementation agrees with our previous observations in rats fed an obesogenic diet (Molinar-Toribio et al., 2015). The reduction in the plasma concentration of triglycerides and cholesterol resulting from ω-3 PUFA supplementation also agrees with previous studies where this treatment reduced the levels of total plasmatic fatty acids in healthy rats (Méndez et al., 2013). The effects of ω-3 PUFAs on the lipid profile seem to be related to the upregulation of the expression of genes encoding proteins involved in fatty acid oxidation and downregulation of genes encoding proteins necessary for lipid synthesis (Lombardo & Chicco, 2006).

We also report here a moderate effect of D-fagomine in lowering fasting blood insulin concentration, which is only significantly evident when comparing the FG and the ω-3 groups (Table 2). This observation is consistent with the outcome of previous studies with SD rats fed a high-fat, high-sucrose diet (Molinar-Toribio et al., 2015; Ramos-Romero et al., 2014).

Inflammatory status is another variable that may be influenced by dietary habits and ageing. The anti-inflammatory effect of EPA and DHA in both humans and animal models of disease is well documented (Calder, 2006). We show here that EPA/DHA 1:1 reduces the levels of several ARA-derived pro-inflammatory lipid mediators (Figure 2). This reduction is explained by the displacement of the pro-inflammatory ω-6

pathway towards the ω-3 pathway, as both metabolic pathways share several oxygenases (cyclooxygenase and lipoxygenases) (Calder, 2006). D-Fagomine also reduced the levels of pro-inflammatory mediators (11HETE and 12HETE). In this latter case, the action may be ascribed to a eubiotic effect of the iminosugar on gut microbiota, as we have previously suggested (Ramos-Romero et al., 2018). The combination of supplements resulted in a significant reduction in the levels of all the mediators tested (Figure 2).

As it is becoming increasingly evident that fat accumulation, glycemia, low-grade inflammation and gut microbiota are all interconnected (Cani et al., 2007; Schwiertz et al., 2009; Turnbaugh & Gordon, 2009; Turnbaugh et al., 2006), we examined the changes in relevant bacterial groups experienced by our experimental animals. The population of Bacteroidetes, the main gut microbiota phylum, was seen to reduce significantly over time in the control animals and there was a trend (P = 0.1) for D-fagomine to counteract this change (Figure 3A). This is consistent with the reduction in weight gain in the D-fagomine-supplemented animals, as the lean phenotype has been associated with increased populations of Bacteroidetes in rodents and humans (Turnbaugh & Gordon, 2009; Turnbaugh et al., 2006). We next examined the genera *Prevotella* and *Bacteroides*, which are subgroups of Bacteroidetes. In humans, diets that are high in complex carbohydrates and dietary fiber have been associated with dominance of the genus *Prevotella*; whereas high fat/protein diets have been connected with higher levels of the genus *Bacteroides* (De Filippo et al., 2010; Wu et al., 2011). Human subjects with a high *Prevotella/Bacteroides* ratio appear to lose more body fat when on diets that are high in fiber than subjects with a low *Prevotella*/*Bacteroides* ratio (Hjorth et al., 2018). In agreement with this, the consumption of barley kernel-based bread resulted in both improved glucose metabolism and increased populations of *Prevotella*, particularly *P. copri* (Kovatcheva-Datchary et al., 2015). Those same authors also offered evidence of a cause and effect relationship between *Prevotella* and glucose metabolism efficiency in the host, as germ-free mice transplanted with microbiota from responders had improved glucose tolerance and showed increased populations of *Prevotella*, compared to mice given microbiota from non-responders (Kovatcheva-Datchary et al., 2015). Here, we report that the populations of *Prevotella* dropped significantly in SD rats over the 21-week period of the intervention (Figure 3D) in both the CTL and ω-3 groups. Meanwhile, theses populations remained stable in animals supplemented with

D-fagomine, independently of ω-3 PUFA supplementation (Figure 3D). Those animals (the FG and FG + ω-3 groups) had the lowest body weight gain. As the proportion of *Prevotella* in gut microbiota is directly related to the intake of dietary fiber and to improved glucose tolerance (Kovatcheva-Datchary et al., 2015), we suggest that D-fagomine exerts a fiber-like action which affects microbiota-related fat accumulation and weight gain. Both ω-3 PUFAs and D-fagomine appear to induce a slight increase of *Bacteroides* which was statistically significant in the case of the double supplementation (the FG + ω-3 group) (Figure 3E). The group supplemented only with D-fagomine presented a *Prevotella* to *Bacteroides* ratio that was significantly higher than those of the other groups (Figure 3F). Meanwhile, in the FG + ω-3 group, the *Prevotella*/*Bacteroides* ratio was not statistically different from that in the CTL group; this is probably because of the additive effect of D-fagomine and ω-3 PUFAs on the populations of *Bacteroides* (Figure 3E). The physiological significance of the observation that D-fagomine and ω-3 PUFAs may induce an increase in the populations of *Bacteroides* is something to be examined in future studies.

A reduction in the populations of some putatively beneficial bacteria such as Lactobacilli and Bifidobacteria is a risk factor for the development of many intestinal conditions, including diarrhea, obesity, irritable bowel syndrome and inflammatory bowel disease (Gareau, Sherman, & Walker, 2010). Numbers of bacterial species of the genera *Bifidobacterium* and *Lactobacillus* are negatively correlated with adiposity, microbe-derived inflammation and obesity (Cani et al., 2007; Million et al., 2012). Our results show that D-fagomine tended to promote the growth of Bifidobacteria, while ω-3 PUFAs tended to increase the populations of Lactobacilli (Figure 3G, H). These differences were statistically significant when the two individually supplemented groups were compared. The results for individual supplementation are in agreement with previous reports by us and others. For instance, ω-3 PUFAs increased the populations of Lactobacilliales and Bifidobacteriales while reducing ω-6 PUFA-induced inflammation in mice (Ghosh et al., 2013); and D-fagomine partially counteracted the loss of these two groups in WKY rats over time (Hereu et al., 2018). We show here that combined supplementation with D-fagomine and EPA/DHA may contribute to host homeostasis by maintaining the relative populations of putatively beneficial Bifidobacteriales and Lactobacilliales at levels similar to those of the CTL group.

We also observed differences in fecal SCFAs, which are products of bacterial fermentation. The two groups supplemented with ω-3 PUFAs presented significantly lower concentrations of acetate ($P < 0.001$) (Table 3). It has been reported that acetate may counteract obesity-induced low-grade inflammation by upregulating anti-inflammatory regulatory T cells and by reducing the production of cytokines and chemokines (Kobayashi et al., 2017; Smith et al., 2013; Soliman, Combs, & Rosenberger, 2013). As fecal acetate content was lower in EPA/DHA-supplemented animals, our results seem to imply that this variable does not contribute to the possible effect of ω-3 PUFAs on host homeostasis. A more careful examination of the literature may lead to the opposite conclusion, as it has been shown that low levels of acetate in feces are inversely correlated with intestinal absorption (Vogt & Wolever, 2003); therefore, they may be an indication of higher bioavailability. In fact, high fecal acetate has been associated with gut dysbiosis, obesity and hypertension (de la Cuesta-Zuluaga et al., 2018). Similarly, lower levels of excreted SCFAs together with higher Bacteroidetes/Firmicutes ratios have consistently been associated with the lean healthy phenotype, compared to metabolically altered phenotypes (Canfora, Jocken, & Blaak, 2015). Thus, the anti-inflammatory action of ω-3 PUFAs may in part be mediated by an increase in acetate absorption in the intestinal tract. As we did not record any significant intergroup differences in the populations of Firmicutes, which are supposed to include the main SCFA-producing gut microorganisms, we suggest that minor species may be affected by ω-3 PUFAs, independently of the action of D-fagomine. This is another point worth exploring in future studies.

CONCLUSIONS

This paper presents the first evidence that bacteria of the genus *Prevotella*, which are associated with functional effects on glucose metabolism, may mediate the microbiota-related effects of D-fagomine on host homeostasis. D-Fagomine also helped to stabilize the populations of Bacteroidetes and Bifidobacteriales, while ω-3 PUFAs (EPA/DHA 1:1) tended to stabilize the populations of Lactobacilliales in SD rats fed a standard diet. The two supplements show complementary effects which result in stabilization of putatively beneficial gut bacteria, and reductions of both weight gain and pro-inflammatory mediators.

AUTHOR INFORMATION

Corresponding author

Phone: (+34) 934006100, Fax: (+34) 932045904
e-mail: sara.ramos@iqac.csic.es

Acknowledgements

English language revision by Christopher Evans is appreciated.

Funding

This research was supported by the Spanish Ministry of Economy and Competitiveness (via grant AGL2013-49079-C2-1,2-R and a graduate fellowship to M. Hereu: BES2014-068592).

REFERENCES

Abdallah Ismail, N., Ragab, S. H., Abd Elbaky, A., Shoeib, A. R., Alhosary, Y., & Fekry, D. (2011). Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Archives of Medical Science*, 7(3), 501-507.

Alkhatib, A., Tsang, C., Tiss, A., Bahorun, T., Arefanian, H., Barake, R., . . . Tuomilehto, J. (2017). Functional foods and lifestyle approaches for diabetes prevention and management. *Nutrients*, 9(12), 1310.

Amézqueta, S., Galán, E., Fuguet, E., Carrascal, M., Abian, J., & Torres, J. L. (2012). Determination of D-fagomine in buckwheat and mulberry by cation exchange HPLC/ESI-Q-MS. *Analytical and Bioanalytical Chemistry*, 402(5), 1953-1960.

Bucolo, G., & David, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clinical Chemistry*, 19(5), 476-482.

Calder, P. C. (2006). n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *The American Journal of Clinical Nutrition*, 83(6 Suppl), 1505S-1519S.

Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature reviews. Endocrinology*, 11(10), 577-591.

Cani, P. D., Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R. G., Tuohy, K. M., . . . Delzenne, N. M. (2007). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia*, 50(11), 2374-2383.

Costantini, L., & Molinari, R. (2017). Impact of omega-3 fatty acids on the gut microbiota. International Journal of *Molecular Sciences*, 18(12), 2645.

Dasilva, G., Pazos, M., Gallardo, J. M., Rodriguez, I., Cela, R., & Medina, I. (2014). Lipidomic analysis of polyunsaturated fatty acids and their oxygenated metabolites in plasma by

solid-phase extraction followed by LC-MS. *Analytical and Bioanalytical Chemistry*, 406(12), 2827-2839.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505(7484), 559-563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., . . . Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America*, 107(33), 14691-14696.

de la Cuesta-Zuluaga, J., Mueller, N. T., Alvarez-Quintero, R., Velasquez-Mejia, E. P., Sierra, J. A., Corrales-Agudelo, V., . . . Escobar, J. S. (2018). Higher fecal short-chain fatty acid levels are associated with gut microbiome dysbiosis, obesity, hypertension and cardiometabolic disease risk factors. *Nutrients*, 11(1), 51.

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., . . . Gordon, J. I. (2013). The long-term stability of the human gut microbiota. *Science*, 341(6141), 1237439.

Gareau, M. G., Sherman, P. M., & Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature reviews. Gastroenterology & Hepatology*, 7(9), 503-514.

Ghosh, S., DeCoffe, D., Brown, K., Rajendiran, E., Estaki, M., Dai, C., . . . Gibson, D. L. (2013). Fish oil attenuates omega-6 polyunsaturated fatty acid-induced dysbiosis and infectious colitis but impairs LPS dephosphorylation activity causing sepsis. *PLoS One*, 8(2), e55468.

Gómez, L., Molinar-Toribio, E., Calvo-Torras, M. Á., Adelantado, C., Juan, M. E., Planas, J. M., . . . Torres, J. L. (2012). D-Fagomine lowers postprandial blood glucose and modulates bacterial adhesion. *British Journal of Nutrition*, 107(12), 1739-1746.

Haakensen, M., Dobson, C. M., Deneer, H., & Ziola, B. (2008). Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *International Journal of Food Microbiology*, 125(3), 236-241.

Hartman, A. L., Lough, D. M., Barupal, D. K., Fiehn, O., Fishbein, T., Zasloff, M., & Eisen, J. A. (2009). Human gut microbiome adopts an alternative

state following small bowel transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 106(40), 17187-17192.

Hereu, M., Ramos-Romero, S., García-González, N., Amézqueta, S., & Torres, J. L. (2018). Eubiotic effect of buckwheat D-fagomine in healthy rats. *Journal of Functional Foods*, 50, 120-126.

Hildebrandt, M. A., Hoffmann, C., Sherrill-Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., . . . Wu, G. D. (2009). High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology*, 137(5), 1716-1724.

Hjorth, M. F., Roager, H. M., Larsen, T. M., Poulsen, S. K., Licht, T. R., Bahl, M. I., . . . Astrup, A. (2018). Pre-treatment microbial *Prevotella*-to-*Bacteroides* ratio, determines body fat loss success during a 6-month randomized controlled diet intervention. *International Journal of Obesity*, 42(3), 580-583.

Kobayashi, M., Mikami, D., Kimura, H., Kamiyama, K., Morikawa, Y., Yokoi, S., . . . Iwano, M. (2017). Short-chain fatty acids, GPR41 and GPR43 ligands, inhibit TNF-alpha-induced MCP-1 expression by modulating p38 and JNK signaling pathways in human renal cortical epithelial cells. *Biochemical and Biophysical Research Communications*, 486(2), 499-505.

Koletsky, S. (1975). Pathologic findings and laboratory data in a new strain of obese hypertensive rats. *The American Journal of Pathology*, 80(1), 129-142.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., . . . Backhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of *Prevotella*. *Cell Metabolism*, 22(6), 971-982.

Koyama, M., & Sakamura, S. (1974). The structure of a new piperidine derivative from buckwheat seeds (*Fagopyrum esculentum* Moench). *Agricultural and Biological Chemistry*, 38(5), 1111-1112.

Lombardo, Y. B., & Chicco, A. G. (2006). Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. *The Journal of Nutritional Biochemistry*, 17(1), 1-13.

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., . . . Calhau, C. (2016). High-fat diet-induced obesity rat model: a comparison between

Wistar and Sprague Dawley rat. *Adipocyte*, 5(1), 11-21.

Méndez, L., Pazos, M., Gallardo, J. M., Torres, J. L., Pérez-Jiménez, J., Nogués, R., . . . Medina, I. (2013). Reduced protein oxidation in Wistar rats supplemented with marine omega 3 PUFAs. *Free Radical Biology and Medicine*, 55, 8-20.

Million, M., Maraninchi, M., Henry, M., Armougom, F., Richet, H., Carrieri, P., . . . Raoult, D. (2012). Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii*. *International Journal of Obesity*, 36(6), 817-825.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Gómez, L., Taltavull, N., Nogués, M. R., . . . Torres, J. L. (2015). D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food & Function*, 6(8), 2614-2619.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Romeu, M., Giralt, M., Taltavull, N., . . . Torres, J. L. (2015). Effect of n-3 PUFA supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model of the metabolic syndrome. *British Journal of Nutrition*, 113(6), 878-887.

Mujico, J. R., Baccan, G. C., Gheorghe, A., Diaz, L. E., & Marcos, A. (2013). Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice. *The British Journal of Nutrition*, 110(4), 711-720.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9), e45.

Portune, K. J., Benitez-Paez, A., Del Pulgar, E. M., Cerrudo, V., & Sanz, Y. (2017). Gut microbiota, diet, and obesity-related disorders-The good, the bad, and the future challenges. *Molecular Nutrition & Food Research*, 61(1).

Poudyal, H., Panchal, S. K., Diwan, V., & Brown, L. (2011). Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action. *Progress in Lipid Research*, 50(4), 372-387.

Queipo-Ortuno, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., . . . Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One*, 8(5), e65465.

Ramos-Romero, S., Hereu, M., Atienza, L., Casas, J., Taltavull, N., Romeu, M., . . . Torres, J. L. (2018). Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-induced prediabetes. *Molecular Nutrition & Food Research*, 62(16), e1800373.

Ramos-Romero, S., Hereu, M., Molinar-Toribio, E., Almajano, M. P., Mendez, L., Medina, I., . . . Torres, J. L. (2017). Effects of the combination of omega-3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats. *Food Research International*, 97, 364-371.

Ramos-Romero, S., Molinar-Toribio, E., Gómez, L., Pérez-Jiménez, J., Casado, M., Clapés, P., . . . Torres, J. L. (2014). Effect of D-fagomine on excreted enterobacteria and weight gain in rats fed a high-fat high-sucrose diet. *Obesity*, 22(4), 976-979.

Robertson, R. C., Seira Oriach, C., Murphy, K., Moloney, G. M., Cryan, J. F., Dinan, T. G., . . . Stanton, C. (2017). Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood. *Brain, Behavior, and Immunity*, 59, 21-37.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2009). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity*, 18(1), 190-195.

Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly, Y. M., . . . Garrett, W. S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*, 341(6145), 569-573.

Soliman, M. L., Combs, C. K., & Rosenberger, T. A. (2013). Modulation of inflammatory cytokines and mitogen-activated protein kinases by acetate in primary astrocytes. *Journal of Neuroimmune Pharmacology*, 8(1), 287-300.

Turnbaugh, P. J., & Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *The Journal of Physiology*, 587(17), 4153-4158.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), 1027-1031.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine*, 1(6), 6ra14.

Vogt, J. A., & Wolever, T. M. (2003). Fecal acetate is inversely related to acetate absorption from the human rectum and distal colon. *The Journal of Nutrition*, 133(10), 3145-3148.

Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, 67(6), 2578-2585.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., . . . Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science*, 334(6052),105-108.

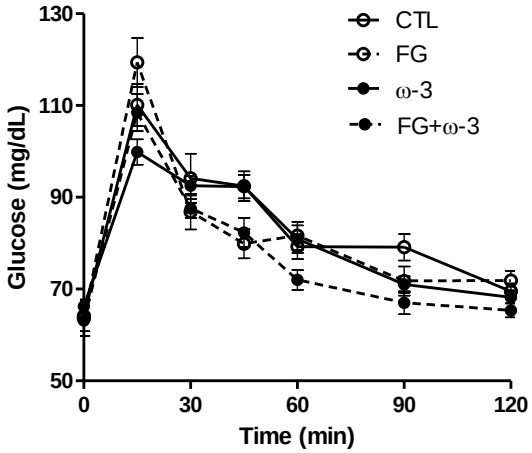


Figure SM1. Glycemic response of the different groups (CTL, FG, ω-3 and FG + ω-3) of Sprague Dawley rats fed a standard diet for 18 weeks. Curves of OGTT after ingestion of a single dose of glucose (1 g/kg body weight). The data represent means with their standard errors. Comparisons were conducted using two-way ANOVA and Tukey's post-hoc test.

3.3 PAPER 3

TITLE: Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-induced prediabetes

AUTHORS: Sara Ramos-Romero†, Mercè Hereu†, Lidia Atienza, Josefina Casas, Núria Taltavull, Marta Romeu, Susana Amézqueta, Gabriel Dasilva, Isabel Medina and Josep Lluís Torres (†contributed equally to this work)

JOURNAL: Molecular Nutrition and Food Research, 2018, 62(16), e1800373

IMPACT FACTOR (2017): 5.15

CLASSIFICATION AND CATEGORY: FOOD SCIENCE & TECHNOLOGY 5/133 (Q1)

TIMES CITED: 2

REFERENCE: Ramos-Romero, S., Hereu, M., Atienza, L., Casas, J., Taltavull, N., Romeu, M., Amézqueta, S., Dasilva, G., Medina, I. & Torres, J. L. (2018). Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-induced prediabetes. *Molecular Nutrition & Food Research*, 62(16):e1800373.

PARTICIPATION OF MERCÈ HEREU IN PAPER 3:

Mercè Hereu performed the animal intervention, the biometric determinations, the evaluation of glycemic status and the qRT-PCR experiments. Mercè Hereu also analyzed the data and collaborated in writing the first draft of the paper.

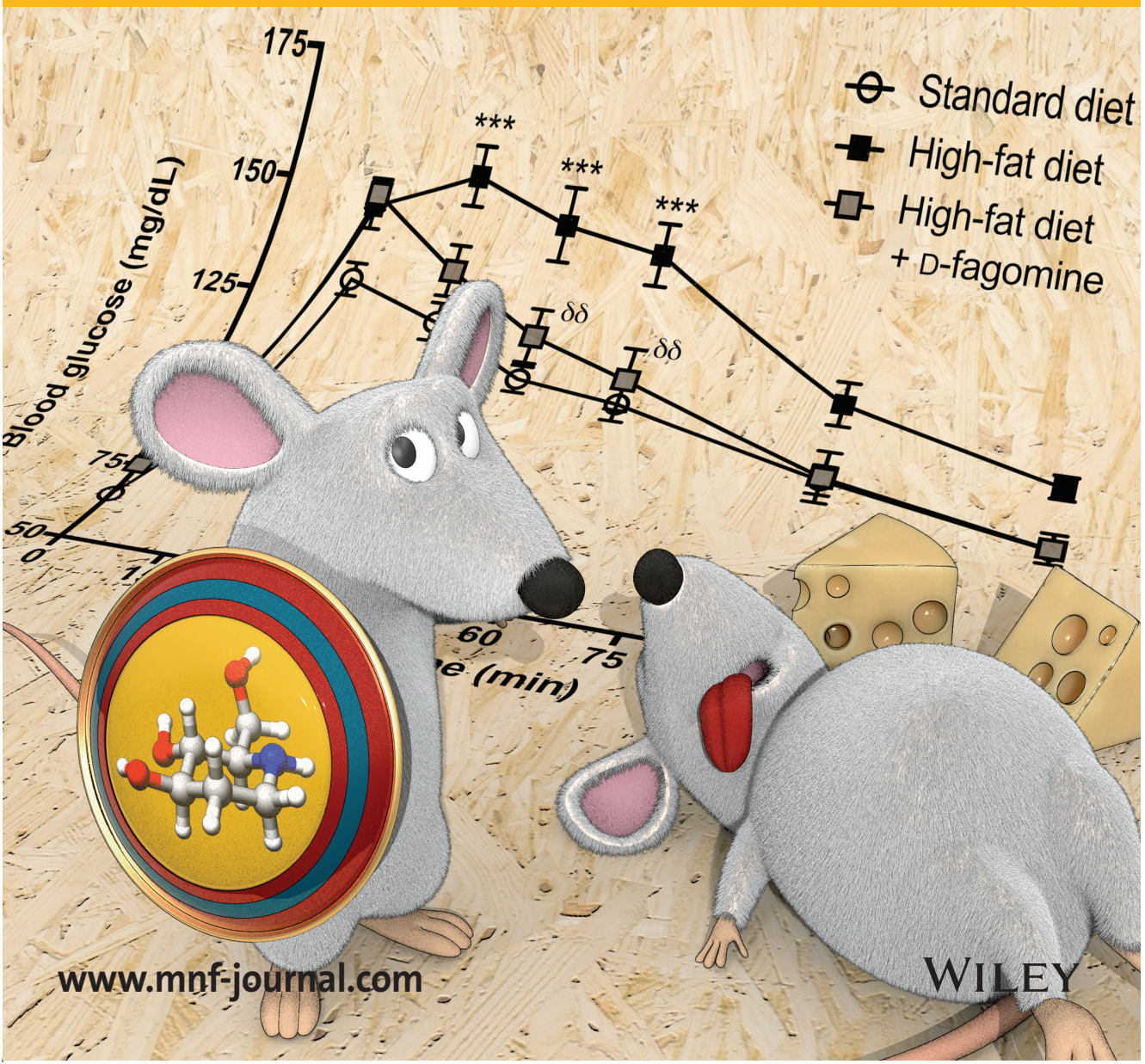
Sara Ramos Romero

Thesis Supervisor

ISSN 1613-4125 · MNFRCV 62 (16) (2018) · Vol. 62 · No. 16 · August 2018

Molecular Nutrition & Food Research

16 | 18



www.mnf-journal.com

WILEY

Molecular Nutrition & Food Research
www.mnf-journal.com

Functional Effects of the Buckwheat Iminosugar D-Fagomine on Rats with Diet-Induced Prediabetes

Sara Ramos-Romero, Mercè Hereu, Lidia Atienza, Josefina Casas, Núria Taltavull, Marta Romeu, Susana Amézqueta, Gabriel Dasilva, Isabel Medina, and Josep L. Torres*

Scope: The goals of this work are to test if D-fagomine, an iminosugar that reduces body weight gain, can delay the appearance of a fat-induced prediabetic state in a rat model and to explore possible mechanisms behind its functional action.

Methods and results: Wistar Kyoto rats were fed a high-fat diet supplemented with D-fagomine (or not, for comparison) or a standard diet (controls) for 24 weeks. The variables measured were fasting blood glucose and insulin levels; glucose tolerance; diacylglycerols as intracellular mediators of insulin resistance in adipose tissue (AT), liver, and muscle; inflammation markers (plasma IL-6 and leptin, and liver and AT histology markers); eicosanoids from arachidonic acid as lipid mediators of inflammation; and the populations of Bacteroidetes, Firmicutes, Enterobacteriales, and Bifidobacteriales in feces. It was found that D-fagomine reduces fat-induced impaired glucose tolerance, inflammation markers, and mediators (hepatic microgranulomas and lobular inflammation, plasma IL-6, prostaglandin E_2, and leukotriene B_4) while attenuating the changes in the populations of Enterobacteriales and Bifidobacteriales.

Conclusion: D-Fagomine delays the development of a fat-induced prediabetic state in rats by reducing low-grade inflammation. We suggest that the anti-inflammatory effect of D-fagomine may be linked to a reduction in fat-induced overpopulation of minor gut bacteria.

1. Introduction

The World Health Organization estimated that 422 million adults suffered from diabetes in 2014 and 1.5 million deaths could be directly attributed to this pathology in just one year (2012). Most of the population suffering from diabetes is affected by type 2 diabetes (T2D). T2D is preceded by insulin resistance (IR): a reduced capacity to internalize glucose from the bloodstream as a result of insensitivity to insulin that may result from genetic predisposition, physical inactivity, and/or obesity in both rats and humans.[1] IR brings about an increase in pancreatic insulin secretion from a greater number or size of pancreatic β-cells, which compensates the low insulin sensitivity. Then, if IR proceeds further into diabetes, a drop in insulin secretion follows, with subsequent increased fasting glucose levels and impaired glucose tolerance (IGT; high glucose levels 2 h after ingestion) as a consequence of a loss and dedifferentiation of pancreatic β-cells.[1] Three main mechanisms have been proposed to explain the pathogenesis of IR in different organs: endoplasmic reticulum stress with the activation of the unfolded protein response, ectopic lipid accumulation with impairment of intracellular signaling patterns by particular lipid mediators, and systemic inflammation.[2] More recently, systemic inflammation, IR, and obesity have been linked to shifts in the populations of gut microbiota (gut "dysbiosis").[3]

Dr. S. Ramos-Romero, M. Hereu, Prof. J. L. Torres
Institute of Advanced Chemistry of Catalonia (IQAC-CSIC)
Barcelona 08034, Spain
E-mail: sara.ramos@iqac.csic.es
Dr. S. Ramos-Romero
Department of Cell Biology, Physiology and Immunology
Faculty of Biology
University of Barcelona
Barcelona 08028, Spain
Dr. L. Atienza
Department of Pathology
Puerta del Mar University Hospital
Cádiz 11009, Spain
Dr. J. Casas
Research Unit on Bioactive Molecules (RUBAM)
Department of Biomedicinal Chemistry
Institute of Advanced Chemistry of Catalonia (IQAC-CSIC) and CIBEREHD
Barcelona 08034, Spain
Dr. N. Taltavull, Dr. M. Romeu
Facultat de Medicina i Ciències de la Salut
Universitat Rovira i Virgili
Reus 43201, Spain
Dr. S. Amézqueta
Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina
Universitat de Barcelona
Barcelona 08028, Spain
Dr. G. Dasilva, Prof. I. Medina
Instituto de Investigaciones Marinas (IIM-CSIC)
Vigo 36208, Spain

DOI: 10.1002/mnfr.201800373

The major bacterial phyla in distal gut microbiota are Bacteroidetes and Firmicutes. A reduction in the ratio between these two phyla has been related to weight gain by the host.[4,5] Also, an increase in the population of Enterobacteriales has been associated with diet-induced obesity.[6] A common antecedent that may link dysbiosis, obesity, and IR is the induction of plasmatic endotoxemia,[7] which may trigger low-grade inflammation and/or changes in energy harvest capacity.[8,9] Other minor gut bacterial subgroups such as Lactobacillus and Bifidobacterium may help to maintain host homeostasis.[10] Specifically, high levels of Bifidobacterium reduce diet-induced IR and inflammation.[11,12]

D-Fagomine (1,2-dideoxynojirimycin) is an iminosugar: a carbohydrate analog that includes an endocyclic nitrogen instead of oxygen.[13] D-Fagomine is naturally present in buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) and can be found in several buckwheat-based foodstuffs such as noodles, pancakes, fried dough, beer, cookies, and bread.[14] D-Fagomine lowers postprandial blood glucose in sucrose/starch loading tests[15] and it reduces elevated plasma insulin concentrations induced by a high-fat high-sucrose diet in the short term (9 weeks).[16]

This study examines the long-term functional effect of D-fagomine on a fat-induced prediabetic state and explores possible molecular mechanisms behind its action.

2. Experimental Section

2.1. Animals

A total of 27 male Wistar Kyoto (WKY) rats from Envigo (Indianapolis, IN, USA), aged 8–9 weeks were used. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals, and were under license from the Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2. Experimental Design: Data and Sample Collection

The rats were kept under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h light–12 h dark cycle. They were randomly divided into three dietary groups ($n = 9$ per group): the standard (STD) group, fed an STD diet (2014 Teklad Global 14% Protein) from Envigo; the high-fat (HF) group fed an HF diet (TD.08811 45% kcal Fat) from Envigo; and the group fed the HF diet supplemented with 0.96 g of D-fagomine (>98% from Bioglane SLNE; Barcelona, Spain) per kg of feed (HF+FG group). The dose of D-fagomine corresponded to that used in postprandial sucrose/starch loading tests (2 mg g^{-1} sucrose).[15] All the groups were fed ad libitum with free access to water.

Feed consumption was monitored daily and body weight was measured weekly throughout the experiment. Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors, assigning 4 kcal g^{-1} protein, 9 kcal g^{-1} fat, and 4 kcal g^{-1} available carbohydrate.

Fecal samples were collected by abdominal massage at weeks 9, 20, and 24. The energy content of the feces from week 20 was determined by differential scanning calorimetry (25–600 °C in an O_2 atmosphere, 10 °C min^{-1}) by means of a thermogravimetric analyzer TGA/SDTA 851e (Mettler Toledo; Columbus, OH, USA) with an integrated SDTA signal.

At weeks 10 and 16, blood samples were collected from the saphenous vein after overnight fasting, and plasma was separated by centrifugation and stored at −80 °C until analysis.

At the end of the experiment, the rats were fasted overnight and anesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg per kg body weight, respectively). Blood was collected by cardiac puncture, then plasma was immediately obtained by centrifugation and stored at −80 °C until analysis. Perigonadal adipose tissue (AT), liver, and quadriceps (muscular tissue) were removed, weighed, and cut into small pieces. One part of the liver was fixed in 10% formalin for histological analysis. The rest of the liver as well as the muscle and AT samples were washed with 0.9% NaCl solution and stored at −80 °C for diacylglycerol (DAG) analysis.

2.3. Plasma Lipid Profile

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured using a spectrophotometric method and the corresponding kits from Spinreact (Girona, Spain) as described by Bucolo et al.[17,18]

2.4. Plasma Insulin, Glucose, and Oral Glucose Tolerance test

Plasma insulin levels were measured using MILLIPLEX xMAP multiplex technology on a Luminex xMAP instrument (Millipore, Austin, TX, USA) at weeks 10 and 16. MILLIPLEX Analyst 5.1 (VigeneTech, Carlisle, PA, USA) software was used for data analysis. The standard curve was generated in the range 69–50 000 pg mL^{-1}.

At weeks 13 and 21, an oral glucose tolerance test (OGTT) was performed on fasted animals. A solution of glucose (1 g per kg body weight) was administered to the rats by oral gavage. Blood glucose concentration was measured by the enzyme electrode method using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care AG; Basel, Switzerland) before the experiment and 15, 30, 45, 60, 90, and 120 min after glucose intake. Fasting glucose concentration was measured by the same method at weeks 10 and 16.

2.5. Diacylglycerols in Perigonadal Adipose Tissue, Liver, and Muscle

Frozen samples were weighted and sonicated (SFX150 Sonifier; Emerson Industrial Automation, St. Louis, MO, USA) until total homogenization. DAG extracts were prepared and analyzed using the method described by Simbari et al.[19] with some modifications. The mixtures were fortified with an internal standard (1,3–17:0 D5 DG, Avanti Polar Lipids Inc., Alabaster, AL, USA; 200 pmol) and incubated overnight at 48 °C. After solvent evaporation, the samples were suspended in methanol, centrifuged

(9390 g, 3 min), and the supernatants were loaded into an Acquity UPLC system connected to an LCT Premier orthogonal accelerated time-of-flight mass spectrometer (Waters, Milford, MA, USA), which was operated in positive ESI mode (LC-TOF-MS). Full-scan spectra from 50 to 1500 Da were acquired, and individual spectra were summed to produce data points of 0.2 s each. Mass accuracy and precision were maintained by using an independent reference spray (leucine enkephalin) via the LockSpray interference. A C8 Acquity UPLC-bridged ethylene hybrid 100 × 2.1 mm inner diameter, 1.7 μm column (Waters) was used in the separation step. The samples (8 μL) were eluted with a binary system consisting of 0.2% v/v formic acid, 2 mM ammonium formate in water [A] and in methanol [B] at 30 °C under linear gradient conditions: 0 min, 80% B; 3 min, 90% B; 6 min, 90% B; 15 min, 99% B; 18 min, 99% B; 20 min, 80% B; and 22 min, 80% B. The flow rate was 0.3 mL min^{-1}. Quantification was carried out using the extracted ion chromatogram of each compound, across 50 mDa windows. The linear range was determined by injecting mixtures of internal standards. DGA content was calculated as DAG 16:0, 16:0 equivalents.

2.6. Liver and Adipose Tissue Histology

Fixed liver and AT were dehydrated in alcohol and embedded in paraffin (Panreac Quimica SLU; Barcelona, Spain), then cut into 3 μm thick slices, using a steel knife mounted in a microtome (HM 355S Rotary Microtome; Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained with hematoxylin (hematoxylin solution modified in accordance with Gill III for microscopy; Merck KGaA, Darmstadt, Germany)/eosin (Pharmacy Service of Puerta del Mar Hospital, Cádiz, Spain) then viewed under a light microscope (NIKON Eclipse 80i; NIKON Corporation, Minato, Japan). Variables were graded following the method described by Taltavull et al.[20] using observation of the entire field of the tissue preparations. Liver: steatosis, 0 (<5%), 1 (5–33%), 2 (33–66%), or 3 (>66%); steatosis localization, 0 (absence), 1 (periportal), and 2 (non-zonal); lobular inflammation with lymphoplasmacytic inflammatory infiltration, 0 (absence), 1 (1–2 foci), 2 (2–4 foci), or 3 (>4 foci); and the presence of microgranulomas, 0 (absence) or 1 (presence). AT: adipocyte hypertrophy, 0 (absence) or 1 (presence); macrophages 0 (absence) or 1 (presence); mast cells, 0 (absence) or 1 (presence); and adipose tissue inflammation with lymphoplasmacytic inflammatory infiltration, 0 (absence) or 1 (presence).

2.7. Biomarkers and Lipid Mediators of Inflammation in Plasma

Plasma IL-6 and leptin levels were measured using MILLIPLEX xMAP multiplex technology (Millipore) on a Luminex xMAP instrument.

Plasma lipopolysaccharides (LPS) concentration was estimated by reaction with Limulus amoebocyte extract: LAL kit end point-QCL1000 (Cambrex BioScience, Walkersville, MD). Plasma samples collected at the end of the study under sterile conditions were diluted 70-fold and heated for 20 cycles of 10 min at 68 °C and 10 min at 4 °C each. An internal control for LPS recovery was included.

Lipid mediators derived from arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were determined in plasma using a method modified from Dasilva et al.[21] Briefly, plasma samples (90 μL) were diluted in cold 0.05% butylated hydroxytoluene (BHT) in methanol/water (3:7) (1 mL) and spiked with the internal standard (12HETE-d8, Cayman Chemicals; Ann Arbor, MI, USA). Then, the samples were centrifuged (Avanti J25, Beckman Coulter; Brea, CA, USA) (800 g, 10 min) at 4 °C. The supernatants were purified by solid-phase extraction.

The LC-MS/MS analyzer used was an Agilent 1260 Series (Agilent; Palo Alto, CA, USA) chromatograph coupled to an LTQ Velos Pro dual-pressure linear ion trap mass spectrometer (Thermo Fisher; Rockford, IL, USA) operated in negative ESI mode. A C18-Symmetry 150 × 2.1 mm inner diameter, 3.5 μm column (Waters) was used with a C18 4 × 2 mm guard cartridge (Phenomenex; Torrance, CA, USA) in the separation step. Samples (10 μL) were eluted with a binary system of 0.02% formic acid in water [A] and in methanol [B]. The gradient was: 1 min, 60% B; 2 min, 60% B; 12 min, 80% B; 13 min, 80% B; 23 min, 100% B; 25 min, 100% B; and 30 min, 60% B. The flow rate was 0.2 mL min^{-1}. LC-MS/MS details are provided in Table S1, Supporting Information.

2.8. Fecal Microbiota

The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales, Bifidobacteria, and Lactobacilliales were estimated from fecal DNA by quantitative real-time PCR (qRT-PCR). DNA was extracted from the feces using QIAamp DNA Stool Mini Kit from Qiagen (Hilden, Germany) and its concentration was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Scientific; Waltham, MA, USA). All DNA samples were diluted to 20 ng μL^{-1}. The qRT-PCR experiments were carried out using a LightCycler 480 II (Roche; Basel, Switzerland).

Each qRT-PCR well was run in triplicate and contained a total of 20 μL: 18 μL of Master Mix (10 μL of 2X SYBR, 1 μL of each [forward and reverse] corresponding primer, and 6 μL of water) and 2 μL of DNA sample. All reactions were paralleled by analysis of a nontemplate control (water) and a positive control. The primers and annealing temperatures are detailed in Table S2, Supporting Information.

The qRT-PCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at primer-specific annealing temperature (Table S2, Supporting Information), and 30 s at 72 °C (extension). Following amplification, to determine the specificity of the qRT-PCR, melting curve analysis was carried out by treatment for 2 s at 95 °C, 15 s at 65 °C, and then continuous increase of temperature up to 95 °C (0.11 °C s^{-1}), with five fluorescence recordings per degree Celsius. The relative DNA abundances for the different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated in triplicate) by considering Cp values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, according to the equation: $[DNA_a]/[DNA_b] = 2^{Cpb\text{-}Cpa}$.[22] Total bacteria was normalized as 16S rRNA gene copies per mg of wet feces (copies per mg).

Table 1. Mean feed and energy intakes, energy excreted in feces and final body weight of WKY rats fed the experimental diets for 24 weeks.

	STD		HF		HF+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake [g per day per 100 g body weight]	4.8	0.7	3.0*	0.7	2.9*	0.5
Energy intake[a] [kcal per day per 100 g body weight]	14.3	0.2	17.5*	0.2	19.0*	0.2
Energy in feces [ks °C g^{-1}][b]	327.0	17.6	381.6	29.5	426.9*	18.4
Body weight at week 24 [g]]	416.4	12.9	537.9***	15.1	499.9** †	15.7

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs STD group; †$P = 0.06$ vs HF group; [a] Estimated as metabolizable energy based on the Atwater factors: 4 kcal g^{-1} protein, 9 kcal g^{-1} fat, and 4 kcal g^{-1} available carbohydrate; [b] Integrated SDTA signal proportional to energy in feces from week 20.

2.9. Statistical Analysis

All data manipulation, statistical analysis, and figure construction were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The results of the quantitative measurements are expressed as mean values with their standard errors (SEM). Normal distributions and the heterogeneity of data were evaluated and their statistical significance was determined by one- or two-way ANOVA, and Tukey's multiple comparison test was used for mean comparisons. The results from qualitative measurements (histology) are expressed in frequencies (percentage of animals that present the variable, or do not) and their statistical significance was determined using contingency tables and χ^2 statistics. Differences were considered significant when $P < 0.05$ and were considered to indicate a tendency when $0.05 < P < 0.1$.

3. Results

3.1. Feed Intake, Body Weight, and Lipid Profile

Feed/energy intake and body weight were monitored throughout the study (**Table 1**; Figure S1, Supporting Information). Rats fed the two high-energy-dense diets (HF and HF+FG) consumed significantly less feed ($P < 0.05$) and more energy ($P < 0.05$) than those in the STD group (Table 1). Based on feed intake, the mean daily dose of D-fagomine was 2.9 mg per 100 g body weight. As observed in previous studies, D-fagomine supplementation did not modify feed intake.[16] The energy excreted, proportional to the SDTA signal obtained by thermal analysis, was similar in both the STD and HF groups and significantly higher ($P < 0.05$) in animals fed HF and D-fagomine. This result may be explained by the inhibitory activity of D-fagomine on intestinal disaccharidases,[15] which would result in the excretion of some undigested sucrose.

Body weight was similar in all the groups at the beginning (236.1 g, SEM 3.2). After 7 weeks, body weight in the HF group (374.3 g, SEM 10.0) was significantly higher ($P < 0.05$) than in the STD group (320.3 g, SEM 9.1); while the body weight increase in the HF+FG group only reached statistical difference ($P < 0.05$) with respect to the STD group 5 weeks later: after 12 weeks of diet (Figure S1, Supporting Information). At the end of the study, the HF group gained 29% more weight than those given the STD diet (537.9 g, SEM 15.1 vs 416.4 g, SEM 12.9 STD group) while animals supplemented with D-fagomine showed a tendency to gain less weight (20%: 499.9 g, SEM 15.7, $P = 0.06$ vs the HF group) (Table 1). The plasma lipid profile presented values within normal ranges with some differences between groups (Table S3, Supporting Information).

3.2. Glycemic Status

Fasting plasma glucose and insulin were measured at weeks 10, 16, and at the end of the study (**Figure 1**). Fasting glucose levels in the HF group were higher ($P < 0.001$) than those in the STD group (Figure 1A) already from week 10. D-Fagomine supplementation reduced this increase from week 16 to levels similar to those in the STD group ($P < 0.05$ vs the HF group at week 21; Figure 1A). Fasting glucose levels were below 80 mg dL^{-1} in all the groups at all times. Fasting plasma insulin was higher in both groups fed the HF diet at weeks 10 and 16 ($P < 0.05$; Figure 1B). At the end of the study (week 24), insulin levels in the HF group dropped significantly ($P < 0.05$) while the group supplemented with D-fagomine still presented significantly higher insulin concentrations ($P < 0.01$; Figure 1B).

The OGTT was performed twice during the study, after 13 and 21 weeks (**Figure 2**). In the first test, the levels of postprandial glucose in the HF group were significantly ($P < 0.001$) higher than those in the other two groups (STD and HF+FG) 30, 45, and 60 min after administration, with levels of ≥140 mg dL^{-1} (Figure 2A). The area under the curve (AUC) corresponding to the HF group was significantly greater ($P < 0.001$) than that for the STD and HF+FG groups, which presented no significant differences. By the end of the study (week 21, Figure 2B), plasma glucose concentrations in the group supplemented with D-fagomine were still lower than those in the HF group, but only significantly lower ($P < 0.05$) 30 min after glucose intake. The AUC for the HF+FG and STD groups were not significantly different.

3.3. Biomarkers and Lipid Mediators of Inflammation in Plasma

Plasma concentration of IL-6 after 10 and 16 weeks of intervention was higher in animals fed HF diets ($P < 0.05$) than in animals fed the STD diet (**Table 2**). D-Fagomine showed a tendency ($P = 0.07$) to reduce the levels of plasma IL-6 at week 16. The plasma leptin concentration was higher in both groups fed HF diets ($P < 0.01$) than in the STD group.

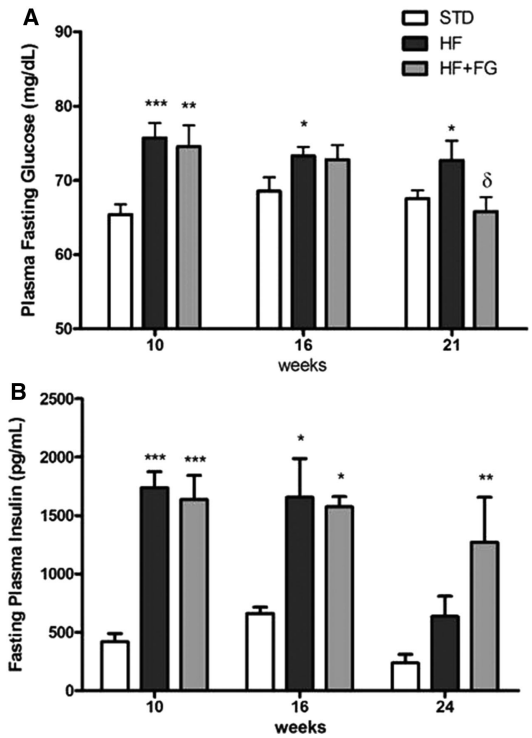


Figure 1. Plasma levels of A) fasting glucose and B) insulin in WKY rats fed standard (STD), high-fat (HF), and high-fat supplemented with D-fagomine (HF+FG) diets at weeks 10, 16, and 21 or 24. Concentrations are represented as means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs STD group, $^{\delta}P < 0.05$ vs HF group.

LPS concentration increased significantly in HF+FG group (Table 2) compared to the STD group.

The levels of ARA-derived pro-inflammatory eicosanoids as well as eicosanoids and docosanoids derived from EPA and DHA, respectively, were measured by LC-MS/MS in plasma samples collected at the end of the study (Table 2). The plasma concentration of prostaglandin E2 (PGE2) significantly increased in the HF group ($P < 0.05$) compared to the STD group. The levels of pro-inflammatory PGE_2 and leukotriene B4 (LTB4) were similar in the STD and HF+FG groups. No differences were detected in the levels of other eicosanoids or docosanoids (Table 2).

3.4. Diacylglycerols in Perigonadal Adipose Tissue, Liver, and Muscle

IR-related intracellular signaling lipid mediators DAGs were measured in perigonadal AT, liver, and muscle by LC-TOF-MS from the samples taken at the end of the study (**Figure 3**). There was no increase in the amounts of total DAGs or in some of the relevant structures, namely DAG 34:1 (putatively 1-palmitoyl-2-oleoyl-*sn*-glycerol), DAG 36:2 (putatively 1-stearoyl-2-linoleoyl-*sn*-glycerol), and DAG 38:4 (putatively 1-stearoyl-2-arachidonoyl-*sn*-glycerol) in the HF group compared to the STD one. The levels of total DAGs in AT as well as of DAG 34:1 in AT and DAG 38:4 in AT and muscle were even significantly lower in the HF group. D-Fagomine supplementation did not induce any significant change in the levels of DAGs with respect to the HF group.

3.5. Liver and Adipose Tissue Histology

Steatosis, lobular inflammation, and microgranulomas were determined in liver by histology (**Figure 4**). Neither high-fat diet induced steatosis to any significant extent. The inflammation and microgranulomas of animals fed HF were significantly higher ($P < 0.001$) than those of the STD and HF+FG groups (Figure 4D). The livers sections obtained from animals in the HF group showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels (e.g., Figure 4B). In contrast, such infiltration was scarce and smaller in extent in sections from livers pertaining to the group supplemented with D-fagomine (e.g., Figure 4C).

Adipocytes were larger in the AT of animals fed the HF diets than the STD group (Figure S2A,B,D, Supporting Information); while no differences were detected between the HF and HF+FG groups (Figure S2B,C, Supporting Information). AT inflammation was not detected in any of the groups (Figure S2G, Supporting Information).

3.6. Subpopulations of Gut Microbiota

The relative proportions of several bacterial groups of the gut microbiota were evaluated at weeks 9 and 24 (**Figure 5**). The Bacteroidetes:Firmicutes ratio (Figure 5F) was significantly reduced ($P < 0.01$) in both high-fat diet groups and the presence of D-fagomine in the diet made no difference. The proportion of Enterobacteriales (Figure 5C,G) significantly ($P < 0.05$) increased in the HF group with respect to the STD group. The increase observed in the group supplemented with D-fagomine was not significant. The relative populations of Bifidobacteriales decreased as the animals grew older ($P < 0.01$ vs STD week 9; Figure 5D,H) and some differences were detected between the groups. The HF diet significantly reduced the population of Bifidobacteriales already at week 9 ($P < 0.05$) independently of supplementation while D-fagomine showed a tendency to counteract the age- and diet-related losses of Bifidobacteriales that was only significant ($P < 0.05$) at the end of the intervention (week 24).

4. Discussion

The present study examines the long-term functional effects of D-fagomine on the preservation of glucose/insulin homeostasis and explores possible mechanisms of action for them in a rat model of diet-induced prediabetes. The prediabetic state was induced in male Wistar Kyoto rats by feeding them an HF diet and the effects of D-fagomine were observed over a period of 24 weeks. In agreement with preceding short-term studies (5 and 9 weeks)

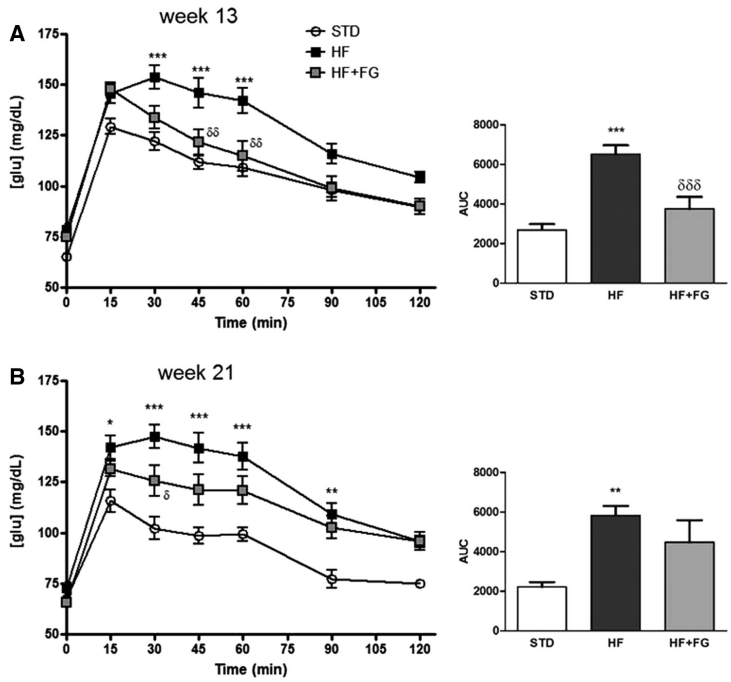


Figure 2. Time-course and area under curve (AUC) of plasma glucose concentration after administration of a single dose of glucose (1 g per kg body weight) to WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet at weeks A) 13 and B) 21. Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs STD group, $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$ and $^{\delta\delta\delta}P < 0.001$ vs HF group.

Table 2. Plasma biomarkers and lipid mediators of inflammation.

		STD		HF		HF + FG	
		Mean	SEM	Mean	SEM	Mean	SEM
IL-6 [pg mL^{-1}]	Week 10	47.3	19.6	193.9*	26.6	176.2	59.3
	Week 16	44.4	24.4	215.4**	47.4	149.6$^{*\,\dagger}$	20.8
Leptin [pg mL^{-1}]	Week 24	2444.8	303.7	8511.0**	1389.8	7984.3**	1490.7
LPS [EU mL^{-1}]	Week 24	2.4	0.4	4.9	2.1	8.00***	0.9
Eicosanoids from ARA (ppb)							
PGE_2	Week 24	14.4	1.7	23.3*	2.1	18.4	3.4
LTB_4	Week 24	3.5	0.7	4.4	0.7	2.7$^{\delta}$	0.3
11HETE	Week 24	8.2	1.1	10.2	1.4	7.8	1.1
Eicosanoids from EPA (ppb)							
12HpEPE	Week 24	14606.6	8327.7	3845.3	469.2	5034.2	956.1
12HEPE	Week 24	38.5	1.9	41.8	4.0	39.8	2.2
5HEPE	Week 24	4.4	0.1	4.5	0.1	4.3	0.0
Docosanoids from DHA (ppb)							
17HDoHE	Week 24	16.8	2.7	17.2	1.3	14.2	1.3
11HDoHE	Week 24	14.8	0.0	14.9	0.0	14.8	0.0
4HDoHE	Week 24	11.5	0.6	12.6	0.7	10.7	0.5

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs STD group; $^{\delta}P < 0.05$ vs HF group; $\dagger P = 0.07$ vs HF group. HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid, HETE, hydroxyeicosatetraenoic acid; HpEPE, hydroperoxyeicosapentaenoic acid.

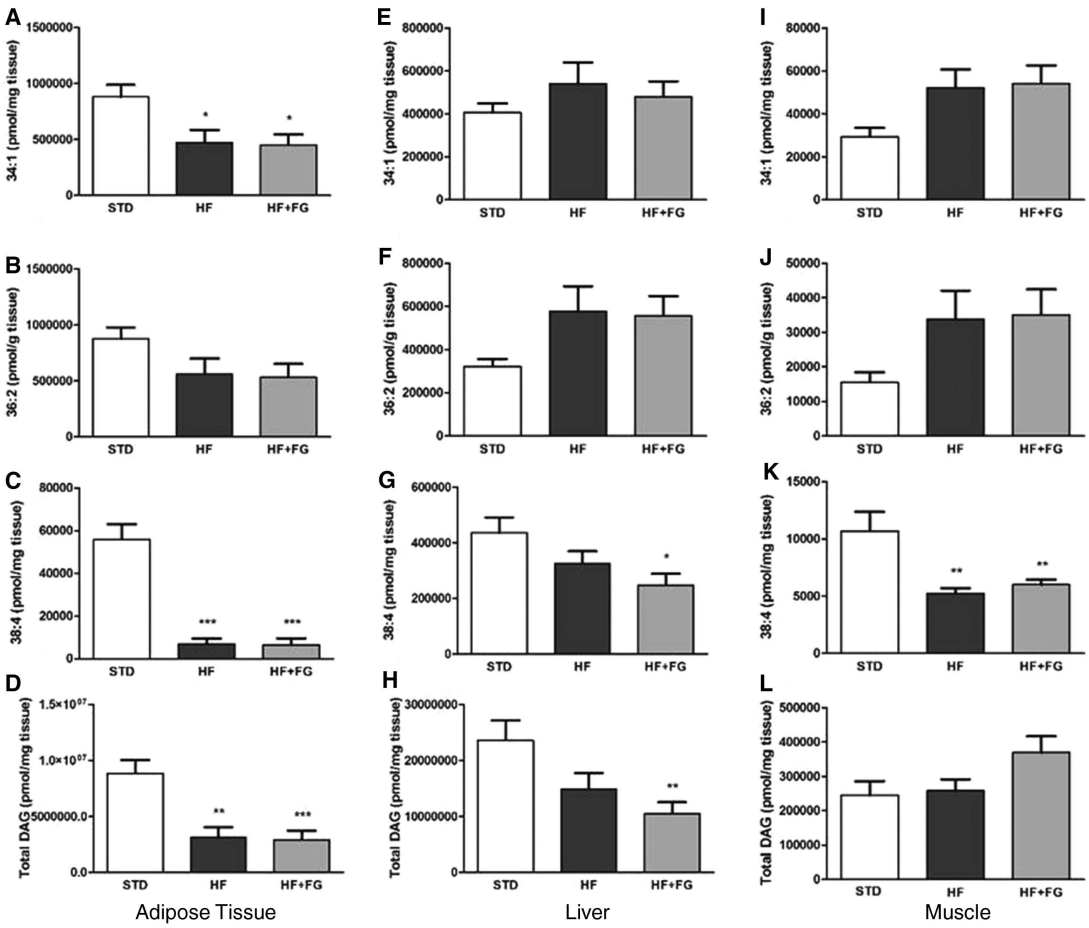


Figure 3. Levels of DAG A,E,I) 34:1, B,F,J) 36:2, C,G,K) 38:4, and D,H,L) total DAGs in WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet for 24 weeks. Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs STD group.

in Sprague-Dawley rats,[16,23] D-fagomine partially counteracted the body weight gain induced by the HF diet in the long term (Figure S1, Supporting Information). Plasma insulin levels also increased in animals fed the HF diet after 10 weeks of intervention (Figure 1). High plasma insulin levels define the first of the five stages of diabetes proposed by Weir and Bonner-Weir for both rats and humans.[1] This compensation stage is characterized by increased overall rates of insulin secretion, via a greater number or size of pancreatic β-cells, in response to the loss of insulin sensitivity in tissues. D-Fagomine, which had shown a tendency to reduce insulin levels in the short term,[16,23] did not have an influence on insulin levels after 10 weeks of our intervention (Figure 1B).

Later on, at the end of the study, the levels of fasting insulin in the HF group (without supplementation) dropped significantly (Figure 1B), while fasting glucose levels were still moderately high (Figure 1A). This situation is compatible with the second stage in the diabetes progression, which is characterized by a loss of β-cell mass and disruption of pancreatic function.[1] Animals supplemented with D-fagomine did not seem to reach this second prediabetic stage, as their insulin levels remained high (Figure 1B) and their fasting glucose levels were similar to those of the STD group (Figure 1A). The second prediabetic state is also compatible with the IGT recorded in rats fed the HF diet, which already showed the classic plateau-like prediabetic curve after just 13 weeks. D-Fagomine counteracted this fat-induced IGT pattern as the supplemented rats removed glucose from their blood at a normal rate (Figure 2A,B). By the end of the study (21 weeks), the AUC for the HF+FG and STD groups were still not significantly different. The evidence presented here, together with our previous observations of the short-term reduction in the early increase of fasting insulin concentration,[16,23] shows that rats supplemented with D-fagomine always seem to be one step behind in the development of diet-induced prediabetes.

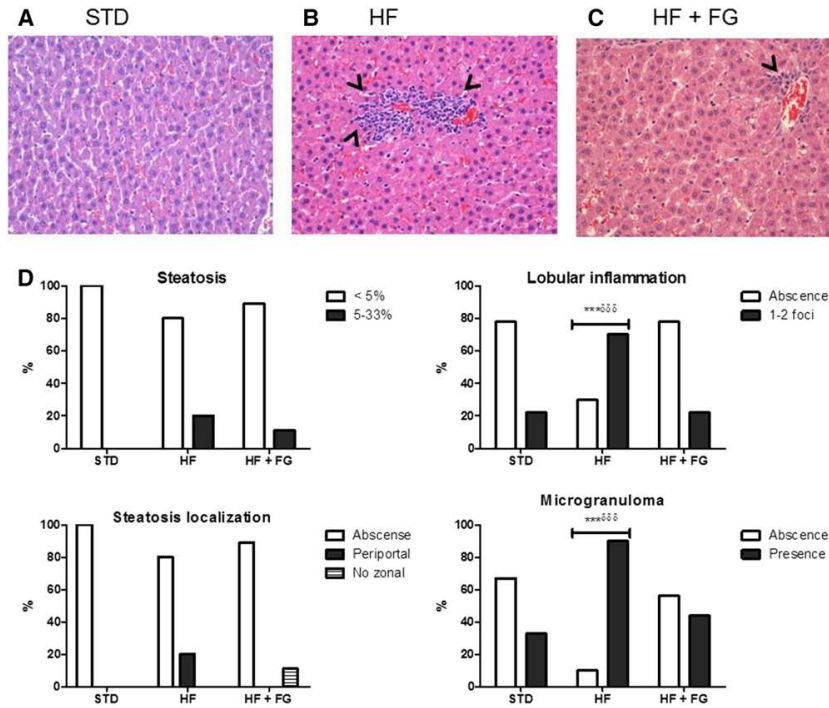


Figure 4. Liver histological cuts (20X) stained with hematoxylin–eosin from WKY rats fed a A) standard (STD), B) high-fat (HF), or C) high-fat supplemented with D-fagomine (HF+FG) diet for 24 weeks, and D) the histology summary. The STD cut (A) shows normal liver anatomy. The HF cut (B) shows lobular inflammation with lymphoplasmacytic inflammatory infiltration (arrows) around blood vessels (red). The HF+FG cut (C) shows slight inflammatory infiltration around a centrilobular vein (red). D) Values are in frequencies (percentage of animals that present the variable, or do not). Comparisons were performed using χ^2 statistics. $^{***}P < 0.001$ vs STD group; $^{\delta\delta\delta}P < 0.001$ vs HF+FG group.

We next considered through what mechanism or mechanisms D-fagomine exerts this functional metabolic effect. As D-fagomine reduced IGT more dramatically than it reduced body weight gain, we hypothesized that it may delay the development of diabetes in Wistar Kyoto rats by a mechanism that is not directly dependent on lipid accumulation. The results from our DAG analysis and histological study support this explanation. IR has been linked to ectopic fat through the action of DAGs, which are intermediates of lipid metabolism with the capacity to impair intracellular insulin signaling in both liver and muscle.[2] It has been proposed that DAGs interrupt the translocation of the glucose transporter GLUT4 to the plasma membrane by modifying the phosphorylation pattern of the intracellular insulin receptor substrate (IRS) after attaching to protein kinase C.[24] We evaluated total DAGs and the levels of selected molecular species in these two organs as well as in AT (Figure 3). As there is no information to date as to what particular DAG species might impair insulin signaling, particular DAGs were chosen on the basis of their selective interaction with the cellular PKC–Ca_2^+ signaling network.[25] Systemic IR in our model does not seem to be triggered by DAG-mediated impairment of insulin signaling, as the levels of total and selected DAGs in AT, liver, and muscle did not increase in either of the groups fed the high-fat diet (Figure 3); in fact, DAG levels were even lower in some instances. The liver histology supported the hypothesis that direct lipid-mediated loss of insulin sensitivity was probably not a triggering factor of the early prediabetic stage in our model, as significant steatosis was not detected (Figure 4D). In contrast, strong lymphocyte infiltration indicated inflammation around the blood vessels (Figure 4B) which was greatly attenuated in animals supplemented with D-fagomine (Figure 4C). Lobular inflammation and numbers of microgranulomas were significantly higher in the HF group than in the STD group, while these levels in animals supplemented with D-fagomine were no different from those in the STD group (Figure 4D). The histology did not detect any sign of inflammation in AT and D-fagomine did not have any observable effect of fat-induced adipocyte hypertrophy (Figure S3, Supporting Information). All these results suggest that D-fagomine has a functional effect on HF diet-induced low-grade systemic inflammation that is independent of lipid accumulation. This hypothesis is supported by our additional measurements of the systemic inflammatory marker IL-6 and its related eicosanoid PGE_2, a strong pro-inflammatory secondary metabolite from the oxidation of ARA catalyzed by cyclooxygenase-2 (COX-2)[26] which induces the production of IL-6 via macrophages.[27] The levels of IL-6 and PGE_2 were significantly higher in the HF group than in the STD group and were significantly reduced in the HF+FG group (Table 2). Also, the levels of LTB_4, another pro-inflammatory ARA-derived metabolite, were significantly lower in the supplemented group compared to the HF group (Table 2). The fact that no

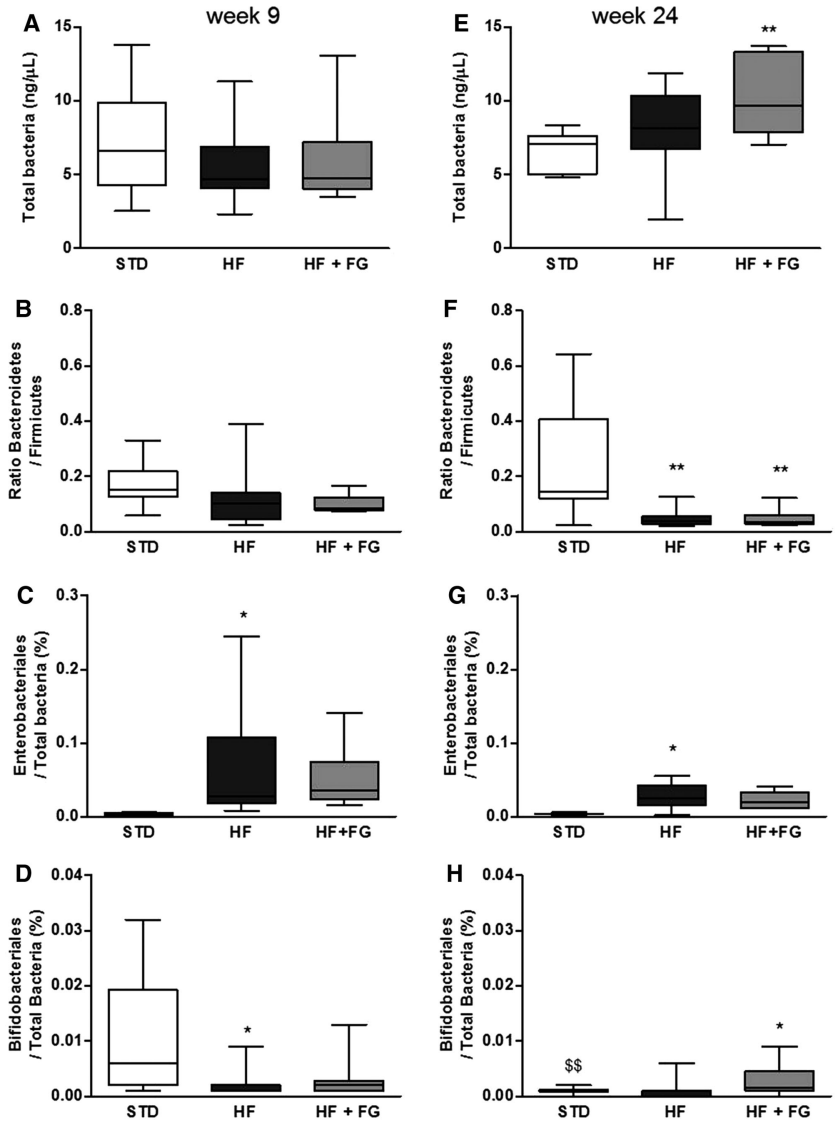


Figure 5. Excreted intestinal bacteria measured by qRT-PCR and expressed as percentages of total bacteria in fecal samples from WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF + FG) diet, after 9 and 24 weeks of nutritional intervention. Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ vs STD group; $^{\$\$}P < 0.01$ vs STD group week 9.

differences were detected in the levels of putatively anti-inflammatory EPA- and DHA-derived eicosanoids and docosanoids (Table 2) suggests that D-fagomine exerts its functional effect when inflammation first occurs and not by activating anti-inflammatory pathways.

We next turned to how D-fagomine counteracts fat-induced low-grade inflammation. Gut microbiota may be the answer, or at least part of the answer. Gut dysbiosis is known to induce endotoxemia and low-grade inflammation in the host[3] through disruption of the intestinal barrier properties and release of pro-inflammatory molecules, such as LPS, into the bloodstream.[28] Chronic subclinical inflammation has been associated with insulin insensitivity[29] and suggested as a link between gut dysbiosis and early IR.[7,11,28] We have already suggested that the effect of D-fagomine on body weight gain and glycemic status may be related to a reduction in the overgrowth of gut Enterobacteriales induced by a high-energy-dense diet in the short term (up to 5 weeks).[23] The results presented here show that

this explanation may hold in the long term, as the population of Enterobacteriales in the STD and HF+FG groups experienced no significant changes throughout this study (Figure 5C,G). Moreover, D-fagomine also showed a tendency to counteract the reduction in Bifidobacterium induced by the HF diet and age (HF and STD groups at week 24, Figure 5D,H). The action of D-fagomine on Bifidobacterium may be connected to its capacity to eliminate Enterobacteriales, as the populations of these subgroups appear to be inversely related.[30] The hypothesis that D-fagomine exerts its anti-inflammatory and anti-diabetic action by balancing the populations of Enterobacteria and Bifidobacteria is backed by previous observations by others that link Enterobacteria to endotoxemia[6] and Bifidobacteria to a reduced impact of fat on diet-induced diabetes.[11] Bifidobacteria have also been inversely associated with obesity and age.[31]

We also evaluated variations in the populations of Bacteroidetes and Firmicutes: the main two bacterial phyla in the intestinal tract (Figure 5B,F). A reduction in the Bacteroidetes:Firmicutes ratio has been related to a shift from lean to fat phenotypes in both rats and humans.[9,32] Low levels of fasting-induced adipose factor (Fiaf) and phosphorylated AMP-activated protein kinase (AMPK) may be responsible for the lipid accumulation effect associated with changes in gut microbiota.[33] We show here that an HF diet can reduce the Bacteroidetes:Firmicutes ratio concomitantly with a significant gain in body weight (Figure 5B,F; Figure S1, Supporting Information) while D-fagomine does not appear to modify this change (Figure 5B,F). This observation confirms that the moderate effect of D-fagomine on weight gain might be associated with the contribution from minor components of gut microbiota (e.g., Enterobacteriales) rather than with changes in the main phyla.

The observation that the effects of D-fagomine on fat-induced changes in Enterobacteriales and Bifidobacteriales were moderate compared to the more dramatic effects on glucose tolerance and inflammation suggests that other microorganisms may be involved. We have also recorded an increase of plasma LPS in the D-fagomine supplemented group (Table 2). Although LPS are usually associated with the stimulation of the inflammatory response, there is evidence that different LPS produced by different bacteria could either stimulate or actively inhibit inflammation.[34] A more thorough examination of the composition of gut microbiota, the gut barrier function, blood LPS composition, and the role of other mediators (e.g., biliary acids) in animals fed HF diets supplemented, or not, with D-fagomine would be the next step to take along this line of enquiry. Thus, D-fagomine may help to shed more light on the complex relationships between gut microbiota and metabolic alterations.

The mean daily dose of D-fagomine (29 mg per kg body weight) consumed by the rats in this study would translate to 4.6 mg per kg body weight in humans by following the conversion proposed by Reagan-Shaw et al.[35] This dose could be reached by supplementing the diet rather than relying on the D-fagomine content in buckwheat-based foodstuffs.[14]

In summary, a very early effect of D-fagomine against fat-induced systemic low-grade inflammation would explain why animals fed D-fagomine are always one step behind in the progression of prediabetes: first against the loss of insulin sensitivity, then against loss of β-cell mass and disruption of pancreatic function. This effect may be attributed, at least in part, to a tendency to counteract the changes induced by a high-fat diet in the populations of gut bacterial subgroups such as Enterobacteriales and Bifidobacteriales.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

S.R.-R. and M.H. contributed equally to this work. This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (grant number AGL2013-49079-C2-1, 2-R and graduate fellowship BES2014-068592 to M.H.). The authors thank Sonia Pérez-Rentero for the calorimetry analysis and Eva Dalmau for the DAG analysis. Language revision by Christopher Evans is appreciated. S.R.-R., I.M., M.R., and J.L.T. conceived and designed the research; S.R.-R., M.H., N.T., and S.A. supervised and performed the animal intervention, the biometric determinations, the evaluation of glycemic status and the qRT-PCR experiments; L.A. performed the histology; S.R.-R. and J.C. performed the DAG determinations; G.D. and I.M. determined the lipid mediators of inflammation; S.R.-R. and M.H. analyzed the data; and S.R.-R. and J.L.T. wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

diabetes, iminocyclitol, iminosugars, inflammation, microbiota, obesity

Received: April 17, 2018
Revised: June 19, 2018
Published online:

[1] G. C. Weir, S. Bonner-Weir, *Diabetes* **2004**, *53*, S16.
[2] V. T. Samuel, G. I. Shulman, *Cell* **2012**, *148*, 852.
[3] P. D. Cani, S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. Neyrinck, D. M. Lambert, G. G. Muccioli, N. M. Delzenne, *Gut* **2009**, *58*, 1091.
[4] E. E. Canfora, J. W. Jocken, E. E. Blaak, *Nat. Rev. Endocrinol.* **2015**, *11*, 577.
[5] R. E. Ley, P. J. Turnbaugh, S. Klein, J. I. Gordon, *Nature* **2006**, *444*, 1022.
[6] C. B. de la Serre, C. L. Ellis, J. Lee, A. L. Hartman, J. C. Rutledge, H. E. Raybould, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2010**, *299*, G440.
[7] P. D. Cani, J. Amar, M. A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A. M. Neyrinck, F. Fava, K. M. Tuohy, C. Chabo, A. Waget, E. Delmee, B. Cousin, T. Sulpice, B. Chamontin, J. Ferrieres, J. F. Tanti, G. R. Gibson, L. Casteilla, N. M. Delzenne, M. C. Alessi, R. Burcelin, *Diabetes* **2007**, *56*, 1761.
[8] A. Schwiertz, D. Taras, K. Schafer, S. Beijer, N. A. Bos, C. Donus, P. D. Hardt, *Obesity* **2010**, *18*, 190.
[9] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, J. I. Gordon, *Nature* **2006**, *444*, 1027.

[10] W. Jia, H. Li, L. Zhao, J. K. Nicholson, *Nat. Rev. Drug Discov.* **2008**, *7*, 123.
[11] P. D. Cani, A. M. Neyrinck, F. Fava, C. Knauf, R. G. Burcelin, K. M. Tuohy, G. R. Gibson, N. M. Delzenne, *Diabetologia* **2007**, *50*, 2374.
[12] T. K. Le, T. Hosaka, T. T. Le, T. G. Nguyen, Q. B. Tran, T. H. Le, X. D. Pham, *Biomed. Res.* **2014**, *35*, 303.
[13] N. Asano, E. Tomioka, H. Kizu, K. Matsui, *Carbohydr. Res.* **1994**, *253*, 235.
[14] S. Amézqueta, E. Galán, I. Vila-Fernández, S. Pumarola, M. Carrascal, J. Abian, L. Ribas-Barba, L. Serra-Majem, J. L. Torres, *Food Chem.* **2013**, *136*, 1316.
[15] L. Gómez, E. Molinar-Toribio, M. Á. Calvo-Torras, C. Adelantado, M. E. Juan, J. M. Planas, X. Cañas, C. Lozano, S. Pumarola, P. Clapés, J. L. Torres, *Br. J. Nutr.* **2012**, *107*, 1739.
[16] E. Molinar-Toribio, J. Pérez-Jiménez, S. Ramos-Romero, L. Gómez, N. Taltavull, M. R. Nogués, A. Adeva, O. Jaúregui, J. Joglar, P. Clapés, J. L. Torres, *Food Funct.* **2015**, *6*, 2614.
[17] G. Bucolo, H. David, *Clin. Chem.* **1973**, *19*, 476.
[18] L. Méndez, M. Pazos, J. M. Gallardo, J. L. Torres, J. Pérez-Jiménez, R. Nogués, M. Romeu, I. Medina, *Free Radic. Biol. Med.* **2013**, *55*, 8.
[19] F. Simbari, J. McCaskill, G. Coakley, M. Millar, R. M. Maizels, G. Fabriás, J. Casas, A. H. Buck, *J. Extracell. Vesicles* **2016**, *5*, 30741.
[20] N. Taltavull, M. Muñoz-Cortés, L. Lluís, M. Jové, À. Fortuño, E. Molinar-Toribio, J. L. Torres, M. Pazos, I. Medina, M. R. Nogués, *Lipids Health Dis.* **2014**, *13*, 31.
[21] G. Dasilva, M. Pazos, J. M. Gallardo, I. Rodríguez, R. Cela, I. Medina, *Anal. Bioanal. Chem.* **2014**, *406*, 2827.
[22] M. W. Pfaffl, *Nucleic Acids Res.* **2001**, *29*, e45.
[23] S. Ramos-Romero, E. Molinar-Toribio, L. Gómez, J. Pérez-Jiménez, M. Casado, P. Clapés, B. Piña, J. L. Torres, *Obesity* **2014**, *22*, 976.
[24] T. O. Eichmann, A. Lass, *Cell. Mol. Life Sci.* **2015**, *72*, 3931.
[25] A. Nadler, G. Reither, S. Feng, F. Stein, S. Reither, R. Mueller, C. Schultz, *Angew. Chem. Int. Ed.* **2013**, *52*, 6330.
[26] P. C. Calder, *Am. J. Clin. Nutr.* **2006**, *83*, 1505s.
[27] D. Bagga, L. Wang, R. Farias-Eisner, J. A. Glaspy, S. T. Reddy, *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1751.
[28] P. D. Cani, R. Bibiloni, C. Knauf, A. M. Neyrinck, A. M. Neyrinck, N. M. Delzenne, R. Burcelin, *Diabetes* **2008**, *57*, 1470.
[29] A. Festa, R. D'Agostino, G. Howard, L. Mykkanen, R. P. Tracy, S. M. Haffner, *Circulation* **2000**, *102*, 42.
[30] M. Roberfroid, G. R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M. J. Davicco, L. Leotoing, Y. Wittrant, N. M. Delzenne, P. D. Cani, A. M. Neyrinck, A. Meheust, *Br. J. Nutr.* **2010**, *104* (Suppl 2), S1.
[31] S. Arboleya, C. Watkins, C. Stanton, R. P. Ross, *Front. Microbiol.* **2016**, *7*, 1204.
[32] P. J. Turnbaugh, J. I. Gordon, *J. Physiol.* **2009**, *587*, 4153.
[33] F. Bäckhed, J. K. Manchester, C. F. Semenkovich, J. I. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 979.
[34] T. Vatanen, A. D. Kostic, E. d'Hennezel, H. Siljander, E. A. Franzosa, M. Yassour, R. Kolde, H. Vlamakis, T. D. Arthur, A.-M. Hamalainen, A. Peet, V. Tillmann, R. Uibo, S. Mokurov, N. Dorshakova, J. Ilonen, S. M. Virtanen, S. J. Szabo, J. A. Porter, H. Lahdesmaki, C. Huttenhower, D. Gevers, T. W. Cullen, M. Knip, R. J. Xavier, *Cell* **2016**, *165*, 842.
[35] S. Reagan-Shaw, M. Nihal, N. Ahmad, *FASEB J.* **2008**, *22*, 659.

SUPPLEMENTARY MATERIAL (SM) INCLUDED IN THE DIGITAL VERSION OF PAPER 3

Table SM1. LC-MS/MS experimental details

Compound	Retention Time (min)	MS/MS parameters	
		Collision energy (eV)	Quantification transition (*m/z*)
PGE_2	9.09	20	351→315
LTB_4	13.79	27	335→195
11HETE	22.09	30	319→167
12HpEPE	17.80	25	333→315
12HEPE	18.72	27	317→179
5HEPE	20.47	25	317→255
17HDoHE	21.94	27	343→245
11HDoHE	23.20	27	343→149
4HDoHE	23.64	27	343→281

The identification of the lipid mediators was done with the help of the full ion product spectra recorded in the range from 90 to 400 *m/z* units. To corroborate the identification and to quantify the analytes, the most intense and selective MS/MS transitions, obtained after direct infusion of individual standard solutions (5 μg/mL, 20 μL/min), were chosen. The linear dynamic range was determined with standards for each compound.

Table SM2. qRT-PCR primers and conditions

Target bacteria	Positive control	Annealing temperature (°C)	Sequence (5'-3')	Reference
Total Bacteria	[a]	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(Hartman et al., 2009)
Bacteroidetes	*Bacteroides fragilis*	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	(Abdallah Ismail et al., 2011)
Firmicutes	*Lactobacillus brevis*	52	F: AGA GTT TGA TCC TGG CTC R: ATT ACC GCG GCT GCT GG	(Haakensen, Dobson, Deneer, & Ziola, 2008)
Enterobacteriales	*Escherichia coli* M15	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	(Hartman et al., 2009)
Bifidobacteriales	*Bifidobacterium longum*	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	(Queipo-Ortuno et al., 2013)

[a] Positive control of total bacteria was the strain with which the result was rated.

Table SM3. Plasma lipid profile (mg/dL) in WKY rats after 24 weeks of intervention.

	STD		HF		HF+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Cholesterol	135.2	3.7	139.4	5.1	150.4*	3.4
HDL-cholesterol	48.7	1.1	47.3	1.3	50.3	1.2
LDL-cholesterol	23.2	1.6	24.1	1.3	29.4*$^{\delta}$	1.1
Triglycerides	63.1	5.6	92.4**	7.1	116.4*	16.4

* $P < 0.05$, ** $P < 0.01$ *vs* STD group, $^{\delta}$ $P < 0.05$ *vs* HF group

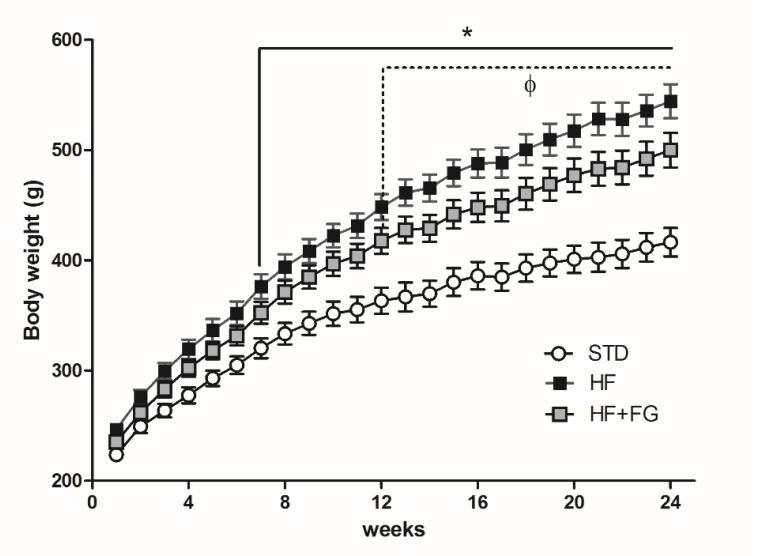


Figure SM1. Body weight of rats fed a standard (STD, ○), high-fat (HF, ■), or high-fat supplemented with D-fagomine (HF+FG, □) diet for 24 weeks. Data are presented as means with their standard errors. Comparisons were performed using the two-way ANOVA test. *$P < 0.05$ HF *vs* STD group, ϕ $P < 0.05$ HF+FG *vs* STD group.

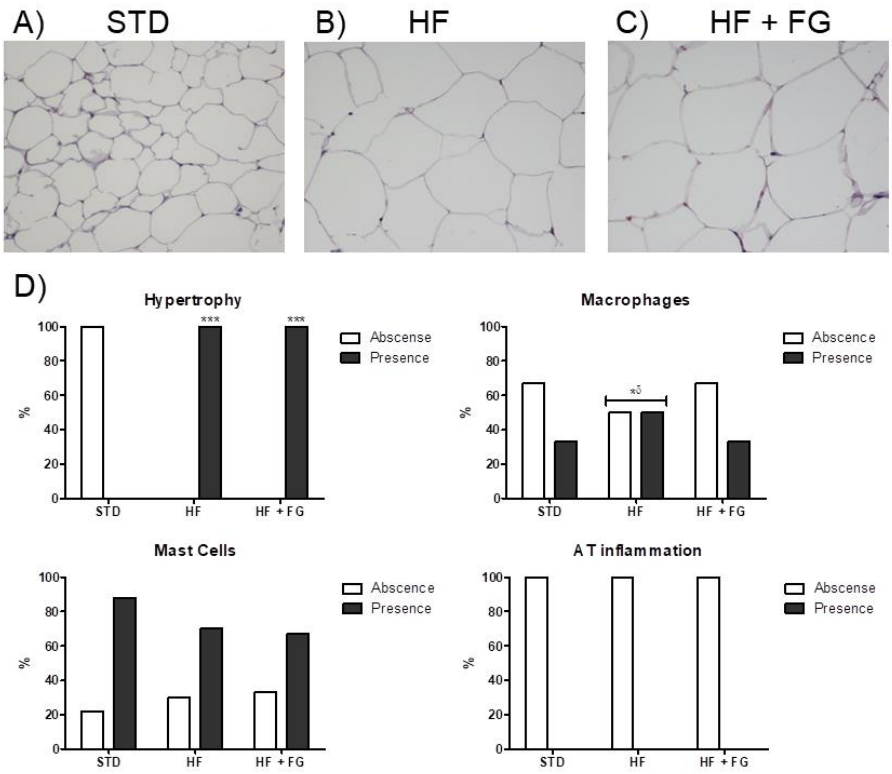


Figure SM2. Adipose tissue histological cuts (20X) stained with hematoxylin-eosin from WKY rats fed a A) standard (STD), B) high-fat (HF), or C) high-fat supplemented with D-fagomine (HF+FG) diet for 24 weeks and D) histology summary. Values are in frequencies (percentage of animals that present or not the variable). Comparisons were performed using χ^2 statistics. * $P < 0.05$ and *** $P < 0.001$ *vs* STD group; $^{\delta}$ $P < 0.05$ *vs* HF+FG group.

3.4 PAPER 4

TITLE: Effects of combined buckwheat D-fagomine and fish omega-3 PUFAs on the gut microbiota and diabetis risk factors in Sprague Dawley rats fed a high-fat diet

AUTHORS: Mercè Hereu, Sara Ramos-Romero, Cristina Busquets, Susana Amézqueta, Bernat Miralles-Pérez, Maria Rosa Nogués, Lucía Méndez, Isabel Medina and Josep Lluís Torres

JOURNAL: Submitted to Scientific Reports

IMPACT FACTOR (2017): 4.12

CLASSIFICATION AND CATEGORY: MULTIDISCIPLINARY SCIENCES 12/64 (Q1)

PARTICIPATION OF MERCÈ HEREU IN PAPER 4:

Mercè Hereu supervised and performed the animal intervention, the biometric determinations, the evaluation of glycemic status, the LC/ESI-MS/MS determinations, the qRT-PCR experiments, and the gas chromatography determinations. Mercè Hereu also analyzed the data and wrote the paper.

Sara Ramos Romero

Thesis Supervisor

Effects of combined buckwheat D-fagomine and fish omega-3 PUFAs on gut microbiota and diabetes risk factors in Sprague Dawley rats fed a high-fat diet

Mercè Hereu[1], Sara Ramos-Romero[1,2*], Cristina Busquets[1], Lidia Atienza[3], Susana Amézqueta[4], Bernat Miralles-Pérez[5], Maria Rosa Nogués[5], Lucía Méndez[6], Isabel Medina[6], Josep Lluís Torres[1]

[1] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

[2] Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

[3] Department of Pathology Puerta del Mar University Hospital, Cádiz, Spain

[4] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina

(IBUB), Universitat de Barcelona, Barcelona, Spain

[5] Universitat Rovira i Virgili, Unitat de Farmacologia, Facultat de Medicina i Ciències de la Salut, Reus, Spain

[6] Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

ABSTRACT

Food bioactive compounds are potential tools that can be used to prevent changes in gut microbiota associated with Western diets high in saturated fat and refined sugars. The aim of this study is to explore the possible additive effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota, and related risk factors, during early stages in the development of fat-induced pre-diabetes. Male Sprague Dawley (SD) rats were fed a standard diet (control), or a high-fat (HF) diet supplemented with D-fagomine, ω-3 PUFAs (EPA/DHA 1:1), a combination of both, or neither, for 24 weeks. The animals not receiving ω-3 PUFAs were given an equivalent dose of soy bean oil. The main variables measured were weight gain, visceral fat, fasting glucose and glucose tolerance, plasma insulin and leptin, liver inflammation, fecal and cecal short-chain fatty acids (SCFAs) and urine F_{2t}-isoprostanes. Gut bacterial subgroups were estimated in fecal and cecal DNA by qRT-PCR. We found that the animals supplemented with both D-fagomine and ω-3 PUFAs tended to gain less weight and to accumulate less fat than those in the non-supplemented HF group and those given only ω-3 PUFAs. Thus, the combined supplements counteracted the high-fat-induced incipient IR, as well as liver inflammation, while increasing the cecal SCFA content, the Bacteroidetes/Firmicutes ratio and the gut populations of both Bifidobacteriales and Lactobacilliales. The functional effects of the

combination of D-fagomine and EPA/DHA 1:1 against gut dysbiosis and the very early metabolic alterations induced by a high-fat Westernized diet are mainly those of D-fagomine complemented by the anti-inflammatory action of ω-3 PUFAs.

INTRODUCTION

There is mounting evidence that diet affects homeostasis by modulating the composition of gut microbiota in both rodents and humans (Hildebrandt et al., 2009; Portune, Benitez-Paez, Del Pulgar, Cerrudo, & Sanz, 2017; Turnbaugh et al., 2009). Early insulin resistance (IR) and impaired glucose tolerance (IGT), the first manifestations of diet-induced metabolic disorders that can lead to type 2 diabetes (Weir & Bonner-Weir, 2004), might be triggered by intestinal barrier alterations induced by unbalanced microbiota (dysbiosis) (Janssen & Kersten, 2017). Dietary changes can rapidly modify gut microbial composition (David et al., 2014; Hildebrandt et al., 2009). Fat- or carbohydrate-restricted diets increase the populations of the order Bacteroidetes while reducing body weight (Ley, Turnbaugh, Klein, & Gordon, 2006). At the genus level, a high intake of fat and protein is associated with increased levels of *Bacteroides*; whereas a high fiber intake is associated with increased levels of *Prevotella* (De Filippo et al., 2010; Wu et al., 2011). The mechanisms by which gut microbiota interact with host physiology are increasingly being revealed.

Lipopolysaccharides (LPS: a component of the bacterial cell wall), short-chain fatty acids (SCFAs: end products of the fermentation of dietary fiber) and bile acids are possible mediators linking gut bacteria to IR and adipose tissue function (Janssen & Kersten, 2017). Food components such as prebiotic fiber may prevent changes in gut microbiota associated with obesity and metabolic disorders (Portune et al., 2017).

D-Fagomine (1,2-dideoxynojirimycin) is an iminosugar: a carbohydrate analog with a nitrogen atom in place of the endocyclic oxygen that was first isolated from seeds of buckwheat (*Fagopyrum esculentum*) (Koyama & Sakamura, 1974). D-Fagomine lowers post-prandial blood glucose in sucrose/starch loading tests in healthy rats through the inhibition of intestinal disaccharidases (Gómez et al., 2012) and it reduces weight gain, low-grade inflammation and IGT in rats fed a high-fat diet (Ramos-Romero et al., 2018; Ramos-Romero et al., 2014). D-Fagomine also promotes diversity in gut microbiota by increasing the populations of Bacteroidetes in healthy rats, while also mitigating the age-related reduction in the populations of the putatively beneficial bacteria Lactobacillus and Bifidobacterium (Hereu, Ramos-Romero, García-González, Amézqueta,

& Torres, 2018) and it helps to stabilize the populations of *Prevotella* in the intestinal tract of healthy rats while reducing weight gain independently of ω-3 PUFA supplementation (Hereu, sent).

Eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are the major ω-3 PUFAs in fish oil. EPA and DHA reduce risk factors (elevated plasma cholesterol and triglycerides, oxidative stress (OS), and high blood pressure) for cardiovascular diseases (Poudyal, Panchal, Diwan, & Brown, 2011), and other pathologies that involve inflammation (Calder, 2006). The possible contribution of changes in gut microbiota to the anti-inflammatory activity of ω-3 PUFAs is poorly documented. It has been shown that EPA and DHA increase the populations of putatively beneficial gut Lactobacillus and Bifidobacteria in mice fed a high-fat diet (Mujico, Baccan, Gheorghe, Diaz, & Marcos, 2013; Robertson et al., 2017). When combined with proanthocyanidins or D-fagomine, EPA/DHA 1:1 helps to stabilize the populations of Bifidobacteriales and Lactobacilliales in healthy rats (Ramos-Romero et al., 2017)(Hereu, sent).

The aim of this study is to explore the possible complementary or additive effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota and risk factors for diabetes during early stages in the development of fat-induced pre-diabetes in rats.

MATERIALS AND METHODS

Animals, experimental design and sample collection

Male Sprague Dawley (SD) rats from Envigo (Indianapolis, IN, USA), aged 8-9 weeks were used. As we wanted to detect early effects of the supplementations, SD rats were preferred over other strains, such as Wistar rats, because they take longer to develop IGT (Marques et al., 2016). The rats (n = 45) were housed (n = 3 per cage) under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h light-12 h dark cycle. They were randomly divided into 5 groups (n = 9 per group): the control group (STD), fed a standard diet (2014 Teklad Global 14% Protein Diet from Envigo); the high-fat group (HF), fed only a high-fat diet (TD.08811 45% kcal fat diet from Envigo) with no supplementation; a group fed the high-fat diet supplemented with D-fagomine (Envigo custom designed, manufactured by Mucedola srl; Settimo Milanese, Italy) (HF+FG); a group fed the high-fat diet supplemented with ω-3 PUFAs (HF+ω-3); and a group fed the high-fat diet supplemented with both D-fagomine and ω-3 PUFAs (HF+FG+ω-3 group). D-Fagomine (> 98%) manufactured by Bioglane SLNE (Barcelona, Spain) was generously provided by

Taihua Shouyue (HK) International Co. Ltd (Hong Kong, China). It was included in the feed at a proportion of 0.96 g/kg feed, as in previous studies (Gómez et al., 2012; Ramos-Romero et al., 2018). ω-3 PUFAs (EPA/DHA 1:1) were obtained by mixing the appropriate quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and EnerZona Omega 3 RX (Milan, Italy). ω-3 PUFAs were administered by oral gavage using a gastric probe once a week at a dose of 0.8 mL oil per kg of body weight. The dose and EPA/DHA proportions used were previously determined (Molinar-Toribio et al., 2015). To compensate for the stress of probing and the excess of calories from fish oil in the HF+ω-3 and HF+FG+ω-3 groups, the animals in the groups STD, HF and HF+FG were administered p.o. soybean oil at the same dose at the same time. All the groups were fed *ad libitum* with free access to water.

Feed consumption was monitored daily and body weight was measured weekly throughout the experiment. Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors, assigning 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

After overnight fasting, blood samples were collected from the saphenous vein after weeks 9, 14 and 23 of the experiment. Plasma was separated by centrifugation and stored at −80 °C until analysis. After week 21, urine was collected by placing the rats in metabolic cages; while after week 23, fecal samples were collected by abdominal massage.

At the end of the experiment (week 24), the rats were fasted overnight and anesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg per kg of body weight, respectively). Perigonadal fat, liver and cecum were removed, weighed and stored at −80 °C. One portion of the liver was fixed in 4% formalin for histological analysis.

All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals, and were licensed by the regional Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

Glycemic status

Fasting blood glucose and plasma insulin levels were measured after weeks 9, 14 and 23 in fasted animals. Blood glucose concentration was measured by the enzyme electrode method, using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care; Basel, Switzerland). Plasma insulin levels were measured using the rat/mouse insulin ELISA kit from Millipore Corporation (Billerica, MA, USA). The HOMA (Homeostatic Assessment Model)

index was calculated as fasting insulin (µU/mL) × fasting glucose (mmol/L) / 22.5 (Matthews et al., 1985). Insulin units (IU) were calculated using the conversion 1 IU = 0.0347 mg insulin.

The oral glucose tolerance test (OGTT) was performed after week 20 on fasted animals. A solution of glucose (1 g/kg body weight) was administered by oral gavage, and blood glucose concentration was measured 15, 30, 45, 60, 90 and 120 min after glucose intake by the enzyme electrode method.

Plasma leptin levels were measured using MILLIPLEX xMAP multiplex technology on a Luminex xMAP instrument (Millipore, Austin, TX, USA) after week 23. MILLIPLEX Analyst 5.1 (VigeneTech; Carlisle, PA, USA) software was used for data analysis.

Isoprostanes in urine

F_{2t}-isoprostanes (F_{2t}-IsoPs) were determined in urine samples by LC/ESI-MS/MS following a previously reported procedure (Molinar-Toribio et al., 2015) with some modifications. Urine samples (500 µL) were acidified, β-glucuronidase (90 U/mL) (Sigma; Saint Louis, MO, USA) was added, and the mixtures were incubated for 2 h at 37 °C. After the addition of the internal standard [$^2H_4$]15-F_{2t}-IsoP (Cayman; Ann Arbor, MO, USA) (100 µL, 10 µg/L), F_{2t}-IsoPs were purified by SPE in a C18 Sep-Pak cartridge (Waters, Mildford, MA, USA). F_{2t}-IsoPs were analyzed using an Agilent 1260 chromatograph fitted with a Mediterranea Sea 18 column (10 cm x 2.1 mm id., 2.2 µm particle size) (Teknokroma; Barcelona, Spain) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems; Foster City, CA, USA). The instrument was operated in the negative ion mode with a Turbo V source to obtain MS/MS data. Separation was achieved with a binary system consisting of 0.1% aqueous formic acid [A] and formic acid in acetonitrile [B], at 40 °C, with an increasing linear gradient (v/v) of [B]: 0 min, 10% B; 7 min, 50% B; 7.1 min, 100% B; 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at a flow rate of 700 µL/min. F_{2t}-IsoPs were detected by MS/MS multiple reaction monitoring. Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The LOQ was 0.4 µg/L for 15-F_{2t}-IsoP and 2 µg/L for 5-F_{2t}-IsoP. The results were expressed as nanograms per milligram of creatinine, to correct for urine dilution. Creatinine levels in urine were determined via a colorimetric method using a commercial kit (Creatinine-J, Spinreact; Girona, Spain) by measuring absorbance at 492 nm.

Subpopulations of gut microbiota

The relative populations of selected bacterial phyla, orders and genera were estimated in fecal and cecal DNA by quantitative real-time

polymerase chain reaction (qRT-PCR). DNA was extracted from fecal and cecal samples using a QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany). Its concentration was quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific; Waltham, MA, USA) and all DNA samples were diluted to 20 ng/µL. The qRT-PCR experiments were carried out using a LightCycler® 480 II (Roche, Basel, Switzerland) in triplicate. Each qRT-PCR well contained a total of 20 µL: DNA (2 µL) and a master mix (18 µL) made of 2X SYBR (10 µL), the corresponding forward and reverse primer (1 µL each), and water (6 µL) purified using a Milli-Q system (Millipore Corporation; Billerica, MA, USA). All the reactions were paralleled by analysis of a non-template control (water) and a positive control (Supplementary Material, Table 1) from DSMZ (Braunschweig, Germany). The qRT-PCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at the primer-specific annealing temperature (Supplementary Material, Table 1), and 30 s at 72 °C (extension). The specificity of the qRT-PCR reactions was assessed by melting curve analysis which consisted of heating to 95 °C and maintaining this temperature for 2 s, then cooling to 65 °C and maintaining this temperature for 15 s, and running a temperature gradient from 65 °C to 95 °C at a rate of 0.11 °C/s, with five fluorescence recordings per °C. The relative DNA abundances for each bacterial subgroup were calculated from the second derivative maximum of their respective amplification curves (C*p*, calculated in triplicate) by considering C*p* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation: $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ (Pfaffl, 2001). Amounts of total bacteria were normalized as 16S rRNA gene copies per mg of wet feces (copies/mg).

Short-chain fatty acids

SCFAs were analyzed in feces and in cecal content by gas chromatography using a previously described method (Schwiertz et al., 2009) with some modifications. Briefly, the samples were freeze-dried and weighed (~50 mg dry matter) and a solution (1.5 mL) containing the internal standard 2-ethylbutiric acid (6.67 mg/L) and oxalic acid (2.97 g/L) in acetonitrile/water 3:7 was added. Then, SCFAs were extracted for 10 min using a rotating mixer. The suspension was centrifuged (5 min, 12,880 g) in a 5810R centrifuge (Eppendorf; Hamburg, Germany) and the supernatant passed through a 0.45 µm nylon filter. An aliquot of the supernatant (0.7 mL) was diluted to 1 mL with acetonitrile/water 3:7. SCFAs were analyzed using a Trace2000 gas chromatograph coupled to a flame ionization detector (ThermoFinnigan; Waltham, MA, USA) equipped with an Innowax 30 m × 530 µm × 1

µm capillary column (Agilent; Santa Clara, CA, USA). Chrom-Card software was used for data processing. Helium was used as the carrier gas with a linear velocity of 5 mL/min. GC oven temperature was programmed as follows: 80 °C (hold 1 min) to 120 °C at 15 °C/min (hold 4 min) to 130 °C at 5 °C/min (hold 4 min) to 235 °C at 8 °C/min (hold 4 min). FID detection was performed at a base temperature of 240 °C. Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The precision (RSD < 15%) and recovery (> 70%) of the method were adequate and both inter- and intra-day reproducible.

Liver histology

Fixed livers were dehydrated in alcohol and embedded in paraffin (Panreac Quimica SLU; Barcelona, Spain), then cut into 3 µm-thick slices, using a steel knife mounted in a microtome (HM 355S Rotary Microtome, Thermo Fisher Scientific; Waltham, MA, USA). Sections were stained with hematoxylin (hematoxylin solution modified in accordance with Gill III for microscopy, Merck KGaA; Darmstadt, Germany)/eosin (Pharmacy Service of Puerta del Mar Hospital; Cadiz, Spain) then viewed under a light microscope (NIKON Eclipse 80i, NIKON Corporation; Minato, Japan). Variables were graded following the method described by Taltavull et al. (Taltavull et al., 2014) using observation of the entire field of the tissue preparations: steatosis, 0 (absence) or 1 (presence); and lobular inflammation, 0 (absence), 1 (1–2 foci), 2 (2–4 foci), or 3 (>4 foci).

Statistical analysis

All data manipulation and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software; San Diego, CA, USA). The results are expressed as means with their standard errors (SEM). The normal distributions and heterogeneity of the data were evaluated, and statistical significance was determined by one-way ANOVA and the Tukey multiple-comparisons test or by two-way ANOVA. Differences were considered significant when $P \leq 0.05$ and were considered to indicate a tendency when $0.05 < P \leq 0.1$.

RESULTS

Feed and energy intakes, body weight, perigonadal fat, plasma leptin and urine F_{2t}-isoprostanes

All the rats fed the high-fat diet consumed less feed but more energy than those in the STD group (Table 1), whether they are supplemented with D-fagomine and/or ω-3 PUFAs or not.

At the beginning of the study, the mean body weight was 323.9 ± 6.3 g (Supplementary Material, Figure 1A, B). At the end of the study (after 24 weeks) the HF group showed a tendency ($P = 0.07$) to gain more weight than the STD group (Table 1). The groups supplemented D-fagomine (HF+FG and HF+FG+ω-3) tended ($P = 0.1$ and $P = 0.08$, respectively) to gain less weight than those in the HF group (Table 1). This tendency was only evident after week 22 for the single supplementation with D-fagomine; while the body weight of the group supplemented with both D-fagomine and ω-3 PUFAs tended to be lower from the beginning of the experiment and it seemed to drift towards higher values at the end (Supplementary Material, Figure 1B).

The HF group and the rats supplemented only with ω-3 PUFAs showed significantly higher ($P < 0.05$) perigonadal fat deposition than the STD group; while the groups supplemented with D-fagomine presented a level of perigonadal fat similar to the STD group (Figure 1B).

Plasma leptin concentration (Figure 1C) was higher ($P < 0.01$) in all 4 HF groups than in the STD group; but both D-fagomine and ω-3 PUFAs partially prevented this effect.

The animals fed the high-fat diet supplemented with ω-3 PUFAs had significantly ($P < 0.05$) lower concentrations of total urine F_{2t}-IsoPs (5-F_{2t}-IsoP plus 15-F_{2t}-IsoP: markers of systemic OS) after 21 weeks, than rats in the STD group (Figure 1D). The levels of F_{2t}-IsoPs were similar among the supplemented groups.

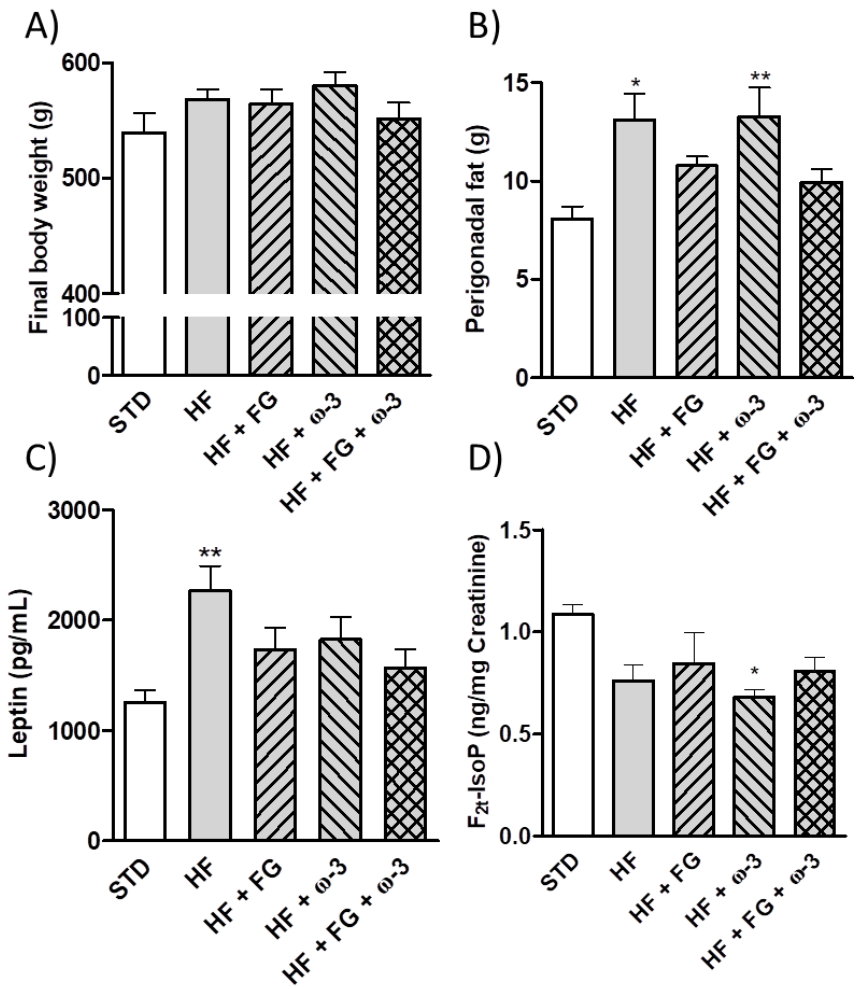


Figure 1.- Final body weight (A), perigonadal fat (B), plasma leptin (C) and urine F_{2t}-IsoPs (D) of SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet for 24 weeks (A-C) or 21 weeks (D). Data are presented as means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$, ** $P < 0.01$ *vs* STD.

Table 1.- Feed and energy intakes, and final body weight gain, of SD rats fed a high-fat diet supplemented, or not, with D-fagomine, ω-3 PUFAs or a combination of both, for 24 weeks.

	STD		HF		HF+FG		HF+ω-3		HF+FG+ω-3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/day/100 g body weight)	4.6	0.5	3.3***	0.1	3.0***	0.1	3.2***	0.1	3.4***	0.1
Energy intake£ (kcal/day/100 g body weight)	13.3	1.4	15.3***	0.5	15.0***	0.1	15.7***	0.5	16.1***	0.1
Body weight gain (%)	67.3	4.1	78.9$^{\beta}$	4.6	74.2$^{\alpha}$	2.2	76.4	5.7	71.5$^{\phi}$	3.3

£ Energy intake is estimated as metabolizable energy based on Atwater factors, which assign 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrates.

Data are presented as means with their standard errors; n = 9 per group. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. β P = 0.07, *** P < 0.001 vs STD; and α P = 0.1, ϕ P = 0.08 vs HF.

Glycemic status

Plasma fasting glucose and insulin were measured after weeks 9, 14 (Supplementary Material, Figure 2) and 23 (Figure 2) of the study. Fasting glucose levels were below 80 mg/dL in all the groups at all times. Fasting glucose levels in the 4 HF groups were higher (P < 0.05) than those in the STD group after 14 weeks of intervention; while plasma insulin was significantly elevated already after week 9 (Supplementary Material, Figure 2) and remained so up to the end of the study (Figure 2). The group supplemented only with D-fagomine presented insulin levels similar to the STD group already after week 9 (Supplementary Material, Figure 2). At the end of the study (week 23), only the HF group presented significantly higher insulin concentrations than those of STD group (Figure 2B).

HOMA-IR is an indicator of IR that accounts for the levels of fasting plasma glucose and insulin levels. HOMA-IR increased in all the HF groups (P < 0.05 *vs* STD group) except in the group supplemented with D-fagomine, as early as week 9 and for the entire experiment (Supplementary Material, Figure 2). At the end of the study (week 23), the animals

supplemented with D-fagomine (HF+FG and HF+FG+ω-3) presented a HOMA-IR similar to those in the STD group (Figure 2D).

The OGTT was performed at week 20, near the end of the experiment (Supplementary Material, Figure 3). Although there were no significant differences in the area under the curve between groups, a close examination to the response curves clearly reveals that the combination of D-fagomine and ω-3 PUFAs generated a glucose response similar to that in the control animals (Supplementary Material, Figure 3C). The postprandial blood glucose concentration 60 min after glucose load was statistically similar in the doubly supplemented rats (HF+FG+ω-3 group) and those in the STD group, while being higher in the HF+ω-3 group (Figure 2C).

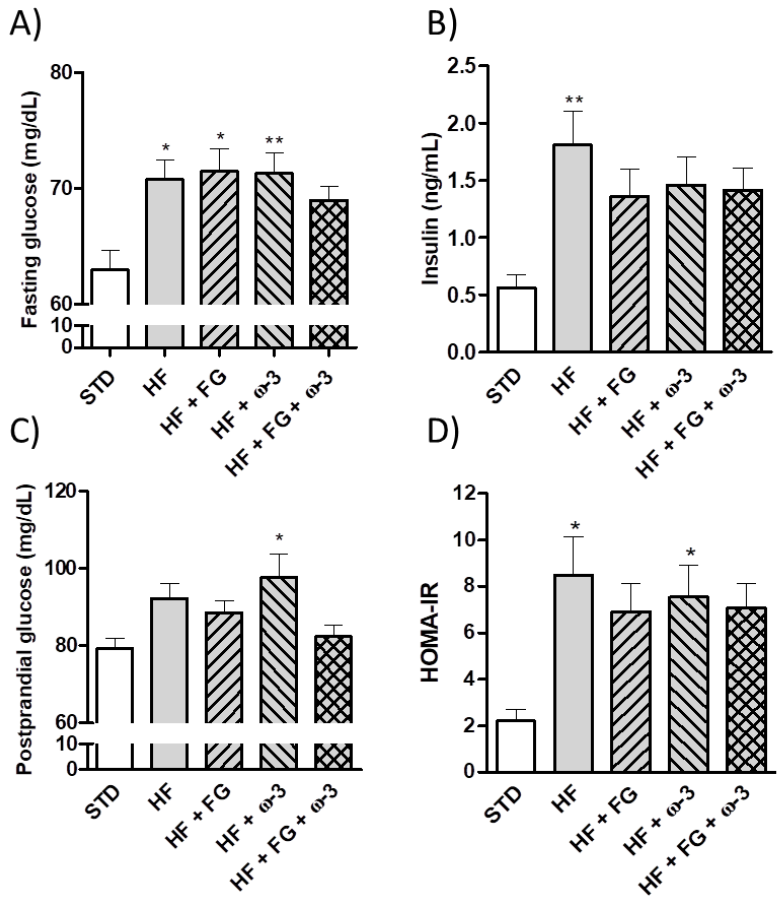


Figure 2.- Plasma levels of fasting glucose (A) and insulin (B), postprandial glucose 60 min after glucose intake (C) and calculated HOMA-IR (D) in SD rats fed a standard (STD), high-fat (HF), high-fat

supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet after 20 weeks (C) or 23 weeks (A, B, D). Data are presented as means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$, ** $P < 0.01$ *vs* STD.

Subpopulations of gut microbiota

The proportions of the major bacterial phyla (Bacteroidetes and Firmicutes), selected orders (Lactobacilliales, Bifidobacteriales and Enterobacteriales) and genera (*Prevotella* and *Bacteroides*) were estimated in fecal and cecal DNA (Figures 3 and 4, respectively) at the end of the study (week 23-24).

In fecal samples, the proportion of Bacteroidetes (Figure 3A) significantly ($P < 0.01$) decreased and the proportion of Firmicutes (Figure 3B) significantly ($P < 0.001$) increased in rats from groups HF and HF+ω-3, with respect to the STD group. The proportion of Bacteroidetes and Firmicutes in animals supplemented with D-fagomine (the HF+FG and HF+FG+ω-3 groups) was similar to that in animals in the STD group. The percentage of Lactobacilliales (Figure 3D) was significantly ($P < 0.05$) higher in the HF+FG group than in all other groups; while the population of Bifidobacteriales (Figure 3E) was significantly ($P < 0.05$) higher in both groups supplemented with D-fagomine (HF+FG and HF+FG+ω-3) compared to the other groups. The proportion of Enterobacteriales (Figure 3F) was significantly ($P < 0.05$) higher in the HF group with respect to the STD group, and all the supplementations partially reversed this change. Significant differences between groups in the relative populations of the genera *Prevotella* and *Bacteroides* were detected. The proportions of *Prevotella* (Figure 3G) and *Bacteroides* (Figure 3H) were significantly ($P < 0.05$) lower in all 4 HF groups with respect to the STD group. The supplementation with D-fagomine (the HF+FG and HF+FG+ω-3 groups) partially counteracted the reduction in the relative populations of *Prevotella* and *Bacteroides* (Figure 3G, H).

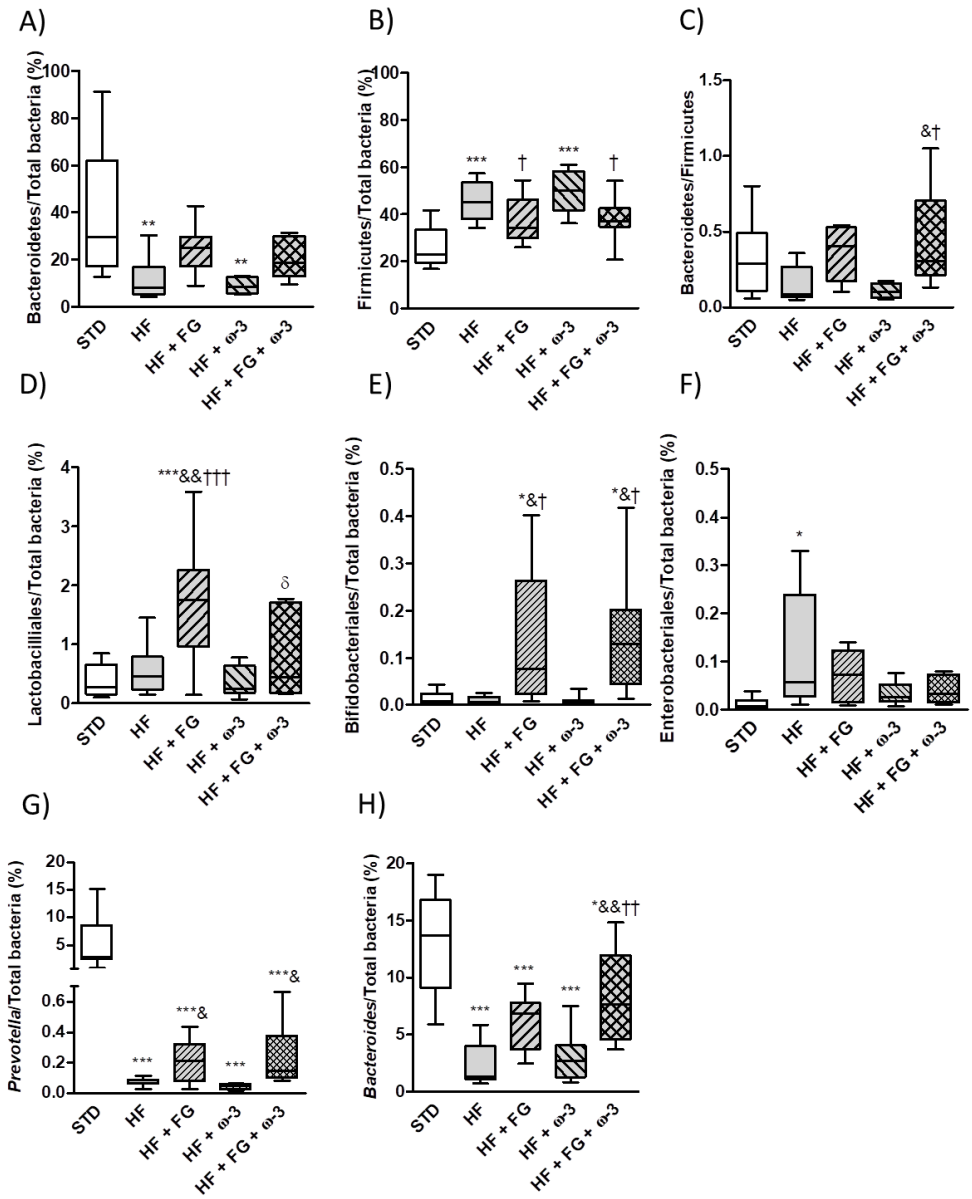


Figure 3.- Relative gut microbial populations in fecal samples from SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet after 23 weeks. Bacteroidetes (A), Firmicutes (B), Bacteroidetes/Firmicutes ratio (C), Lactobacilliales (D), Bifidobacteriales (E), Enterobacteriales (F), *Prevotella* (G) and *Bacteroides* (H). Data are presented as means with their standard error. Comparisons were made using one-way ANOVA followed by Tukey's

post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs* STD; $^{\&}$ $P < 0.05$, $^{\&\&}$ $P < 0.01$ *vs* HF; $^{\delta}$ $P < 0.05$ *vs* HF+FG; and † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ *vs* HF+ω-3.

In cecal samples, the proportion of Bacteroidetes significantly ($P < 0.01$) increased in the group of animals supplemented with both D-fagomine and ω-3 PUFAs, with respect to all the other groups (Figure 4A). The proportion of Firmicutes significantly ($P < 0.05$) increased in all the HF groups with respect to the STD group, except in the group supplemented with both D-fagomine and ω-3 PUFAs (Figure 4B). The highest Bacteroidetes/Firmicutes ratio resulted from the combined supplementation (the HF+FG+ω-3 group, Figure 4C). The percentage of Lactobacilliales (Figure 4D) was significantly ($P < 0.01$) higher in the HF+FG group than in the HF and HF+ω-3 groups; while the population of Bifidobacteriales (Figure 4E) in both D fagomine-supplemented groups was not significantly different from that in the STD group. The proportion *Bacteroides* was significantly ($P < 0.05$) higher in animals supplemented with both D-fagomine and ω-3 PUFAs than in the other groups, except for the group supplemented only with D-fagomine.

Animals in the 4 HF groups had significantly lower ($P < 0.001$) cecum content than those in the STD group. The group supplemented with both D-fagomine and EPA/DHA 1:1 had significantly higher ($P < 0.01$) cecum content than the HF group, and it was similar in the STD group (Figure 4I).

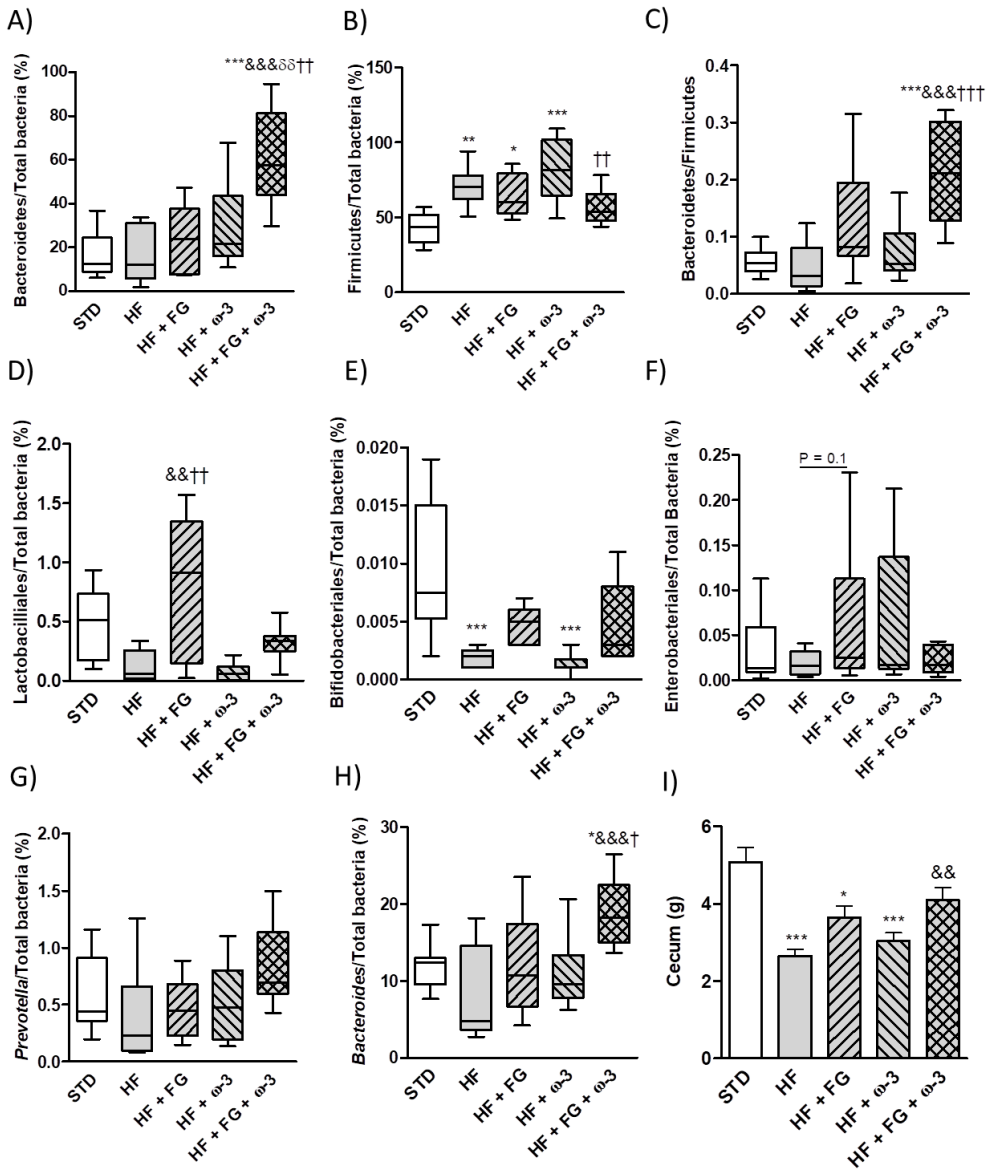


Figure 4.- Relative gut microbial populations in cecal samples of SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet after 24 weeks. Bacteroidetes (A), Firmicutes (B), Bacteroidetes/Firmicutes ratio (C), Lactobacilliales (D), Bifidobacteriales (E), Enterobacteriales (F), *Prevotella* (G) and *Bacteroides* (H). Cecum weight (I). Data are presented as means with their standard error. Comparisons were made using one-way ANOVA

followed by Tukey's post-hoc test. * P < 0.05, ** P < 0.01, *** P < 0.001 *vs* STD; $^{\&\&}$ P < 0.01, $^{\&\&\&}$ P < 0.001 *vs* HF; $^{\delta\delta}$ P < 0.01 *vs* HF+FG; and † P < 0.05, †† P < 0.01, ††† P < 0.001 *vs* HF+ω-3.

Short-chain fatty acids

SCFAs were determined in feces (Table 2) and in the cecal content (Table 3) at the end of the study (week 23-24).

In fecal samples, the concentrations of all SCFAs were lower in the 4 HF groups than in the STD group. The major SCFA (acetic acid) was significantly reduced (P < 0.05) in all HF groups except the group supplemented only with D-fagomine.

In cecal samples, the concentration of acetic acid was significantly reduced (P < 0 .001) in all 4 HF groups; while the concentration of butyric acid was significantly higher (P < 0.01) in all these groups than in the STD group. The levels of isobutyric acid were significantly reduced (P < 0.05) in the HF group without supplementation and the group supplemented only with ω-3, but not in the 2 D-fagomine supplemented groups. The concentration of isovaleric acid was significantly higher (P < 0.05) in the HF+FG group than in the HF group; while levels of valeric acid were higher (P < 0.001) in all the supplemented groups (HF+FG, HF+ω-3 and HF+FG+ω-3) than in the STD group.

Table 2.- Fecal short-chain fatty acids (SCFAs) after week 23 of the study.

	STD		HF		HF+FG		HF+ω-3		HF+FG+ω-3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	115.2	12.8	77.2**	2,6	90.7	10.9	84.9*	5.9	79.8**	3.9
Propionic acid	13.4	0.9	5.7***	0.5	8.6*	1.6	4.7***	0.9	4.0***	0.8
Isobutyric acid	2.7	0.2	1.0***	0.2	1.4***	0.2	1.3***	0.3	1.6**	0.7
Butyric acid	17.3	2.4	8.5**	1.1	6.9***	1.6	7.2***	1.2	6.4***	1.7
Isovaleric acid	1.7	0.2	0.6***	0.1	0.9*	0.2	0.4***	0.3	0.5***	0.3
Valeric acid	1.4	0.1	0.8**	0.0	0.9*	0.1	0.6***	0.3	0.6***	0.3
TOTAL SCFAs	151.9	9.3	93.9**	3.2	109.7	13.8	99.4*	2.1	93.0**	1.7

Data are presented as means with their standard errors; n = 9 per group. SCFA amounts are given in millimoles per kilogram feces. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. * P < 0.05, ** P < 0.01, *** P < 0.001 *vs* STD.

Table 3.- Cecal short-chain fatty acids (SCFAs) at the end of the study (24 weeks).

	STD		HF		HF+FG		HF+ω-3		HF+FG+ω-3	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	159.9	18.5	75.5***	9.2	84.3***	10.0	88.6***	10.4	74.5***	7.7
Propionic acid	32.4	3.7	25.5	2.6	32.4	3.2	32.4	4.2	34.0	5.4
Isobutyric acid	3.7	0.3	2.1*	0.1	3.4	0.3	2.6*	0.2	3.5	0.4
Butyric acid	11.8	1.0	24.7**	2.0	24.7**	2.6	33.0***	2.8	27.1***	3.4
Isovaleric acid	3.2	0.3	1.9	0.2	3.6$^{\&}$	0.4	2.7	0.4	3.2	0.6
Valeric acid	1.9	0.3	3.4	0.2	4.6***	0.4	4.7***	0.4	4.4***	0.4
TOTAL SCFAs	213.1	23.5	133.3*	12.7	153.2	14.1	164.2	17.1	146.8	16.3

Data are presented as means with their standard errors; n = 9 per group. SCFA amounts are given in millimoles per kilogram cecum. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test. * P < 0.05, ** P < 0.01, *** P < 0.001 *vs* STD; and $^{\&}$ P < 0.05 *vs* HF.

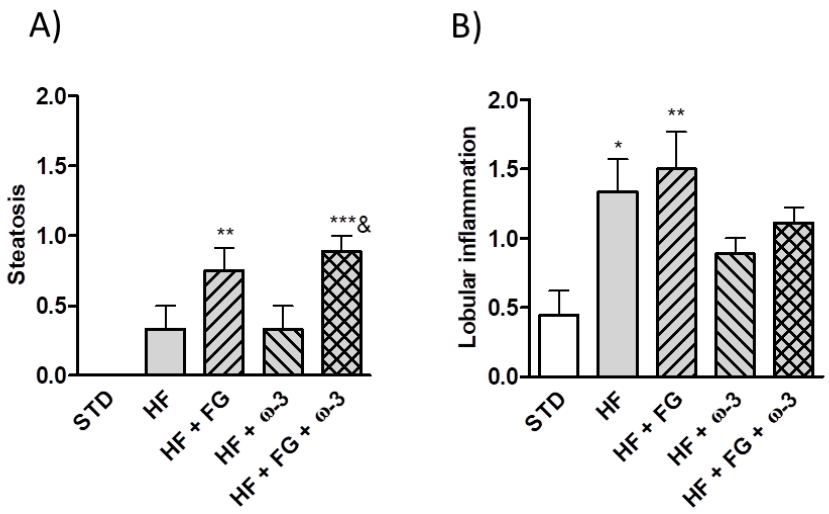


Figure 5.- Liver histology. Estimation of variables in SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet at the end of the study (week 24). Steatosis (A) and lobular inflammation (B). Scores are presented as means with their standard error. Comparisons were made using one-way ANOVA followed by Tukey's post-hoc test. * P < 0.05, ** P < 0.01, *** P < 0.001 *vs* STD; and $^{\&}$ P < 0.05 *vs* HF.

Liver histology

Steatosis and lobular inflammation were determined by histology (Figure 5). The livers of animals supplemented with D-fagomine presented higher ($P < 0.05$) steatosis than those from the STD group (Figure 5A) and the livers from animals supplemented with both D-fagomine and ω-3 PUFAs presented higher ($P < 0.05$) steatosis than those from rats in the HF and HF+ω-3 groups. Lobular inflammation of animals in the HF and HF+FG groups was higher ($P < 0.05$) than for those in the STD group (Figure 5B).

DISCUSSION

The present study examines the effects of the combination of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on SD rats at a very early stage in the development of fat-induced pre-diabetes. SD rats aged 8-9 weeks and fed a high-fat diet tended ($P = 0.07$) to gain more weight than those fed a standard diet over a period of 24 weeks (Table 1) and they also developed incipient IR as assessed by HOMA-IR (Figure 2D). These metabolic effects are less evident than those we reported for Wistar Kyoto (WKY) rats, which significantly ($P < 0.001$) gained more weight when fed a high-fat diet over the same period of time (Ramos-Romero et al., 2018). WKY rats presented IGT with blood glucose levels around 150 mg/dL, already at week 13 of the intervention (Ramos-Romero et al., 2018); while here, blood glucose concentration in SD rats fed a high-fat diet was lower than 120 mg/dL even at week 20 (Supplementary Material, Figure 3). These observations coincide with the results published by other authors showing that: i) even though SD rats gain more weight than Wistar rats, the differences between the STD group and the 4 HF groups are lower in SD rats; and ii) SD rats fed a high-fat diet develop IGT later than Wistar rats do (Marques et al., 2016). Therefore, differences in weight gain and effects on glucose metabolism are harder to detect in SD rats than in Wistar or WKY rats. This has to be taken into consideration when interpreting the results of the present study.

In apparent contradiction with our previous observations with WKY rats (Ramos-Romero et al., 2018), D-fagomine did not reduce the weight gain induced by a high-fat diet (Table 1 and Supplementary Material, Figure 1A) when administered alone; while it tended to reduce perigonadal fat (Figure 1B). The supplementation with EPA/DHA 1:1 had no effect on this induced body weight gain or perigonadal fat, when administered alone (Table 1 and Supplementary Material, Figure 1B). Surprisingly, combined D-fagomine and ω-3 PUFAs showed a tendency ($P = 0.08$) to reduce body weight (Table 1 and Supplementary

Material, Figure 1A, B). The pro- and anti-inflammatory effects of the different agents in this experimental set-up may provide an explanation for these apparently surprising results. High-fat diets induce a state of systemic low-grade inflammation, probably initiated by toxic bacterial components such as LPS which leak from an altered intestinal barrier (Sanmiguel, Gupta, & Mayer, 2015). We previously showed that D-fagomine counteracted fat-induced weight gain while lowering the levels of inflammatory markers in plasma and liver and we postulated that these effects might be attained by re-balancing the intestinal microbiota (Ramos-Romero et al., 2018). One possible explanation for the lack of effect of D-fagomine on SD rats fed a high-fat diet reported here is that the animals not supplemented with ω-3 PUFAs (STD, HF and HF+FG groups) were administered an equivalent dose of soybean oil to compensate for both the stress of probing and the excess of calories. Soybean oil, which is rich in the ω-6 PUFA linoleic acid (LA), might have counteracted the putative microbiota-related anti-inflammatory effect of D-fagomine. LA may increase the levels of inflammatory prostaglandins and cytokines by entering the ARA metabolic pathway (for a review see (Naughton, Mathai, Hryciw, & McAinch, 2016)). This will not happen in the case of ω-3 PUFA supplementation, as EPA and DHA are converted into less inflammatory and even anti-inflammatory metabolites (for a review on the actions of ω-6 and ω-3 PUFAs see (Innes & Calder, 2018)). Therefore, the anti-inflammatory-related weight-lowering effect of D-fagomine would be reinforced instead of counteracted by the accompanying lipid (EPA/DHA in the HF+FG+ω-3 group). These events would occur downstream of the fat-induced microbial-triggered toxicity and they are consistent with the liver histology results obtained. All the groups presented some lobular inflammation (Figure 5) which was significantly higher in the HF and HF+FG groups ($P < 0.05$ and 0.01 *vs* STD, respectively). LA may have contributed to liver inflammation in the HF+FG group while EPA/DHA 1:1 might have partially counteracted the pro-inflammatory effect of the saturated fats in the diet. The levels of visceral adiposity in the experimental groups corresponded to the levels of leptin, except in the HF+ω-3 group (Figure 1C). The anorexigenic hormone leptin is mainly secreted by adipocytes and it is directly related to adipose tissue mass (Considine et al., 1996).

The high-fat diet promoted a statistically significant elevation in plasma insulin levels ($P < 0.01$) (Figure 2B) and HOMA-IR ($P < 0.05$) (Figure 2D), which are consistent with a

physiological compensation for reduced insulin sensitivity: the very first stage in the progression to diabetes (Weir & Bonner-Weir, 2004). SD rats fed the high-fat diet showed only an incipient tendency to develop IGT, evidenced by the postprandial levels of plasma glucose 60 min after the glucose load in the OGTT (Figure 2C). The combination of D-fagomine and EPA/DHA 1:1 was the most effective supplementation against diet-induced IR and IGT as evidenced by the observations that HOMA-IR and the 60 min postprandial glucose levels in the HF+FG+ω-3 group were statistically similar to the values in the STD group (Figure 2C, D and Supplementary Material, Figure 3C). When the supplements were administered separately, only D-fagomine showed a capacity to counteract the increase of HOMA-IR (Figure 2D).

In agreement with our results with WKY rats (Ramos-Romero et al., 2018), fat accumulation and IR did not increase systemic OS as assessed by the levels of urine F_{2t}-IsoPs (Figure 1D). This result is also consistent with the observation that the generation of mitochondrial ROS occurs later than IR as a consequence of hyperglycemia, as observed in skeletal muscle of mice fed a high-fat high-sucrose diet (Bonnard et al., 2008).

As there is mounting evidence that alterations in gut microbiota (dysbiosis) and intestinal barrier dysfunction may be the first steps leading to dietary fat-induced chronic systemic low-grade inflammation, visceral fat accumulation and IR (Duan et al., 2018), we checked the effects of D-fagomine, ω-3 PUFAs and their combination on relevant bacterial subgroups. At the *phyla* level, D-fagomine counteracted the high-fat diet-induced changes in the excreted populations of Bacteroidetes and in both excreted and cecal populations of Firmicutes (Figures 3A, B and 4B). These results confirm our previous observations in WKY rats (Ramos-Romero et al., 2018) and they agree with an association between a high Bacteroidetes/Firmicutes ratio and the lean phenotype in both rodents and humans (Turnbaugh & Gordon, 2009; Turnbaugh et al., 2006). ω-3 PUFAs did not affect the excreted populations of Bacteroidetes and Firmicutes, either when administered alone or when combined with D-fagomine (Figure 3A, B, C). The combination of both supplements resulted in the highest Bacteroidetes/Firmicutes ratio in the cecum (Figure 4A, B, C).

We next evaluated the changes in the populations of the putatively beneficial Lactobacilliales, Bifidobacteriales and the putatively harmful Enterobacteriales. D-Fagomine promoted the growth of Lactobacilliales and Bifidobacteriales (Figure 3D and 3E, respectively) in agreement with our

previous study in rats fed a standard diet (Hereu et al., 2018). Bifidobacteria have been shown to protect gut barrier function by preserving mucosal permeability and preventing translocation of pathogenic enterobacteria, such as *E. coli* (Caplan et al., 1999). As bacterial toxicity may be the main cause of fat-induced low-grade inflammation, D-fagomine might be exerting an anti-inflammatory action by fostering gut colonization with bifidobacteria. D-Fagomine may also contribute a direct action against putatively harmful enterobacteria as it is capable of inhibiting adhesion of *E. coli* to the intestinal mucosa (Gómez et al., 2012). The exclusion of enterobacteria from the intestinal wall would explain the tendency for D-fagomine to increase the population of this subgroup in the cecal content (Figure 4F). Neither the excess of fat nor the supplementation with ω-3 PUFAs had any significant effect on lactobacilli or bifidobacteria.

It is known that part of the beneficial health effects of a well-balanced gut microbiota is mediated by SCFAs, which are products of microbial fermentation of dietary fiber (den Besten et al., 2013). The total excreted SCFAs were lower in all the HF groups than in the STD group. This may be due to the fact that cellulose is almost the only source of fiber in the high-fat diet, which has been proved to yield amounts of SCFAs as low as those generated by fiber-free diets (den Besten et al., 2013). High-fiber low-fat diets are known to generate large amounts of fecal SCFAs (mainly acetic acid) compared to fiber-poor diets (Filippo et al., 2010). D-Fagomine partially counteracted the decrease of acetic acid and total SCFAs in rats fed the high-fat diet (Table 2). This effect could be explained by D-fagomine promoting the growth of SCFA-producing bacteria. We recently reported that D-fagomine stabilizes the populations of the genus *Prevotella* in the intestinal tract of SD rats fed a standard diet while reducing weight gain (Hereu, sent). In the present study, D-fagomine showed some capacity to counteract the high-fat-induced reduction in the populations of *Prevotella* (Figure 3G). As bacteria from this genus are capable of fermenting complex polysaccharides from the diet in a process that is mechanistically linked to functional effects on glucose metabolism (Kovatcheva-Datchary et al., 2015), we hypothesize that D-fagomine might collaborate with dietary fiber in promoting the growth of beneficial bacteria. This effect is necessarily weak in the present case because the high-fat diet is low in fermentable fiber. The weight of the cecum is another indication of gut microbial activity, as rats supplemented with dietary fiber have been reported to have both cecal content and cecal tissue increased already after just two weeks of

intervention (Dalby, Ross, Walker, & Morgan, 2017; Jakobsdottir, Xu, Molin, Ahrne, & Nyman, 2013). Here, the cecal content in the HF group was significantly reduced with respect to the STD group while the cecal weight in the 2 groups supplemented with D-fagomine was similar to that in the STD group. The present results with bacterial populations and their fermentation products provide evidence that D-fagomine may contribute to the prevention of metabolic alterations by promoting balanced gut microbiota, whether it is combined with ω-3 PUFAs or not. The combination of supplements might be more effective than the individual supplementations in some cases (e.g. the Bacteroidetes/Firmicutes ratio and cecal content).

The main limitation of this study is that even though SD rats are appropriate for the detection of the early metabolic effects resulting from a high-fat diet, the differences between the treated groups and the controls are too small to reach statistical significance. Despite this limitation, the picture that emerges from this study, together with our previous reports, clearly points towards a functional role for D-fagomine in the prevention of risk factors for diet-induced pre-diabetes which is reinforced by the action of ω-3 PUFAs via complementary mechanisms.

In conclusion, the very early metabolic alterations induced by a Westernized high-fat diet in SD rats may be reduced by the combined action of D-fagomine and ω-3 PUFAs. The functional effects of the combination are mainly those of D-fagomine complemented by those of EPA/DHA 1:1, which may be attributed to its anti-inflammatory activity (See Table 4 for a summary). The combined supplements counteract high-fat-induced weigh gain, visceral fat, incipient IR, and liver inflammation; while increasing the cecal content, the Bacteroidetes/Firmicutes ratio and the gut populations of both Bifidobacteriales and Lactobacilliales

.

Table 4.- Qualitative summary of the main effects of diet and supplementations in SD rats compared to rats fed a standard diet.

	Fasting Glucose	Fasting Insulin	HOMA-IR	60 min Postprandial Glucose	Weight gain	B/F [£] ratio	Bifidobacteriales
HF	↑	↑	↑	↑	↑ [β]	≈	≈
HF+FG	↑	≈	≈	≈	≈ [α]	≈	↑
HF+ω-3	↑	≈	↑	↑	≈	≈	≈
HF+FG+ω-3	≈	≈	≈	≈	≈ [ϕ]	↑	↑

[£] Bacteroidetes/Firmicutes; [β] P = 0.07 *vs* STD; [α] P = 0.1 *vs* HF; and [ϕ] P = 0.08 *vs* HF

AUTHOR INFORMATION

Corresponding author

Phone: (+34) 934006100, Fax: (+34) 932045904
e-mail: sara.ramos@iqac.csic.es

Acknowledgements

English language revision by Christopher Evans is appreciated.

Funding

This research was supported by the Spanish Ministry of Economy and Competitiveness (via grant numbers AGL2013-49079-C2-1,2-R and AGL2017-83599-R, and a graduate fellowship to M. Hereu: BES2014-068592).

REFERENCES

Abdallah Ismail, N., Ragab, S. H., Abd Elbaky, A., Shoeib, A. R., Alhosary, Y., & Fekry, D. (2011). Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Archives of Medical Science, 7*(3), 501-507.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M.-A., Morio, B., . . . Rieusset, J. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *The Journal of Clinical Investigation, 118*(2), 789-800.

Calder, P. C. (2006). n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *The American Journal of*

Clinical Nutrition, 83(6 Suppl), 1505S-1519S.

Caplan, M. S., Miller-Catchpole, R., Kaup, S., Russell, T., Lickerman, M., Amer, M., . . . Thomson, R., Jr. (1999). Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterology, 117*(3), 577-583.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., . . . Caro, J. F. (1996). Serum immunoreactive leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine, 334*(5), 292-295.

Dalby, M. J., Ross, A. W., Walker, A. W., & Morgan, P. J. (2017). Dietary uncoupling of gut microbiota and energy harvesting from obesity and glucose tolerance in mice. *Cell reports, 21*(6), 1521-1533.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., . . . Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America, 107*(33), 14691-14696.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research, 54*(9), 2325-2340.

Duan, Y., Zeng, L., Zheng, C., Song, B., Li, F., Kong, X., & Xu, K. (2018). Inflammatory links between high fat diets and diseases. *Frontiers in Immunology, 9*, 2649.

Filippo, C., Cavalieri, D., Paola, M., Ramazzotti, M., Poullet, J. B., & Massart, S. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America, 107*(33), 14691-14696.

Gómez, L., Molinar-Toribio, E., Calvo-Torras, M. Á., Adelantado, C., Juan, M. E., Planas, J. M., . . . Torres, J. L. (2012). D-Fagomine lowers postprandial blood glucose and modulates bacterial

adhesion. *British Journal of Nutrition, 107*(12), 1739-1746.

Haakensen, M., Dobson, C. M., Deneer, H., & Ziola, B. (2008). Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *International Journal of Food Microbiology, 125*(3), 236-241.

Hartman, A. L., Lough, D. M., Barupal, D. K., Fiehn, O., Fishbein, T., Zasloff, M., & Eisen, J. A. (2009). Human gut microbiome adopts an alternative state following small bowel transplantation. *Proceedings of the National Academy of Sciences of the United States of America, 106*(40), 17187-17192.

Hereu, M., Ramos-Romero, S., García-González, N., Amézqueta, S., & Torres, J. L. (2018). Eubiotic effect of buckwheat D-fagomine in healthy rats. *Journal of Functional Foods, 50*, 120-126.

Hildebrandt, M. A., Hoffmann, C., Sherrill-Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., . . . Wu, G. D. (2009). High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology, 137*(5), 1716-1724.

Innes, J. K., & Calder, P. C. (2018). Omega-6 fatty acids and inflammation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 132*, 41-48.

Jakobsdottir, G., Xu, J., Molin, G., Ahrne, S., & Nyman, M. (2013). High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects. *PLoS One, 8*(11), e80476.

Janssen, A. W., & Kersten, S. (2017). Potential mediators linking gut bacteria to metabolic health: a critical view. *The Journal of Physiology, 595*(2), 477-487.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., . . . Backhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of *Prevotella*. *Cell Metabolism, 22*(6), 971-982.

Koyama, M., & Sakamura, S. (1974). The structure of a new piperidine derivative from buckwheat seeds (*Fagopyrum esculentum* Moench). *Agricultural and Biological Chemistry, 38*(5), 1111-1112.

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature, 444*(7122), 1022-1023.

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., . . . Calhau, C.

(2016). High-fat diet-induced obesity rat model: a comparison between Wistar and Sprague-Dawley rat. *Adipocyte, 5*(1), 11-21.

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia, 28*(7), 412-419.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Gómez, L., Taltavull, N., Nogués, M. R., . . . Torres, J. L. (2015). D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food & Function, 6*(8), 2614-2619.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Romeu, M., Giralt, M., Taltavull, N., . . . Torres, J. L. (2015). Effect of n-3 PUFA supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model of the metabolic syndrome. *British Journal of Nutrition, 113*(6), 878-887.

Mujico, J. R., Baccan, G. C., Gheorghe, A., Diaz, L. E., & Marcos, A. (2013). Changes in gut microbiota due to supplemented fatty acids in diet-induced obese mice. *The British Journal of Nutrition, 110*(4), 711-720.

Naughton, S. S., Mathai, M. L., Hryciw, D. H., & McAinch, A. J. (2016). Linoleic acid and the pathogenesis of obesity. *Prostaglandins & Other Lipid Mediators, 125*, 90-99.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), e45.

Portune, K. J., Benitez-Paez, A., Del Pulgar, E. M., Cerrudo, V., & Sanz, Y. (2017). Gut microbiota, diet, and obesity-related disorders-the good, the bad, and the future challenges. *Molecular Nutrition & Food Research, 61*(1).

Poudyal, H., Panchal, S. K., Diwan, V., & Brown, L. (2011). Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action. *Progress in Lipid Research, 50*(4), 372-387.

Queipo-Ortuno, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., . . . Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One, 8*(5), e65465.

Ramos-Romero, S., Hereu, M., Atienza, L., Casas, J., Taltavull, N., Romeu, M., . . . Torres, J. L. (2018). Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-Induced prediabetes. *Molecular Nutrition & Food Research*, 62(16), e1800373.

Ramos-Romero, S., Hereu, M., Molinar-Toribio, E., Almajano, M. P., Mendez, L., Medina, I., . . . Torres, J. L. (2017). Effects of the combination of omega-3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats. *Food Research International, 97*, 364-371.

Ramos-Romero, S., Molinar-Toribio, E., Gómez, L., Pérez-Jiménez, J., Casado, M., Clapés, P., . . . Torres, J. L. (2014). Effect of D-fagomine on excreted enterobacteria and weight gain in rats fed a high-fat high-sucrose diet. *Obesity, 22*(4), 976-979.

Robertson, R. C., Seira Oriach, C., Murphy, K., Moloney, G. M., Cryan, J. F., Dinan, T. G., . . . Stanton, C. (2017). Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood. *Brain, Behavior, and Immunity, 59*, 21-37.

Sanmiguel, C., Gupta, A., & Mayer, E. A. (2015). Gut Microbiome and Obesity: A plausible explanation for obesity. *Current Obesity Reports, 4*(2), 250-261.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2009). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity, 18*(1), 190-195.

Taltavull, N., Muñoz-Cortés, M., Lluís, L., Jové, M., Fortuño, À., Molinar-Toribio, E., . . . Nogués, M. R. (2014). Eicosapentaenoic acid/docosahexaenoic acid 1:1 ratio improves histological alterations in obese rats with metabolic syndrome. *Lipids in Health and Disease, 13*, 31.

Turnbaugh, P. J., & Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *The Journal of Physiology, 587*(17), 4153-4158.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature, 444*(7122), 1027-1031.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine, 1*(6), 6ra14.

Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 67*(6), 2578-2585.

Weir, G. C., & Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes, 53*, S16-S21.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., . . . Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science,334*(6052), 105-108.

SUPPLEMENTARY MATERIAL (SM) INCLUDED IN THE DIGITAL VERSION OF PAPER 4

Table SM1.- Quantitative real-time PCR primers and conditions

Target bacteria	Annealing temperature (°C)	Sequences (5'-3')	Positive Control	Reference
Total Bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(a)	(Hartman et al., 2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	*Bacteroides fragilis*	(Abdallah Ismail et al., 2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	*Ruminococcus productus*	(Haakensen, Dobson, Deneer, & Ziola, 2008)
Lactobacillales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus acidophylus*	(Walter et al., 2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	*Bifidobacterium longum*	(Queipo-Ortuno et al., 2013)
Enterobacteriales	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	*Escherichia coli M15*	(Hartman et al., 2009)
Prevotella	60	F: CAG CAG CCG CGG TAA TA R: GGC ATC CAT CGT TTA CCG T	*Prevotella copri*	(Schwiertz et al., 2009)
Bacteroides	60	F: GGT TCT GAG AGG AGG TCC C R: GCT GCC TCC CGT AGG AGT	*Bacteroides fragilis*	(Schwiertz et al., 2009)

[a] Positive control of Total Bacteria was the same as those the result was rated with.

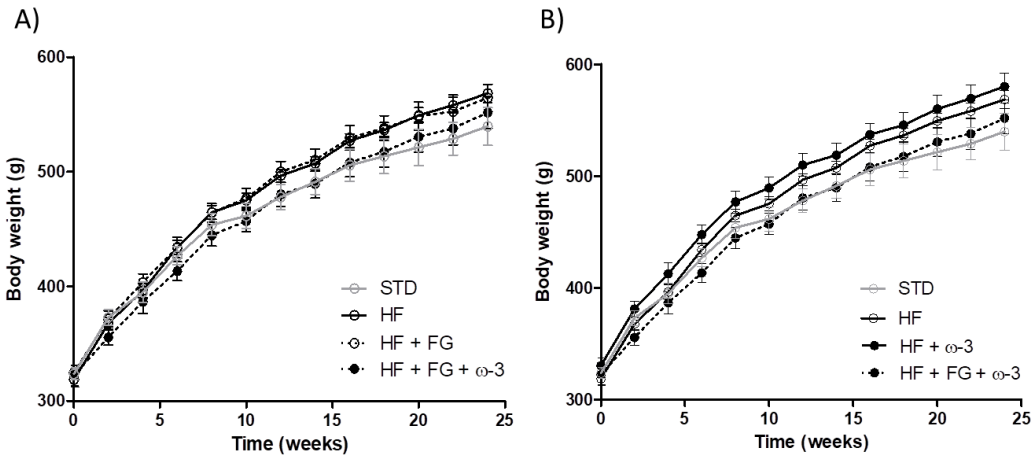


Figure SM1.- Body weight (A, B) of SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet for 24 weeks. Data are presented as means with their standard errors.

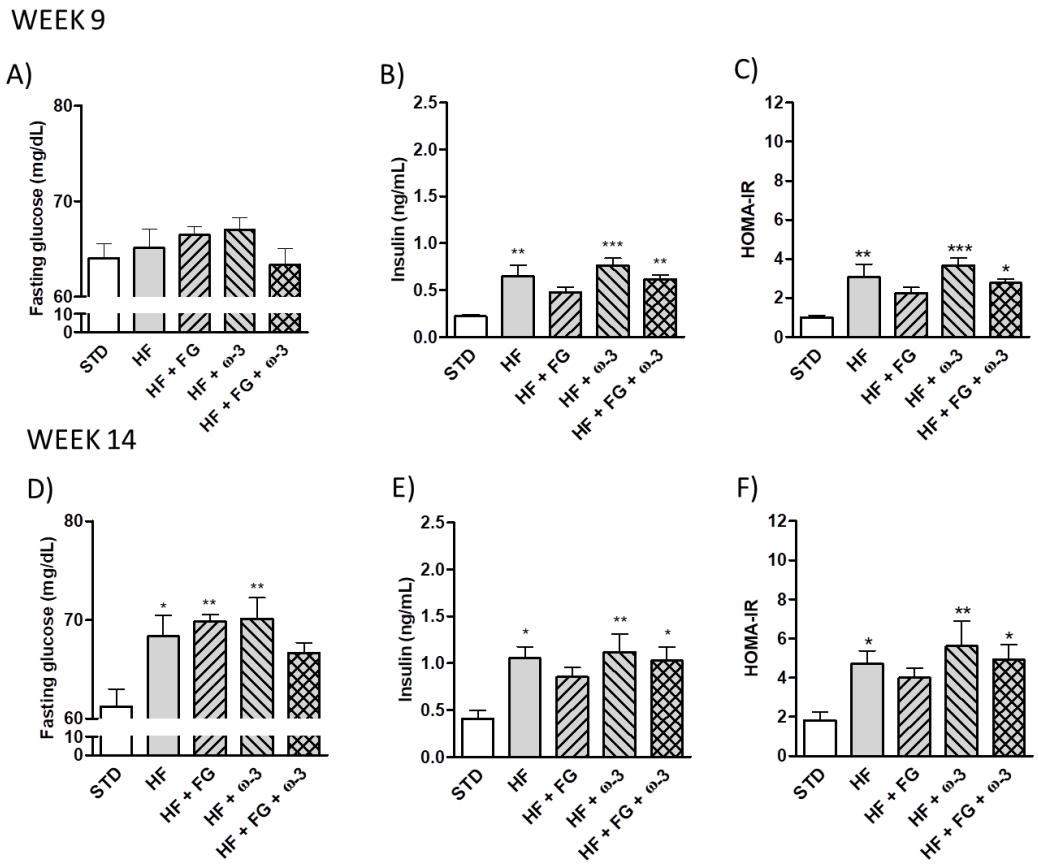


Figure SM2.- Plasma levels of fasting glucose (A and D), insulin (B and E) and calculated HOMA-IR (C and F) in SD rats fed a standard (STD), high-fat (HF), high-fat supplemented with D-fagomine (HF+FG), high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) or high-fat supplemented with both D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet, after weeks 9 (A, B and C) and 14 (D, E and F). Data are presented as means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* STD.

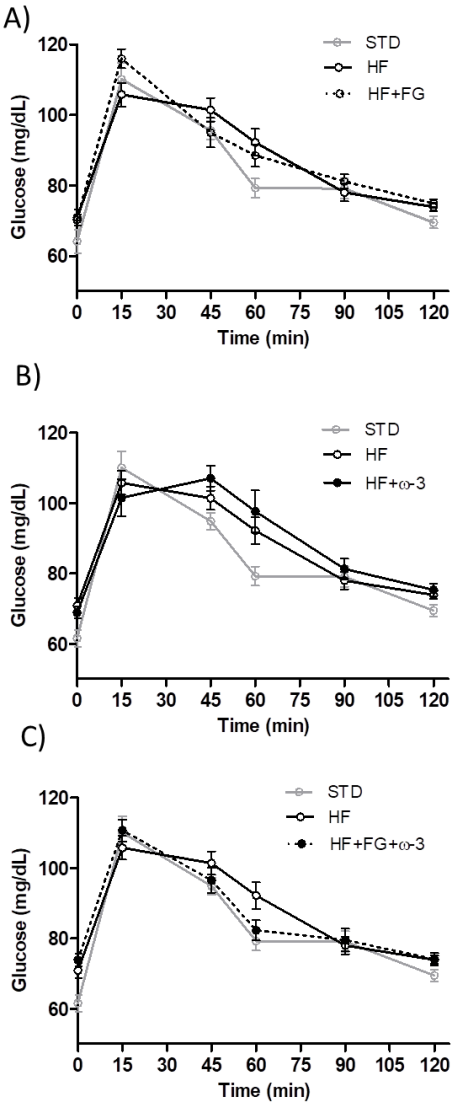


Figure SM3.- Glycemic response (OGTT curves) in SD rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet (A); STD, HF or high-fat supplemented with EPA/DHA (1:1) (HF+ω-3) diet (B); and STD, HF or high-fat supplemented with D-fagomine and EPA/DHA (1:1) (HF+FG+ω-3) diet (C) after ingestion of a single dose of glucose (1 g/kg body weight) at week 20 of the intervention. Data are presented as means with their standard errors.

4 SUMMARY OF RESULTS

This thesis consisted of exploring the effects of D-fagomine, ω-3 PUFAs (EPA/DHA 1:1) and their combination on i) rats given a standard diet (**PAPERS 1** and **2**) and ii) rat models of fat-induced pre-diabetes (**PAPERS 3** and **4**). The thesis is based on previous post-graduate work by the candidate included as Annex (**PAPERS A1** and **A2**). Paper **A1** explored the efficiency of the combination between ω-3 PUFAs (EPA/DHA 1:1) and polyphenols (grape seed extract, GSE) in Wistar Kyoto (WKY) rats fed a standard diet. The results show that the combination maintained the standard proportions of *Lactobacillus*, *Bifidobacterium* and SCFAs, while also providing the cardiovascular benefits of ω-3 PUFAs. The rat model of pre-diabetes used in **PAPER 3** of the main body of this thesis was defined in **PAPER A2**. The paper compared the pre-diabetic state generated in WKY rats by an excess of either fat or sucrose (glucose + fructose). The results show that saturated fat triggered IR and IGT before sucrose did. The mechanisms involved in both cases were different. Fat triggered low-grade inflammation probably originated in gut dysbiosis while fructose stimulated liver steatosis and the biosynthesis of putatively toxic lipid mediators (DAGs). As our research group is interested in the relationship between gut microbiota and IR/IGT through low-grade inflammation, fat was preferred over sucrose for the definition of a rat model of pre-diabetes.

The thesis then focused on the activity of D-fagomine, a new possible dietary supplement using the high-fat model defined in **PAPER A2** and explored the activity of its combination with ω-3 PUFAs. The results reported in **PAPERS 1** and **3** were generated by an intervention with WKY rats fed a standard diet or a high-fat diet supplemented (or not) with D-fagomine (0.096% w/w in the feed) for 24 weeks. The results reported in **PAPERS 2** and **4** were generated with Sprague Dawley (SD) rats fed a standard diet or a high-fat diet supplemented (or not) with D-fagomine (0.096% w/w in the feed), ω-3 PUFAs EPA/DHA (1:1) (0.8 mL/kg body weight administed weekly by oral gavage) or a combination of both supplementations for 24 weeks. The reason for using SD rats instead of WKY rats in **PAPERS 2** and **4** is that SD rats develop IR/IGT later than WKY rats, therefore it was considered that they might provide a better model for the detection of very early events in the development of pre-diabetes.

To facilitate the reader's understanding of this summary, the abbreviations used for the experimental groups are given in the following table:

Group by diet and supplementation	Abbreviation
Standard diet	STD or CTL (**PAPER 2**)
Standard diet + D-fagomine	FG
Standard diet + ω-3 PUFAs	ω-3
Standard diet + D-fagomine + ω-3 PUFAs	FG + ω-3
High-fat diet	HF
High-fat diet + D-fagomine	HF + FG
High-fat diet + ω-3 PUFAs	HF + ω-3
High-fat diet + D-fagomine + ω-3 PUFAs	HF + FG + ω-3

The main results of this thesis are summarized below:

4.1 Effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on feed and energy intakes, and body weight

The standard diet supplemented with D-fagomine did not modify the feed or energy intakes (**PAPERS 1** and **2**). WKY rats fed the standard diet showed the same body weight gain whether supplemented with D-fagomine or not (**PAPER 1**) while SD rats fed a standard diet supplemented with D-fagomine (FG and FG + ω-3 groups) gained less weight than those non-supplemented with the iminosugar (CTL and ω-3 groups) after 21 weeks of intervention (**PAPER 2**). Accordingly, these animals supplemented with D-fagomine showed a tendency to store less perigonadal fat than those non-supplemented or supplemented with only ω-3 PUFAs (**PAPER 2**).

Rats fed the high-fat diet consumed less feed but more energy than those fed the standard diet (**PAPERS 3** and **4**). The supplementations with D-fagomine and/or ω-3 PUFAs did not modify intakes with respect to the non-supplemented controls (**PAPERS 1-4**).

WKY rats fed a high-fat diet gained more weight than those given the standard diet while animals supplemented with D-fagomine showed a tendency ($P = 0.06$) to gain less weight than those in the HF group (**PAPER 3**). SD rats fed a high-fat diet showed a tendency ($P = 0.07$) to gain more weight than those fed the standard diet (**PAPER 4**). The HF groups supplemented D-fagomine (HF + FG and HF + FG + ω-3) tended ($P = 0.1$ and $P = 0.08$, respectively) to gain less weight than those in the HF group (**PAPER 4**).

Unlike ω-3 PUFAs, D-fagomine appears to help to reduce body weight gain under an obesogenic diet (**PAPERS 3** and **4**). The results are more evident in WKY rats because even though SD rats reach higher body weight than WKY rats, the differences between the groups given standard and high-fat diets are lower in SD rats.

4.2 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota

Major microbiota phyla: In WKY rats, D-fagomine clearly increased the populations of Bacteroidetes in feces over the whole experiment when administered together with the standard diet. This effect was also detected in the cecal content at the end of the intervention (**PAPER 1**). No such an effect was detected in rats given a high-fat diet (**PAPER 3**).

In SD rats fed a high-fat diet, the Bacteroidetes/Firmicutes ratio in animals supplemented with D-fagomine (HF + FG and HF + FG + ω-3 groups) was similar to that in animals fed the standard diet. The highest Bacteroidetes/Firmicutes ratio resulted from the combined supplementation (HF + FG + ω-3 group) (**PAPER 4**).

Minor microbiota orders: The relative populations of Bifidobacteriales and Lactobacillales decreased as the animals grew older (**PAPERS 1** and **2**) or with the high-fat diet (**PAPERS 3** and **4**), while the proportion of Enterobacteriales significantly increased with the high-fat diet (**PAPERS 3** and **4**). D-Fagomine partially counteracted the age- and diet-related losses of Bifidobacteriales in all cases (**PAPERS 1, 2, 3** and **4**) whether in the presence of ω-3 PUFAs or not. ω-3 PUFAs had an effect mainly on Lactobacillales in healthy rats given a standard diet (**PAPER 2**).

Genera: The populations of both *Prevotella* and *Bacteroides* significantly decreased with time (**PAPER 2**) and also in animals fed a high-fat diet (**PAPER 4**). The supplementation with D-fagomine significantly increased the proportion of *Prevotella* in animals fed a standard diet (**PAPER 2**) and partially counteracted the reduction of this genus in rats fed a high-fat diet (**PAPER 4**).

4.3 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on short-chain fatty acids

In WKY rats fed a standard diet, D-fagomine significantly reduced the concentration of excreted acetic and isobutyric acids, and also the total content of excreted SCFAs (**PAPER 1**). In SD rats fed a standard diet, the supplementation with ω-3 PUFAs reduced SCFA content, particularly the concentration of acetic acid, with respect to the other groups (**PAPER 2**).

The concentrations of all SCFAs were lower in rats fed a high-fat diet than in rats fed the standard diet (**PAPER 4**). The major SCFA (acetic acid) was significantly reduced in all groups fed the HF diet except the group supplemented with D-fagomine (**PAPER 4**).

4.4 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on the glycemic status

In WKY rats, plasma fasting glucose levels in the HF group were higher than those in the STD group (**PAPER 3**). D-Fagomine reduced this increase to levels similar to those in the STD group. Plasma fasting insulin was higher in both groups fed the high-fat diet. At the end of the study, insulin levels in the HF group dropped significantly while the group supplemented with D-fagomine still presented significantly higher insulin concentrations than the STD group. The oral glucose tolerance test (OGTT) was performed twice during the study. In the first test, the levels of postprandial glucose in the HF

group were significantly higher than those in the other two groups (STD and HF + FG) 30, 45, and 60 min after administration, with levels higher than 140 mg/dL. By the end of the study, plasma glucose concentrations in the group supplemented with D-fagomine were still lower than those in the HF group, but only significantly lower 30 min after glucose load.

The levels of fasting glucose in SD rats were similar in all supplemented groups and below 80 mg/dL (**PAPER 4**). At the end of the study, the animals given a high-fat diet presented a Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) value higher than those fed the standard diet while the animals supplemented with D-fagomine (HF + FG and HF + FG + ω-3 groups) presented HOMA-IR values similar to those in the STD group.

D-Fagomine had lowering effects on IR and IGT in rats. These metabolic effects were more evident in WKY than in SD rats. ω-3 PUFAs did not counteract the changes induced by the high-fat diet.

4.5 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on biomarkers and lipid mediators of inflammation

In SD rats fed a STD diet, plasma concentration of the ARA-derived lipid mediators hydroxyeicosatetraenoic acids (HETEs) were significantly reduced in the groups supplemented with ω-3 PUFAs with respect to the control group (**PAPER 2**). D-Fagomine by itself only reduced 12HETE.

In WKY rats fed a high-fat diet, the plasma concentration of IL-6 was higher than in animals fed the standard diet (**PAPER 3**). The plasma concentration of PGE2 significantly increased in the HF group compared to the STD group. The levels of pro-inflammatory PGE2 and LTB4 were similar between the STD and HF+FG groups (**PAPER 3**).

4.6 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on liver tissue

The liver sections obtained from WKY rats fed a high-fat diet showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels. In contrast, such infiltration was scarce and smaller in sections from livers pertaining to the group supplemented with D-fagomine (**PAPER 3**).

In SD rats, the livers of animals fed a high-fat diet supplemented or not with D-fagomine (together with soybean oil) presented higher lobular inflammation than those fed the STD diet. The animals

supplemented with ω-3 PUFAs (HF + ω-3 and HF + FG + ω-3 groups) did not present significant lobular inflammation (**PAPER 4**).

4.7 General view of the results

Taken together (**PAPERS 1, 2, 3** and **4**) the effects on microbiota and variables associated to metabolic alterations detected in both WKY and SD rats under either standard or obesogenic diets may be assigned to the iminosugar D-fagomine with some contribution of ω-3 PUFAs via their anti-inflammatory activity.

5 GENERAL DISCUSSION

This thesis focuses on the effects of D-fagomine alone or in combination with ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota and related risk factors for diabetes with the goal of assessing the capacity of these supplements to maintain a healthy status over time under standard or obesogenic diets.

Functional food components are supposed to preserve bodily functions. They may provide health benefits for healthy subjects following a healthy lifestyle as well as for people belonging to risk groups such as obese or pre-diabetic populations. The first part of this thesis explored the effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on rats given a standard diet as a model for healthy subjects (**PAPERS 1** and **2**). The second part explored the effects of the same supplements on rats with fat-induced pre-diabetes as models for people at risk of suffering from diabetes and cardiovascular diseases (**PAPERS 3** and **4**). Significant changes and tendencies recorded in this thesis may give us clues to the putative beneficial effects of D-fagomine alone or in combination with ω-3 PUFAs.

5.1 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on beneficial gut bacteria over time in rats given a standard diet

The composition and activity of the gut microbiota evolves throughout life, from birth to old age and their diversity and balance is fundamental for host health (Nicholson et al., 2012). In humans, age-related differences in gut microbiota composition include an increase in the total number of facultative anaerobe bacteria, mainly Enterobacteriales; and a reduction in the populations of species belonging to the phylum Bacteroidetes, such as *Prevotella*, as well as of the health-promoting Lactobacillales and Bifidobacteriales (Woodmansey, 2007). A decline in the populations of some putatively beneficial bacteria such as *Lactobacillius* and *Bifidobacterium* is a risk factor for the development of many intestinal disorders, including diarrhea, irritable bowel syndrome and inflammatory bowel disease (Gareau et al., 2010). Moreover, bacterial species from these orders are negatively correlated with adiposity, systemic inflammation and obesity (Cani et al., 2007b). The reduction in numbers and species of many beneficial bacteria, such as *Bifidobacterium*, as well as shifts in the dominant bacterial species can help to understand the decreased functionality of the microbiota in elderly people.

One of the goals of this thesis is elucidate the effect of D-fagomine on the gut microbiota in healthy rats over time. Our first results indicate that D-fagomine increased the populations of excreted Bacteroidetes, Bifidobacteriales and Lactobacilliales in rats fed a standard diet over a period of 24

weeks (**PAPER 1**). Then, we corroborated that the populations of Bacteroidetes and *Prevotella* remained stable in rats supplemented with D-fagomine, while they dropped significantly over time in rats not supplemented with the iminosugar (**PAPER 2**). We also tested whether its combination with ω-3 PUFAs (EPA/DHA 1:1) had any additive or complementary effect and our findings indicate that the combination may contribute the host homeostasis by maintaining the relative populations of Bifidobacteriales and Lactobacilliales at levels similar to those of the control group (**PAPER 2**).

D-Fagomine, alone or in combination with ω-3 PUFAs (EPA/DHA 1:1) might contribute to the maintenance of the intestinal health by preserving diversity and mitigating the age-related reduction of some beneficial bacteria. Therefore, the combination between D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) may have an eubiotic effect on the composition of intestinal microbiota that may be complementary to that of prebiotics and probiotics.

5.2 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on gut microbiota in relation to body weight gain in rats given standard or obesogenic diets

As there is mounting evidence that dysbiosis, fat accumulation, glycaemia and weight gain are connected (Schwiertz et al., 2009; Turnbaugh & Gordon, 2009; Turnbaugh et al., 2006) we checked the effects of D-fagomine, ω-3 PUFAs (EPA/DHA 1:1) and their combination on relevant bacterial subgroups. The lean phenotype and the level of functional diversity in the gut microbiome have been associated with increased populations of Bacteroidetes in rodents and humans (Turnbaugh & Gordon, 2009; Turnbaugh et al., 2009; Turnbaugh et al., 2006). Our results indicate that rats supplemented with D-fagomine presented higher Bacteroidetes/Firmicutes ratio than those not supplemented with the iminosugar (**PAPERS 1** and **4**). This is in agreement with the observation that D-fagomine was effective at reducing body weight gain in rats under either standard or obesogenic diets (**PAPERS 2, 3** and **4**). EPA/DHA 1:1 did not significantly affect body weight gain and did not modify the Bacteroidetes/Firmicutes ratio (**PAPERS 2** and **4**).

The proportion of the genus *Prevotella* in gut is directly related to the intake of complex carbohydrates and dietary fiber and to improved glucose tolerance (Kovatcheva-Datchary et al., 2015). Human subjects with high *Prevotella/Bacteroides* ratio appear to be more susceptible to lose body fat on diets high in fiber than subjects with a low *Prevotella*/*Bacteroides* ratio (Hjorth et al., 2018). D-Fagomine also presented a capacity to stabilize the populations of bacteria of the genus *Prevotella* (**PAPERS 2** and **4**). The iminosugar helped to maintain the populations of *Prevotella* in the

intestinal tract of rats fed a standard diet while reducing weight gain (**PAPER 2**), and partially counteracted the high-fat induced reduction of *Prevotella* (**PAPER 4**). ω-3 PUFAs did not have any effect on the populations of *Prevotella* (**PAPER 4**).

It is known that part of the beneficial health effects of a well-balanced gut microbiota is mediated by SCFAs, which are products of microbial fermentation of dietary fiber (den Besten et al., 2013). High-fiber low-fat diets are known to generate high amounts of fecal SCFAs (mainly acetic acid) compared to poor-fiber diets (Filippo et al., 2010). D-Fagomine partially counteracted the decrease of acetic acid and total SCFAs in rats fed the high-fat diet (**PAPER 4**). This effect could be explained by a growth-promoting activity of D-fagomine on SCFA-producing bacteria. We hypothesize that D-fagomine might collaborate with dietary fiber in promoting the growth of beneficial bacteria. This effect is necessarily weak in animals fed the high-fat diet, which is low in fermentable fiber (**PAPER 4**). D-Fagomine reduced the fecal concentration of acetic acid in rats fed the STD diet (**PAPER 1**), and ω-3 PUFAs EPA/DHA 1:1 also reduced the levels of this acid (**PAPER 2**). We hypothesize that the reduction in fecal SCFAs may actually reflect an increase in acetate absorption in the intestinal tract as it has been shown that low levels of acetate in feces are inversely correlated with intestinal absorption (Vogt & Wolever, 2003). This would explain, at least in part, the reduction in pro-inflammatory mediators in the EPA/DHA 1:1 supplemented animals (**PAPER 2**)

Taken together these results are suggesting that D-fagomine might exert a fiber-like action affecting microbiota-related fat accumulation and weight gain. ω-3 PUFAs do not significantly affect these variables while they may contribute an anti-inflammatory effect through SCFA producing bacteria.

5.3 Effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on fat-induced low-grade inflammation and insulin resistance and its possible relationship with gut dysbiosis

As the prevention of diet-induced type-2 diabetes is a pivotal goal of the research project in which this thesis is integrated, **PAPERS 3** and **4** consisted of exploring the effect of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on a rat model of fat-induced pre-diabetes.

A pre-diabetic state was induced to WKY rats (**PAPER 3**) or SD rats (**PAPER 4**) by a HF diet over a period of 24 weeks. Despite all of the information available on the induction of IR and IGT by fat in rat models, the molecular mechanisms behind this action are still largely unknown. First, we observed that SD rats fed a standard diet reach higher body weight than WKY rats while, when fed a high-fat diet, the weight gain of SD rats is lower than that of WKY rats, probably because at the same age SD

rats weigh more than WKY ones. Second, fat induced IR and IGT appear sooner in WKY rats than in SD rats. IR is manifested by elevated plasma insulin levels defined as the first of the five stages of diabetes proposed by Weir and Bonner-Weir for both rats and humans (Weir & Bonner-Weir, 2004). This compensation stage is characterized by increased overall rates of insulin secretion in response to the loss of insulin sensitivity in tissues. The second stage in the diabetes progression is characterized by a loss of β-pancreatic cell mass and disruption of pancreatic function and consequently the levels of fasting insulin drops, while fasting glucose levels are still moderately high (Weir & Bonner-Weir, 2004). The second pre-diabetic state is compatible with the IGT recorded in WKY rats fed the HF diet, which already showed the classic plateau-like pre-diabetic curve (**PAPER 3**). In contrast, SD rats fed the HF diet showed only an incipient tendency to develop IGT evidenced by the postprandial levels of plasma glucose 60 min after glucose load in the OGTT while presenting significantly high fasting plasma insulin and HOMA-IR (**PAPER 4**). This elevated plasma insulin concentration while glucose tolerance is still unaltered characterizes the first step in the development of diabetes (Weir & Bonner-Weir, 2004). The differences between rat strains described here coincide with the results of other authors showing that i) even though SD rats gain more weight than Wistar rats, the differences between the groups given standard and high-fat diets are lower in SD rats and ii) SD rats fed a high-fat diet develop IGT later than Wistar rats do (Marques et al., 2016). Therefore, differences in weight gain and effects on glucose metabolism are harder to detect in SD rats than in WKY rats. This has to be taken into consideration when assessing the efficacies of the supplementation evaluated in the present thesis.

WKY rats fed a high-fat diet supplemented with D-fagomine did not seem to reach the second pre-diabetic stage, as their insulin levels remained high while presenting fasting glucose levels similar to those in animals fed the standard diet and showing reduced IGT compared to high-fat fed rats (stage 1) (**PAPER 3**). In SD rats D-fagomine was effective at delaying stage 1 as plasma insulin and HOMA-IR remained statistically similar to the values in the control group (**PAPER 4**). Its combination with EPA/DHA 1:1 was, at least, as effective as the single supplementation. The postprandial glucose levels 60 min after glucose load in the OGTT in the HF + FG + ω-3 group presented values in similar to those in the STD group (**PAPER 4**). The evidence presented here (**PAPERS 3** and **4**), together with other previous observations in our group (Molinar-Toribio et al., 2015; Ramos-Romero et al., 2014) shows that rats supplemented with D-fagomine always seem to be one step behind in the development of diet-induced pre-diabetes.

We also explored possible mechanisms by which D-fagomine might exert this functional effect. Three mechanisms have been proposed to explain the pathogenesis of IR in different organs: ectopic lipid accumulation, endoplasmic reticulum stress and systemic inflammation (Samuel & Shulman, 2012). The first mechanism has been linked to the action of diacylglycerols (DAGs), which are intermediates of lipid metabolism with the capacity to interrupt the translocation of the glucose transporter GLUT4 to the cell membrane (Samuel & Shulman, 2012) (See Figure 4 in the Introduction). IR in our WKY model does not seem to be triggered by DAG-mediated impairment of insulin signaling, as the levels of DAGs in adipose tissue, liver, and muscle were not higher in high-fat fed animals (**PAPER 3**). Also, liver histology supported the hypothesis that the lipid-mediated loss of insulin sensitivity was not a triggering factor of the pre-diabetic stage, as significant steatosis was not detected (**PAPER 3**). In contrast, high-fat fed WKY rats presented strong lymphocyte infiltration indicating inflammation around the blood vessels in the liver and they also presented elevated levels of pro-inflammatory interleukin-6 (IL-6) and PGE2. Thus, all these results suggest that IR in our WKY model is probably triggered by systemic low-grade inflammation.

We then asked how D-fagomine may counteract fat-induced low-grade inflammation. There is mounting evidence that dysbiosis and intestinal barrier dysfunction may be the first steps leading to dietary fat-induced chronic systemic low-grade inflammation and IR (Duan et al., 2018). An excess of gram-negative bacteria such as *E. coli* and other Enterobacteriales is known to induce endotoxemia and low-grade inflammation in the host through disruption of the intestinal barrier properties and release of pro-inflammatory molecules, such as LPS, into the bloodstream (Cani et al., 2009). In contrast, *Bifidobacterium* species have been shown to protect the gut barrier function by preserving mucosal permeability and preventing translocation of pathogenic Enterobacteriales, such as *E. coli* (Caplan et al., 1999). As bacterial toxicity may be the main cause of fat-induced low-grade inflammation, we hypothesized that D-fagomine might exert its anti-inflammatory action by balancing the populations of these two related mucosa-associated orders and fostering gut colonization with *Bifidobacterium* species.

Our results show that D-fagomine consistently increased the populations of excreted Bifidobacteriales under either standard or obesogenic diets (**PAPERS 1, 2, 3** and **4**). D-Fagomine reduced the excreted populations of potentially harmful Enterobacteriales in pre-diabetic high-fat fed rats (**PAPERS 3** and **4**). *Bifidobacterium* spp. have been shown to protect the gut barrier function by preserving mucosal permeability and preventing translocation of pathogenic Enterobacteriales,

such as *E. coli* (Caplan et al., 1999). As bacterial activation of the host's immune system may be the main cause fat-induced low-grade inflammation, D-fagomine might be exerting an anti-inflammatory action by fostering gut colonization with *Bifidobacterium* species. D-Fagomine might be contributing to the activity of Bifidobacteriales against putatively harmful Enterobacteriales by reducing their capacity to colonize the intestine. This hypothesis is supported by a previous study, which shows that D-fagomine inhibits the adhesion of *E. coli*, but not of *Bifidobacterium* species to the intestinal mucosa (Gómez et al., 2012). Fimbriated strains such most members of Enterobacteriales express mannose-specific lectins in the tip of their fimbriae that bind to membrane carbohydrates. As D-fagomine is structurally similar to lectin-binding sugars such as mannose, it may be hampering bacterial adhesion by blocking lectins.

In conclusion, D-fagomine delays the progression of pre-diabetes. This activity could be explained by its action balancing the populations of Bifidobacteriales and Enterobacteriales and consequently improving the gut barrier function, reducing the translocation of bacteria and endotoxins that are capable of inducing systemic inflammation and IR.

5.4 Influence of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on inflammation markers

As IR is probably triggered by systemic low-grade inflammation in our models, we explored the contribution of D-fagomine and the well-known anti-inflammatory compounds ω-3 PUFAs (EPA/DHA 1:1) to reducing systemic inflammation. The inflammatory status is another variable that may be influenced by age and dietary habits. Inflammation is part of the normal host response to infection and injury. However, excessive or inappropriate inflammation contributes to a range of acute and chronic diseases and is characterized by the production of inflammatory cytokines, ARA-derived eicosanoids and other inflammatory agents such as ROS. Our results show that D-fagomine reduced the levels of some pro-inflammatory secondary metabolites from the oxidation of ARA (HETEs) in rats fed a standard diet (**PAPER 2**) as well as, the levels of other systemic inflammatory markers such as IL-6 and its related eicosanoid PGE2 in rats fed a high-fat diet (**PAPER 3**). These results, together with the fact that no differences were detected in the levels on anti-inflammatory EPA and DHA-derived eicosanoids and docosanoids (**PAPER 3**), is suggesting that D-fagomine exerted its functional effect early in the progression of low-grade inflammation by precluding the formation of pro-inflammatory signals rather than by activating anti-inflammatory pathways. This preventative anti-inflammatory effect of D-fagomine may be connected to its balancing effect of gut microbiota. The possible implication of D-fagomine in the physiological pathways connecting microbiota, low-grade

inflammation and IR should be further explored in future research. The role of EPA and DHA as anti-inflammatory agents may be complementary to that of D-fagomine as they have been shown to be effective on already declared inflammatory processes. EPA and DHA compete with the pro-inflammatory ARA for common oxygenases with the result of favouring the synthesis of anti-inflammatory lipid mediators such as resolvins (Calder, 2013) (for a review on the actions of ω-6 and ω-3 PUFAs see (Innes & Calder, 2018)) (Figures 10 and 11 in the Introduction). In agreement with this we found lower levels of ARA-derived pro-inflammatory lipid mediators in rats fed a standard diet and supplemented with EPA/DHA 1:1 while D-fagomine did not show any effect on most of the lipid intermediates tested (**PAPER 2**).

The complementary or additive effect of the two supplementations on low-grade inflammation was evidenced in the last manuscript of this thesis (**PAPER 4**). In contrast with the results on high-fat fed WKY rats, we observed that D-fagomine did not have any effect on the body weight of high-fat fed SD rats when administered alone while it showed a tendency to reduce body weight when combined D-fagomine and ω-3 PUFAs. One possible explanation for the lack of effect of D-fagomine on high-fat fed SD rats is that the animals not supplemented with ω-3 PUFAs (STD, HF and HF+FG groups) were administered an equivalent dose of soy bean oil to compensate for both the stress of probing and the excess of calories. Soy bean oil, which is rich in linoleic acid (LA) might have counteracted the putative microbiota-related anti-inflammatory effect of D-fagomine. LA is a ω-6 PUFA that may increase the levels of inflammatory prostaglandins and cytokines by entering the ARA metabolic pathway (for a review see (Naughton et al., 2016) (Figures 10 and 11 in the Introduction). This will not happen in the case of ω-3 PUFA supplementation as EPA and DHA are converted into less inflammatory and even anti-inflammatory metabolites. Therefore, the anti-inflammatory effect of D-fagomine would be reinforced by the ω-3 PUFAs (EPA/DHA 1:1) in the doubly supplemented group. These events would occur downstream the fat-induced microbial-triggered toxicity.

The results of this thesis together with our previous reports point clearly towards a functional role for D-fagomine in the maintenance of the intestinal health by preserving gut microbial diversity and mitigating the age-related reduction of some beneficial bacteria as well as in the prevention of risk factors for diet-induced pre-diabetes reinforced by the action of ω-3 PUFAs by complementary mechanisms.

6 CONCLUSIONS

In healthy animals:

- D-Fagomine stabilized the population of Bacteroidetes, reduced the loss of Lactobacilliales and Bifidobacteriales, and *Prevotella* with time and reduced the levels of some excreted SCFAs in rats given a standard diet. ω-3 PUFAs did not present a clear effect on gut microbiota on rats under the same conditions.

- D-Fagomine reduced weight gain when administered either alone or together with ω-3 PUFAs (EPA/DHA 1:1) in SD rats given a standard diet. This effect was not detected in WKY rats.

- The combination between D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) reduced the levels of plasma ARA-derived pro-inflammatory lipid mediators (HETEs) in rats given a standard diet.

- D-Fagomine and ω-3 PUFAs (EPA/DHA 1:1) showed complementary effects in rats given a standard diet: reduction of weight gain, stabilization of putatively beneficial gut bacteria, and reduction of pro-inflammatory mediators.

In animals with pre-diabetes:

- D-Fagomine counteracted IGT and maintained the levels of plasma insulin in high-fat fed rats.

- D-Fagomine counteracted fat-induced systemic low-grade inflammation in rats as monitored by liver histology (lymphocyte infiltration, lobular inflammation) and by measuring plasma ARA-derived mediators of inflammation, notably PGE2 and LTB4.

- D-Fagomine showed a tendency to counteract the changes induced by a high-fat diet in the populations of gut Bifidobacteriales and Enterobacteriales in rats.

- D-Fagomine showed a tendency to reduce weight gain either alone or together with ω-3 PUFAs (EPA/DHA 1:1) in rats given a high-fat diet.

- The combination between D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) counteracted incipient IR, and liver lobular inflammation in rats given a high-fat diet.

- The combination between D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) increased the cecal content, the Bacteroidetes/Firmicutes ratio as well as the populations of gut Bifidobacteriales and Lactobacilliales in rats given a high-fat diet.

The general picture resulting from this thesis is that the functional effects of D-fagomine and ω-3 PUFAs (EPA/DHA 1:1) on rats fed either standard or high-fat diets consist mainly on delaying low-grade inflammation and its consequences, notably insulin resistance. D-Fagomine may exert its anti-inflammatory activity at a very early stage by maintaining a well-balanced gut microbiota. EPA/DHA 1:1 may contribute its competition with pro-inflammatory ARA for common enzymes in their metabolic pathways as well as an effect on some beneficial bacteria.

7 REFERENCES

Alakomi, H. L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology, 66*(5), 2001-2005.

Amézqueta, S., Galán, E., Vila-Fernández, I., Pumarola, S., Carrascal, M., Abian, J., . . . Lluís Torres, J. (2013). The presence of D-fagomine in the human diet from buckwheat-based foodstuffs. *Food Chemistry, 136*(3-4), 1316-1321.

Amézqueta, S., & Torres, J. L. (2016). Advances in the analysis of iminocyclitols: Methods, sources and bioavailability. *Talanta, 151*, 157-171.

Asano, N. (2003a). Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology, 13*(10), 93R-104R.

Asano, N. (2003b). Naturally occurring iminosugars and related compounds: structure, distribution, and biological activity. *Current Topics in Medicinal Chemistry, 3*(5), 471-484.

Asano, N., Kato, A., Miyauchi, M., Kizu, H., Tomimori, T., Matsui, K., . . . Molyneux, R. J. (1997). Specific alpha-galactosidase inhibitors, N-methylcalystegines--structure/activity relationships of calystegines from Lycium chinense. *European Journal of Biochemistry, 248*(2), 296-303.

Asano, N., Kizu, H., Oseki, K., Tomioka, E., Matsui, K., Okamoto, M., & Baba, M. (1995). N-alkylated nitrogen-in-the-ring sugars: conformational basis of inhibition of glycosidases and HIV-1 replication. *Journal of Medicinal Chemistry, 38*(13), 2349-2356.

Asano, N., Yamashita, T., Yasuda, K., Ikeda, K., Kizu, H., Kameda, Y., . . . Ryu, K. S. (2001). Polyhydroxylated alkaloids isolated from mulberry trees (Morusalba L.) and silkworms (Bombyx mori L.). *Journal of Agricultural and Food Chemistry, 49*(9), 4208-4213.

Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., . . . Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America, 101*(44), 15718-15723.

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science, 307*(5717), 1915-1920.

Backhed, F., Manchester, J. K., Semenkovich, C. F., & Gordon, J. I. (2007). Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proceedings of the National Academy of Sciences of the United States of America, 104*(3), 979-984.

Berkowitz, A. (2007). *Clinical Pathophysiology Made Ridiculously Simple*. Miami, USA: MedMaster.

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., & Pini, E. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PloS One, 5*(5), e10667.

Biesalski, H. K., Dragsted, L. O., Elmadfa, I., Grossklaus, R., Muller, M., Schrenk, D., . . . Weber, P. (2009). Bioactive compounds: definition and assessment of activity. *Nutrition, 25*(11-12), 1202-1205.

Blandino, G., Inturri, R., Lazzara, F., Di Rosa, M., & Malaguarnera, L. (2016). Impact of gut microbiota on diabetes mellitus. *Diabetes & Metabolism, 42(5),* 303-315.

Brashers, V. (2006). *Clinical Applications of Pathophysiology. An Evidence-Based Approach* (3 ed.). Philadelphia, USA: Elsevier.

Butters, T. D., Dwek, R. A., & Platt, F. M. (2005). Imino sugar inhibitors for treating the lysosomal glycosphingolipidoses. *Glycobiology, 15*(10), 43R-52R.

Caesar, R., Tremaroli, V., Kovatcheva-Datchary, P., Cani, P. D., & Backhed, F. (2015). Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. *Cell Metabolism, 22*(4), 658-668.

Calder, P. C. (2004). n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clinical Science, 107*(1), 1-11.

Calder, P. C. (2006). n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *The American Journal of Clinical Nutrition, 83*(6 Suppl), 1505S-1519S.

Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *British Journal of Clinical Pharmacology, 75*(3), 645-662.

Calder, P. C., & Yaqoob, P. (2009). Understanding omega-3 polyunsaturated fatty acids. *Postgraduate Medicine, 121*(6), 148-157.

Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature reviews. Endocrinology, 11*(10), 577-591.

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., . . . Burcelin, R. (2007a). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes, 56*(7), 1761-1772.

Cani, P. D., Bibiloni, R., Knauf, C., Neyrinck, A. M., Neyrinck, A. M., Delzenne, N. M., & Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes, 57*(6), 1470-1481.

Cani, P. D., & Delzenne, N. M. (2011). The gut microbiome as therapeutic target. *Pharmacology & Therapeutics, 130*(2), 202-212.

Cani, P. D., Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R. G., Tuohy, K. M., . . . Delzenne, N. M. (2007b). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia, 50*(11), 2374-2383.

Cani, P. D., Possemiers, S., Van de Wiele, T., Guiot, Y., Everard, A., Rottier, O., . . . Delzenne, N. M. (2009). Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut, 58*(8), 1091-1103.

Caplan, M. S., Miller-Catchpole, R., Kaup, S., Russell, T., Lickerman, M., Amer, M., . . . Thomson, R., Jr. (1999). Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterology, 117*(3), 577-583.

Castillo, J. A., Calveras, J., Casas, J., Mitjans, M., Vinardell, M. P., Parella, T., . . . Clapes, P. (2006). Fructose-6-phosphate aldolase in organic synthesis: preparation of D-fagomine, N-alkylated derivatives, and preliminary biological assays. *Organic Letters, 8*(26), 6067-6070.

Chen, T., Long, W., Zhang, C., Liu, S., Zhao, L., & Hamaker, B. R. (2017). Fiber-utilizing capacity varies in Prevotella- versus Bacteroides-dominated gut microbiota. *Scientific Reports, 7*, 2594.

Cummings, J. H., & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *The Journal of Applied Bacteriology, 70*(6), 443-459.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., . . . Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America, 107*(33), 14691-14696.

de la Serre, C. B., Ellis, C. L., Lee, J., Hartman, A. L., Rutledge, J. C., & Raybould, H. E. (2010). Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *American Journal of Physiology Gastrointestinal and Liver Physiology, 299*(2), G440-448.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research, 54*(9), 2325-2340.

Duan, Y., Zeng, L., Zheng, C., Song, B., Li, F., Kong, X., & Xu, K. (2018). Inflammatory Links Between High Fat Diets and Diseases. *Frontiers in Immunology, 9*, 2649.

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., . . . Gordon, J. I. (2013). The long-term stability of the human gut microbiota. *Science, 341*(6141), 1237439.

Favier, C. F., Vaughan, E. E., De Vos, W. M., & Akkermans, A. D. (2002). Molecular monitoring of succession of bacterial communities in human neonates. *Applied and Environmental Microbiology, 68*(1), 219-226.

Feller, S. E., & Gawrisch, K. (2005). Properties of docosahexaenoic-acid-containing lipids and their influence on the function of rhodopsin. *Current Opinion in Structural Biology, 15*(4), 416-422.

Filippo, C., Cavalieri, D., Paola, M., Ramazzotti, M., Poullet, J. B., & Massart, S. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America, 107*(33), 14691-14696.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes, 3*(4), 289-306.

Foulis, A. K., McGill, M., & Farquharson, M. A. (1991). Insulitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes, and interferon-gamma containing cells. *The Journal of Pathology, 165*(2), 97-103.

Gareau, M. G., Sherman, P. M., & Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature reviews. Gastroenterology & Hepatology, 7*(9), 503-514.

Gómez, L., Molinar-Toribio, E., Calvo-Torras, M. Á., Adelantado, C., Juan, M. E., Planas, J. M., . . . Torres, J. L. (2012). D-Fagomine lowers postprandial blood glucose and modulates bacterial adhesion. *British Journal of Nutrition, 107*(12), 1739-1746.

Hildebrandt, M. A., Hoffmann, C., Sherrill-Mix, S. A., Keilbaugh, S. A., Hamady, M., Chen, Y. Y., . . . Wu, G. D. (2009). High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology, 137*(5), 1716-1724.

Hjorth, M. F., Roager, H. M., Larsen, T. M., Poulsen, S. K., Licht, T. R., Bahl, M. I., . . . Astrup, A. (2018). Pre-treatment microbial Prevotella-to-Bacteroides ratio, determines body fat loss success during a 6-month randomized controlled diet intervention. *International Journal of Obesity, 42*(3), 580-583.

Hopkins, M. J., & Macfarlane, G. T. (2002). Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. *Journal of Medical Microbiology, 51*(5), 448-454.

Hopkins, M. J., Sharp, R., & Macfarlane, G. T. (2001). Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. *Gut, 48*(2), 198-205.

Innes, J. K., & Calder, P. C. (2018). Omega-6 fatty acids and inflammation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 132*, 41-48.

Inouye, S., Tsuruoka, T., & Nida, T. (1966). The structure of nojirimycin, a piperidinose sugar antibiotic. *The Journal of Antibiotics, 19*(6), 288-292.

Janssen, A. W., & Kersten, S. (2017). Potential mediators linking gut bacteria to metabolic health: a critical view. *The Journal of Physiology, 595*(2), 477-487.

Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *The Journal of Clinical Investigation, 106*(4), 473-481.

Kampmann, U., Madsen, L. R., Skajaa, G. O., Iversen, D. S., Moeller, N., & Ovesen, P. (2015). Gestational diabetes: A clinical update. *World Journal of Diabetes, 6*(8), 1065-1072.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Reviews. Microbiology, 2*(2), 123-140.

Kovatcheva-Datchary, P., Nilsson, A., Akrami, R., Lee, Y. S., De Vadder, F., Arora, T., . . . Backhed, F. (2015). Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. *Cell Metabolism, 22*(6), 971-982.

Koyama, M., & Sakamura, S. (1974). The structure of a new piperidine derivative from buckwheat seeds (*Fagopyrum esculentum* Moench). *Agricultural and Biological Chemistry, 38*(5), 1111-1112.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America, 102*(31), 11070-11075.

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature, 444*(7122), 1022-1023.

Lou, D.-S., Zou, F.-M., Yan, H., & Gui, Z.-Z. (2011). Factors influencing the biosynthesis of 1-deoxynojirimycin in *Morus alba* L. *African Journal of Agricultural Research, 6*(13), 2998-3006.

Mariat, D., Firmesse, O., Levenez, F., Guimarăes, V., Sokol, H., & Doré, J. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology, 9, 123*.

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., . . . Calhau, C. (2016). High-fat diet-induced obesity rat model: a comparison between Wistar and Sprague-Dawley rat. *Adipocyte, 5*(1), 11-21.

McEwen, B. J., Morel-Kopp, M. C., Chen, W., Tofler, G. H., & Ward, C. M. (2013). Effects of omega-3 polyunsaturated fatty acids on platelet function in healthy subjects and subjects with cardiovascular disease. *Seminars in Thrombosis and Hemostasis, 39*(1), 25-32.

Melmed, S., Polonsky, K., Larsen, P., & Kronenberg, H. (2017). *Williams Tratado de Endocrinologia* (13 ed.). Madrid, Spain: Elsevier.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Gómez, L., Taltavull, N., Nogués, M. R., . . . Torres, J. L. (2015). D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food & Function, 6*(8), 2614-2619.

Naughton, S. S., Mathai, M. L., Hryciw, D. H., & McAinch, A. J. (2016). Linoleic acid and the pathogenesis of obesity. *Prostaglandins & Other Lipid Mediators, 125*, 90-99.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science, 336*(6086), 1262-1267.

Pérez-Escamilla, R., Obbagy, J. E., Altman, J. M., Essery, E. V., McGrane, M. M., Wong, Y. P., . . . Williams, C. L. (2012). Dietary energy density and body weight in adults and children: a systematic review. *Journal of the Academy of Nutrition and Dietetics, 112*(5), 671-684.

Portune, K. J., Benítez-Páez, A., Del Pulgar, E. M., Cerrudo, V., & Sanz, Y. (2017). Gut microbiota, diet, and obesity-related disorders-The good, the bad, and the future challenges. *Molecular Nutrition & Food Research, 61*(1).

Ramos-Romero, S., Molinar-Toribio, E., Gómez, L., Pérez-Jiménez, J., Casado, M., Clapés, P., . . . Torres, J. L. (2014). Effect of D-fagomine on excreted Enterobacteria and weight gain in rats fed a high-fat high-sucrose diet. *Obesity, 22*(4), 976-979.

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., . . . Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. *The British Journal of Nutrition, 104 Suppl 2*, S1-63.

Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. *Life Sciences, 203*, 255-267.

Salzman, N. H., Underwood, M. A., & Bevins, C. L. (2007). Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. *Seminars in Immunology, 19*(2), 70-83.

Samuel, V. T., & Shulman, G. I. (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell, 148*(5), 852-871.

SanGiovanni, J. P., & Chew, E. Y. (2005). The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Progress in Retinal and Eye Research, 24*(1), 87-138.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2009). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity, 18*(1), 190-195.

Stubbs, C. D., & Smith, A. D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica et Biophysica Acta, 779*(1), 89-137.

Swann, J. R., Want, E. J., Geier, F. M., Spagou, K., Wilson, I. D., Sidaway, J. E., . . . Holmes, E. (2011). Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proceedings of the National Academy of Sciences of the United States of America, 108 Suppl 1*, 4523-4530.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in Immunology, 121*, 91-119.

Thiruvoipati, T., Kielhorn, C. E., & Armstrong, E. J. (2015). Peripheral artery disease in patients with diabetes: Epidemiology, mechanisms, and outcomes. *World Journal of Diabetes, 6*(7), 961-969.

Turnbaugh, P. J., & Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity. *The Journal of Physiology, 587*(17), 4153-4158.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., . . . Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature, 457*(7228), 480-484.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature, 444*(7122), 1027-1031.

Vogt, J. A., & Wolever, T. M. (2003). Fecal acetate is inversely related to acetate absorption from the human rectum and distal colon. *The Journal of Nutrition, 133*(10), 3145-3148.

Walker, R. W., Clemente, J. C., Peter, I., & Loos, R. J. F. (2017). The prenatal gut microbiome: are we colonized with bacteria in utero? *Pediatric Obesity, 12 Suppl 1*, 3-17.

Watson, A. A., Fleet, G. W., Asano, N., Molyneux, R. J., & Nash, R. J. (2001). Polyhydroxylated alkaloids -- natural occurrence and therapeutic applications. *Phytochemistry, 56*(3), 265-295.

Weir, G. C., & Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes, 53*, S16-S21.

WHO. (2000). Obesity: Preventing and Managing the Global Epidemic. World Health Organization, Geneva.

WHO. (2016). Global report on diabetes. World Health Organization, Geneva.

Wiktorowska-Owczarek, A., Berezinska, M., & Nowak, J. Z. (2015). PUFAs: Structures, metabolism and functions. *Advances in Clinical and Experimental Medicine, 24*(6), 931-941.

Winchester, B. G. (2009). Iminosugars: from botanical curiosities to licensed drugs. *Tetrahedron: Asymmetry, 20*(6), 645-651.

Woodmansey, E. J. (2007). Intestinal bacteria and ageing. *Journal of Applied Microbiology, 102*(5), 1178-1186.

Woodmansey, E. J., McMurdo, M. E., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiology, 70*(10), 6113-6122.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., . . . Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science, 334*(6052), 105-108.

8 ANNEXES

8.1 OTHER PUBLICATIONS

8.1.1 PAPER A1

TITLE: Effects of the combination of ω-3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats

AUTHORS: Sara Ramos-Romero, **Mercè Hereu**, Eunice Molinar-Toribio, María Pilar Almajano, Lucía Méndez, Isabel Medina, Núria Taltavull, Marta Romeu, Maria Rosa Nogués and Josep Lluís Torres

JOURNAL: Food Research International, 2017, 97, 364-371

IMPACT FACTOR (2017): 3.52

CLASSIFICATION AND CATEGORY: FOOD SCIENCE & TECHNOLOGY 14/133 (Q1)

TIMES CITED: 7

REFERENCE: Ramos-Romero, S., Hereu, M., Molinar-Toribio, E., Almajano, M. P., Méndez, L., Medina, I., Taltavull, N., Romeu, M., Nogués, M. R. & Torres, J. L. (2017). Effects of the combination of ω-3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats. *Food Research International*, 97, 364-371.

Food Research International 97 (2017) 364–371

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effects of the combination of ω-3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats

Sara Ramos-Romero[a,*], Mercè Hereu[a], Eunice Molinar-Toribio[a,1], María Pilar Almajano[b], Lucía Méndez[c], Isabel Medina[c], Núria Taltavull[d], Marta Romeu[d], Maria Rosa Nogués[d], Josep Lluís Torres[a]

[a] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain
[b] Chemical Engineering Department, Technical University of Catalonia, Barcelona, Spain
[c] Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain
[d] Faculty of Medicine and Health Science, Universitat Rovira i Virgili, Reus, Spain

ARTICLE INFO

Keywords:
Microbiota, proanthocyanidins
EPA/DHA
Lactobacillus
Bifidobacterium

ABSTRACT

ω-3 Polyunsaturated fatty acids (PUFAs) reduce risk factors for cardiovascular diseases (CVD) and other pathologies that involve low-grade inflammation. They have recently been shown to exert complementary functional effects with proanthocyanidins. As the reduction of health-promoting gut bacteria such as lactobacilli and bifidobacteria has been linked to a number of alterations in the host, the aim of this study was to determine whether PUFAs and proanthocyanidins also cooperate in maintaining well-balanced microbiota. To this end, rats were supplemented for 6 months with eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) 1:1 (16.6 g/kg feed); proanthocyanidin-rich grape seed extract (GSE, 0.8 g/kg feed); or both. Plasma adiponectin, cholesterol, and urine nitrites were measured. Gut bacterial subgroups were evaluated in fecal DNA by qRT-PCR. Short-chain fatty acids (SCFAs) were determined in feces by gas chromatography. Body and adipose tissue weights were found to be higher in the animals given ω-3 PUFAs, while their energy intake was lower. Plasma cholesterol was lower in ω-3 PUFA supplemented groups, while adiponectin and urine nitrites were higher. ω-3 PUFAs reduced the population of Lactobacillales and *L. acidophilus* after 6 months of supplementation. GSE significantly reduced *L. plantarum* and *B. longum*. The combination of ω-3 PUFAs and GSE maintained the health-promoting bacteria at levels similar to those of the control group. Acetic acid was increased by the ω-3 PUFA individual supplementation, while the combination with GSE kept this value similar to the control value. In conclusion, while individual supplementations with ω-3 PUFAs or GSE modify the populations of Lactobacillus, Bifidobacterium and microbial products (SCFAs), their combination maintains the standard proportions of these bacterial subgroups and their function while also providing the cardiovascular benefits of ω-3 PUFAs.

1. Introduction

ω-3 Polyunsaturated fatty acids (ω-3 PUFAs) and polyphenols are bioactive compounds present in common foodstuffs (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011; Tapiero, Ba, Couvreur, & Tew, 2002). Eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are the major ω-3 PUFAs of marine origin. Different ω-3 PUFAs may have different effects, many of them related to the prevention of cardiovascular diseases (CVD). We recently showed that mixtures of EPA and DHA at different proportions have different effects on CVD risk factors in rats: EPA/DHA 1:1 and 2:1 decrease inflammation, while oxidative stress is more effectively reduced by a 1:2 mixture in obese hypertense rats (Molinar-Toribio et al., 2015). The 1:1 mixture was the most effective at reducing protein carbonylation (Méndez et al., 2013) and CVD risk markers (Lluís et al., 2013) in rats fed a standard diet. The molecular mechanism through which ω-3 PUFAs exert their preventive effects include shift of lipids from the ω-6 to the ω-3 metabolic pathway and the modulation of genes associated with both lipid catabolism and anabolism (Poudyal, Panchal, Diwan, & Brown, 2011). Consequently, ω-3 PUFAs help maintain the

* Corresponding author at: Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona, 18-26, 08034 Barcelona, Spain.
E-mail addresses: sara.ramos@iqac.csic.es (S. Ramos-Romero), merce.hereu@iqac.csic.es (M. Hereu), eice7@hotmail.com (E. Molinar-Toribio), m.pilar.almajano@upc.edu (M.P. Almajano), luciamendez@iim.csic.es (L. Méndez), medina@iim.csic.es (I. Medina), nuria.taltavull@urv.cat (N. Taltavull), marta.romeu@urv.cat (M. Romeu), mariarosa.nogues@urv.cat (M.R. Nogués), josepllluis.torres@iqac.csic.es (J.L. Torres).
[1] Present address: Department of Biochemistry, Faculty of Natural Sciences, Exact and Technology, University of Panama, Panama City, Panama

http://dx.doi.org/10.1016/j.foodres.2017.04.024
Received 20 February 2017; Received in revised form 6 April 2017; Accepted 23 April 2017
Available online 25 April 2017

normal levels of systemic biomarkers of CVD, such as plasma cholesterol and triglycerides, oxidative stress, and blood pressure (Poudyal et al., 2011). Despite hypertension and other CVD risk factors having been linked to gut dysbiosis (Yang et al., 2015) and the possibility that they may be averted by probiotics (Miremadi, Sherkat, & Stojanouska, 2016), the effect of ω-3 PUFAs on gut microbiota has hardly been studied. Studies of the effects of ω-3 PUFAs on microbiota have mainly focused on the major bacterial phyla Bacteroidetes and Firmicutes. ω-3 PUFAs from flaxseed seem to decrease the proportion of Bacteroidetes (Liu, Hougen, Vollmer, & Hiebert, 2012), and those from fish oil lower the population of Firmicutes (Yu, Zhu, Pan, Shen, Shan, & Das, 2014). A reduction in the Bacteroidetes/Firmicutes ratio has been linked to weight gain and other metabolic conditions, such as insulin resistance, in part by the synthesis of short-chain fatty acids (SCFAs) (Canfora, Jocken, & Blaak, 2015): end products of the fermentation of indigestible dietary components of the host diet.

Polyphenols are a family of phytochemicals and are widespread in plant foods; they have a common structure including at least two phenol groups (Bravo, 1998; Quideau et al., 2011). Dietary polyphenols and their metabolites exert a beneficial effect through a combination of mechanisms that may include the reduction of inflammation and oxidative stress (Pan, Lai, & Ho, 2010; Salvadó, Casanova, Fernández-Iglesias, Arola, & Bladé, 2015), as well as inhibition of intestinal glycosidases and of glucose transporters that reduce postprandial glycaemia (Williamson, 2013). Moreover, polyphenols can modulate the intestinal microbial composition and modify the metabolic activity of gut bacteria in humans (Duda-Chodak, 2012). Proanthocyanidins are oligomeric and polymeric flavan-3-ols composed mainly of (*epi*)catechin and its gallic acid esters. (−)-epicatechin and (+)-catechin from tea and cocoa protect commensal anaerobes and probiotics such as *Bifidobacterium* spp. and *Lactobacillus* spp., and they also inhibit potential pathogenic bacteria in humans (Lee, Jenner, Low, & Lee, 2006). Similarly, flavan-3-ols from grape products reduce the growth of *Clostridium* spp., while increasing the populations of other subgroups such as Lactobacillus, in rats (Dolara et al., 2005). This effect on Lactobacillus as well as on other groups such as Bifidobacterium has also been reported in humans after daily intake of red wine for one month (Queipo-Ortuño et al., 2012).

It has recently been shown that ω-3 PUFAs and proanthocyanidins exert collaborative functional effects such as a reduction of plasma insulin, leptin, and perigonadal fat accumulation in obese rats (Ramos-Romero et al., 2016), and decrease C-reactive protein concentration in rats with hypercholesterolemia (Sekhon-Loodu et al., 2014). As alterations in these variables have been associated with a reduction in health-promoting gut bacteria such as lactobacilli and bifidobacteria (Arboleya, Watkins, Stanton, & Ross, 2016; Cani et al., 2007; Guardamagna et al., 2014), we decided to study whether ω-3 PUFAs and proanthocyanidins also cooperate in maintaining the levels of putatively beneficial gut bacteria. Thus, the aim of this study is to explore the possible complementary function of a combination of dietary ω-3 PUFAs (EPA/DHA 1:1) and proanthocyanidins in a grape seed extract (GSE) on the proportions of health-promoting bacteria in rats.

2. Materials and methods

2.1. Animals

A total of twenty-eight female Wistar-Kyoto rats from Charles River Laboratories (Wilmington, MA, USA), aged 8–10 weeks, were used. Female rats were chosen for consistency with our previous studies of ω-3 PUFAs (Lluís et al., 2013; Molinar-Toribio et al., 2015; Ramos-Romero et al., 2016; Taltavull et al., 2014). All animal handling was carried out in the morning to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines (EU Directive 2010/63/EU) for the care and management of laboratory animals, and were approved by the CSIC Subcommittee for Bioethical Issues (reference no. CEEA-12-007).

Table 1
Composition of experimental diets.

Ingredients (g)	STD	ω-3	GSE	ω-3 + GSE
Flour[a]	1000.0	1000.0	1000.0	1000.0
tert-butylhydroquinone	0.08	0.08	0.08	0.08
Porcine gelatin	25.0	25.0	25.0	25.0
Soybean lecithin	6.0	6.0	6.0	6.0
Soybean oil	17.4	–	17.4	–
ω-3 PUFAs[b]	–	17.4	–	17.4
Grajfnol®	–	–	0.88	0.88
Protein (% by weight)	16.4	16.4	16.4	16.4
Carbohydrate (% by weight)	46.6	46.6	46.5	46.5
Fat (% by weight)	6.2	6.2	6.2	6.2
Energy from protein (%)	21.3	21.3	21.3	21.3
Energy from carbohydrate (%)	60.5	60.5	60.5	60.5
Energy from fat (%)	18.2	18.2	18.2	18.2
Total energy density (kcal/g)[c]	3.1	3.1	3.1	3.1

[a] Standard flour (Teklad Global 2014).
[b] The amount of EPA/DHA was 25 mg/kg body weight.
[c] Energy density is estimated as *metabolizable energy* based on the Atwater factors, assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

2.2. Experimental design

The rats were housed two to three per cage under controlled conditions of humidity (60%), and temperature (22 °C ± 2 °C) with a 12 h light-12 h dark cycle. The rats were randomly divided into 4 dietary groups (n = 7/group): STD, the control group; ω-3, a group supplemented with EPA/DHA 1:1 (16.6 g/kg feed); GSE, a group supplemented with 0.84 g GSE/kg feed; and ω-3 + GSE, a group supplemented with both EPA/DHA 1:1 and GSE. The experimental diets were pelletized in-house by lyophilization from frozen emulsions. To prevent oxidation and contamination by fungi, the dry pellets were vacuum-packed and stored at 4 °C until use. To ensure that all the diets were isocaloric, appropriate amounts of soybean oil was added to the feed preparations that were not supplemented with ω-3 PUFAs. To guarantee a proper mixture of the different components and an adequate consistency of the final pellet, soybean lecithin and porcine gelatin were added. The feed compositions are shown in Table 1. Following the reported conversion of animal doses into human equivalent doses (Reagan-Shaw, Nihal, & Ahmad, 2008), the ω-3 PUFA supplementation was equivalent to 0.15 mL ω-3 PUFAs/kg human and day; and GSE supplementation was equivalent to 6.7–9.5 mg GSE/kg human and day. The standard diet Global 2014 was from Harlan Teklad Inc. (Indianapolis, IN, USA). Porcine gelatin type A 240/60 was from Juncà (Girona, Spain). Soybean lecithin Topcithin 50™ was from Cargill (Barcelona, Spain). EPA/DHA 1:1 was obtained by mixing appropriate quantities of the commercial fish oils AFAMPES 121 EPA (A.F.A.M.S.A., Vigo, Spain), EnerZona Omega 3 RX (Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, S.L., Barcelona, Spain). The EPA/DHA 1:1 ratio was chosen on the basis of previous results (Méndez et al., 2013; Molinar-Toribio et al., 2015). Soybean oil, obtained from unrefined organic soya beans (first cold pressing), was from Clearspring Ltd. (London, UK). The GSE Grajfnol® was from JF-Natural Products (Tianjin, China). The GSE consisting of fine Grajfnol® powder contains 98% grape seed (poly)phenols with the following composition: total proanthocyanidins (UV), ≥ 95%; oligomeric proanthocyanidins, ≥ 60%; procyanidin dimer B2 (HPLC), ≥ 1.8%; ash, − 1.5%; weight loss on drying, − 5.0%. All the groups had free access to water and feed.

2.3. Data and sample collection

Body weight and feed intake were measured after 0, 6, 12, 18 and

24 weeks of the experiment. Feed intake per kg of body weight and day was estimated by dividing the total intake per cage by the total weight of the animals in that cage and the number of days. Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors, assigning 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

For urine collection, during weeks 14–16 of the experiment, the rats were randomized and placed in metabolic cages and deprived of food for 18 h and all the urine produced during that period was collected. Fecal samples were collected by abdominal massage at week 23 at the same time in the morning.

At the end of the experiment, the rats were fasted overnight and anesthetized intraperitoneally with ketamine and xylacine (80 and 10 mg/kg body weight, respectively). Blood was collected by cardiac puncture and stored at − 80 °C until analysis. Perigonadal adipose tissue samples were washed with 0.9% NaCl solution, dried and weighed.

2.4. CVD-related markers in plasma and urine

Systolic and diastolic blood pressures were measured after week 24 of the experiment. The rats were restrained in a rat pocket and maintained at 32 °C. Systolic and diastolic blood pressure were measured by the tail-cuff method, using a non-invasive automatic blood pressure analyzer (Panlab; Barcelona, Spain) as described (Bunag, 1973). To obtain stable responses and to reduce variability associated with circadian rhythms, the operations were performed in a quiet place and always at the same time in the morning. Data are presented as the mean of four measurements.

Plasma total cholesterol, HDL-cholesterol (HDLc), LDL-cholesterol (LDLc), and triglycerides were measured using a spectrophotometric method and the corresponding kits from Spinreact (Girona, Spain) as described by Bucolo *et al* (Bucolo & David, 1973; Méndez et al., 2013). Plasma adiponectin was measured using the ELISA kit from Millipore (Billerica, MA, USA).

The stable end product of NO, NO_2^-, was quantified in urine by a modification of the Griess reaction. Briefly, the urine was lyophilized from frozen samples and concentrated 5-fold. The concentrates (50 μL) were mixed with sulfanilamide 1% in 1.2 N HCl (60 μL) and 0.3% aqueous N-(1-naphthyl)ethylene-diamine dihydrochloride 0.3% (60 μL) for 10 min at room temperature. Absorbance was measured spectrophotometrically at 550 nm. The concentration of NO_2^- was calculated using a calibration curve made using $NaNO_2$.

2.5. Fecal microbial subgroups

The levels of total bacteria, the Bacteroidetes and Firmicutes phyla, the Lactobacillales and Bifidobacteriales orders, and the *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium longum* species were determined in fecal DNA by quantitative real-time polymerase chain reaction (qRT-PCR).

Total DNA was extracted from feces using the QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany). The DNA concentration was quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). All DNA samples were diluted to 20 ng/μL.

qRT-PCR cycling conditions were as follows: 5 s at 95 °C; then 45 cycles of: 5 s at 95 °C, 30 s at the primer-specific annealing temperature (Table 2), and 30 s at 72 °C (extension).

Following amplification, to determine the specificity of the qRT-PCR reactions, melting curve analysis was carried out by treatment for 2 s at 95 °C and for 15 s at 65 °C, followed by a temperature gradient up to 95 °C at a rate of 0.11 °C/s, with five fluorescence recordings per °C. The relative DNA abundances for the different genes were calculated from the second derivative maximum of their respective amplification curves (C*p*, calculated in triplicate), considering C*p* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation: $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ (Pfaffl, 2001). Total bacteria were normalized as 16S rDNA gene copies per mg of wet feces (copies/mg).

2.6. Short-chain fatty acids in feces

SCFAs (acetic acid, propionic acid, butyric acid and pentanoic acid) were analyzed by gas chromatography in feces after week 23 using the method proposed by Schwiertz (Schwiertz et al., 2010) with some modifications. Briefly, fecal samples were freeze-dried and weighed (~50 mg dry matter with 10^{-4} g precision). A solution of oxalic acid (0.1 M) and the internal standard, caproic acid (0.1 mM), in acetonitrile/water 3:7, was added to each sample (0.025 mL/mg dry feces). Then, SCFAs were extracted using a horizontal shaker (30 min, 4 °C) and the suspension was centrifuged (15 min, 16,000 g, 4 °C). The supernatant was passed through a nylon filter (0.45 μm) into a GC vial. SCFAs were analyzed using a Shimazdu (Kyoto, Japan) gas chromatograph (GC2025) with an automatic injector (AOC20i) at 240 °C, with a flame ionization detector (Shimazdu 2025) at 240 °C, equipped with an HP-Innowax capillary column (Agilent, Santa Clara, CA, USA) (30 m × 0.25 mm i.d. × 0.25 μm f.d.). The injection volume was 1 μL, the carrier gas was helium at a flow of 1 mL/min and the mode of injection was splitless. The oven temperature program was 50 °C (3 min) then slope 8 °C/min to 180 °C (0 min) and slope 50 °C/min to 200 °C (5 min). Other conditions were: gas helium flow, 30 mL/min; hydrogen flow, 40 mL/min; and airflow 400 mL/min.

2.7. Statistical analysis

The results are expressed as mean values with their standard errors (SEM). After verifying the variance equality and the normal distribution, statistical significance was determined by ANOVA for repeated measures of body weight or one-way ANOVA with each group as variable, and Tukey's multiple comparison test was used for mean comparisons. Differences were considered significant when $P < 0.05$. All data manipulation and statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Feed/energy intakes, body weight and perigonadal adipose tissue

The rats fed the ω-3 PUFAs enriched diets (ω-3 and ω-3 + GSE groups) had consumed significantly ($P < 0.05$) less feed and energy than the STD group after 24 weeks (Supplementary material Table S1).

Body weight was similar for all groups at the beginning of the experiment (144.0 g, SEM 2.6). After 24 weeks of the diets, the animals supplemented with ω-3 PUFAs had significantly higher ($P < 0.05$) body weights (ω-3 275.7 g, SEM 9.0 g; ω-3 + GSE 275.6 g, SEM 8.1) than the other groups (STD 254.4 g, SEM 5.4; and GSE 262.1 g, SEM 4.5) (Fig. 1a). Similarly, perigonadal fat was significantly higher in animals supplemented with ω-3 PUFAs ($P < 0.01$) than in non-supplemented and GSE-supplemented rats (Fig. 1b).

3.2. Risk factors of CVD

Blood pressure and plasma total cholesterol, HDLc, LDLc, triglycerides and adiponectin were measured after 24 weeks of supplementation (Table 3). Systolic and diastolic blood pressures were similar between the groups at the end of the study. Plasma cholesterol was significantly ($P < 0.01$) lower in animals supplemented with ω-3 PUFAs (ω-3 and ω-3 + GSE groups) than in the other two groups (STD and GSE). All the groups presented similar concentrations of plasma HDLc and triglycerides. Plasma LDLc levels presented some differences (Table 3), but they were all below 1.28 mmol/L, which may be considered a reference

Table 2
qRT-PCR primers and conditions.

Target bacteria	Annealing temp. (°C)	Sequence (5′-3′)	Positive control	Ref.
Total bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	[a]	(Hartman et al., 2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	*Bacteroides fragilis*	(Ismail et al., 2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	*Lactobacillus brevis*	(Haakensen, Dobson, Deneer, & Ziola, 2008) (Muhling, Woolven-Allen, Murrell, & Joint, 2008)
Lactobacillales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus acidophilus*	(Walter et al., 2001)
L. acidophillus	64	F: AGC TGA ACC AAC AGA TTC AC R: ACT ACC AGG GTA TCT AAT CC	*Lactobacillus acidophillus*	(Walter et al., 2001)
L. plantarum	55	F: GCC GCC TAA GGT GGG ACA GAT R: TTA CCT AAC GGT AAA TGC GA	*Lactobacillus plantarum*	(Walter et al., 2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	*Bifidobacterium longum*	(Queipo-Ortuno et al., 2013)
B. longum	50	F: GTT CCC GAC GGT CGT AGA G R: GTG AGT TCC CGG CAT AAT CC	*Bifidobacterium longum*	(R. F. Wang, Cao, & Cerniglia, 1996)

[a] Positive control of Total Bacteria was the same as those the result was rated with.

value (Ihedioha, Noel-Uneke, & Ihedioha, 2013). Plasma adiponectin was higher in the groups supplemented with ω-3 PUFAs than in the STD group; while this difference was significant only for animals supplemented with both ω-3 PUFAs and GSE.

Urine nitrites were similar between the groups and slightly higher ($P = 0.123$) in animals fed ω-3 PUFAs compared to those in the STD group (Table 3).

3.3. Proportions of fecal bacterial populations

The proportions of the major bacterial phyla (Fig. 2), and selected probiotics (Fig. 3) were determined in fecal DNA. The percentages of Bacteroidetes and Firmicutes were similar in the groups supplemented with ω-3 PUFAs and/or GSE (Fig. 2a, b).

The population of Lactobacillales was significantly ($P < 0.01$) lower in the ω-3 group than in the STD group; while supplementation with proanthocyanidins (GSE and ω-3 + GSE groups) did not modify the proportion of lactobacilli (Fig. 3a). The percentage of Bifidobacteriales was similar in all the groups (Fig. 3d). The proportion of *L. acidophilus* was lower ($P < 0.001$ vs STD) in animals supplemented with ω-3 PUFAs, independently of the presence of proanthocyanidins (Fig. 3b). The proportions of *L. plantarum* (Fig. 3c) and *B. longum* (Fig. 3e) were lower ($P < 0.05$ vs STD) in animals supplemented with GSE; while the combination of GSE with ω-3 PUFAs did not affect the levels of these populations.

Table 3
CDV risk factors in rats supplemented with ω-3 PUFAs and/or GSE for 24 weeks.

	STD		ω-3		GSE		ω-3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Systolic pressure (mmHg)	123.5	4.0	123.2	2.4	114.8	3.6	117.2	3.1
Diastolic pressure (mmHg)	84.0	4.0	96.2	7.0	94.6	7.4	91.3	2.1
Plasma cholesterol (mmol/L)	4.7^{a}	0.2	3.8^{bc}	0.1	4.4^{ab}	0.1	3.5^{c}	0.2
Plasma HDLc (mmol/L)	1.5	0.1	1.6	0.1	1.5	0.1	1.5	0.1
Plasma LDLc (mmol/L)	0.4^{a}	0.1	0.4^{a}	0.0	0.7^{b}	0.0	0.6^{c}	0.0
Plasma triglycerides (mmol/L)	1.7	0.1	1.7	0.1	1.9	0.1	1.6	0.1
Plasma adiponectin (μg/mL)	21.7^{a}	2.9	30.0^{ab}	2.1	22.0^{a}	2.8	32.1^{bc}	1.5
Urine nitrites (ng/mL)	1.3	0.3	2.5	0.9	1.5	0.1	2.1	0.2

Means with different letters differ, $P < 0.05$. Comparisons were performed using one-way ANOVA and Tukey's post-hoc tests.

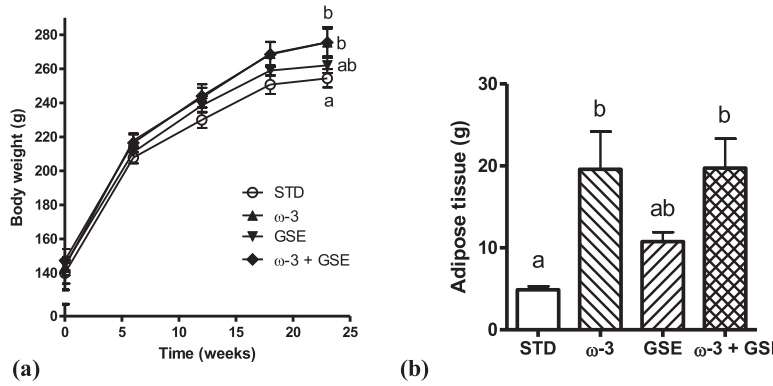


Fig. 1. Body weight (a) and perigonadal fat (b) of rats fed the different diets for 24 weeks: STD, ○; ω-3, ▲; GSE, ▼; ω-3 + GSE, ♦. The curves corresponding to the ω-3 and ω-3 + GSE groups are superimposed. The data represent means with their standard errors. Comparisons were performed using two-way ANOVA for repeated measures (a) or one-way ANOVA followed by Tukey's post-hoc test (b). Means with different letters differ, $P < 0.05$.

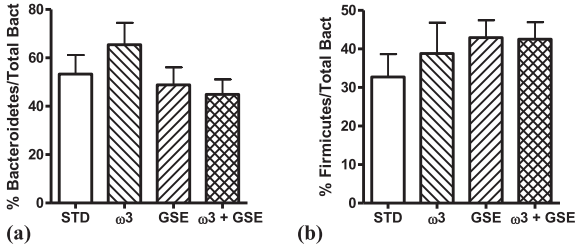


Fig. 2. Bacteroidetes (a) and Firmicutes (b) in fecal samples from rats fed the different diets (STD, ω-3, GSE, or ω-3 + GSE) for 23 weeks. The data represent means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test.

Table 4
Short-chain fatty acids determined in feces from rats supplemented with ω-3 PUFAs and/or GSE for 23 weeks.

	STD		ω-3		GSE		ω-3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	5.54[a]	1.26	9.10[b]	1.36	6.03[a]	1.62	4.48[a]	1.89
Propionic acid	1.18	0.43	1.47	0.54	1.11	0.51	1.24	0.63
Butyric acid	0.26	0.07	0.31	0.11	0.30	0.14	0.24	0.14
Valeric acid	0.15[ab]	0.07	0.21[a]	0.08	0.09[b]	0.05	0.10[b]	0.02

SCFA content is expressed as mmol of caproic acid equivalents/g dry feces. Means with different letters differ, $P < 0.05$. Comparisons were performed using one-way ANOVA and Tukey's post-hoc tests.

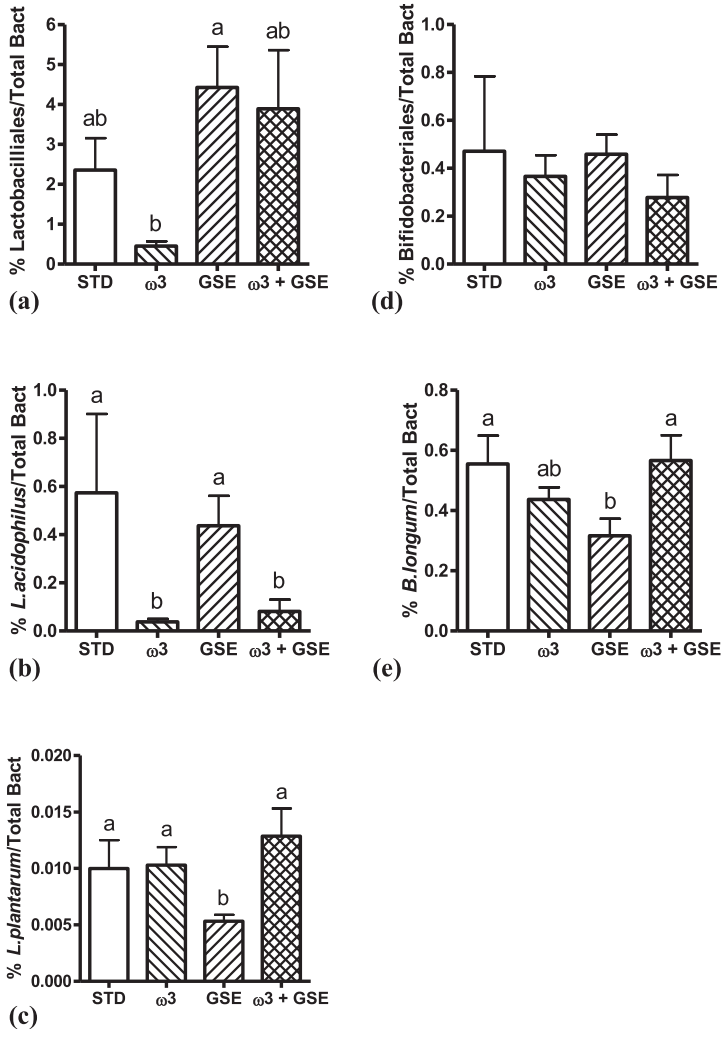


Fig. 3. Lactobacillales (a), *Lactobacillus acidophilus* (b), *Lactobacillus plantarum* (c), Bifidobacteriales (d), and *Bifidobacterium longum* (e) in fecal samples from rats fed the different diets (STD, ω-3, GSE or ω-3 + GSE) for 23 weeks. The data represent means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. Means with different letters differ, $P < 0.05$.

3.4. Short-chain fatty acids

SCFAs were determined in feces from rats supplemented for 23 weeks (Table 4). The animals supplemented only with ω-3 PUFAs showed increased SCFA content with respect to the other groups; with the concentration of acetic acid being significantly higher ($P < 0.05$). The valeric acid concentration was significantly ($P < 0.05$) lower in the groups supplemented with GSE.

4. Discussion

The present study focuses on the effect of the combination of ω-3 PUFAs (EPA/DHA 1:1) and proanthocyanidins (mostly oligomers consisting of 2 to 4 (−)-epicatechin units in GSE) on gut health-promoting commensal bacteria, and the possible influence of their combination on the functional effects of ω-3 PUFAs on CVD risk factors. The 1:1 proportion of ω-3 PUFAs was chosen on the basis of previous results with healthy and rats affected by CVD (Lluís et al., 2013; Méndez et al., 2013; Molinar-Toribio et al., 2015). In the present study, we show that GSE may enhance the effects of ω-3 PUFAs on CVD risk factors. The reduction in plasma cholesterol by ω-3 PUFAs agrees with previous studies where fish oil supplementation decreased total plasmatic fatty acids in healthy rats (Méndez et al., 2013). Plasma cholesterol is inversely related to adiponectin levels, as reverse cholesterol transport is accelerated by adiponectin through increasing high-density lipoprotein assembly in the liver (Matsuura et al., 2007). Here, we show that plasma adiponectin increased in animals fed ω-3 PUFAs, and increased even further when GSE was added to the supplementation (Table 3). Plasma adiponectin levels are related to the endothelial vasodilation response (Ouchi et al., 2003), probably because adiponectin stimulates the production of NO (Z. V. Wang & Scherer, 2008), which partially agrees with our results from urine nitrites (ω-3 groups show nonsignificant higher values than the STD group).

Besides CVD risk factors, the first observable effects of ω-3 PUFAs were changes in feed intake and body weight, independently of GSE supplementation. Our results for feed intake corroborate the satiety effect induced by ω-3 PUFAs, previously reported by other authors (Parra et al., 2008). Despite the fact that ω-3 PUFAs reduced feed intake, they increased body weight gain in our animals (Fig. 1), an effect also detected in normoweight women who consume ω-3 PUFAs (Iso et al., 2001). The body weight increase induced by ω-3 PUFA intake in normoweight individuals could be related to a post-prandial increase of the chylomicron response (Griffo et al., 2014) and to a faster gastric emptying of fat, associated with modified patterns of cholecystokinin and GLP-1 release (Robertson et al., 2002). Moreover, ω-3 PUFAs may promote fat accumulation through the stimulation of adipocyte growth and differentiation via expression of peroxisome proliferator-activated receptor γ (PPARγ) (Chambrier et al., 2002). The weight gain observed in lean animals supplemented with ω-3 PUFAs is in apparent contradiction with the observations of other authors who report the anti-obesogenic effect of ω-3 PUFAs in obese individuals (Buckley & Howe, 2010; Lorente-Cebrián et al., 2013). This anti-obesogenic effect of ω-3 PUFAs seems to be related to a reduction in obesity-associated low-grade inflammation (Calder, 2013; Flock, Rogers, Prabhu, & Kris-Etherton, 2013). This state has been linked to metabolic endotoxemia, commonly derived from gut dysbiosis, in turn typically induced by an energy-dense diet (Kaliannan, Wang, Li, Kim, & Kang, 2015). The anti-obesogenic effect of ω-3 PUFAs may only be evident within a pro-inflammatory environment and not in normoweight individuals with well-balanced microbiota. Fat gain induced by ω-3 PUFAs in lean rats may be viewed as a beneficial contribution to the physiological role of adipose tissue as a fuel reservoir that keeps fat from being deposited in organs.

A reduction in the populations of probiotic bacteria is a risk factor for the development of many intestinal conditions, including diarrhea, obesity, irritable bowel syndrome, inflammatory bowel disease, and even tumors (Gareau, Sherman, & Walker, 2010; Guarner & Malagelada, 2003). Our results show that ω-3 PUFAs and proanthocyanidins induced changes in the proportions of several subgroups and species of probiotics (Fig. 3). The reduction of Lactobacillales induced by ω-3 PUFAs is in agreement with the fact that polyunsaturated fatty acids reduce the adherence of most probiotic lactobacilli (Kankaanpaa, Yang, Kallio, Isolauri, & Salminen, 2004). Specifically, the incorporation of a given PUFA into bacterial fatty acids is clearly observed when lactobacilli are cultured in broth supplemented with that particular fatty acid, and these changes seem to influence microbial adhesion to intestinal surfaces (Kankaanpaa et al., 2004). Proanthocyanidins in GSE counteracted this reduction in Lactobacillales, possibly by promoting the growth of lactobacilli as observed in rats given tea and wine phenolics (Dolara et al., 2005; Lee et al., 2006). The effects of polyphenols on the adhesion and viability of probiotics are complex, and depend on the chemical structure of the phenolic compound and on the membrane composition (proteins, enzymes and lipids) of any particular species (Bustos et al., 2012; Parkar, Stevenson, & Skinner, 2008; Parkar, Trower, & Stevenson, 2013). These pleiotropic effects can modify cell permeability and finally result in the loss of protons, other ions, and macromolecules (Bustos et al., 2012). Specifically, epicatechin and other proanthocyanidin monomers inhibit the growth of *Lactobacillus rhamnosus* (Parkar et al., 2008) and the adhesion of both *Lactobacillus plantarum* and *Lactobacillus acidophillus* to enterocytes (Bustos et al., 2012) *in vitro*. This inhibitory effect on some Lactobacillus species is in agreement with our results, which show a reduction in the populations of *Lactobacillus plantarum* and *Bifidobacterium longum* as result of GSE supplementation. Combined supplementation with ω-3 PUFAs and GSE also counteracted the effect of ω-3 PUFAs on the excreted concentration of acetic acid, an SCFA product of bacterial metabolism. Acetic acid contributes to lipogensis via acetyl-CoA and regulates levels of plasma cholesterol (Fushimi et al., 2006), in agreement with the present results on body weight gain (Fig. 1) and cholesterolemia (Table 3). Higher concentrations of SCFAs in feces have been observed in overweight and obese rats and humans (Byrne, Chambers, Morrison, & Frost, 2015; Canfora et al., 2015; Schwiertz et al., 2010).

5. Conclusions

In conclusion, supplementation of rats with ω-3 PUFAs increases their weight gain and perigonadal fat, reduces the populations of several probiotics and increases the levels of acetic acid, a product of bacterial metabolism. Combined supplementation of ω-3 PUFAs and proanthocyanidins from grape seed counteracts the effects of the ω-3 PUFAs on health-promoting lactobacillus and bifidobacterium, and on acetic acid, while maintaining their beneficial effects on cholesterolemia.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.04.024.

Funding

This research was supported by the Spanish Ministry of Science and Innovation (through grants AGL2009-12374-C03-01, -02 and -03; and AGL2013-49079-C2-1,2-R). The Spanish Ministry of Economics and the Panamanian Government awarded graduate fellowships to M.H. (BES2014-068592) and E.M.-T. (SENACYT/IFRHU), respectively.

Author contributions

S.R.-R. and E. M.-T. performed the *in vivo* experiments; S.R.-R. and M.H. performed the qRT-PCR experiments; M.P.A. performed the SCFA experiments; N.T. and M.R. evaluated the CVD risk factors; S.R.-R. and L.M. analyzed the data; S.R.-R. and J.L.T. wrote the paper; S.R.-R., M.R.N., I.M. and J.L.T. conceived and designed the experiments. All

authors have approved the final article.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

Language revision by Christopher Evans is appreciated.

References

Arboleya, S., Watkins, C., Stanton, C., & Ross, R. P. (2016). Gut bifidobacteria populations in human health and aging. *Frontiers in Microbiology*, *7*. http://dx.doi.org/10.3389/fmicb.2016.01204.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, *56*(11), 317–333.

Buckley, J. D., & Howe, P. R. C. (2010). Long-chain omega-3 polyunsaturated fatty acids may be beneficial for reducing obesity—a review. *Nutrients*, *2*(12), 1212–1230. http://dx.doi.org/10.3390/nu2121212.

Bucolo, G., & David, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clinical Chemistry*, *19*(5), 476–482.

Bunag, R. D. (1973). Validation in awake rats of a tail-cuff method for measuring systolic pressure. *Journal of Applied Physiology*, *34*(2), 279–282.

Bustos, I., García-Cayuela, T., Hernández-Ledesma, B., Peláez, C., Requena, T., & Martínez-Cuesta, M. C. (2012). Effect of flavan-3-ols on the adhesion of potential probiotic lactobacilli to intestinal cells. *Journal of Agricultural and Food Chemistry*, *60*(36), 9082–9088. http://dx.doi.org/10.1021/jf301133g.

Byrne, C. S., Chambers, E. S., Morrison, D. J., & Frost, G. (2015). The role of short chain fatty acids in appetite regulation and energy homeostasis. *International Journal of Obesity*, *39*(9), 1331–1338. http://dx.doi.org/10.1038/ijo.2015.84.

Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *British Journal of Clinical Pharmacology*, *75*(3), 645–662. http://dx.doi.org/10.1111/j.1365-2125.2012.04374.x.

Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews. Endocrinology*, *11*(10), 577–591. http://dx.doi.org/10.1038/nrendo.2015.128.

Cani, P. D., Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R. G., Tuohy, K. M., ... Delzenne, N. M. (2007). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia*, *50*(11), 2374–2383. http://dx.doi.org/10.1007/s00125-007-0791-0.

Chambrier, C., Bastard, J. P., Rieusset, J., Chevillotte, E., Bonnefont-Rousselot, D., Therond, P., ... Vidal, H. (2002). Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor gamma. *Obesity Research*, *10*(6), 518–525. http://dx.doi.org/10.1038/oby.2002.70.

Dolara, P., Luceri, C., De Filippo, C., Femia, A. P., Giovannelli, L., Caderni, G., ... Cresci, A. (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, *591*(1–2), 237–246. http://dx.doi.org/10.1016/j.mrfmmm.2005.04.022.

Duda-Chodak, A. (2012). The inhibitory effect of polyphenols on human gut microbiota. *Journal of Physiology and Pharmacology*, *63*(5), 497–503.

Flock, M. R., Rogers, C. J., Prabhu, K. S., & Kris-Etherton, P. M. (2013). Immunometabolic role of long-chain omega-3 fatty acids in obesity-induced inflammation. *Diabetes-Metabolism Research and Reviews*, *29*(6), 431–445. http://dx.doi.org/10.1002/dmrr.2414.

Fushimi, T., Suruga, K., Oshima, Y., Fukiharu, M., Tsukamoto, Y., & Goda, T. (2006). Dietary acetic acid reduces serum cholesterol and triacylglycerols in rats feda cholesterol-rich diet. *British Journal of Nutrition*, *95*(5), 916–924. http://dx.doi.org/10.1079/bjn20061740.

Gareau, M. G., Sherman, P. M., & Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature Reviews Gastroenterology & Hepatology*, *7*(9), 503–514. http://dx.doi.org/10.1038/nrgastro.2010.117.

Griffo, E., Di Marino, L., Patti, L., Bozzetto, L., Annuzzi, G., Cipriano, P., ... Rivellese, A. A. (2014). Test meals rich in marine long-chain n-3 polyunsaturated fatty acids increase postprandial chylomicron response. *Nutrition Research*, *34*(8), 661–666. http://dx.doi.org/10.1016/j.nutres.2014.07.005.

Guardamagna, O., Amaretti, A., Puddu, P. E., Raimondi, S., Abello, F., Cagliero, P., & Rossi, M. (2014). Bifidobacteria supplementation: Effects on plasma lipid profiles in dyslipidemic children. *Nutrition*, *30*(7–8), 831–836. http://dx.doi.org/10.1016/j.nut.2014.01.014.

Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet*, *361*(9356), 512–519. http://dx.doi.org/10.1016/s0140-6736(03)12489-0.

Haakensen, M., Dobson, C. M., Deneer, H., & Ziola, B. (2008). Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *International Journal of Food Microbiology*, *125*(3), 236–241. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.04.002.

Hartman, A. L., Lough, D. M., Barupal, D. K., Fiehn, O., Fishbein, T., Zasloff, M., & Eisen, J. A. (2009). Human gut microbiome adopts an alternative state following small bowel transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(40), 17187–17192.

Ihedioha, J. I., Noel-Uneke, O. A., & Ihedioha, T. E. (2013). Reference values for the serum lipid profile of albino rats (*Rattus norvegicus*) of varied ages and sexes. *Comparative Clinical Pathology*, *22*(1), 93–99.

Ismail, N. A., Ragab, S. H., Elbaky, A. A., Shoeib, A. R., Alhosary, Y., & Fekry, D. (2011). Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Archives of Medical Science*, *7*(3), 501–507. http://dx.doi.org/10.5114/aoms.2011.23418.

Iso, H., Rexrode, K. M., Stampfer, M. J., Manson, J. E., Colditz, G. A., Speizer, F. E., ... Willett, W. C. (2001). Intake of fish and omega-3 fatty acids and risk of stroke in women. *Jama-Journal of the American Medical Association*, *285*(3), 304–312. http://dx.doi.org/10.1001/jama.285.3.304.

Kaliannan, K., Wang, B., Li, X. Y., Kim, K. J., & Kang, J. X. (2015). A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Scientific Reports*, *5*, 11276. http://dx.doi.org/10.1038/srep11276.

Kankaanpaa, P., Yang, B., Kallio, H., Isolauri, E., & Salminen, S. (2004). Effects of polyunsaturated fatty acids in growth medium on lipid composition and on physicochemical surface properties of lactobacilli. *Applied and Environmental Microbiology*, *70*(1), 129–136. http://dx.doi.org/10.1128/aem.70.1.129-136.2004.

Lee, H. C., Jenner, A. M., Low, C. S., & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in Microbiology*, *157*(9), 876–884. http://dx.doi.org/10.1016/j.resmic.2006.07.004.

Liu, T., Hougen, H., Vollmer, A. C., & Hiebert, S. M. (2012). Gut bacteria profiles of *Mus musculus* at the phylum and family levels are influenced by saturation of dietary fatty acids. *Anaerobe*, *18*(3), 331–337. http://dx.doi.org/10.1016/j.anaerobe.2012.02.004.

Lluís, L., Taltavull, N., Muñoz-Cortés, M., Sánchez-Martos, V., Romeu, M., Giralt, M., ... Nogués, M. R. (2013). Protective effect of the omega-3 polyunsaturated fatty acids: Eicosapentaenoic acid/docosahexaenoic acid 1:1 ratio on cardiovascular disease risk markers in rats. *Lipids in Health and Disease*, *12*, 140. http://dx.doi.org/10.1186/1476-511x-12-140.

Lorente-Cebrián, S., Costa, A. G. V., Navas-Carretero, S., Zabala, M., Martínez, J. A., & Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: A review of the evidence. *Journal of Physiology and Biochemistry*, *69*(3), 633–651. http://dx.doi.org/10.1007/s13105-013-0265-4.

Matsuura, F., Oku, H., Koseki, M., Sandoval, J. C., Yuasa-Kawase, M., Tsubakio-Yamamoto, K., ... Yamashita, S. (2007). Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochemical and Biophysical Research Communications*, *358*(4), 1091–1095. http://dx.doi.org/10.1016/j.bbrc.2007.05.040.

Méndez, L., Pazos, M., Gallardo, J. M., Torres, J. L., Pérez-Jiménez, J., Nogués, R., ... Medina, I. (2013). Reduced protein oxidation in Wistar rats supplemented with marine omega 3 PUFAs. *Free Radical Biology and Medicine*, *55*, 8–20. http://dx.doi.org/10.1016/j.freeradbiomed.2012.11.004.

Miremadi, F., Sherkat, F., & Stojanouska, L. (2016). Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics: A review. *Journal of Functional Foods*, *25*, 497–510. http://dx.doi.org/10.1016/j.jff.2016.06.016.

Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Romeu, M., Giralt, M., Taltavull, N., ... Torres, J. L. (2015). Effect of n-3 PUFA supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model of the metabolic syndrome. *British Journal of Nutrition*, *113*(6), 878–887. http://dx.doi.org/10.1017/S0007114514004437.

Muhling, M., Woolven-Allen, J., Murrell, J. C., & Joint, I. (2008). Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME Journal*, *2*(4), 379–392. http://dx.doi.org/10.1038/ismej.2007.97.

Ouchi, N., Ohishi, M., Kihara, S., Funahashi, T., Nakamura, T., Nagaretani, H., ... Matsuzawa, Y. (2003). Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension*, *42*(3), 231–234. http://dx.doi.org/10.1161/01.hyp.0000083488.67550.b8.

Pan, M.-H., Lai, C.-S., & Ho, C.-T. (2010). Anti-inflammatory activity of natural dietary flavonoids. *Food & Function*, *1*(1), 15–31. http://dx.doi.org/10.1039/c0fo00103a.

Parkar, S. G., Stevenson, D. E., & Skinner, M. A. (2008). The potential influence of fruit polyphenols on colonic microflora and human gut health. *International Journal of Food Microbiology*, *124*(3), 295–298. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.03.017.

Parkar, S. G., Trower, T. M., & Stevenson, D. E. (2013). Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*, *23*, 12–19. http://dx.doi.org/10.1016/j.anaerobe.2013.07.009.

Parra, D., Ramel, A., Bandarra, N., Kiely, M., Martínez, J. A., & Thorsdottir, I. (2008). A diet rich in long chain omega-3 fatty acids modulates satiety in overweight and obese volunteers during weight loss. *Appetite*, *51*(3), 676–680. http://dx.doi.org/10.1016/j.appet.2008.06.003.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45.

Poudyal, H., Panchal, S. K., Diwan, V., & Brown, L. (2011). Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action. *Progress in Lipid Research*, *50*(4), 372–387. http://dx.doi.org/10.1016/j.plipres.2011.06.003.

Queipo-Ortuño, M. I., Boto-Ordoñez, M., Murri, M., Gómez-Zumaquero, J. M., Clemente-Postigo, M., Estruch, R., ... Tinahones, F. J. (2012). Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *American Journal of Clinical Nutrition*, *95*(6), 1323–1334. http://dx.doi.org/10.3945/ajcn.111.027847.

Queipo-Ortuño, M. I., Seoane, L. M., Murri, M., Pardo, M., Gómez-Zumaquero, J. M., Cardona, F., ... Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association

with serum leptin and ghrelin levels. *PLoS One*, *8*(5), e65465. http://dx.doi.org/10.1371/journal.pone.0065465.

Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouysegu, L. (2011). Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angewandte Chemie-International Edition*, *50*(3), 586–621. http://dx.doi.org/10.1002/anie.201000044.

Ramos-Romero, S., Molinar-Toribio, E., Pérez-Jiménez, J., Taltavull, N., Dasilva, G., Romeu, M., ... Torres, J. L. (2016). The combined action of omega-3 polyunsaturated fatty acids and grape proanthocyanidins on a rat model of diet-induced metabolic alterations. *Food & Function*, *7*(8), 3516–3523. http://dx.doi.org/10.1039/c6fo00679e.

Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *FASEB Journal*, *22*(3), 659–661. http://dx.doi.org/10.1096/fj.07-9574LSF.

Robertson, M. D., Jackson, K. G., Fielding, B. A., Morgan, L. M., Williams, C. M., & Frayn, K. N. (2002). Acute ingestion of a meal rich in n-3 polyunsaturated fatty acids results in rapid gastric emptying in humans. *American Journal of Clinical Nutrition*, *76*(1), 232–238.

Salvadó, M. J., Casanova, E., Fernández-Iglesias, A., Arola, L., & Bladé, C. (2015). Roles of proanthocyanidin rich extracts in obesity. *Food & Function*, *6*(4), 1053–1071. http://dx.doi.org/10.1039/c4fo01035c.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2010). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)*, *18*(1), 190–195.

Sekhon-Loodu, S., Catalli, A., Kulka, M., Wang, Y., Shahidi, F., & Rupasinghe, H. P. V. (2014). Apple flavonols and n-3 polyunsaturated fatty acid-rich fish oil lowers blood C-reactive protein in rats with hypercholesterolemia and acute inflammation. *Nutrition Research*, *34*(6), 535–543. http://dx.doi.org/10.1016/j.nutres.2014.05.002.

Taltavull, N., Muñoz-Cortés, M., Lluís, L., Jové, M., Fortuño, À., Molinar-Toribio, E., ... Nogués, M. R. (2014). Eicosapentaenoic acid/docosahexaenoic acid 1:1 ratio improves histological alterations in obese rats with metabolic syndrome. *Lipids in Health and Disease*, *13*, 31. http://dx.doi.org/10.1186/1476-511x-13-31.

Tapiero, H., Ba, G. N., Couvreur, P., & Tew, K. D. (2002). Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomedicine & Pharmacotherapy*, *56*(5), 215–222. http://dx.doi.org/10.1016/s0753-3322(02)00193-2.

Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, *67*(6), 2578–2585. http://dx.doi.org/10.1128/aem.67.6.2578-2585.2001.

Wang, R. F., Cao, W. W., & Cerniglia, C. E. (1996). PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Applied and Environmental Microbiology*, *62*(4), 1242–1247.

Wang, Z. V., & Scherer, P. E. (2008). Adiponectin, cardiovascular function, and hypertension. *Hypertension*, *51*(1), 8–14. http://dx.doi.org/10.1161/hypertensionaha.107.099424.

Williamson, G. (2013). Possible effects of dietary polyphenols on sugar absorption and digestion. *Molecular Nutrition & Food Research*, *57*(1), 48–57. http://dx.doi.org/10.1002/mnfr.201200511.

Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., ... Mohamadzadeh, M. (2015). Gut dysbiosis is linked to hypertension. *Hypertension*, *65*(6), 1331–1340. http://dx.doi.org/10.1161/hypertensionaha.115.05315.

Yu, H.-N., Zhu, J., Pan, W.-S., Shen, S.-R., Shan, W.-G., & Das, U. N. (2014). Effects of fish oil with a high content of n-3 polyunsaturated fatty acids on mouse gut microbiota. *Archives of Medical Research*, *45*(3), 195–202. http://dx.doi.org/10.1016/j.arcmed.2014.03.008.

SUPPLEMENTARY MATERIAL (SM) INCLUDED IN THE DIGITAL VERSION OF THE PAPER A1

Table SM1. Feed and energy intakes, and CDV risk factors in rats supplemented with ω-3 PUFAs and/or GSE for 24 weeks.

	STD		ω-3		GSE		ω-3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/kg bw/day)	59.4[a]	2.6	41.4[b]	4.5	56.4[a]	4.2	40.0[b]	3.6
Energy intake[1] (kcal/kg bw/day)	184.0[a]	8.1	128.4[b]	14.1	174.8[a]	13.0	123.9[b]	11.1

[1] Energy intake is estimated as metabolizable energy based on Atwater factors, which assign 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.

Means with different letters differ, $P<0.05$. Comparisons were performed using one-way ANOVA and Tukey's post-hoc test.

8.1.2 PAPER A2

TITLE: Mechanistically different effects of fat and sugar on insulin resistance, hypertension, and gut microbiota in rats

AUTHORS: Sara Ramos-Romero, **Mercè Hereu**, Lidia Atienza, Josefina Casas, Olga Jáuregui, Susana Amézqueta, Gabriel Dasilva, Isabel Medina, Maria Rosa Nogués, Marta Romeu and Josep Lluís Torres

JOURNAL: American Journal of Physiology-Endocrinology and Metabolism, 2018, 314(6), E552–E563

IMPACT FACTOR (2017): 4.02

CLASSIFICATION AND CATEGORY: ENDOCRYNOLOGY & METABOLISM 35/142 (Q1)

TIMES CITED: 8

REFERENCE: Ramos-Romero, S., Hereu, M., Atienza, L., Casas, J., Jáuregui, O., Amézqueta, S., Dasilva, G., Medina, I., Nogués, M. R., Romeu, M. & Torres, J.L. (2018). Mechanistically different effects of fat and sugar on insulin resistance, hypertension, and gut microbiota in rats. *American Journal of Physiology-Endocrinology and Metabolism, 314(6),* E552–E563.

Am J Physiol Endocrinol Metab 314: E552–E563, 2018.
First published January 2, 2018; doi:10.1152/ajpendo.00323.2017.

RESEARCH ARTICLE | *Role of Gut Microbiota and Gut-Brain and Gut-Liver Axes in Physiological Regulation of Inflammation, Energy Balance, and Metabolism*

Mechanistically different effects of fat and sugar on insulin resistance, hypertension, and gut microbiota in rats

Sara Ramos-Romero,[1,2] Mercè Hereu,[1] Lidia Atienza,[3] Josefina Casas,[4] Olga Jáuregui,[5] Susana Amézqueta,[6] Gabriel Dasilva,[7] Isabel Medina,[7] Maria Rosa Nogués,[8] Marta Romeu,[8] and Josep Lluís Torres[1]

[1]*Institute of Advanced Chemistry of Catalonia, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona, Spain;* [2]*Faculty of Biology, University of Barcelona, Barcelona, Spain;* [3]*Department of Pathology, Puerta del Mar University Hospital, Cádiz, Spain;* [4]*Research Unit on Bioactive Molecules, Department of Biomedicinal Chemistry, IQAC-CSIC, Barcelona, Spain;* [5]*Scientific and Technological Centers of the University of Barcelona, Barcelona, Spain;* [6]*Faculty of Chemistry, University of Barcelona, Barcelona, Spain;* [7]*Instituto de Investigaciones Marinas, Vigo, Spain; and* [8]*Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Reus, Spain*

Submitted 14 September 2017; accepted in final form 31 December 2017

Ramos-Romero S, Hereu M, Atienza L, Casas J, Jáuregui O, Amézqueta S, Dasilva G, Medina I, Nogués MR, Romeu M, Torres JL. Mechanistically different effects of fat and sugar on insulin resistance, hypertension, and gut microbiota in rats. *Am J Physiol Endocrinol Metab* 314: E552–E563, 2018. First published January 2, 2018; doi:10.1152/ajpendo.00323.2017.—Insulin resistance (IR) and impaired glucose tolerance (IGT) are the first manifestations of diet-induced metabolic alterations leading to Type 2 diabetes, while hypertension is the deadliest risk factor of cardiovascular disease. The roles of dietary fat and fructose in the development of IR, IGT, and hypertension are controversial. We tested the long-term effects of an excess of fat or sucrose (fructose/glucose) on healthy male Wistar-Kyoto (WKY) rats. Fat affects IR and IGT earlier than fructose through low-grade systemic inflammation evidenced by liver inflammatory infiltration, increased levels of plasma IL-6, PGE_2, and reduced levels of protective short-chain fatty acids without triggering hypertension. Increased populations of gut Enterobacteriales and *Escherichia coli* may contribute to systemic inflammation through the generation of lipopolysaccharides. Unlike fat, fructose induces increased levels of diacylglycerols (lipid mediators of IR) in the liver, urine F_2-isoprostanes (markers of systemic oxidative stress), and uric acid, and triggers hypertension. Elevated populations of Enterobacteriales and *E. coli* were only detected in rats given an excess of fructose at the end of the study. Dietary fat and fructose trigger IR and IGT in clearly differentiated ways in WKY rats: early low-grade inflammation and late direct lipid toxicity, respectively; gut microbiota plays a role mainly in fat-induced IR, and hypertension is independent of inflammation-mediated IR. The results provide evidence that suggests that the combination of fat and sugar is potentially more harmful than fat or sugar alone when taken in excess.

diabetes; hypertension; microbiota; obesity

INTRODUCTION

Obesity and Type 2 diabetes together with hypertension and hypercholesterolemia are the main risk factors of cardiovascular disease (CVD), the leading cause of death worldwide. An unhealthy lifestyle, the combination of a poor diet with physical inactivity, is the single greatest contributor to the appearance of all these warning signs (36). Obesity, insulin resistance (IR, first revealed by high fasting insulinemia), and impaired glucose tolerance (IGT) appear to be the first manifestations of the so-called metabolic syndrome (12), and hypertension is probably the deadliest CVD triggering factor (16).

The relationships between fat accumulation, IR, and hypertension are still unclear in both humans and animal models. Whereas most diet-induced rodent models of obesity and/or hypertension include insulin resistance, they show significant differences. Rats fed a high-fat diet become overweight, but rarely do they develop hypertension unless their diet is supplemented with salt (1, 39). Those fed a high-fructose or high-sucrose (50% fructose) diet may become hypertensive while remaining normal weight (10, 40, 49). There is a wide consensus that ectopic lipid accumulation in key organs, such as liver and muscle, leads to IR, but how particular lipids contribute to systemic IR and the relevance of their location are active areas of research (44). Visceral adipose tissue (AT) has been associated with low-grade inflammation and the metabolic complications of obesity, mainly because it releases free fatty acids and proinflammatory adipokines into the portal vein for direct transport to the liver (9, 14). Meanwhile, particular lipids, such as diacylglycerols (DAGs) and ceramides, may impair insulin signaling in the liver and muscle independently of inflammation by altering the phosphorylation pattern of the insulin receptor substrate: a key protein in the intracellular insulin signaling pathway (13, 44). DAGs may be formed from triacylglycerides (TAGs) by lipolysis or via de novo synthesis of TAGs from free fatty acids (13).

The relationship between IR and hypertension is also a controversial issue. Whereas it has been proposed that IR is the main upstream event leading to hypertension (49), other evidence suggests that different factors may increase blood pressure more decisively than IR (27).

Address for reprint requests and other correspondence: J. L. Torres, IQAC-CSIC, Jordi Girona 18-26, 08034-Barcelona, Spain (e-mail: joseplluis.torres@iqac.csic.es).

As it is becoming evident that diet-induced changes in populations of gut microbiota play a role in the development of obesity and related disorders (42), the study of the crosstalk between the host organism and its associated microbiota is emerging as an active area of research. Specifically, gut microbiota has been shown to trigger IR through the action of proinflammatory products of bacterial metabolism (3).

To substantiate preventive dietary strategies against metabolic alterations, it is important to characterize early predisease events and to define molecular and physiological markers that are suitable for evaluating nutritional or behavioral interventions. As the molecular mechanisms linking lipid accumulation, IR, and hypertension are still largely unknown, we compared two animal models of diet-induced metabolic changes: Wistar-Kyoto (WKY) rats fed a high-fat (HF) or high-sucrose (HS) diet. Both models develop incipient IR and/or IGT, while only the HS-fed rats become hypertensive. This article focuses on the differential mechanisms associated with dietary fat and sugar (fructose) as triggering factors of IR, IGT, and hypertension, and it examines the possible role played by gut microbiota in these effects.

MATERIALS AND METHODS

Animals. A total of 27 male WKY rats (Envigo, Indianapolis, IN), aged 8–9 wk, was used. All animal manipulation was carried out in the morning to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals (directive 2010/63/UE) under license from the regional Catalan authorities (reference no. DAAM7921) and were approved by the Spanish Consejo Superior de Investigaciones Científicas Subcommittee of Bioethical Issues.

Experimental design and sample collection. The rats were housed ($n = 3$ per cage) under controlled conditions of humidity (60%) and temperature (22 ± 2°C), with a 12:12-h light-dark cycle. They were randomly divided into three dietary groups ($n = 9$ per group): the standard (STD) group, fed a standard diet (2014 Teklad global 14% protein diet from Envigo) and mineral water (Ribes, Girona, Spain); the HF group, fed an HF diet (TD 08811 45% kcal fat diet, from Envigo) and mineral water; and the HS group, fed the standard diet and 35% (wt/vol) sucrose solution in mineral water as the only source of liquid intake. The diets were chosen with the aim of mimicking real nutritional conditions in humans who consume a moderate excess of fat or sugar (glucose + fructose). All of the animals were fed ad libitum with free access to water or sucrose solution.

Fecal samples were collected by abdominal massage at the start of the experiment (*time 0*) and after *weeks 1*, *3*, *9*, *12*, *20*, and *24*. The energy content of the feces from *week 20* was determined by differential scanning calorimetry using a TGA/SDTA851e thermobalance (Mettler Toledo, Columbus, OH) with an integrated SDTA signal. After *weeks 10* and *16*, blood samples were collected from the saphenous vein after overnight fasting. After *week 23*, the rats were placed in metabolic cages for urine collection. After *week 24*, they were fasted overnight and anesthetized intraperitoneally with ketamine (Merial Laboratorios, Barcelona, Spain) and xylazine (Química Farmacéutica, Barcelona, Spain) at doses of 80 and 10 mg/kg body wt, respectively. Blood was collected by cardiac puncture, and plasma was immediately obtained by centrifugation. Perigonadal fat, quadriceps muscle, and liver samples were collected, washed with 0.9% NaCl solution, weighed, and immediately frozen in liquid N_2. All the samples were stored at −80°C until analysis.

Biometric measurements. Feed and drink intake, as well as body weight, were measured weekly. Feed and drink intake per day as a function of body weight were estimated by dividing the total intake per cage by the weight of the animals in that cage and the number of days, and then they were averaged over the total number of cages in a group. Fat and fructose intake was calculated from the experimental measurements, and the composition of the feed and drink: fat, 0.04 g/g standard feed (groups STD and HS) and 0.23 g/g HF feed (HF group); and fructose, 0.175 g/ml water (HS group) and 0.17 g/g HF feed (HF group). Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors, assigning 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

Systolic and diastolic blood pressure was measured at 0 min and after *weeks 4*, *9*, *15*, and *22* by the tail-cuff method, using a noninvasive automatic blood pressure analyzer (Harvard Apparatus, Holliston, MA).

Measurement of uric acid. Total urine uric acid was determined by a spectrophotometric method using a uricase/peroxidase kit from BioSystems (Barcelona, Spain) by measuring the absorbance at 520 nm on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). Creatinine levels in urine were determined by a colorimetric method using a commercial kit (C-cromatest Linear Chemicals, Montgat, Spain) by measuring absorbance at 510 nm.

Glycemic status. Fasting blood glucose and plasma insulin levels were measured after *weeks 10* and *16* on fasted animals. Blood glucose concentration was measured by the enzyme electrode method, using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care, Basel, Switzerland); plasma insulin levels were measured using Milliplex xMAP multiplex technology on a Luminex xMAP instrument (Millipore, Austin, TX). The standard curve was generated for the range 69–50,000 pg/ml, using a five-parameter logistic curve fit. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the formula HOMA-IR = fasting blood glucose in mmol/l × fasting plasma insulin in μU/ml ÷ 405 (31). Insulin units (IU) were calculated using the conversion 1 IU = 0.0347 mg insulin. Oral glucose tolerance tests (OGTT) were performed after *weeks 13* and *21* on fasted animals. A solution of glucose (1 g/kg body wt) was administered by oral gavage before the tests, and blood glucose concentration was measured 15, 30, 45, 60, 90 and 120 min after glucose intake.

Histology of the liver. Fixed livers were dehydrated in alcohol and embedded in paraffin, then cut into 3-μm-thick slices, using an HM 355S Rotary Microtome (Thermo Fisher Scientific, Waltham, MA). Sections were stained with hematoxylin (hematoxylin solution modified according to Gill III for microscopy; Merck, Darmstadt, Germany) mixed with eosin (Pharmacy Service of Puerta del Mar Hospital, Cádiz, Spain). The tissue sections were viewed under a Nikon Eclipse 80i light microscope (Nikon, Minato, Japan). Four variables were graded (0-3) following the method described by Taltavull et al. (48): steatosis, steatosis localization, lobular inflammation with lymphoplasmacytic inflammatory infiltration, lobular inflammation with lymphoplasmacytic inflammatory infiltration, and the presence of microgranulomas.

Measurement of diacylglycerols. Frozen samples of liver, muscle, and AT were weighed and sonicated (SFX150 Sonifier; Emerson Industrial Automation, St. Louis, MO) until total homogenization. Diacylglycerol (DAG) extracts were prepared in the presence of BHT (butylated hydroxytoluene, 0.01%) and analyzed using a reported method (46) with some modifications. The mixtures were fortified with an internal standard (1,3–17:0 D5 DG, 200 pmol; Avanti Polar Lipids, Alabaster, AL) and incubated overnight at 48°C. After solvent evaporation, the samples were suspended in methanol, centrifuged (9,390 *g*, 3 min), and the supernatants loaded into an Acquity UPLC system connected to an LCT Premier orthogonal accelerated time-of-flight mass spectrometer (Waters, Milford, MA), operated in positive ESI mode. Full-scan spectra from 50 to 1,500 Da were acquired, and individual spectra were summed to produce data points of 0.2 s each. Mass accuracy and precision were maintained by using an independent reference spray (leucine enkephalin) via the LockSpray interference. A C8 Acquity UPLC-bridged ethylene hybrid 100 × 2.1 mm inner diameter, 1.7 μm column (Waters) was used in the separation

step. The samples (8 μl) were eluted with a binary system consisting of 0.2% (vol/vol) formic acid, 2 mM ammonium formate in water [A] and in methanol [B] under linear gradient conditions: 0 min, 80% B; 3 min, 90% B; 6 min, 90% B; 15 min, 99% B; 18 min, 99% B; 20 min, 80% B; and 22 min, 80% B, at 30°C. The flow rate was 0.3 ml/min. Quantification was carried out using the extracted ion chromatogram of each compound, using 50-mDa windows.

Subpopulations of gut microbiota. The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales, and *Escherichia coli* were estimated from fecal DNA by quantitative real-time PCR (qRT-PCR). DNA was extracted from feces using QIAamp DNA stool mini kit from Qiagen (Hilden, Germany) and quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA). DNA samples were diluted to 20 ng/μl, and qRT-PCR was carried out on a LightCycler 480 II (Roche, Basel, Switzerland) in triplicate. The samples contained DNA (2 μl) and a master mix (18 μl) made of 2× SYBR (10 μl), the corresponding forward and reverse primer (1 μl each), and water (6 μl). All reactions were paralleled by analysis of a nontemplate control (water) and a positive control. The primers and annealing temperatures are detailed in Table 1. Total bacteria were normalized as 16S rRNA gene copies per milligram of wet feces (copies/mg).

Measurement of short-chain fatty acids. Short-chain fatty acids (SCFAs) in feces were analyzed after *week 12* by gas chromatography using a reported method (45), with some modifications. SCFAs were extracted from freeze-dried feces (~50 mg) with a mixture consisting of acetonitrile-water 3:7 (1 ml) and the internal standard 2-ethylbutyric acid (0.1 ml, 100 mg/l) and 0.1 M oxalic acid (0.5 ml), both in the same solvent, for 10 min using a horizontal shaker. Finally, the suspension was centrifuged (12,880 *g*, 5 min) in a 5810R centrifuge (Eppendorf, Hamburg, Germany), and the supernatant passed through a 0.45-μm nylon filter. Aliquots (0.7 ml) were diluted to 1 ml with acetonitrile-water 3:7, and the SCFAs were analyzed using a Trace2000 gas chromatograph (ThermoFinnigan, Waltham, MA) coupled to a flame ionization detector equipped with an Innowax 30 m × 530 μm × 1 μm capillary column (Agilent, Santa Clara, CA). The method showed good selectivity (for acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid), sensitivity, linearity, and accuracy (trueness and precision). To check the method trueness and precision, a recovery study at three concentrations was performed on three different days. Precision (RSD <15%) and recovery (>70%) were adequate, as was intraday reproducible.

Biomarkers and lipid mediators of inflammation. Plasma LPS concentration was estimated by reaction with Limulus amoebocyte extract: LAL kit end point-QCL1000 (Cambrex BioScience, Walkersville, MD). Plasma samples collected at the end of the study under sterile conditions were diluted 70-fold and heated for 20 cycles of 10 min at 68°C and 10 min at 4°C each. An internal control for LPS recovery was included.

Levels of plasma IL-6 were measured using Milliplex xMAP multiplex technology. Liver function was ascertained by measuring the activities of alanine transaminase (AST) and aspartate transaminase (ALT) in plasma by a spectrophometric method using kits from Spinreact (Sant Esteve de Bas, Spain), and it is expressed as the AST/ALT ratio.

Lipid mediators from the metabolism of arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were determined in plasma by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a method modified from Dasilva et al. (6). Erythrocyte-free plasma samples (90 μl) were thawed, diluted in the presence of BHT, and spiked with the internal standard 12HETE-d8 (Cayman Chemicals, Ann Arbor, MI). Then, the samples were centrifuged (800 *g*, 10 min), and the lipids in the supernatants were purified by solid-phase extraction (SPE). The LC-MS/MS analyzer consisted of an Agilent 1260 Series chromatograph (Agilent) coupled to a dual-pressure linear ion trap mass spectrometer LTQ Velos Pro (Thermo Fisher, Rockford, IL) operated in negative ESI mode. A C18-Symmetry 150 × 2.1 mm inner diameter, 3.5 μm column (Waters) with a C18 4 × 2 mm guard cartridge (Phenomenex, Torrance, CA) were used in the separation step. Samples (10 μl) were eluted with a binary system consisting of 0.02% aqueous formic acid [A] and 0.02% formic acid in methanol [B] under gradient conditions of 0 min, 60% B; 2 min, 60% B; 12 min, 80% B; 13 min, 80% B; 23 min, 100% B; 25 min, 100% B; and 30 min, 60% B, at a flow rate of 0.2 ml/min.

Measurement of isoprostanes. F_2-isoprostanes (F_2-IsoPs) were determined in urine samples by LC/ESI-MS/MS following a previously reported procedure (35) with modifications. Urine samples (500 μl) were acidified, β-glucuronidase (90 U/ml) (Sigma, Saint Louis, MO) was added, and the mixtures were incubated for 2 h at 37°C. After the addition of the internal standard [$^2H_4$]15-F_{2t}-IsoP (Cayman, Ann Arbor, MO) (100 μl, 10 μg/l), F_2-IsoPs were purified by SPE. F_2-IsoPs were analyzed using an Agilent 1260 chromatograph fitted with a Mediterranea Sea 18 column (10 cm × 2.1 mm ID, 2.2-μm particle size) (Teknokroma, Barcelona, Spain) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA). The instrument was operated in the negative ion mode with a Turbo V source to obtain MS/MS data. Separation was achieved with a binary system consisting of 0.1% aqueous formic acid [A] and formic acid in acetonitrile [B], at 40°C, with an increasing linear gradient (vol/vol) of [B]: 0 min, 10% B; 7 min, 50% B; 7.1 min, 100% B; 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at a flow rate of 700 μl/min. F_2-IsoPs were detected by MS/MS multiple reaction monitoring. Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The LOQ was 0.4 μg/l for 15-F_{2t}-IsoP and 2 μg/l for 5-F_{2t}-IsoP. The results were expressed as nanograms per milligram of creatinine, to correct for urine dilution.

Statistical analysis. All data manipulation and statistical analysis were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). The results are expressed as means ± SE. Normal distribution and heterogeneity of data were evaluated, and statistical

Table 1. *Quantitative RT-PCR primers and conditions*

Target Bacteria	Positive Control	Annealing Temperature, °C	Sequence (5′-3′)	Reference
Total Bacteria	#	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(18)
Bacteroidetes	*Bacteroides fragilis*	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	(22)
Firmicutes	*Lactobacillus brevis*	52	F: AGA GTT TGA TCC TGG CTC R: ATT ACC GCG GCT GCT GG	(17) (37)
Enterobacteriales	*E. coli* M15	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	(18)
Escherichia coli	*E. coli* M15	61	F: GTT AAT ACC TTT GCT CAT TGA R: ACC AGG GTA TCT AAT CCT GTT	(29)

R, reverse; F, forward. #Positive control of total bacteria was the strain with which the result was rated.

Table 2. *Feed, drink, and energy intakes of rats fed different diets for 24 wk, and residual excreted energy in feces after 20 wk of intervention*

	STD		HF		HS	
	Mean	SE	Mean	SE	Mean	SE
Feed intake, g·day^{-1}·100 g body wt^{-1}	5.4	0.3	4.1*	0.3	2.7*†	0.2
Drink intake, ml·day^{-1}·100 g body wt^{-1}	7.2	0.3	5.5	0.3	10.7*†	0.3
Total energy intake, kcal·day^{-1}100 g body wt^{-1}	15.5	0.8	19.5*	1.2	22.8*	0.8
Energy in feces[a]	306.7	19.4	362.3	22.8	291.8†	17.3

Data are presented as means with their standard errors of the mean; $n = 9$ per group. Comparisons were performed using one-way ANOVA and Tukey's multiple-comparisons test. *$P < 0.05$ vs. STD group; †$P < 0.05$ vs. HF group. [a]Integrated STD signal (kcal·°C^{-1}·g^{-1}) proportional to energy.

significance was determined by two-way ANOVA for repeated measurements or one-way ANOVA, and the Tukey multiple-comparisons test was used for mean comparison. Differences were considered significant when $P < 0.05$.

RESULTS

Feed and drink intakes and energy balance. Feed intake was lower in animals fed the HF diet than in those in the STD group, and even lower in animals given the HS diet (Table 2). Drink intake was higher in animals in the HS group than in those from the other two groups. Energy intake was higher in both the HF and HS groups than in the STD group. Residual energy in feces was similar in the STD and HF groups, and lower in the HS group ($P < 0.05$ vs. HF). The HF animals consumed significantly ($P < 0.001$) more fat than the other two groups throughout the experiment (Fig. 1*A*), while HS rats consumed significantly ($P < 0.001$) more fructose (Fig. 1*B*) per 100 g of body weight than rats in the other groups. As the only source of carbohydrate in the HF diet was sucrose (equimolar fructose and glucose), the rats in this group consumed as much glucose as fructose.

Weight gain and lipid accumulation. Body weight was similar in all of the groups at the beginning (235.9 g, SE 3.6), and no differences were observed between the STD and HS groups during the whole experiment (Fig. 1*C*). After 6 wk, the HF group had significantly ($P < 0.001$) increased body weight (352.0 g, SE 10.8) compared with the STD (305.9 g, SE 7.3) and HS (292.4 g, 9.1) groups, and the differences in weight gain increased until the end of the study (STD: 416.4 g, SE 12.9; HF: 544.3 g, SE 15.5, after *week 24*). Perigonadal AT weight was significantly ($P < 0.001$) higher in the HF group than in the other two groups (Fig. 1*E*).

Blood pressure and urine uric acid. Systolic and diastolic blood pressures (Fig. 1*D*) were similar in the STD and HF groups throughout the experiment. After 23 wk of intervention, systolic blood pressure was significantly higher ($P < 0.001$) in animals given the HS diet than in those given the STD or HF diet. Diastolic blood pressure was higher ($P < 0.05$ vs. STD) in HS-fed animals from *week 9* to *week 23*. Animals fed HS

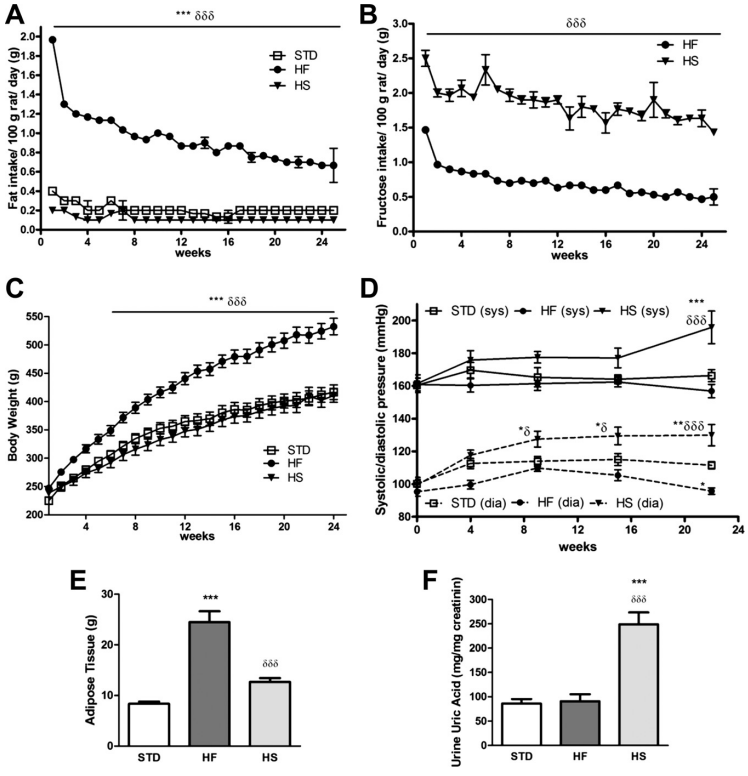


Fig. 1. Fat intake (*A*), fructose intake (*B*), body weight (*C*), blood pressure (*D*), adipose tissue weight (*E*), and uric acid in urine (*F*) of rats ($n = 9$ per group) fed the standard (STD), high-fat (HF), or high-sucrose (HS) diet for 24 wk. Data are presented as means ± SE. Comparisons were conducted using two-way ANOVA (*A–D*) or one-way ANOVA (*E* and *F*) and Tukey's multiple-comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. STD group; δδδ$P < 0.001$ and δ$P < 0.05$ vs. HF group.

Table 3. *Fasting plasma glucose and insulin concentration and calculated HOMA-IR*

	STD		HF		HS	
	Mean	SE	Mean	SE	Mean	SE
Week 10						
Fasting glucose, mg/dl	65.4	1.4	75.7***	2.0	64.8††	2.7
Insulin, pg/ml	417.9	71.4	1737.0***	137.6	584.5†††	105.8
HOMA-IR	1.9	0.3	9.3***	0.7	2.7†††	0.5
Week 16						
Fasting glucose, mg/dl	66.0	1.1	70.8*	1.4	59.3††	3.0
Insulin, pg/ml	661.2	53.9	1654.8*	331.5	1090.1	203.6
HOMA-IR	3.1	0.2	8.4*	1.8	4.7	1.1

Data are presented as means ± SE; $n = 9$ per group. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test.*$P < 0.05$ and ***$P < 0.001$ vs. STD group; ††$P < 0.01$, †††$P < 0.001$ vs. HF group.

presented a significantly ($P < 0.001$) higher concentration of uric acid in urine at *week 23* than those of the STD and HF groups (Fig. 1*F*).

Glycemic status. Fasting blood glucose and plasma insulin concentration were measured after *weeks 10* and *16* of the intervention (Table 3). HOMA-IR is used as an indicator of IR. At both times, both fasting glucose and insulin, as well as HOMA-IR, were significantly higher in the HF group than in the STD group. After 16 wk of intervention, the plasma insulin concentration and HOMA-IR in the HS group were not different from the values in either the STD or the HF group (Table 3). The OGTT was performed twice during the study, after *weeks 13* and *21* (Fig. 2). The increase of the area under the curve (AUC) is an indicator of IGT. After 13 wk, the levels of postprandial glucose in the HF group were higher than those in the other groups (STD and HS), 30 and 60 min after administration (Fig. 2). The AUC corresponding to the HF group was significantly higher ($P < 0.001$) than that in the STD group. By the end of the study (*week 21*), the plasma glucose levels in groups HF and HS were similar and significantly higher than those of the STD group at all the time points (Fig. 2).

Liver histology, total liver triacylglycerols, and biochemical measurement of liver function. An excess of dietary fat triggered lobular inflammation and microgranulomas, while an excess of fructose did not (Fig. 3*A*). The livers of animals fed the HF diet showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels (e.g., Fig. 3*C*). Conversely, an excess of fructose induced significant and highly localized steatosis (Fig. 3*A, D*), while an excess of dietary fat did not (Fig. 3*A, C*). The levels of total triacylglycerols (μmol/g liver) were STD: 46.8, SE 20.1; HF: 80.4, SE 19.7; and HS: 168.1, SE 92.6, with no significant differences between the groups. No liver functional damage resulted from any of the diets, as revealed by the similar AST/ALT ratio in the three groups (STD: 6.7, SE 0.6; HF: 6.3, SE 0.8; and HS: 5.7, SE 1.2).

Diacylglycerols in tissues. The levels of saturated DAGs 38:0 and 40:0, and unsaturated DAGs 34:2, 34:3, 34:4, 36:5, and 40:5, were lower in liver in the HF group than in the STD and/or HS groups (Table 4). The levels of unsaturated DAGs 32:1, 32:2, 34:1, and 36:2 were higher in animals that consumed an excess of fructose than in animals in the other two groups, whereas the levels of DAG 42:12 were lower. The levels of DAGs 36:4, 36:6, 38:1, and 38:6, were lower in both HS and HF groups than in the STD group.

In muscle (Table 5), the levels of DAGs 34:3, 38:3, 38:4, 38:5, and 40:5 were lower in the HF group than in the STD group, whereas the levels of DAG 36:1 were higher. The levels of unsaturated DAG 32:1 were higher in the HS group than in

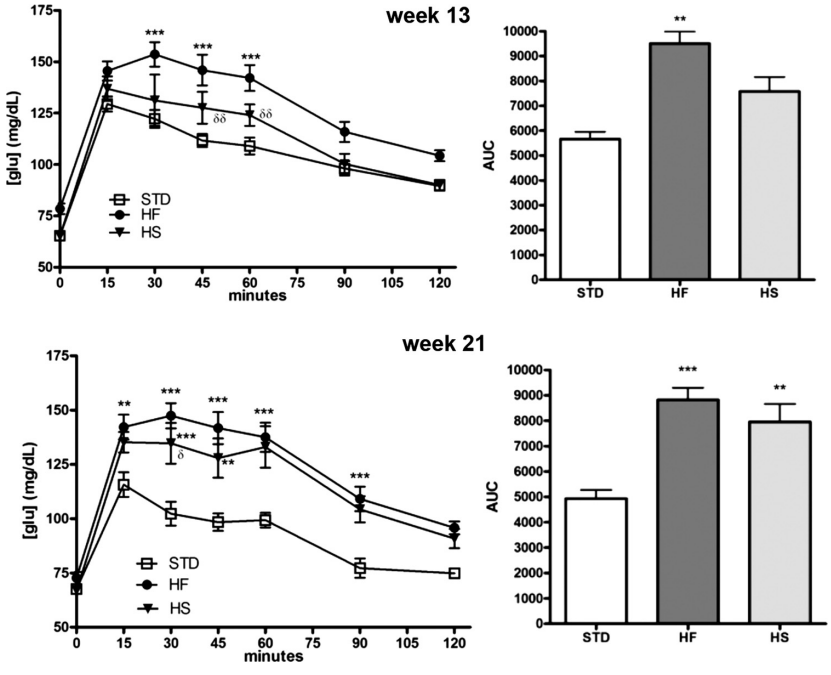


Fig. 2. Glycemic response in rats ($n = 9$ per group) fed the STD, HF, or HS diet. Curves of OGTT after ingestion of a single dose of glucose (1 g/kg body wt) after *weeks 13* and *21* of intervention, and the corresponding areas under the curve (AUC). Values are presented as means ± SE. Comparisons were conducted using two-way ANOVA (OGTT curves) or one-way ANOVA (AUCs) and the Tukey multiple-comparisons test. **$P < 0.01$, and ***$P < 0.001$ vs. STD group; δ$P < 0.05$ and δδ$P < 0.01$ vs. HF group.

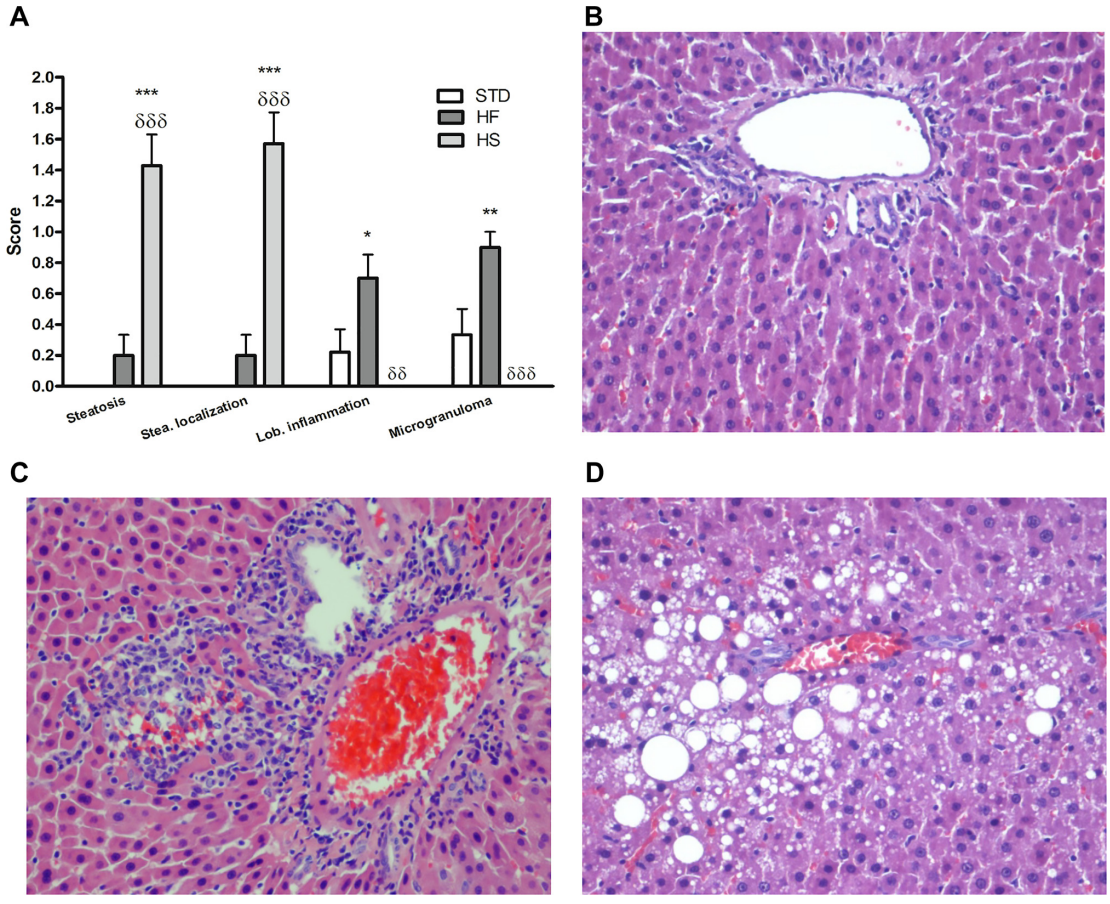


Fig. 3. Liver histology. Estimation of variables (*A*) and cuts stained with hematoxylin and eosin. Rats fed the STD diet present normal liver (*B*; ×20), rats fed the HF diet present inflammatory infiltration within the portal triad without steatosis (*C*; ×20), and rats fed the HS diet present steatosis without inflammatory infiltration (*D*; ×20). Scores are presented as means ± SE. Comparisons were conducted using one-way ANOVA and Tukey's test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. STD group; $\delta\delta P < 0.01$ and $\delta\delta\delta P < 0.001$ vs. HF group.

the other two groups (STD and HF). The levels of DAG 34:1 were higher, and those of 36:4 were lower in both HS and HF groups than in the STD group. In perigonadal AT (Table 6), only the levels of DAG 42:1 were higher in the HF group than in the other two groups (STD and HS). The levels of unsaturated DAGs 32:3, 36:1, 36:2, 38:2, and 40:3 were lower in animals that consumed an excess of fructose than in animals in the STD or HF groups. The levels of DAGs 34:1, 34:2, 34:3, 34:4, 36:3, 36:4, 36:5, 36:6, 38:3, 38:4, 38:5, 38:6, and 40:5 were lower in both HS and HF groups than in the STD group.

Subpopulations of gut microbiota and microbial products. The Bacteroidetes:Firmicutes ratio (Fig. 4*A*) was reduced in HF animals and increased in HS animals compared with STD animals. The proportion of Enterobacteriales and *E. coli* in animals given the HF diet was already significantly increased after *weeks 1* and *3* and tended to decrease gradually afterward (Fig. 4, *B* and *C*). An excess of fructose only increased the populations of Enterobacteriales and *E. coli* at the end of the study (Fig. 4, *B* and *C*).

The levels of acetic, propionic, and isobutyric acids, and total SCFAs were lower in the feces of HF animals than in those fed the STD or HS diet (Table 7).

The HF animals showed a nonsignificant tendency ($P = 0.1$ vs. STD group) toward increased plasma concentration of LPS at the end of the study (Fig. 5*A*). The HS animals did not show any increase in plasma LPS.

Markers and lipid mediators of inflammation. The animals fed the HF diet showed increased plasma concentrations of IL-6 and PGE_2 (EPA metabolite) compared with the STD group at the end of the study (Fig. 5, *B* and *C*). The concentrations of ARA, EPA, and DHA were higher in the HS group than in the STD and HF groups (Fig. 5, *D–F*). No differences were detected in the concentration of LTB_4 (leukotriene B_4) or 12HEPE (12-hydroxyeicosapentaenoic acid), ARA, and EPA metabolites, respectively (Fig. 5, *G* and *H*). The concentration of the DHA metabolite 17HDoHE (17-hydroxy docosahexaenoic acid) was lower in animals fed the HS diet than in those fed the HF diet (Fig. 5*I*).

Table 4. *Liver diacylglycerols by LC-MS*

	STD		HF		HS	
DAGs	Mean	SE	Mean	SE	Mean	SE
32:0	2332	504	2256	447	5224	2063
34:0	7661	1676	8044	1469	17320	8406
36:0	5598	1213	6116	1124	12040	5659
38:0	296.3	53.3	139.1*	24.9	208.9	32.7
40:0	32.5	5.0	14.3**	14.3	22.0	3.3
32:1	64.3	6.8	69.2	12.7	242.6*†	72.1
32:2	15.1	1.5	14.8	2.4	43.6*†	12.5
34:1	405.7	43.0	540.4	100.1	1499.0*†	427.2
34:2	328.8	39.1	139.6	26.6	414.6†	111.1
34:3	88.0	10.7	27.0*	6.0	101.7†	26.7
34:4	17.4	2.3	2.1*	1.4	18.1†	6.5
36:1	85.6	10.1	111.4	19.3	131.0	31.9
36:2	321.4	35.0	576.4	117.5	1296.0*	380.6
36:3	711.1	86.3	398.9	78.5	681.0	182.1
36:4	381.7	48.8	102.1***	18.7	203.0**	34.4
36:5	50.5	6.3	8.3***	2.6	29.6	8.5
36:6	12.2	2.6	0.0***	0.0	1.7**	1.7
38:1	6.3	1.6	0.0***	0.0	1.6*	1.1
38:2	12.2	1.5	9.7	2.4	17.8	4.7
38:3	18.5	5.1	17.6	4.5	21.0	5.2
38:4	436.5	54.8	325.4	44.2	367.5	41.2
38:5	220.1	25.2	131.8	23.0	170.2	35.3
38:6	227.7	27.3	57.8***	10.3	66.7***	10.5
40:5	171.8	27.1	90.6*	17.9	112.9	16.7
42:5	141.9	22.2	80.6	17.4	80.4	10.2
42:12	12.3	1.8	10.1	1.6	3.5**†	1.3

Data are presented as means with their standard errors of the mean; $n = 9$ per group. DAGs are given in nanomoles per gram tissue. DAG amounts are expressed as DAG 16:0, 16:0 equivalents. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. STD group; †$P < 0.05$ vs. HF group.

Isoprostanes as markers of oxidative stress. The animals fed HS showed increased concentrations of 5-F_{2t}-IsoP and 15-F_{2t}-IsoP ($P < 0.05$) compared with the STD and HF groups after 23 wk of intervention (Fig. 6, *A* and *B*).

DISCUSSION

The present study explores some molecular factors behind the differential action of an excess of dietary fat or fructose on normal rats and examines the role that gut microbiota may play in these processes. A prediabetic state was induced to WKY rats by HF or HS[1] diets over a period of 24 wk. Despite all of the information available on the induction of IR and IGT by fat and fructose in rat models, the molecular mechanisms behind this action are still largely unknown. In our models, fat induced a prediabetic state faster than fructose, as evidenced by the results of fasting blood glucose, plasma insulin concentration, HOMA-IR (Table 3), and the OGTT (Fig. 2). Only the animals in the HF group presented both IR (HOMA-IR) and IGT (OGTT). The rats that consumed an excess of sugar showed a tendency toward elevated plasma insulin and significant IGT only at the end of the study (Table 3 and Fig. 2). The differences in timing between the metabolic response to fat and fructose prompted us to explore the changes induced in some molecular factors known to be mechanistically related to the development of IR and IGT. As low-grade inflammation may trigger IR, we measured inflammation markers in the two models. Unlike the rats in the HS group, the livers of HF-fed animals clearly presented inflammatory infiltrations within the portal space (Fig. 3). This is a sign of systemic inflammation that was confirmed by the plasma levels of IL-6 and PGE_2 (Fig. 5, *B* and *C*). IL-6 and other inflammatory markers are elevated in obesity-induced low-grade inflammation-related IR (11, 25); ARA-derived cyclooxygenase (COX)-mediated proinflammatory factor PGE_2 is the predominant prostaglandin in white AT, where it regulates adipose functions (26). PGE_2 also inhibits pancreatic β-cell function and insulin secretion (43), which is an effect characteristic of intermediate stages of diabetes, beyond the initial increase in insulin secretion (IR, compensation stage) (50). Conversely, potentially anti-inflammatory EPA and DHA were elevated in animals given an excess of fructose (Fig. 5, *E* and *F*). EPA and DHA are considered to protect against inflammation mainly because they compete with ARA for the same metabolizing enzymes and also because they generate protectins and resolvins as metabolites (7, 30). So, the HF animals presented systemic inflammation, while the HS diet not only did not trigger early systemic inflammation, but also may have favored anti-inflammatory pathways.

The observation of a nonsignificant trend ($P = 0.1$) toward elevated plasma LPS concentrations (Fig. 5*A*) suggested that microbe-derived endotoxemia might contribute to the low-grade inflammation in HF-fed animals. LPS is a component of the wall of Gram-negative bacteria present in Bacteroidetes

Table 5. *Muscle diacylglycerols by LC-MS*

	STD		HF		HS	
DAGs	Mean	SE	Mean	SE	Mean	SE
32:0	30.8	5.5	29.3	4.1	37.2	5.0
34:0	47.1	14.6	41.2	10.7	47.4	15.5
36:0	10.7	10.4	24.2	8.6	26.3	12.0
38:0	3.8	1.5	2.5	0.9	4.1	1.8
40:0	1.1	0.4	1.2	0.3	1.4	0.3
42:0	0.4	0.2	0.5	0.2	0.7	0.2
32:1	5.9	0.8	9.0	1.5	10.7*	0.8
32:2	1.3	0.3	1.6	0.4	1.9	0.3
34:1	29.3	4.2	52.2*	8.5	54.4*	3.0
34:2	19.2	3.7	12.3	2.3	16.4	1.6
34:3	4.6	0.9	2.4*	0.4	3.4	0.4
36:1	4.0	0.7	8.5*	1.8	4.9	0.5
36:2	15.5	2.9	33.8	8.3	30.9	3.2
36:3	18.9	4.4	13.2	3.2	12.4	2.1
36:4	11.5	2.1	4.6**	0.5	4.9**	0.5
36:5	0.0	0.0	0.0	0.0	0.2*††	0.1
38:1	0.2	0.1	0.3	0.1	0.2	0.1
38:2	0.7	0.3	0.6	0.1	0.8	0.2
38:3	1.7	0.5	0.6*	0.1	0.8	0.2
38:4	10.7	1.7	5.2**	0.5	7.3	1.0
38:5	4.2	0.6	2.7*	0.3	3.2	0.3
38:6	3.5	0.5	2.4	0.5	2.4	0.5
40:5	1.3	0.4	0.1**	0.1	0.6	0.2
42:1	0.1	0.1	0.2	0.1	0.5	0.1

Data are presented as with their standard errors of the mean; $n = 9$ per group. DAGs are given in nanomoles per gram tissue. DAG amounts are expressed as DAG 16:0, 16:0 equivalents. Comparisons were conducted using one-way ANOVA and Tukey's tests. *$P < 0.05$, **$P < 0.01$, and ††$P < 0.01$ vs. HF.

[1] In our high-sucrose model, insulin resistance and probably hypertension are more likely to be triggered by the excess of fructose than by glucose, as glucose can be metabolized and/or stored as glycogen in different organs (e.g., brain, liver, and muscle), and it is carefully controlled by insulin everywhere, while fructose is almost entirely processed, mainly in the liver, and escapes metabolic control by insulin (18).

Table 6. *Adipose tissue diacylglycerols by LC-MS*

	STD		HF		HS	
DAGs	Mean	SE	Mean	SE	Mean	SE
32:0	217.3	30.4	143.4	41.6	137.1	28.6
34:0	50.1	6.2	63.2	21.7	21.1	4.2
38:0	1.0	0.2	0.7	0.3	0.4	0.1
40:0	0.4	0.1	0.3	0.1	0.2	0.1
42:0	0.0	0.0	0.9	0.4	0.0	0.0
32:1	258.3	54.6	281.9	89.9	210.0	48.4
32:2	207.8	44.5	122.3	50.9	70.8	18.4
32:3	26.8	6.9	10.7	5.0	4.2*	1.1
34:1	880.9	106.0	471.6*	112.7	457.8*	81.0
34:2	1338.0	164.7	400.6***	127.2	436.9***	85.7
34:3	1337.0	153.3	449.2***	125.3	404.2***	83.3
34:4	41.7	10.3	6.2***	3.1	4.3***	1.1
36:1	93.8	11.5	213.3	74.7	39.9†	8.6
36:2	874.1	103.3	560.4	139.0	465.2*	83.4
36:3	1337.0	153.2	449.2***	125.3	404.2***	83.3
36:4	41.8	3.1	8.5***	2.0	7.3***	1.0
36:5	120.1	20.0	9.2***	4.3	4.8***	1.6
36:6	2.7	0.6	0.1***	0.1	0.0***	0.0
38:1	4.5	1.2	2.5	2.1	0.5	0.4
38:2	38.4	5.7	18.2	8.9	11.1*	3.0
38:3	77.7	11.1	13.2***	6.5	9.3***	2.5
38:4	55.7	7.3	6.9***	2.5	3.2***	0.5
38:5	63.1	9.8	11.9***	4.3	8.5***	1.8
38:6	2.5	0.3	0.4***	0.1	0.3***	0.0
40:3	0.3	0.1	0.2	0.1	0.0**	0.0
40:5	2.6	0.4	0.1***	0.1	0.0***	0.0
42:1	0.2	0.1	1.0*	0.3	0.2†	0.0

Data are presented as with their standard errors of the mean; $n = 9$ per group. Comparisons were conducted using one-way ANOVA and Tukey's tests. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. STD group; †$P < 0.05$ vs. HF. DAGs are given in nanomoles per gram tissue. DAG amounts are expressed as DAG 16:0, 16:0 equivalents.

and Enterobacteriales but not in Firmicutes. As the population of Bacteroidetes clearly decreased in HF animals with respect to STD rats (Fig. 4*A*), the increase in LPS and proinflammatory mediators detected in the plasma of HF animals (Fig. 5, *A–C*) might originate from Enterobacteriales (Fig. 4*B*), particularly its major member *E. coli* (Fig. 4*C*). This agrees with observations by other authors (8, 15). Additional research should confirm or refute this suggestion. In addition to LPS, other mediators proposed as the link between gut bacteria and metabolic alterations are bile acids, angiopoietin-like protein 4, and SCFAs: products of microbial fermentation of dietary fiber (23). SCFAs acetate and butyrate generated by Firmicutes may contribute to body fat gain through de novo lipogenesis (2). Together with propionate, these SCFAs exhibit a protective effect against inflammation and IR (2). In apparent contradiction, the animals that presented the lowest Bacteroidetes-to-Firmicutes ratio and gained most weight (HF group) generated the lowest amounts of total SCFAs (Table 7). This may be due to the composition of the diet. Whereas the standard diet (fed to the STD and HS groups) contains wheat middlings, ground wheat, and ground corn, the only source of fiber in the HF diet is cellulose (50 g/kg). The lower diversity in the source of fiber may be the cause of the reduced production of protective SCFAs.

As DAGs may induce IR independently of inflammation, we measured their levels in liver, muscle, and AT. We did not detect any significant increase in the DAG profile in the livers of rats fed a HF diet (Table 4), in agreement with the absence of steatosis (Fig. 3*C*). The only DAGs with elevated levels in the HF group were the monounsaturated 34:1 (muscle), 36:1 (muscle), and 42:1 (AT). As lipid-induced IR occurs earlier in liver than in muscle (28), we concluded that direct impairment of insulin signaling by lipid metabolites would not explain the observed early fat-induced systemic IGT. In contrast, the animals given an excess of fructose presented steatosis around the blood vessels (Fig. 3*D*) and clearly elevated DAGs in the liver (Table 4), without gaining more weight or accumulating more perigonadal AT than those given the STD diet (Fig. 1, *C*

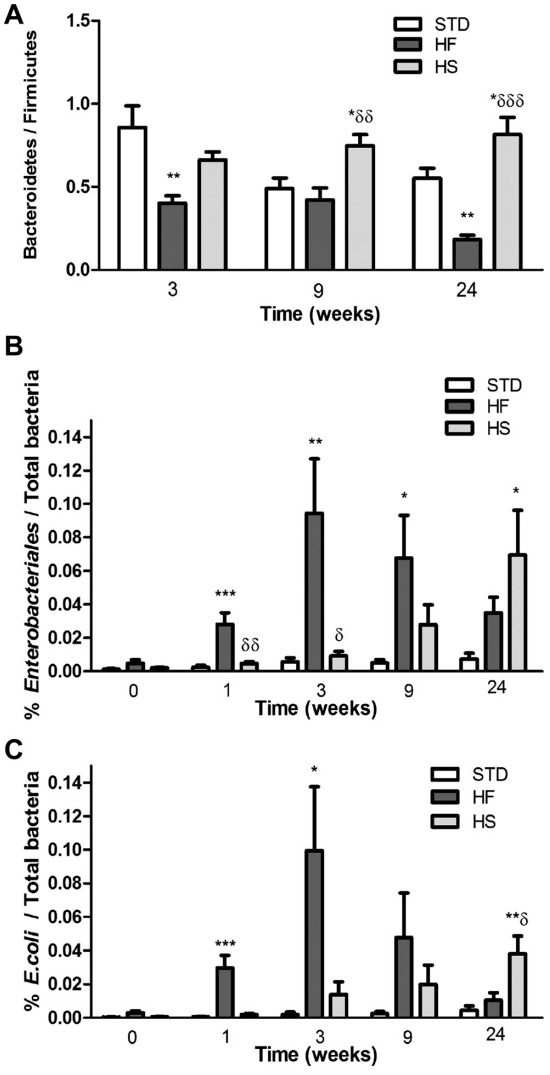


Fig. 4. Excreted intestinal bacteria measured by quantitative RT-PCR and expressed as percentages of total bacteria in fecal samples from rats ($n = 9$ per group) fed a STD, HF, or HS for 24 wk of nutritional intervention. *A*: Bacteroidetes/Firmicutes ratio. *B*: Enterobacteriales. *C*: *Escherichia coli*. Results are presented as means ± SEs. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. STD group; δ$P < 0.05$, δδ$P < 0.01$, and δδδ$P < 0.001$ vs. HF group.

Table 7. *Short-chain fatty acids in feces after 12 weeks*

	STD		HF		HS	
SCFA	Mean	SE	Mean	SE	Mean	SE
Acetic acid	310.9	61.1	149.5*	17.6	265.5	45.6
Propionic acid	27.4	6.2	3.1**	0.6	28.0††	5.7
Isobutyric acid	1.3	0.2	0.2***	0.0	1.1†††	0.1
Butyric acid	17.6	3.3	6.2	1.6	23.1†	5.8
Isovaleric acid	1.0	0.3	0.4	0.1	1.0	0.2
Valeric acid	0.7	0.1	0.4	0.1	1.1†	0.2
Total SCFAs	356.9	66.1	159.4*	19.1	287.2	55.3

Data are presented as means with their standard errors of the mean; $n = 9$ per group. Short-chain fatty acids (SCFAs) amounts are given in millimoles per kilogram feces. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. STD group; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, and $\dagger\dagger\dagger P < 0.001$ vs. HF group.

and *E*). The levels of total triglycerides were also elevated in the HS group, although the differences were not statistically significant, probably due to the fact that the steatosis was highly localized. These observations are consistent with hepatic de novo lipogenesis from fructose (20). It is becoming evident that different DAGs have a very different impact on cellular signaling (13). While there is no information so far as to what particular DAG species might impair insulin signaling, it has been reported that cellular signaling proteins and receptors such as the human transfer receptor potential C3 (TRPC3) are differentially activated by different DAGs and that DAG 36:2 (1-stearoyl-2-linoleoyl-*sn*-glycerol) is one of the active species (21, 38). The amounts of DAGs 36:2 and 34:1 (putatively 1-palmitoyl-2-oleoyl-*sn*-glycerol) were dramatically increased (three- to four-fold) in the livers of HS-fed rats (Table 4), which suggests that DAG 36:2 may play a role in late fructose-induced tendency to IR. DAG 34:1, which does not activate the TRCP3 calcium channel (38), may or may not play a role in our model. In muscle, DAG content is much lower than in the liver, and only the levels of DAG 32:1 were slightly elevated in the muscular tissue of animals given the HS diet and those of DAG 34:1 in both the HF and HS groups (Table 5). As expected, perigonadal AT presented the highest levels of DAGs in all groups (Table 6). None of the significant differences detected corresponded to any increase in DAG levels in the HF or HS group. The overall examination of DAG variations in liver, muscle, and AT showed that the only likely contribution of these lipid metabolites to IR or IGT takes place in the liver of HS-fed rats and that DAGs may not play a significant role in the development of IR or IGT in rats fed an HF diet.

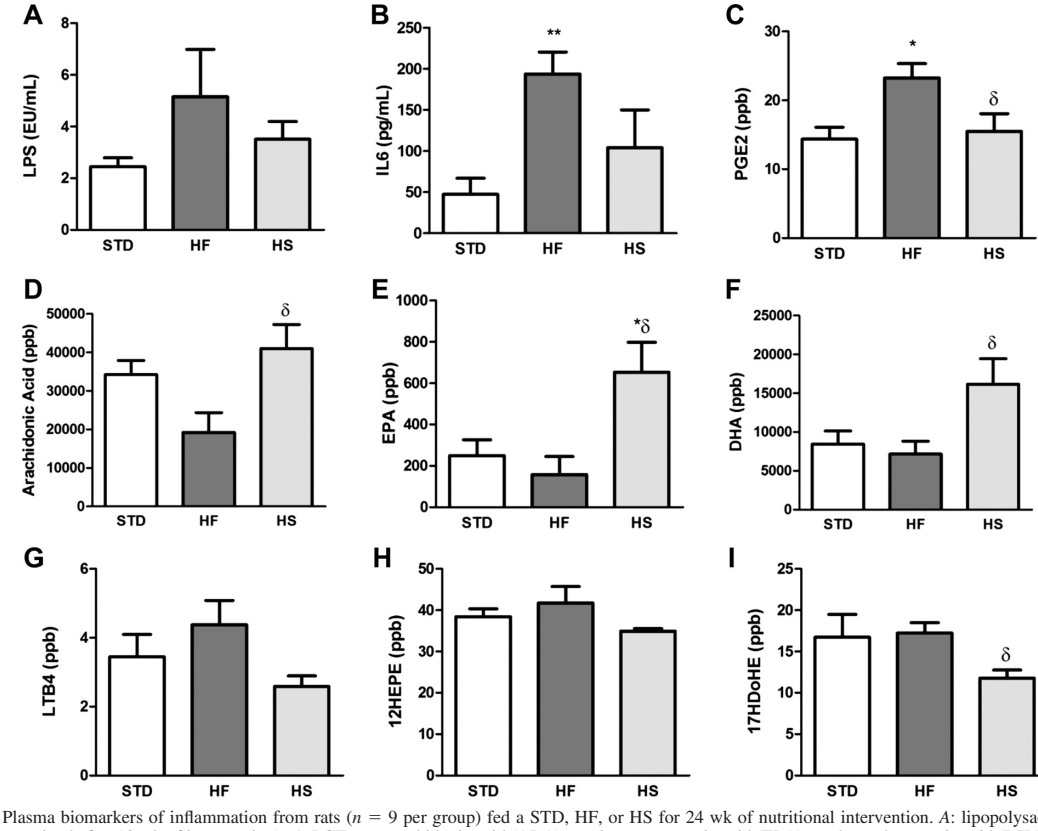


Fig. 5. Plasma biomarkers of inflammation from rats ($n = 9$ per group) fed a STD, HF, or HS for 24 wk of nutritional intervention. *A*: lipopolysaccharide. *B*: IL-6 (determined after 10 wk of intervention). *C*: PGE_2. *D*: arachidonic acid (ARA). *E*: eicosapentaenoic acid (EPA). *F*: docosahexaenoic acid (DHA). *F*: LTB_4. *H*: 12HEPE. *I*: 17HDoHE. Results are presented as means ± SE. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test. $*P < 0.05$ and $**P < 0.01$ vs. STD group; $\delta P < 0.05$ vs. HF group.

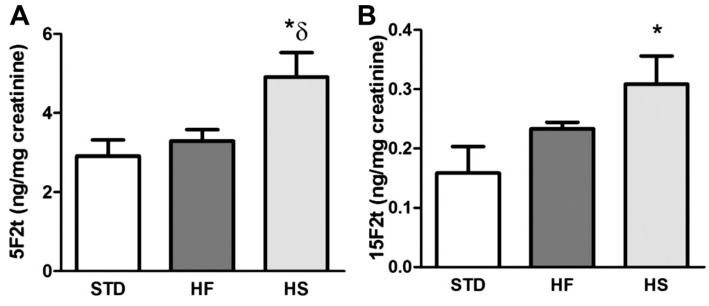


Fig. 6. Isoprostanes in urine from rats ($n = 9$ per group) fed a STD, HF, and HS for 24 wk of nutritional intervention. *A*: 5F2t. *B*: 15F2t. Results are presented as means ± SE. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparisons test. *$P < 0.05$ vs. STD group; δ$P < 0.05$ vs. HF group.

The levels of urine F_2-IsoPs, which are products of free radical-mediated in vivo oxidation of ARA (33) different from COX-derived oxidized prostaglandins, such as PGE_2, were only elevated in animals given an excess of fructose at the end of the study (Fig. 6), consistently with increased levels of ARA (Fig. 5*D*). This implies that an excess of dietary fat did not trigger significant low-grade inflammation-mediated systemic oxidative stress (OS), which may be attributed to the action of the excess of fructose. In agreement with this observation, increased generation of superoxide radical has been detected in the kidneys of high-fructose-fed rats (40). The results also indicate that IR per se does not trigger systemic OS, as the animals given an excess of fat developed IR and IGT for the duration of the entire experiment (Table 3, Fig. 2) while showing IsoP levels similar to those in the STD group (Fig. 6). In contrast with our results, other authors have linked fructose-induced OS to inflammation and hyperinsulinemia (4, 40). Those experiments were performed on Sprague-Dawley rats using shorter intervention times (3–12 wk). In our experiment (WKY rats), if the inflammatory response was associated with OS in an early compensatory stage (stimulation of insulin secretion) (50), it was no longer evident after 24 wk of intervention.

In the present study, only the HS diet induced hypertension, which was statistically significant after 9 wk (diastolic pressure) and 22 wk (systolic pressure) (Fig. 1*D*). As rats in this group consumed significantly more sugar and water than those given the STD or HF diets, the hypertensive effect may be explained, at least in part, by water movement from tissues into the intravascular space caused by elevated levels of blood glucose (41) together with hyperhydration. The high amounts of fructose available in the liver of rats given the HS diet may also have caused elevated blood pressure via the formation of uric acid (32), which is biosynthesized through the degradation of AMP to inosine monophosphate following a fall in intracellular phosphate that originates in the rapid and uncontrolled phosphorylation of fructose (24). This explanation is supported by our observation that the HS group shows significantly higher levels of urine uric acid than the STD and HF groups (Fig. 1*F*). Late mild IR (Table 3) and IGT (Fig. 2) may also contribute to the elevated blood pressure in the HS group, as described in previous studies (49). It should be noted that only hepatic IR (HS group) would in any case bring about hypertension, as the animals showing early IR (HF group, Table 3) were normotensive (Fig. 1*D*). The observation that the populations of Enterobacteriales and particularly *E. coli* only increased in the feces of animals in the HS group (Fig. 4, *B* and *C*) when IGT became significantly high (*week 21*, Fig. 2) suggests a relationship between gut bacteria and late fructose-related metabolic alterations. In this case though, the diet is less likely to induce late changes in the intestine; therefore, the increase in bacterial populations may be a consequence of the systemic action of the diet rather than a cause of it. It has been reported that intestinal uric acid in end-stage renal disease (ESRD) patients increases the populations of bacteria that are able to catabolize uric acid into urea and eventually ammonia (51). As part of the uric acid is excreted through the gut in both humans and rats (19, 47), we hypothesize that uric acid generated by high-fructose-fed rats (Fig. 1*F*) may contribute to the increase in the populations of gut Enterobacteriales (Fig. 4*B*), which are microorganisms known to use urease to metabolize urea (5, 34). To test this hypothesis, it may be worth monitoring changes in the gut populations of microorganisms known to metabolize uric acid into urea. The late increase in Enterobacteriales might trigger further inflammation.

Concluding remarks. This paper examines two rat models of prediabetes with clearly differentiated mechanisms that can be used to test the effects of drugs and food ingredients. As the effects induced are mild, the models are particularly suited for testing functional food ingredients. A high-fat diet induced obesity and fast IR and IGT via low-grade inflammation in WKY rats. In contrast, a high-sucrose (fructose + glucose) diet induced IGT later than the HF diet, by processes triggered by de novo liposynthesis from fructose, probably mediated by active DAGs in the liver. Neither low-grade inflammation (HF group) nor localized steatosis (HS group) severely affected liver function, as assessed by measuring the AST/ALT ratio. Hypertension in HS-fed rats may be due to causes other than systemic IR that include an excess of water/glucose intake and the generation of uric acid from fructose. The development of IR coincides with increased populations of *E. coli* in the intestinal tract that may contribute to low-grade inflammation in HF-fed animals or may be a consequence of the metabolic alterations of an excess of fructose in HS-fed animals. The results show that fat and sugar trigger metabolic alterations by largely independent mechanisms and underscore the potentially harmful effect of the excessive intake of both of these nutrients together.

ACKNOWLEDGMENTS

The language revision by Christopher Evans and technical assistance by Eva Dalmau are appreciated.

GRANTS

This research was supported by the Spanish Ministry of Economy and Competitiveness (Grant AGL2013-49079-C2-1,2-R and a graduate fellowship to M. Hereu: BES2014-068592) and CSIC (grant to G. Dasilva).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.R.-R., I.M., M.R.N., and J.L.T. conceived and designed research; S.R.-R. and M.H. supervised and performed animal intervention, biometric determinations, evaluation of glycemic status, measurement of uric acid, LPS and IL-6, and qRT-PCR experiments; L.A. performed histology; S.R.-R. and J.C. performed DAG determinations; M.H., S.A. and O.J. performed SCFA and IsoP measurements; G.D. and I.M. determined lipid mediators of inflammation; M.R. and M.R.N. evaluated liver function; S.R.-R., M.H., L.A., J.C., O.J., S.A., G.D., and M.R. analyzed data; S.R.-R. and J.L.T. interpreted results of experiments; S.R.-R. and L.A. prepared figures; S.R.-R. and J.L.T. edited and revised manuscript; S.R.-R., M.H., L.A., J.C., O.J., S.A., G.D., I.M., M.R.N., M.R., and J.L.T. approved final version of manuscript; J.L.T. drafted manuscript.

REFERENCES

1. **Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz-Schughart LA, Schölmerich J, Bollheimer LC.** Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *J Mol Endocrinol* 36: 485–501, 2006. doi:10.1677/jme.1.01909.
2. **Canfora EE, Jocken JW, Blaak EE.** Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat Rev Endocrinol* 11: 577–591, 2015. doi:10.1038/nrendo.2015.128.
3. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R.** Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761–1772, 2007. doi:10.2337/db06-1491.
4. **Castro MC, Massa ML, Arbeláez LG, Schinella G, Gagliardino JJ, Francini F.** Fructose-induced inflammation, insulin resistance and oxidative stress: A liver pathological triad effectively disrupted by lipoic acid. *Life Sci* 137: 1–6, 2015. doi:10.1016/j.lfs.2015.07.010.
5. **D'Orazio SEF, Collins CM.** The plasmid-encoded urease gene cluster of the family Enterobacteriaceae is positively regulated by UreR, a member of the AraC family of transcriptional activators. *J Bacteriol* 175: 3459–3467, 1993. doi:10.1128/jb.175.11.3459-3467.1993.
6. **Dasilva G, Pazos M, Gallardo JM, Rodríguez I, Cela R, Medina I.** Lipidomic analysis of polyunsaturated fatty acids and their oxygenated metabolites in plasma by solid-phase extraction followed by LC-MS. *Anal Bioanal Chem* 406: 2827–2839, 2014. doi:10.1007/s00216-014-7701-3.
7. **Dasilva G, Pazos M, García-Egido E, Pérez-Jiménez J, Torres JL, Giralt M, Nogués MR, Medina I.** Lipidomics to analyze the influence of diets with different EPA:DHA ratios in the progression of Metabolic Syndrome using SHROB rats as a model. *Food Chem* 205: 196–203, 2016. doi:10.1016/j.foodchem.2016.03.020.
8. **de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE.** Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *Am J Physiol Gastrointest Liver Physiol* 299: G440–G448, 2010. doi:10.1152/ajpgi.00098.2010.
9. **Després J-P, Lemieux I.** Abdominal obesity and metabolic syndrome. *Nature* 444: 881–887, 2006. doi:10.1038/nature05488.
10. **DiNicolantonio JJ, O'Keefe JH.** Hypertension due to toxic white crystals in the diet: should we blame salt or sugar? *Prog Cardiovasc Dis* 59: 219–225, 2016. doi:10.1016/j.pcad.2016.07.004.
11. **Donath MY, Shoelson SE.** Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* 11: 98–107, 2011. doi:10.1038/nri2925.
12. **Eckel RH, Alberti KGMM, Grundy SM, Zimmet PZ.** The metabolic syndrome. *Lancet* 375: 181–183, 2010. doi:10.1016/S0140-6736(09)61794-3.
13. **Eichmann TO, Lass A.** DAG tales: the multiple faces of diacylglycerol–stereochemistry, metabolism, and signaling. *Cell Mol Life Sci* 72: 3931–3952, 2015. doi:10.1007/s00018-015-1982-3.
14. **Fabbrini E, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, Okunade A, Klein S.** Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc Natl Acad Sci USA* 106: 15430–15435, 2009. doi:10.1073/pnas.0904944106.
15. **Fåk F, Ahrné S, Molin G, Jeppsson B, Weström B.** Microbial manipulation of the rat dam changes bacterial colonization and alters properties of the gut in her offspring. *Am J Physiol Gastrointest Liver Physiol* 294: G148–G154, 2008. doi:10.1152/ajpgi.00023.2007.
16. **Franklin SS, Wong ND.** Hypertension and cardiovascular disease: contributions of the framingham heart study. *Glob Heart* 8: 49–57, 2013. doi:10.1016/j.gheart.2012.12.004.
17. **Haakensen M, Dobson CM, Deneer H, Ziola B.** Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *Int J Food Microbiol* 125: 236–241, 2008. doi:10.1016/j.ijfoodmicro.2008.04.002.
18. **Hartman AL, Lough DM, Barupal DK, Fiehn O, Fishbein T, Zasloff M, Eisen JA.** Human gut microbiome adopts an alternative state following small bowel transplantation. *Proc Natl Acad Sci USA* 106: 17187–17192, 2009. doi:10.1073/pnas.0904847106.
19. **Hatch M, Vaziri ND.** Enhanced enteric excretion of urate in rats with chronic renal failure. *Clin Sci (Lond)* 86: 511–516, 1994. doi:10.1042/cs0860511.
20. **Havel PJ.** Dietary fructose: implications for dysregulation of energy homeostasis and lipid/carbohydrate metabolism. *Nutr Rev* 63: 133–157, 2005. doi:10.1111/j.1753-4887.2005.tb00132.x.
21. **Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G.** Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature* 397: 259–263, 1999. doi:10.1038/16711.
22. **Abdallah Ismail N, Ragab SH, Abd Elbaky A, Shoeib AR, Alhosary Y, Fekry D.** Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Arch Med Sci* 7: 501–507, 2011. doi:10.5114/aoms.2011.23418.
23. **Janssen AW, Kersten S.** Potential mediators linking gut bacteria to metabolic health: a critical view. *J Physiol* 595: 477–487, 2017. doi:10.1113/JP272476.
24. **Johnson RJ, Nakagawa T, Sanchez-Lozada LG, Shafiu M, Sundaram S, Le M, Ishimoto T, Sautin YY, Lanaspa MA.** Sugar, uric acid, and the etiology of diabetes and obesity. *Diabetes* 62: 3307–3315, 2013. doi:10.2337/db12-1814.
25. **Kaliannan K, Wang B, Li XY, Kim KJ, Kang JX.** A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Sci Rep* 5: 11276, 2015. doi:10.1038/srep11276.
26. **Kimple ME, Neuman JC, Linnemann AK, Casey PJ.** Inhibitory G proteins and their receptors: emerging therapeutic targets for obesity and diabetes. *Exp Mol Med* 46: e102, 2014. doi:10.1038/emm.2014.40.
27. **Klein AV, Kiat H.** The mechanisms underlying fructose-induced hypertension: a review. *J Hypertens* 33: 912–920, 2015. doi:10.1097/HJH.0000000000000551.
28. **Kraegen EW, Clark PW, Jenkins AB, Daley EA, Chisholm DJ, Storlien LH.** Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40: 1397–1403, 1991. doi:10.2337/diab.40.11.1397.
29. **Malinen E, Kassinen A, Rinttilä T, Palva A.** Comparison of real-time PCR with SYBR Green I or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* 149: 269–277, 2003. doi:10.1099/mic.0.25975-0.
30. **Massey KA, Nicolaou A.** Lipidomics of oxidized polyunsaturated fatty acids. *Free Radic Biol Med* 59: 45–55, 2013. doi:10.1016/j.freeradbiomed.2012.08.565.
31. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC.** Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28: 412–419, 1985. doi:10.1007/BF00280883.
32. **Mazzali M, Hughes J, Kim YG, Jefferson JA, Kang DH, Gordon KL, Lan HY, Kivlighn S, Johnson RJ.** Elevated uric acid increases blood pressure in the rat by a novel crystal-independent mechanism. *Hypertension* 38: 1101–1106, 2001. doi:10.1161/hy1101.092839.
33. **Milne GL, Yin H, Morrow JD.** Human biochemistry of the isoprostane pathway. *J Biol Chem* 283: 15533–15537, 2008. doi:10.1074/jbc.R700047200.
34. **Mobley HL, Hausinger RP.** Microbial ureases: significance, regulation, and molecular characterization. *Microbiol Rev* 53: 85–108, 1989.

35. **Molinar-Toribio E, Pérez-Jiménez J, Ramos-Romero S, Gómez L, Taltavull N, Nogués MR, Adeva A, Jáuregui O, Joglar J, Clapés P, Torres JL.** D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food Funct* 6: 2614–2619, 2015. doi:10.1039/C5FO00591D.
36. **Mozaffarian D.** Dietary and policy priorities for cardiovascular disease, diabetes, and obesity. A comprehensive review. *Circulation* 133: 187–225, 2016. doi:10.1161/CIRCULATIONAHA.115.018585.
37. **Mühling M, Woolven-Allen J, Murrell JC, Joint I.** Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME J* 2: 379–392, 2008. doi:10.1038/ismej.2007.97.
38. **Nadler A, Reither G, Feng S, Stein F, Reither S, Müller R, Schultz C.** The fatty acid composition of diacylglycerols determines local signaling patterns. *Angew Chem Int Ed Engl* 52: 6330–6334, 2013. doi:10.1002/anie.201301716.
39. **Nascimento AR, Machado M, de Jesus N, Gomes F, Lessa MA, Bonomo IT, Tibiriçá E.** Structural and functional microvascular alterations in a rat model of metabolic syndrome induced by a high-fat diet. *Obesity (Silver Spring)* 21: 2046–2054, 2013. doi:10.1002/oby.20358.
40. **Oudot C, Lajoix AD, Jover B, Rugale C.** Dietary sodium restriction prevents kidney damage in high fructose-fed rats. *Kidney Int* 83: 674–683, 2013. doi:10.1038/ki.2012.478.
41. **Palmer BF, Clegg DJ.** Electrolyte and acid-base disturbances in patients with diabetes mellitus. *N Engl J Med* 373: 548–559, 2015. doi:10.1056/NEJMra1503102.
42. **Portune KJ, Benítez-Páez A, Del Pulgar EMG, Cerrudo V, Sanz Y.** Gut microbiota, diet, and obesity-related disorders-The good, the bad, and the future challenges. *Mol Nutr Food Res* 61: 1600252, 2017. doi:10.1002/mnfr.201600252.
43. **Robertson RP.** Eicosanoids as pluripotential modulators of pancreatic islet function. *Diabetes* 37: 367–370, 1988. doi:10.2337/diab.37.4.367.
44. **Samuel VT, Shulman GI.** Mechanisms for insulin resistance: common threads and missing links. *Cell* 148: 852–871, 2012. doi:10.1016/j.cell.2012.02.017.
45. **Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, Hardt PD.** Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)* 18: 190–195, 2010. doi:10.1038/oby.2009.167.
46. **Simbari F, McCaskill J, Coakley G, Millar M, Maizels RM, Fabriás G, Casas J, Buck AH.** Plasmalogen enrichment in exosomes secreted by a nematode parasite versus those derived from its mouse host: implications for exosome stability and biology. *J Extracell Vesicles* 5: 30,741–30,741, 2016. doi:10.3402/jev.v5.30741.
47. **Sorensen LB, Levinson DJ.** Origin and extrarenal elimination of uric acid in man. *Nephron* 14: 7–20, 1975. doi:10.1159/000180432.
48. **Taltavull N, Muñoz-Cortés M, Lluís L, Jové M, Fortuño A, Molinar-Toribio E, Torres JL, Pazos M, Medina I, Nogués MR.** Eicosapentaenoic acid/docosahexaenoic acid 1:1 ratio improves histological alterations in obese rats with metabolic syndrome. *Lipids Health Dis* 13: 31, 2014. doi:10.1186/1476-511X-13-31.
49. **Tran LT, Yuen VG, McNeill JH.** The fructose-fed rat: a review on the mechanisms of fructose-induced insulin resistance and hypertension. *Mol Cell Biochem* 332: 145–159, 2009. doi:10.1007/s11010-009-0184-4.
50. **Weir GC, Bonner-Weir S.** Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes* 53, *Suppl* 3: S16–S21, 2004. doi:10.2337/diabetes.53.suppl_3.S16.
51. **Wong J, Piceno YM, DeSantis TZ, Pahl M, Andersen GL, Vaziri ND.** Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *Am J Nephrol* 39: 230–237, 2014. doi:10.1159/000360010.

8.1.3 PAPER A3

TITLE: Fate of D-fagomine after oral administration to rats

AUTHORS: Susana Amézqueta, Sara Ramos-Romero, Carolina Martínez-Guimet, Albert Moreno, Mercè Hereu and Josep Lluís Torres

JOURNAL: Journal of Agricultural and Food Chemistry, 2017, 65(22), 4414-4420.

IMPACT FACTOR (2017): 3.41

CLASSIFICATION AND CATEGORY: FOOD SCIENCE & TECHNOLOGY 18/133(Q1)

TIMES CITED: 3

REFERENCE: Amézqueta, S., Ramos-Romero, S., Martínez-Guimet, C., Moreno, A., Hereu, M. & Torres, J.L. (2017). Fate of D-fagomine after oral administration to rats. *Journal of Agricultural and Food Chemistry,* 65(22), 4414-4420.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fate of D-Fagomine after Oral Administration to Rats

Susana Amézqueta,*[,†] Sara Ramos-Romero,[‡] Carolina Martínez-Guimet,[‡] Albert Moreno,[‡] Mercè Hereu,[‡] and Josep Lluís Torres[‡]

[†]Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

[‡]Institute of Advanced Chemistry of Catalonia (IQAC), Spanish National Research Council (CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

ABSTRACT: D-Fagomine is an iminosugar found in buckwheat that is capable of inhibiting the adhesion of potentially pathogenic bacteria to epithelial mucosa and reducing the postprandial blood glucose concentration. This paper evaluates the excretion and metabolism of orally administered D-fagomine in rats and compares outcomes with the fate of 1-deoxynojirimycin. D-Fagomine and 1-deoxynojirimycin show similar absorption and excretion kinetics. D-Fagomine is partly absorbed (41−84%, dose of 2 mg/kg of body weight) and excreted in urine within 8 h, while the non-absorbed fraction is cleared in feces within 24 h. D-Fagomine is partially methylated (about 10% in urine and 3% in feces). The concentration of D-fagomine in urine from 1 to 6 h after administration is higher than 10 mg/L, the concentration that inhibits adhesion of *Escherichia coli*. Orally administered D-fagomine is partially absorbed and then rapidly excreted in urine, where it reaches a concentration that may be protective against urinary tract infections.

KEYWORDS: *D-fagomine, 1-deoxynojirimycin, metabolism, Escherichia coli, urinary tract, mass spectrometry*

INTRODUCTION

D-Fagomine and 1-deoxynojirimycin (DNJ) are polyhydroxylated piperidines, also known as iminocyclitols, azasugars, or iminosugars, that are synthesized by various plants and microorganisms as secondary metabolites.[1,2] D-Fagomine and DNJ can be found in foodstuffs, such as bread, pasta, and biscuits made from buckwheat[3] and tea, snacks, and biscuits made from mulberry.[4] Both of these iminocyclitols are glycosidase inhibitors with the capacity to reduce the postprandial glycemic response after oral administration of either sucrose or starch to rats and humans.[5−8] Because D-fagomine and DNJ reduce the elevation of postprandial blood glucose, they can be used as dietary supplements or functional food components to help maintain short-term homeostasis of blood glucose levels.[9] More recent observations have revealed that iminocyclitols may modify the composition of the gut microbiota by inhibiting bacterial adhesion to the intestinal mucosa.[7] It has been suggested that D-fagomine counteracts the short-term metabolic alterations triggered by a high-energy-dense diet in rats,[10] at least in part through the reduction of a diet-induced excess of gut Enterobacteriales.[11] Thus, D-fagomine might also be administered to prolong maintenance of metabolic homeostasis.

Absorption, distribution, metabolism, and excretion (ADME) studies in animals and humans have been published of naturally and non-naturally occurring bioactive iminocyclitols, such as DNJ, *N*-methyl-DNJ, 1-deoxymannojirimycin (DMJ), 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), *N*-hydroxyethyl-DNJ (miglitol or glyset), and *N*-butyl-DNJ (miglustat or zavesca).[12−22] In rats, DNJ is poorly absorbed in a dose-dependent manner, distributed in the intact form and rapidly excreted in urine.[15,16,18,23] The absorption and distribution of D-fagomine alone has never been reported. When administered as a minor component of an extract from mulberry twigs (Ramulus Mori, Chinese medicine), the D-fagomine absorption rate profile is similar to that of DNJ.[21]

Bioanalytical methods for iminocyclitols have recently been reviewed.[24] Pharmacokinetics is effectively evaluated using radiolabeled derivatives. The technique is extremely sensitive but would fail to identify any putative transformation as a result of metabolism. More selective and safe hyphenated bioanalytical methods have also been developed. In particular, iminocyclitols are analyzed by hydrophilic interaction or cation-exchange liquid chromatography coupled to different mass spectrometry detectors [single quadrupole (Q), triple quadrupole (QqQ), quadrupole ion trap (QTrap), or time of flight (TOF)].[1,15−19,22]

The aim of this paper is to evaluate D-fagomine metabolism and excretion and compare the results with those for DNJ, an iminocyclitol with putatively similar behavior.

MATERIALS AND METHODS

Reagents. A D-fagomine standard (assay of >95%) was provided by Bioglane (Barcelona, Spain). DNJ (assay of >95%) was from Carbosynth (Berkshire, U.K.). 2,5-Hydroxymethyl-3,4-dihydroxypyrrolidine (DMDP), the internal standard, was purchased from IRL (Lower Hutt, New Zealand). Lichrosolv-grade methanol, together with analytical-grade acetic acid and ammonium hydroxide, was obtained from Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC)-grade water (Millipore type I water from Merck) was used to prepare all of the aqueous solutions. Solid-phase extraction (SPE) cartridges for sample purification were Spe-ed, strong cation exchange (SCX) cartridges, 100 mg/mL from Applied Separations

Received: March 6, 2017
Revised: May 10, 2017
Accepted: May 10, 2017
Published: May 10, 2017

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

(Allentown, PA, U.S.A.). Nylon filters (0.45 μm) were obtained from Scharlab (Sentmenat, Spain). Microvette CB 300 K2E dikalium ethylenediaminetetraacetic acid (EDTA) tubes were from Sarstedt (Nümbrecht, Germany); the gastric probe was from Harvard Apparatus (Holliston, MA, U.S.A.); and 25 G needles were from Novico Médica (Barcelona, Spain).

Animals, Diets, and *In Vivo* Tests. A total of 18, male, 8-week-old, Sprague Dawley rats (Janvier, Le Genest-Saint-Isle, France) were housed under controlled conditions of stable humidity (50 ± 10%) and temperature (22 ± 2 °C) with a 12 h light−12 h dark cycle. To minimize circadian rhythm effects, all rat handling was carried out in the morning. After overnight fasting, the rats were divided into six groups, which were given single doses of D-fagomine or DNJ, administered as aqueous solutions (5 mL/kg of body weight) using a gastric probe. Each group received 2.0, 10, or 100 mg/kg of body weight of D-fagomine or DNJ. The rats were then placed in metabolic cages to collect urine and feces at 1, 2, 4, 6, 8, and 24 h after administration. Samples were kept at −80 °C until analysis. After that, the rats were placed in standard cages and the remaining feces were collected by abdominal massage 48 h after administration. The handling of the animals was in full accordance with the European Union guidelines for the care and management of laboratory animals, and the pertinent permission was obtained from the Spanish National Research Council (CSIC) Subcommittee for Bioethical Issues (reference AGL2009-12 374-C03-03, CEEA-12-011, date of approval March 4, 2013).

Extraction and SPE Cleanup. After thawing, aliquots (60 μL) of urine were extracted with 70% aqueous methanol (5 mL) using an orbital shaker (Intelli-mixer RM-2 device from Elmi, Riga, Latvia) for 20 min. Feces were cut longitudinally, and the analytes were extracted from half of the sample also with 70% aqueous methanol (5 mL of solvent/60 mg of feces) using the orbital shaker for 20 min. After extraction, the suspensions were centrifuged in a 5810R centrifuge from Eppendorf (Hamburg, Germany) for 3 min at 8000 rpm and 20 °C, filtered through a 0.45 μm nylon filter (Phenomenex, Torrance, CA, U.S.A.), and the filtrates were diluted with water to a known volume in a volumetric flask (10 mL in the case of urine and variable in the case of feces). Cation-exchange SPE cartridges were conditioned with HPLC-grade methanol (1 mL) and water (1 mL). Then, aliquots from the previous step were loaded onto the cartridges. The aliquot volumes were adjusted to equalize the response of the analysis (urine samples, 2500, 500, and 50 μL from the groups administered the 2.0, 10, and 100 mg/kg of body weight doses, respectively; feces samples, variable volumes depending upon the dilution after the extraction step). SCX resin was then washed with water (4 mL) and vacuum-dried. Next, the analytes were eluted with 2 M aqueous NH_4OH (500 μL). The eluates were spiked with a DMDP solution in methanol (100 μL, 5 mg/L). The solution was evaporated to dryness under a stream of N_2 at 60 °C, and the residue was redissolved in water (500 μL) and filtered through a 0.45 μm nylon filter.

HPLC/ESI−QqQ−MS Analysis. Chromatography was carried out on an Acquity H class system (Waters, Milford, MA, U.S.A.) equipped with a quaternary pump and fitted with a TSK-Gel CM2SW cation-exchange column (25 cm × 4.6 mm inner diameter, 5 μm particle size, Tosoh Bioscience, Tokyo, Japan). The injection volume was 5 μL; the column temperature was 25 °C; and the total analysis time was 30 min. The target compounds were separated with a binary system: 50 mM NH_4CH_3COO (pH 8.5)/methanol (4:1), under isocratic conditions at a flow rate of 0.8 mL/min. Mass spectrometric analysis of the column effluent was carried out on a tandem QqQ Xevo-TQ-S spectrometer (Waters). The electrospray ionization−tandem mass spectrometry (ESI−MS/MS) parameters were: positive polarity, a capillary voltage of 3 kV, a desolvation temperature of 600 °C, a desolvation gas flow of 1000 L/h, 7 bar of nebulizer gas, a cone voltage of 20−45 V depending upon the analyte, and a collision energy of 20−30 V depending upon the analyte (Table 1). Analysis was carried out in the multiple reaction monitoring (MRM) mode using the following quantification transitions: 164 → 80 (DMDP and DNJ), 148 → 86 (D-fagomine), 162 → 100 (monomethyl-D-fagomine), 176 → 114 (dimethyl-D-fagomine), 190 → 128 (trimethyl-D-fagomine), 178 → 94 (monomethyl-DNJ), 192 → 108 (dimethyl-DNJ), and 206 → 122 (trimethyl-DNJ). The retention times of the analytes were 4 min (DNJ), 5.5 min (DMDP), 7.5 min (monomethyl-D-fagomine), 9 min (D-fagomine), 10 min (dimethyl-D-fagomine), 10.5 min (trimethyl-D-fagomine), and 3.7 (monomethyl-DNJ). To confirm the identity of the metabolites, their exact masses were obtained using high-resolution (HR) ESI−TOF−MS on a LCT Premier XE system (Waters) after HR separation using a HPLC Acquity system (Waters) fitted with a TSK-Gel CM2SW column.

Table 1. Cone Voltages and Collision Energies Applied in the ESI−MS/MS Analysis

	cone voltage (V)	collision energy (V)
DMDP	45	20
DNJ	45	20
D-fagomine	35	20
methyl-D-fagomine	20	30
dimethyl-D-fagomine	20	30
trimethyl-D-fagomine	35	30

Standard Solutions. Stock standard solutions of 5 mg/L were prepared by dissolving D-fagomine, DNJ, or DMDP (1.00 mg) in methanol (20 mL) and then diluting 1:9 in methanol. All solutions were stored at −20 °C. To prepare the working standard solutions, the corresponding aliquots of the D-fagomine or DNJ stock solutions were mixed with the DMDP stock solution (100 μL). The solvent was then evaporated to dryness under a stream of N_2, and the residue was dissolved in water (500 μL). These solutions were also stored at −20 °C. The standard solutions were stable at this temperature for a period of at least 6 months.

Validation of the Analytical Method. Because ESI techniques often suffer from matrix effects, the linear MS responses of D-fagomine/DMDP and DNJ/DMDP dissolved in water and matrix-matched solutions were evaluated and the slopes were compared. To prepare the matrix-matched solutions, urine and feces of rats that had still not been administered the iminosugars were used. These samples were subjected to the extraction and purification steps described in the Extraction and SPE Cleanup subsection. In the case of urine, 2500 μL of the extract (the aliquot that contains the highest amount of interfering compounds) was passed through the cartridge. The eluate obtained from the SCX resin was spiked with 100 μL of a DMDP solution in methanol (5 mg/L) and with different volumes of a D-fagomine + DNJ solution in methanol (5 mg/L of both compounds) to prepare a calibration curve in the range of 4−83 mg of D-fagomine/L of urine or kg of feces. Each solution was evaporated to dryness under a stream of N_2 at 60 °C, and the residue was redissolved in water (500 μL) and then filtered through a 0.45 μm nylon filter. Calibration curves were constructed by plotting $A_{\text{D-fagomine or DNJ}}/A_{\text{DMDP}}$ against the D-fagomine or DNJ concentration for each solvent. Then, the signal suppression/enhancement (SSE) was calculated according to eq 1. A SSE value smaller than 100 means that the matrix causes signal suppression, and a higher value means that the matrix causes signal enhancement.[25]

$$\text{SSE (\%)} = \frac{\text{slope}_{\text{matrix-matched calibration curve}}}{\text{slope}_{\text{aqueous standards calibration curve}}} \times 100 \quad (1)$$

In the assessment of linearity, calibration curves were plotted in the range of 1.8−8300 and 8.3−8300 mg/L for D-fagomine and DNJ, respectively. Calibration curves with 11 calibration standards each were prepared across these ranges. All of the calibration standards were prepared using matrix-matched solutions and were spiked with the DMDP standard stock solution (100 μL, 5 mg/L). Linearity was evaluated for every analytical run batch to compensate for ESI variability. The concentration of the metabolites was expressed as DNJ of D-fagomine equivalents as a result of the lack of commercial standards.

A precision and trueness study was carried out with the different matrices. The recovery study was performed by spiking the matrices

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

Table 2. Signal Suppression and Enhancement Effect in Matrix-Matched Solutions of Both Urine and Feces[a]

		D-fagomine/DMDP signal versus D-fagomine concentration	DNJ/DMDP signal versus DNJ concentration
urine	aqueous standards	$y = 0.58x - 0.4$	$y = 0.036x - 0.06$
	matrix-matched standards	$y = 0.099x - 0.3$	$y = 0.0018x - 0.005$
	SSE (%)	17% (signal suppression)	5% (signal suppression)
feces	aqueous standards	$y = 0.103x + 0.1$	$y = 0.0025x - 0.027$
	matrix-matched standards	$y = 0.47x + 1$	$y = 0.010x - 0.08$
	SSE (%)	458% (signal enhancement)	390% (signal enhancement)

[a] y, relative abundance; x, concentration in mg/L or mg/kg; and SSE, signal suppression/enhancement.

with D-fagomine at three different concentrations (67, 330, and 3300 mg/L in urine and 130, 670, and 6700 mg/kg in feces) in triplicate in 3 different days. Three standard solutions of D-fagomine were prepared in methanol (200, 1000, and 10000 mg/L), one for each of the three spiking levels. In the case of urine, the solvent of an aliquot (20 μL) of the corresponding standard was evaporated and the residue containing D-fagomine was suspended in the matrix under study (60 μL of urine). In the case of feces, a portion (60 mg) was spiked with an aliquot (40 μL) of the corresponding standard. The feces samples were processed 24 h after spiking, to ensure complete evaporation of the solvent. Next, the samples were subjected to the purification step. After elution, the eluates were spiked with the DMDP standard stock solution (100 μL, 5 mg/L) to correct for ESI variability. The solution was evaporated to dryness under a stream of N_2 at 60 °C, and the residue was redissolved in water (500 μL) and filtered through a 0.45 μm nylon filter. In parallel, the calibration curves were prepared using matrix-matched standards. Finally, the samples and calibration standards were analyzed by liquid chromatography−tandem mass spectrometry (LC−MS/MS). Recovery was determined by comparing the $A_{\text{D-fagomine}}/A_{\text{DMDP}}$ signal obtained from the spiked samples with the $A_{\text{D-fagomine}}/A_{\text{DMDP}}$ signal from the calibration standards. Precision was evaluated by calculating the relative standard deviations (RSDs) obtained in within and between day recovery experiments. The limit of quantification (LOQ) was established as the concentration at which a recovery value similar to that of the trueness study was obtained with a RSD of <20% when analyzing five spiked samples.

RESULTS AND DISCUSSION

Setup and Validation of the Analytical Method. The analytical method was adapted for biological fluids from a previously described procedure developed for the analysis of plant sources (buckwheat and mulberry).[1] Modifications were introduced in the extraction step, and to improve selectivity and sensibility, QqQ-MS was used instead of Q-MS. First, the MS parameters, such as desolvation temperature, cone voltage, or collision energy, were optimized to improve signal intensity for the analytes under study. Then, selectivity, sensibility, and linearity were checked by preparing the D-fagomine and DNJ calibration curves using DMDP as an internal standard and two solvents: water and the matrix resulting from control urine or feces subjected to the extraction and SPE purification steps. The calibration curves generated with the two matrices showed significant signal suppression/enhancement (Table 2); therefore, the calibration standards used from that point on were prepared using matrix-matched solutions. The assay response (area of the D-fagomine or DNJ peak divided by the area of the DMDP peak) to the D-fagomine or DNJ concentration was linear ($R^2 > 0.99$) in the ranges under study (1.8−8300 mg/L and 8.3−8300 mg/L for D-fagomine and DNJ, respectively). Within and between day precision and trueness were studied using samples of urine and feces spiked with D-fagomine. In the case of urine, the percentage recovery at the three concentrations assayed (96%) was homogeneous (RSD of 12%), which demonstrated the precision and trueness of the analytical procedure. The European Medicines Agency (EMA)[26] recommends a recovery value (trueness) in the range of 85−115% and a RSD value (precision) of under 15% for bioanalytical methods. In the case of feces, within day recovery was 47% (RSD of 10%), outside the EMA suggested interval (85−115%). Intraday recovery was 51% (RSD of 14%), which demonstrated the validity of the method, despite the low recovery. The results in this work are corrected for the recovery

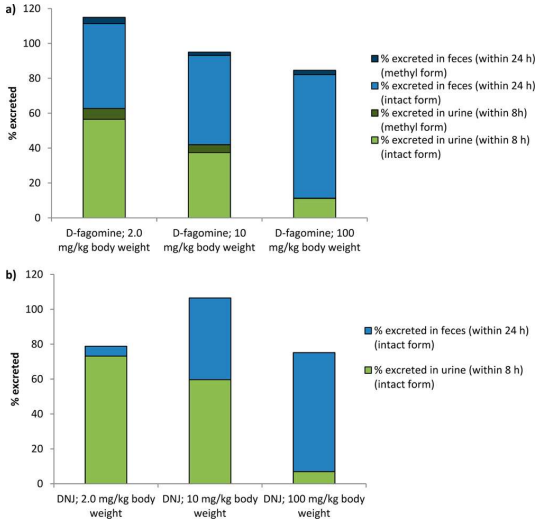


Figure 1. Percentages of (a) D-fagomine and (b) DNJ, including their methyl derivatives, excreted in feces and urine after oral administration at three different doses.

Table 3. Identification of D-Fagomine Metabolites by HPLC/HR-ESI−TOF−MS[a]

m/z	retention time	measured mass	calculated mass	formula	ppm	compound
148	11.7	148.0970	148.0974	$C_6H_{14}NO_3$	−2.0	D-fagomine
162	9.4	162.1129	162.1130	$C_7H_{16}NO_3$	−0.6	methyl-D-fagomine
176	12.7	176.1282	176.1287	$C_8H_{18}NO_3$	−2.8	dimethyl-D-fagomine
190	13.3	190.1441	190.1443	$C_9H_{20}NO_3$	−1.1	trimethyl-D-fagomine

[a] Measurements made from a sample of urine collected 1 h after administration of D-fagomine (250 mg of D-fagomine/kg of body weight) during a preliminary pilot study with Sprague Dawley rats.

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

Table 4. Excretion of D-Fagomine and DNJ in Feces and Urine after Oral Administration

compound (dose)	rat	milligrams administered	milligrams excreted in urine (0−8 h), intact form	milligrams excreted in urine (0−8 h), methylated form	milligrams excreted in feces (6−24 h), intact form	milligrams excreted in feces (6−24 h), methylated form	percentage excreted in urine (0−8 h)	percentage excreted in feces (6−24 h)	total (percentage of feces + percentage of urine)
D-fagomine (2.0 mg/kg of body weight)	1	0.53	0.41	0.04	0.10		84	19	103
	2	0.53	0.30	0.03	0.31	0.02	63	64	127
	3	0.57	0.21	0.02	0.39	0.02	41	71	112
DNJ (2.0 mg/kg of body weight)	4	0.72	0.31				42		42
	5	0.80	0.55		0.09		69	11	80
	6	0.75	0.81		0.05		108	6	114
D-fagomine (10 mg/kg of body weight)	7	2.6	0.9	0.08	0.8	0.02	37	30	67
	8	2.7	1.6	0.25	1.6	0.03	70	60	131
	9	2.8	0.5	0.03	1.8	0.10	18	69	87
DNJ (10 mg/kg of body weight)	10	2.6	2.2		0.6		83	23	105
	11	2.7	1.0		2.0		38	76	113
	12	2.7	1.6		1.1		59	42	101
D-fagomine (100 mg/kg of body weight)	13	26	2.3	0.07	17	0.52	9	66	75
	14	27	1.2	0.03	22	0.53	5	85	90
	15	27	5.4	0.02	18	0.95	20	69	89
DNJ (100 mg/kg of body weight)	16	26	2.4		15		9	56	65
	17	27	1.4		26		5	95	101
	18	27	1.7		14		6	53	60

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

values. The LOQ values were 1.8 mg/L of urine or kg of feces for D-fagomine and 8.3 mg/L of urine or kg of feces for DNJ. DNJ shows a higher LOQ value because its ionization in the MS detector was not as complete as that of D-fagomine.

The results of the validation study show that the new methods met all of the requirements for a bioanalytical process.[26] The methods were selective; the response was linear in the working range; precision and trueness fell within the recommended range except for feces recovery; and the LOQ was much lower than the D-fagomine and DNJ concentrations found after the maximum excretion period. The methods were successfully applied to the determination of D-fagomine, DNJ, and their metabolites in the urine and feces of rats fed D-fagomine or DNJ. The identity of the compounds was confirmed by HPLC/HR-ESI−TOF−MS with samples generated in a previous separate experiment by administration of 250 mg of iminocyclitol/kg of body weight (Table 3).

Absorption and Excretion. The animals were administered 2.0, 10, or 100 mg/kg of body weight of D-fagomine or DNJ (positive control). The dose of 2.0 mg/kg of body weight corresponded to the dose of D-fagomine that reduced the postprandial blood glucose concentration (20% reduction of the area under the curve between 0 and 120 min) after intake of glucose or starch.[7] At this active concentration, part of administered D-fagomine (0.2−0.4 mg) was absorbed and excreted in urine within 8 h, while the rest was recovered in feces within 24 h (Figure 1a and Table 4). Absorption appears to be limited as a result of saturation after a total amount of 3 mg of D-fagomine has been absorbed (Table 4). The highest excretion rate occurred in the period between 2 and 4 h in urine (Figure 2 and Table 5) and in the period between 8 and 24 h in feces (Table 6). D-Fagomine was not detected in feces collected by abdominal massage at 48 h after intake (Table 6). Briefly, the absorbed portion of D-fagomine is cleared in urine

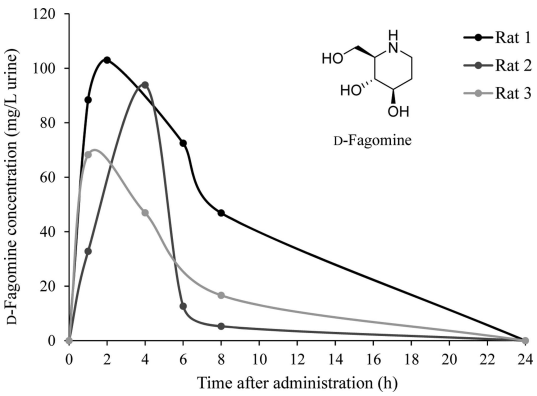


Figure 2. D-Fagomine concentration in urine after oral administration of 2.0 mg/kg of body weight.

Table 6. D-Fagomine and DNJ Concentrations (mg/kg) in Feces Excreted in the 8−24 h Period after Oral Administration[a]

	rat	6−8 h	8−24 h	48 h
D-fagomine (2.0 mg/kg of body weight)	1	–	32	ND
	2	ND	110	ND
	3	ND	170	ND
DNJ (2.0 mg/kg of body weight)	4	ND	ND	ND
	5	ND	71	ND
	6	ND	34	ND
D-fagomine (10 mg/kg of body weight)	7	–	750	ND
	8	–	450	ND
	9	–	890	ND
DNJ (10 mg/kg of body weight)	10	ND	150	ND
	11	ND	650	ND
	12	–	340	ND
D-fagomine (100 mg/kg of body weight)	13	5000	14000	ND
	14	4000	9200	ND
	15	–	8500	ND
DNJ (100 mg/kg of body weight)	16	ND	11000	ND
	17	ND	8100	ND
	18	–	6300	ND

[a]ND, not detected; −, collected at the next time point because the animal did not excrete feces during all collecting periods.

Table 5. D-Fagomine and DNJ Concentrations (mg/L) in Urine during Different Periods after Oral Administration[a]

	rat	0−1 h	1−2 h	2−4 h	4−6 h	6−8 h	8−24 h
D-fagomine (2.0 mg/kg of body weight)	1	88	100	–	72	47	ND
	2	33	–	94	13	traces	ND
	3	68	–	47	ND	17	ND
DNJ (2.0 mg/kg of body weight)	4	160	–	–	–	–	29
	5	430	–	–	–	280	20
	6	350	–	–	–	160	16
D-fagomine (10 mg/kg of body weight)	7	–	410	–	130	16	ND
	8	620	–	–	450	–	ND
	9	66	–	–	65	180	ND
DNJ (10 mg/kg of body weight)	10	580	–	650	99	33	ND
	11	180	–	230	42	ND	ND
	12	350	640	–	440	100	ND
D-fagomine (100 mg/kg of body weight)	13	–	660	380	220	48	<40
	14	–	730	–	490	170	<40
	15	–	2000	360	460	64	<40
DNJ (100 mg/kg of body weight)	16	–	–	1100	–	570	<40
	17	240	–	660	120	63	<40
	18	230	1200	1300	400	90	<40

[a]ND, not detected; −, collected at the next time point because the animal did not excrete urine during all collecting periods.

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

within 8 h, and the non-absorbed portion is cleared in feces within 24 h after intake. The behavior of D-fagomine and DNJ (positive control) was similar (Figure 1 and Tables 4−6), and the absorption under our experimental conditions reached saturation at a total absorbed amount of 3 mg. Because most (around 90% at the three doses) of both of the ingested iminocyclitols is excreted, whether in urine or feces, within the first 24 h after oral administration, these compounds probably do not accumulate in tissues, as already suggested by Nakagawa et al.[15]

The results presented here for DNJ agree with two other studies that also used a pure compound.[15,18] Nakagawa et al.[15] found that about 0.6 mg of DNJ (2%) was excreted in urine within 24 h after a 110 mg/kg of body weight oral administration after fasting.[15] Kim et al.[18] reported that about 4 mg of DNJ administered was excreted in urine within 24 h after a 30 mg/kg of body weight oral administration; the non-absorbed fraction was excreted in feces within 48 h. When DNJ was administered as a component of a plant extract, the excretion profile was similar to that obtained here. Xiao et al. reported that about 2.5 mg of DNJ in a mulberry root bark extract was excreted in urine within the first 4 h.[27] Our results show that D-fagomine is absorbed as fast as DNJ.

Metabolism. It has been reported that DNJ (positive control) is mainly excreted in the intact form.[15,16,18,27] Nakagawa et al. did not detect any degradation products (e.g., oxidized and alkylated products) in plasma and reported that a small signal in the HPLC−MS single ion monitoring chromatogram might correspond to an unidentified metabolite.[15] We found trace amounts of monomethyl-DNJ in urine and several methyl derivatives of D-fagomine. A monomethylated conjugate was present in urine (10%) and feces (3%), at the three doses, within the period of 0−8 h (Figure 1a and Table 4). We also detected trace levels of dimethyl-D-fagomine and trimethyl-D-fagomine in urine, at the three doses, within the period of 0−8 h. In a preliminary experiment with samples obtained by administering a higher dose of D-fagomine (250 mg/kg of body weight), signals compatible with deoxygenated products were detected that were not found at the lower concentrations used here. Metabolism of organic compounds consists mainly of their conversion into more hydrophilic species that can be readily excreted.[28] Phase II conjugation into glucuronides and sulfates are the most common transformations. Iminocyclitols, such as D-fagomine and DNJ, are already highly water-soluble, and they are rapidly excreted without further modification. Methylation is a less common phase II conjugation that reduces water solubility and plays the physiological role of blocking biological activities by modifying chemically active functions, such as those of amines and hydroxyls.[29] In the case of D-fagomine, because only a small percentage is methylated, it is unlikely that methylation serves the purpose of deactivation. Its structure may partially fit the requirements for the enzymes involved in the modification of other chemical species, most likely sugars. The presence of methyl-D-fagomine in feces could be explained by biliary excretion after modification in the liver. Alternatively, methyl-D-fagomine may be generated by the gut microbiota because some species of the phylum Actinobacteria are capable of methylating the terminal units of oligosaccharides.[30]

The presence of intact D-fagomine in urine for several hours after oral ingestion may have important implications for the maintenance of a healthy status in the urinary tract. The concentration of D-fagomine in contact with the tissues along the urinary tract for the period of 1−6 h, in all of the rats tested at any dose (Table 5 and Figure 2), was higher than the concentration (10 mg/L) that inhibits adhesion (95%) of *Escherichia coli* to the intestinal mucosa.[7] Therefore, at the dose that is active at lowering the postprandial glucose concentration by 20% (2.0 mg/kg of body weight, the lowest dose tested), D-fagomine may protect against urinary tract infections. D-Fagomine may also protect the intestinal tract against infections. We recently showed that D-fagomine, at a dose of 23 mg kg^{-1} of body weight day^{-1}, reduced the increase in the population of Enterobacteriales induced by a high-fat, high-sucrose diet.[11]

To summarize, orally administered D-fagomine is rapidly absorbed and excreted in urine within 8 h in rats. The non-absorbed fraction is cleared in feces within 24 h. D-Fagomine is partially methylated (about 10% in urine and 3% in feces). The concentration of D-fagomine in urine at 1−6 h after oral administration of 2.0 mg/kg of body weight is higher than the concentration that inhibits (95%) the adhesion of *E. coli* to epithelial surfaces. Therefore, D-fagomine may protect the urinary tract against infections caused by Enterobacteriales at a dose that is active at reducing the postprandial blood glucose concentration.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +34-934021797. Fax: +34-934021233. E-mail: samezqueta@ub.edu.

ORCID

Susana Amézqueta: 0000-0001-8976-467X

Funding

This research was funded by the Spanish Ministry of Economy and Competitiveness through a research grant (AGL2013-49079-C2-2-R) and a graduate fellowship to Mercè Hereu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Bioglane for providing D-fagomine, E. Galan and L. Gómez for collaborating in the preliminary study, and Dr. E. Prats, Dr. R. Chaler, and D. Fanjul for technical support. Language assistance from Christopher Evans is appreciated.

REFERENCES

(1) Amézqueta, S.; Galán, E.; Fuguet, E.; Carrascal, M.; Abián, J.; Torres, J. L. Determination of D-fagomine in buckwheat and mulberry by cation exchange HPLC/ESI−Q−MS. *Anal. Bioanal. Chem.* **2012**, *402*, 1953−1960.

(2) Watson, A. A.; Fleet, G. W. J.; Asano, N.; Molyneux, R. J.; Nash, R. J. Polyhydroxylated alkaloids - Natural occurrence and therapeutic applications. *Phytochemistry* **2001**, *56*, 265−295.

(3) Amézqueta, S.; Galán, E.; Vila-Fernández, I.; Pumarola, S.; Carrascal, M.; Abian, J.; Ribas-Barba, L.; Serra-Majem, L.; Torres, J. L. The presence of D-fagomine in the human diet from buckwheat-based foodstuffs. *Food Chem.* **2013**, *136*, 1316−1321.

(4) Yoshihashi, T.; Do, H. T. T.; Tungtrakul, P.; Boonbumrung, S.; Yamaki, K. Simple, selective, and rapid quantification of 1-deoxynojirimycin in mulberry leaf products by high-performance anion-exchange chromatography with pulsed amperometric detection. *J. Food Sci.* **2010**, *75*, 246−250.

(5) Kimura, T.; Nakagawa, K.; Kubota, H.; Kojima, Y.; Goto, Y.; Yamagishi, K.; Oita, S.; Oikawa, S.; Miyazawa, T. Food-grade mulberry powder enriched with 1-deoxynojirimycin suppresses the elevation of

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

postprandial blood glucose in humans. *J. Agric. Food Chem.* **2007**, *55*, 5869−5874.
(6) Kong, W. H.; Oh, S. H.; Ahn, Y. R.; Kim, K. W.; Kim, J. H.; Seo, S. W. Antiobesity effects and improvement of insulin sensitivity by 1-deoxynojirimycin in animal models. *J. Agric. Food Chem.* **2008**, *56*, 2613−2619.
(7) Gómez, L.; Molinar-Toribio, E.; Calvo-Torras, M. Á.; Adelantado, C.; Juan, M. E.; Planas, J. M.; Cañas, X.; Lozano, C.; Pumarola, S.; Clapés, P.; Torres, J. L. D-Fagomine lowers postprandial blood glucose and modulates bacterial adhesion. *Br. J. Nutr.* **2012**, *107*, 1739−1746.
(8) Bioglane SLNE. Effects of added D-fagomine on glycaemic responses to sucrose. *ClinicalTrials.gov*; National Library of Medicine: Bethesda, MD, 2000; NCT01811303, https://clinicaltrialbase.com/study/NCT01811303/?search-input=NCT01811303 (accessed Dec 31, 2016).
(9) Giugliano, D.; Ceriello, A.; Esposito, K. Glucose metabolism and hyperglycemia. *Am. J. Clin. Nutr.* **2008**, *87*, 217S−222S.
(10) Molinar-Toribio, E.; Pérez-Jiménez, J.; Ramos-Romero, S.; Gómez, L.; Taltavull, N.; Nogués, M. R.; Adeva, A.; Jáuregui, O.; Joglar, J.; Clapés, P.; Torres, J. L. D-Fagomine attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food Funct.* **2015**, *6*, 2614−2619.
(11) Ramos-Romero, S.; Molinar-Toribio, E.; Gómez, L.; Pérez-Jiménez, J.; Casado, M.; Clapés, P.; Piña, B.; Torres, J. L. Effect of D-fagomine on excreted enterobacteria and weight gain in rats fed a high-fat high-sucrose diet. *Obesity* **2014**, *22*, 976−979.
(12) Faber, E. D.; Oosting, R.; Neefjes, J. J.; Ploegh, H. L.; Meijer, D. K. F. Distribution and elimination of the glycosidase inhibitors 1-deoxymannojirimycin and *N*-methyl-1-deoxynojirimicyn in the rat *in vivo*. *Pharm. Res.* **1992**, *09*, 1442−1450.
(13) Ahr, H. Pharmacokinetics of miglitol: Absorption, distribution, metabolism, and excretion following administration to rats, dogs, and man. *Arzneimittelforschung* **1997**, *47*, 734−745.
(14) Li, X.; Wang, Y.; Wang, J.; Fawcett, J. P.; Zhao, L.; Gu, J. Determination of miglitol in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 247−251.
(15) Nakagawa, K.; Kubota, H.; Kimura, T.; Yamashita, S.; Tsuzuki, T.; Oikawa, S.; Miyazawa, T. Occurrence of orally administered mulberry 1-deoxynojirimycin in rat plasma. *J. Agric. Food Chem.* **2007**, *55*, 8928−8933.
(16) Nakagawa, K.; Kubota, H.; Tsuzuki, T.; Kariya, J.; Kimura, T.; Oikawa, S.; Miyazawa, T. Validation of an ion trap tandem mass spectrometric analysis of mulberry 1-deoxynojirimycin in human plasma: Application to pharmacokinetic studies. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 2210−2213.
(17) Guitton, J.; Coste, S.; Guffon-Fouilhoux, N.; Cohen, S.; Manchon, M.; Guillaumont, M. Rapid quantification of miglustat in human plasma and cerebrospinal fluid by liquid chromatography coupled with tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 149−154.
(18) Kim, J. Y.; Kwon, H. J.; Jung, J. Y.; Kwon, H. Y.; Baek, J. G.; Kim, Y. S.; Kwon, O. Comparison of absorption of 1-deoxynojirimycin from mulberry water extract in rats. *J. Agric. Food Chem.* **2010**, *58*, 6666−6671.
(19) Spieker, E.; Wagner-Redeker, W.; Dingemanse, J. Validated LC−MS/MS method for the quantitative determination of the glucosylceramide synthase inhibitor miglustat in mouse plasma and human plasma and its application to a pharmacokinetic study. *J. Pharm. Biomed. Anal.* **2012**, *59*, 123−129.
(20) Attimarad, M. V.; Nair, A. B.; Aldhubaib, B. E. Development of liquid chromatographic method for the simultaneous determination of metformin and miglitol in human plasma: Application to pharmacokinetic studies. *J. Iran. Chem. Soc.* **2015**, *12*, 1629−1636.
(21) Yang, S.; Wang, B.; Xia, X.; Li, X.; Wang, R.; Sheng, L.; Li, D.; Liu, Y.; Li, Y. Simultaneous quantification of three active alkaloids from a traditional Chinese medicine Ramulus Mori (Sangzhi) in rat plasma using liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2015**, *109*, 177−183.
(22) Jain, R.; Lukram, O.; Dwivedi, A. Ultra-performance liquid chromatography electrospray ionization−tandem mass spectrometry method for the estimation of miglitol in human plasma using metformin as the internal standard. *Drug Test. Anal.* **2011**, *3*, 255−262.
(23) Nakagawa, K.; Ogawa, K.; Higuchi, O.; Kimura, T.; Miyazawa, T.; Hori, M. Determination of iminosugars in mulberry leaves and silkworms using hydrophilic interaction chromatography-tandem mass spectrometry. *Anal. Biochem.* **2010**, *404*, 217−222.
(24) Amézqueta, S.; Torres, J. L. Advances in the analysis of iminocyclitols: Methods, sources and bioavailability. *Talanta* **2016**, *151*, 157−171.
(25) Panuwet, P.; Hunter, R. E.; D'Souza, P. E.; Chen, X.; Radford, S. A.; Cohen, J. R.; Marder, M. E.; Kartavenka, K.; Ryan, P. B.; Barr, D. B. Biological matrix effects in quantitative tandem mass spectrometry-based analytical methods: Advancing biomonitoring. *Crit. Rev. Anal. Chem.* **2016**, *46*, 93−105.
(26) European Medicines Agency (EMA). *Guideline on Bioanalytical Method Validation*; EMA: London, U.K., July 2011; EMEA/CHMP/EWP/192217/2009.
(27) Xiao, B. X.; Wang, Q.; Fan, L. Q.; Kong, L. T.; Guo, S. R.; Chang, Q. Pharmacokinetic mechanism of enhancement by Radix Pueraria flavonoids on the hyperglycemic effects of Cortex Mori extract in rats. *J. Ethnopharmacol.* **2014**, *151*, 846−851.
(28) Levsen, K.; Schiebel, H. M.; Behnke, B.; Dötzer, R.; Dreher, W.; Elend, M.; Thiele, H. Structure elucidation of phase II metabolites by tandem mass spectrometry: An overview. *J. Chromatogr. A* **2005**, *1067*, 55−72.
(29) Muthana, S. M.; Campbell, C. T.; Gildersleeve, J. C. Modifications of glycans: Biological significance and therapeutic opportunities. *ACS Chem. Biol.* **2012**, *7*, 31−43.
(30) Cantoni, G. L. Biological methylation. Selected aspects. *Annu. Rev. Biochem.* **1975**, *44*, 435−451.

DOI: 10.1021/acs.jafc.7b01026
J. Agric. Food Chem. 2017, 65, 4414−4420

8.2 COMMUNICATIONS TO CONFERENCES

- D-Fagomine balances gut bacteroidales and reduces weight gain in rats fed a high-fat high-sucrose diet (ORAL COMMUNICATION)
  Ramos-Romero S, Amézqueta S, Hereu M, Muñoz D, Casado M, Piña B,Torres JL.
  2nd World Congress on Targeting Microbiota. Institut Pasteur, Paris, France. October 16-17, 2014.

- D-Fagomine reduces weight gain and high glycaemia while attenuating the gut microbiota imbalance induced by an energy-dense diet (POSTER)
  Ramos-Romero S, Amézqueta S, Hereu M, Muñoz D, Casado M, Piña B, Torres JL.
  Proceedings of the 2nd EFSA Scientific Conference. Milan, Italy, October 14–16, 2015.

- Prebiotic-like effect of D-fagomine in gut microbiota (ORAL COMMUNICATION)
  Hereu M, Ramos-Romero S, Moreno A, Amézqueta S, Torres JL.
  International Scientific Conference Probiotics and Prebiotics IPC2016. Budapest, Hungary. June 21-23, 2016.

- Changes in gut microbiota subgroups differently related to progression of obesity, hypertension and insulin resistance in rats (ORAL COMMUNICATION)
  Ramos-Romero S, Hereu M, Atienza L, García N, Amézqueta S, Torres JL.
  International Scientific Conference Probiotics and Prebiotics IPC2016. Budapest, Hungary. June 21-23, 2016.

- D-Fagomine, a new functional ingredient in the making (ORAL COMMUNICATION)
  Torres JL, Hereu M, Clapés P, Amézqueta S, Ramos-Romero S.
  The 1st International Congress on Food Bioactives & Health. Norwick, United Kingdom. September 13-15, 2016.

- Proanthocyanidins counteract the reduction of probiotics associated with ω-3 supplementation in rats (POSTER)

  Ramos-Romero S, Hereu M, Molinar-Toribio E, Pérez-Jiménez J, Almajano MP, Taltavull N, Romeu M, Nogués MR, Torres JL.

  The 1st International Congress on Food Bioactives & Health. Norwick, United Kingdom. September 13-15, 2016.

- On the evaluation of D-fagomine excretion after oral intake by LC-MS/MS (ORAL COMMUNICATION)

  Amézqueta S, Ramos-Romero S, Martínez-Guimet C, Moreno A, Hereu M, Torres JL.

  International Symposium on Pharmaceutical and Biomedical Analysis (PBA). Madrid, Spain. July 2-5, 2017.

- The influence of combined buckwheat D-fagomine and fish omega-3 PUFAs on beneficial gut bacteria in rats (SUBMITTED TO ORAL COMMUNICATION)

  Hereu M, Ramos-Romero S, Marín-Valls R, Amézqueta S and Torres JL.

  13th European Nutrition Conference - FENS. Dublin, Ireland. October 15-18, 2019.