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EFFECTS OF ALGAE AND ALGAE EXTRACTS ON LIPID METABOLISM. IMPLICATIONS IN HEPATIC STEATOSIS

ALGEN ETA ALGA-ESTRAKTUEN EFEKTUAK LIPIDOEN METABOLISMOAN. INPLIKAZIOAK GIBEL-ESTEATOSIAN

DOCTORAL THESIS

DOKTORETZA-TESIA

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Abbreviations/Laburdurak

A	ACADL	acyl-CoA dehydrogenase/azil-Ako deshidrogenasa
	ACC	acetyl-CoA carboxylase/azetil-AKo karboxilasa
	ACO	acyl-CoA oxidase/azil-Ako oxidasa
	ACS	acyl-CoA synthetase/azil-Ako sintetasa
	ACTA2	actin alpha 2 smooth muscle/aktina alfa 2 muskulu leuna
	ALP	alkaline phosphatase/fosfatasa alkalinoa
	ALT	alanine aminotransferase/alanina aminotransferasa
	AMPK	AMP-activated protein kinase/AMPk aktibaturiko proteina kinasa
	AST	aspartate aminotransferase /aspartato aminotransferasa
	ATP	adenosine triphosphate/adenosina trifosfatoa
C	C/EBPα	CCAAT-enhancer-binding protein α/CCAAT-ra lotzen den α proteina indartzailearen
	C/EBPβ	CCAAT-enhancer-binding protein β/CCAAT-ra lotzen den β proteina indartzailea
	ChREBP	carbohydrate-response element binding protein/karbohidratoen elementu erantzuleari lotzen zaion proteina
	COL1A1	collagen type I alpha 1 chain/I kolagenoa, alfa 1 katea
	CPT-1	carnitine palmitoyltransferase-1/1 karnitina aziltransferasa
	CPT-2	carnitine-acylcarnitine translocase/2 karnitina palmitoiltransferasa
	CS	citrate synthase/zitrato sintasa
D	DEXA	dexamethasone/dexametasona
	DGAT	diacylglycerol acyltransferase/diazilglizerol aziltransferasa
	DGAT2	diacylglycerol acyltransferase 2/2 diazilglizerol aziltransferasa
	DMEM	Dulbecco's Modified Eagle Medium
E	EB	Europar Batasuna
	EGGA	esterifikatu gabeko gantz-azidoa
	EU	European Union
F	F4/80	adhesion G protein-coupled receptor E1/atxikidura G proteinari lotutako E1 hartzailea

	FA	fatty acid
	FABP	fatty acid binding protein/gantz-azidoei lotutako proteina
	FABP1	fatty acid binding protein 1/1 gantz-azidoei lotutako proteina
	FAS	fatty acid synthase/gantz-azido sintasa
	FAT/CD36	fatty acid translocase/gantz-azido translokasa
	FATP	fatty acid transport protein/gantz-azido proteina garraiatzailea
	FATP2	fatty acid transport protein 2/2 gantz-azido proteina garraiatzailea
	FATP5	fatty acid transport protein 5/5 gantz-azido proteina garraiatzailea
	FBS	fetal bovine serum/behi-fetuaren seruma
G	G3P	glycerol-3-phosphate/glizerol-3-fosfatoa
	GGEA	gibel gantzatsu ez-alkoholikoa
	GJH	Garapen Jasangarrirako Helburua
	GPx	glutathione peroxidase/glutatioi peroxidasa
	GSH	reduced glutathione/erreduzitutako glutatioia
	GSSG	oxidized glutathione/oxidatutako glutatioia
H	HOMA-IR	homeostatic model assessment for insulin resistance/intsulinaren erresistentzia balioztatzeko modelo homeostatikoa
	HSC	hepatic stellate cell
I	IL-1β	interleukin-1β/1β interleukina
	IL-6	interleukin-6/6 interleukina
L	LPS	lipopolysaccharide
M	MCP-1	monocyte chemoattractant protein-1/1 proteina monozito-kimioerakarlea
	MDA	malondialdehyde/malondialdehidoa
	MMP9	matrix metallopeptidase 9/matrizeko 9 metalopeptidasa
	mRNA	messenger ribonucleic acid/azido erribonukleiko mezularia
	MTP	microsomal triglyceride transfer protein/triglizeridoen proteina garraiatzaile mikrosomala
N	NADPH	nicotinamide adenine dinucleotide phosphate/nikotinamida adenina dinukleotido fosfatoa
	NAFLD	non-alcoholic fatty liver disease
	NAS	NAFLD Activity Score

	NASH	non-alcoholic steatohepatitis
	NEFA	non-esterified fatty acid
	NRF1	nuclear respiratory factor 1/1 arnasketa faktore nuklearra
P	PGC1α	peroxisome proliferator-activated receptor-gamma coactivator 1 α/ peroxisomen ugalketak aktibatutako gamma hartzailearen 1 α koaktibatzailea
	PPARα	peroxisome proliferator-activated receptor α/peroxisomen ugalketak aktibatutako α hartzailea
	PPARγ	peroxisome proliferator-activated receptor γ/peroxisomen ugalketak aktibatutako γ hartzailearen
	PUFA	polyunsaturated fatty acid
R	RNA	ribonucleic acid/azido erribonukleikoa
S	ROS	reactive oxygen species/oxigenoaren espezie erreaktiboak
	R-QUICKI	revised quantitative insulin sensitivity check index/intsulinaren sentikortasun indize kuantitatibo berrikusia
	SAT	subcutaneous adipose tissue
	SDG	Sustainable Development Goal
	SIRT1	sirtuin 1/1 sirtuina
	SOD	superoxide dismutase/superoxido dismutasa
	SREBP-1c	sterol regulatory element binding protein-1, c isoform/ esterola doitzen duen elementura loturiko 1 proteina, c isoforma
T	TFAM	mitochondrial transcription factor A/mitokondrioetako A transkripzio faktorea
	TG	triglyceride
	TGFB1	transforming growth factor beta 1/beta 1 hazkuntza-faktore eraldatzailea
	TIMP1	TIMP metallopeptidase inhibitor 1/TIMP metalopeptidasaren 1 inhibitzailea
	TNF-α	tumour necrosis factor-α/α tumore-nekrosiaren faktorea
	TROLOX	total antioxidant capacity/ahalmen antioxidatzaile totala
V	VAT	visceral adipose tissue
	VLDL	very low-density lipoprotein/dentsitate oso baxuko lipoproteina
W	WAT	white adipose tissue

Section 1
1. atala

1. State of the art

1.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a condition characterised by excessive fat accumulation in the liver occurring in subjects who consume little or no alcohol (< 20 g/day in women, < 30 g/day in men) (1) and it is considered the hepatic manifestation of metabolic syndrome. The term NAFLD has been used for decades, however, it has been widely recognised that the term "non-alcoholic" overemphasizes the absence of alcohol consumption as its diagnosis criteria and it does not effectively represent the metabolic dysfunction at its core. Thus, in 2020 a change in nomenclature to metabolic dysfunction-associated fatty liver disease (MAFLD) was proposed to better recognize the heterogeneous metabolic risk factors associated with it (2). However, the term "fatty" was perceived as stigmatising by certain individuals, hence, more recently, in 2023, a proposal to redefine it as metabolic dysfunction-associated steatotic liver disease (MASLD) was driven (3). However, owing to the lack of consensus among all expert committees (4,5) the term NAFLD will be used throughout the present Doctoral Thesis.

NAFLD is the leading cause of chronic liver diseases worldwide. Its global prevalence among adults is estimated to be around 30 %, being higher among males (40 %) than females (26 %). This data also varies among different countries and global regions and it is higher in patients with metabolic diseases such as central obesity and type 2 diabetes mellitus (6,7). In fact, prevalence rates among patients with severe obesity amounts to 90 % and 75 % in patients with type 2 diabetes mellitus (8). Nonetheless, there is a subset of lean or normal weight individuals that develop NAFLD, but they usually present increased visceral adiposity and insulin resistance as metabolic disorders. In this line, global rates of NAFLD have substantially increased over the last three decades and following the current trends, it is projected that these rates will continue to grow in parallel with the current epidemics of obesity and diabetes (6,7).

NAFLD encompasses a variety of disorders, ranging from simple steatosis to more severe non-alcoholic steatohepatitis (NASH) which can course with or without fibrosis and that can progress to cirrhosis and hepatocellular carcinoma (8) (**Figure 1**). Simple steatosis is defined as an excessive lipid accumulation within hepatocytes exceeding 5 % of liver weight or 5 % of hepatocytes containing lipid vacuoles (9) and it is the most common manifestation of the disease (8). The accumulation of triglycerides is not harmful itself, however, this situation still increases the risk of developing NASH, because triglycerides can still be hydrolysed to non-esterified fatty acids (NEFAs) and overwhelm the liver's ability to metabolize them (10,11). Moreover, the excess of NEFAs can act as a substrate for the generation of lipotoxic lipid species such as ceramides and diacylglycerols (12). NASH is a more severe form of the disease characterized by steatosis accompanied by inflammation and hepatocyte injury or ballooning (10). Most patients with NAFLD do not develop NASH, in fact, 10-20 % of

affected individuals progress to this stage (8). Furthermore, progression of liver steatosis into NASH is not linear and can manifest periods of stability and regression (13). However, it must be stated that the development of NASH is associated with increased mortality due to increased liver-cancer and cardiovascular-related mortality (10). Long-term tissue damage leads to fibrosis and subsequently, to cirrhosis, which can drive to the development of hepatocellular carcinoma (11).

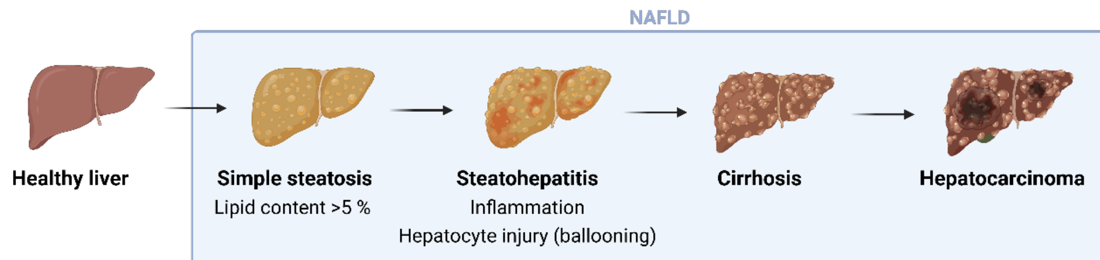


Figure 1. Progression of non-alcoholic fatty liver disease (NAFLD).

For many years, the "two-hit" theory was used to explain the pathophysiology of NAFLD. Based on this theory, the "first hit" involves hepatic fat accumulation driven by insulin resistance, sedentary lifestyle and hypercaloric diets. This state would increase the liver's susceptibility to other harmful factors, known as the "second hit" including oxidative stress and favouring the development of inflammation (14). Nonetheless, this theory fails to capture the complex and heterogeneous mechanisms that favour the development and progression of the disease. Instead, the "multiple-hit" theory provides a more accurate explanation of NAFLD pathogenesis. These hits include insulin resistance, altered production of adipokines, dietary components, mitochondrial dysfunction, oxidative stress, gut microbiota and related metabolites as well as genetic and epigenetic factors (15–17).

NAFLD is usually asymptomatic and it can progress to advances stages silently; consequently, it is often underdiagnosed. Currently, liver biopsy and histology remain the "gold standard" for the diagnosis of this condition, allowing for the classification of stage severity. The NAFLD Activity Score (NAS) and the Steatosis, Activity, Fibrosis (SAF) algorithm are the most frequently used histological scoring systems for NAFLD. Both evaluate histologic steatosis, lobular inflammation and hepatocellular ballooning, and significantly correlate in diagnosing definitive NASH; however, when it comes to borderline NASH, the two systems seem to have differing interpretations (18). The utilization of these histological analysis-based systems presents certain drawbacks, including invasiveness that may result in complications, inter-observer variability of individual pathological features and a high cost, thus it is only indicated in selected cases. Nowadays, abdominal ultrasound, an imaging technique, is used as first-line test to assess hepatic steatosis, but it has low sensitivity to detect mild steatosis. Since fibrosis is the strongest predictor of mortality, the use of techniques that allow distinguishing NASH from early fibrosis is critical. Magnetic resonance elastography and transient elastography (Fibroscan) show the ability to evaluate the stage of fibrosis

non-invasively, however, their accuracy decreases in patients with obesity and have a high cost. In view of this situation, there is an increasing interest in developing non-invasive reliable scoring systems that enable to determine the likehood of advanced fibrosis in NAFLD. These systems include Fibrosis-4 (FIB-4), NAFLD Fibrosis Score (NFS), Hepamet Fibrosis Score (HFS) and Platelet Ratio Index (APRI), however, their sensitivity is rather modest and discrepancies can be noticed in the outcomes (19). In this line, novel biomarkers that allow differentiating among simple steatosis, NASH and fibrosis are being discovered, for example, non-coding RNAs are gaining substantial attention as emerging diagnostic biomarkers (19,20).

Regarding NAFLD treatment, given the close relationship between an unhealthy dietary pattern and the development of obesity, insulin resistance and NAFLD, lifestyle modification is considered the first line therapeutic option for the management of the disease in order to achieve an appropriate body weight by increasing physical activity and through dietary modifications (21). In fact, achieving a reduction of >10 % in body weight through a hypocaloric diet combined with exercise, has shown to be able to reduce NAS (22). However, although it has proven to be valid in most cases, patients show very low adherence, especially in the long term, which is the main limitation of this approach.

Concerning pharmacotherapy, although several clinical trials are undergoing, there is no specific and effective pharmacological treatment approved yet. In fact, the available drug therapies can be grouped in weight loss medications (e.g. lipase inhibitors), antidiabetic drugs (e.g. sodium-glucose transporter-2 (SGLT-2) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, peroxisome proliferator-activated receptors (PPAR) agonists), antioxidants (e.g. vitamin E) and antifibrotic agents (e.g. apoptosis signal-regulating kinase 1 inhibitors (ASK1)) (8,23). In this context, the scientific community is searching for new molecules that could serve as alternative o complementary therapies for the management of NAFLD.

1.1.1. Development of simple steatosis: involvement of insulin resistance and obesity

Insulin resistance and fat accumulation in the liver are strongly associated. In fact, insulin resistance is considered the main pathogenic event linked to the development of hepatic steatosis. In normal situations, insulin promotes glucose uptake and triglyceride synthesis, and suppresses triglyceride hydrolysis. However, in situations where plasma glucose levels are constantly elevated, the tissues in the body become resistant to insulin signalling and the action of insulin is impaired. Regarding insulin-resistant adipose tissue, the anti-lipolytic effect of insulin is suppressed, with the subsequent increase in triglyceride breakdown, that leads to an excessive release of NEFAs (along with glycerol), that can be taken up by the liver (24). Visceral or intra-abdominal fat deserves a special mention, because even lean subjects can display abnormal visceral fat accumulation that leads to reduced insulin sensitivity (25). As far as liver is concerned, insulin resistance and the subsequent hyperinsulinemia, modulate hepatic lipid metabolism by stimulating fatty acid synthesis (24), as it will be more detailed in the section dedicated to *de novo* lipogenesis. Furthermore, insulin fails to suppress hepatic

gluconeogenesis, and thus, the liver continues to synthetize glucose, adding to the circulating a glucose surplus (26) and contributing to the pool of substrates for hepatic *de novo* lipogenesis.

Another pathological situation that favours the development of hepatic steatosis is obesity. Obesity is characterised by the excessive expansion of white adipose tissue, with the subsequent increase in plasma concentrations of NEFAs, and thus increased hepatic availability. Furthermore, it is worth mentioning that adipose tissues from different anatomical locations contribute differently to the circulating NEFAs. Visceral adipose tissue (VAT) shows a higher lipolytic rate than subcutaneous adipose tissue (SAT) due to the lower presence insulin receptors than in SAT. Due to this fact, the mobilization capacity of NEFAs is greater in VAT than in SAT. On the other hand, VAT is drained by the hepatic portal vein directly into the liver, which increases the flow of NEFAs than can enter this viscera, and thus stimulating triglyceride synthesis (27).

Obesity is often associated with insulin resistance due to adipose tissue dysfunction. During the development of obesity, the expansion of white adipose tissue depots can be driven by two mechanisms, the increase in size of existing adipocytes (hypertrophy) or the differentiation of adipocyte precursors into mature adipocytes (hyperplasia), also known as adipogenesis. The way adipose tissue expands has a significant impact on individual's metabolic health, in fact, while during hyperplasia adipose tissue function is maintained, during hypertrophy, adipocytes experience hypoxic stress due to inappropriate remodelling of extracellular matrix. In this last scenario, the release of pro-inflammatory cytokines is increased (e.g. tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) or monocyte chemoattractant protein-1 (MCP-1)), thus promoting infiltration of immune cells, and adipokine secretion is impaired (decreased adiponectin and increased leptin levels) (27,28). As a consequence of these mechanisms, adipose tissue becomes dysfunctional, which is associated with the development of systemic insulin resistance. Moreover, hypertrophic adipocytes exhibit increased lipolytic rate that results in a higher flux of NEFAs to the liver trough the portal vein, leading ectopic fat accumulation in the liver, which also leads to insulin resistance (16,29).

1.1.2. Physiological liver lipid metabolism and alterations in non-alcoholic fatty liver disease

Liver plays a key role in lipid metabolism. Under a normal physiological status, the liver processes large quantities of fatty acids, and only relatively low quantities of triglycerides (less than 5 %) are stored in cytoplasmic lipid droplets (30). Hepatic steatosis is a consequence of impaired hepatic lipid metabolism, more specifically, derived from the imbalance between lipid acquisition (fatty acid uptake, *de novo* lipogenesis and liposynthesis) and removal (fatty acid oxidation and triglyceride-rich very low-density lipoprotein (VLDL) secretion). Disruption of metabolic pathways involved in lipid metabolism lead to excessive fat accumulation in liver, the hallmark of NAFLD (10,31) (**Figure 2**).

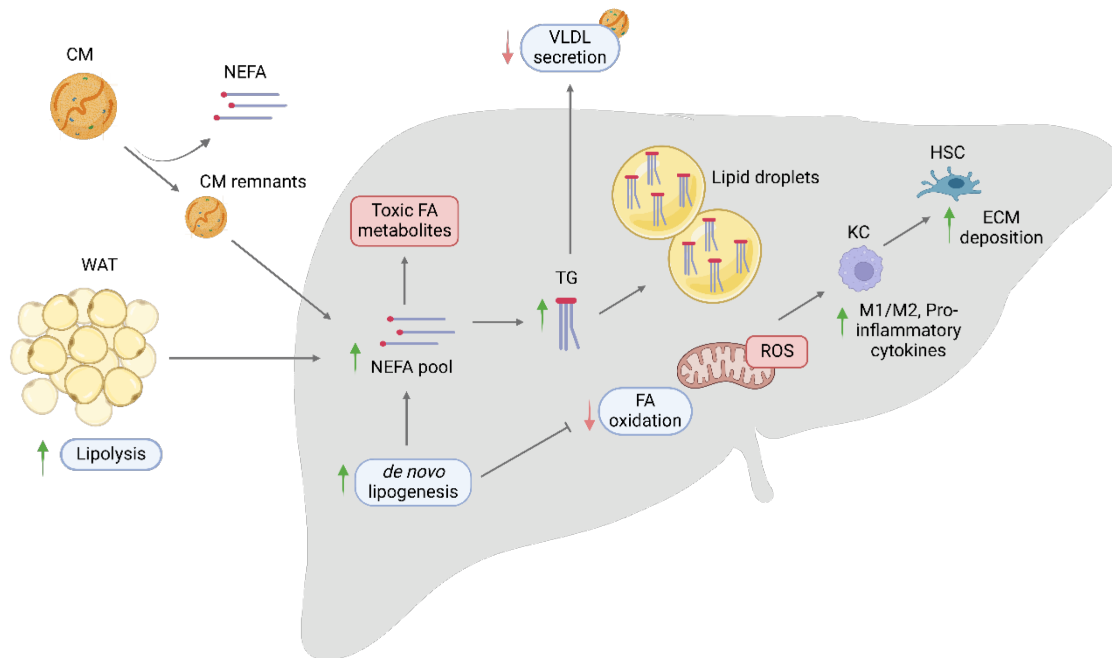


Figure 2. Alterations in lipid metabolism and other pathogenic pathways involved in the progression of NAFLD. CM: chylomicron, ECM: extracellular matrix, FA: fatty acid, HSC: hepatic stellate cell, KC: Kupffer cell, NEFA: non-esterified fatty acid, TG: triglyceride, ROS: reactive oxygen species, VLDL: very low-density lipoprotein, WAT: white adipose tissue. Small green and orange arrows mean "increased" and "decreased" respectively. Grey arrows indicate activation and t-shape grey lines indicate inhibition.

Pathways associated with accumulation of intrahepatic fat: fatty acid uptake, fatty acid synthesis and triglyceride esterification

Fatty acids that contribute to the liver triglyceride accumulation can derive from three different sources: dietary lipids from lipoprotein remnants, NEFAs released by adipose tissue and *de novo* lipogenesis (32).

Dietary fatty acids can arise from the spill over from chylomicrons or from chylomicron remnants. NEFAs can also be released by adipose tissue. In obesity, due to the increase in the size of adipose tissue, the flux of NEFAs released from this tissue increases (33). In the case of insulin resistance, adipose tissue lipolysis is not suppressed and consequently, increases the plasma NEFA flux that is delivered to the liver, among other tissues and organs (31).

i. Fatty acid uptake

Plasmatic NEFAs can be taken up into hepatocytes through specific transporters, including, fatty acid transport proteins (FATP). In particular, fatty acid transport protein 2 (FATP2) and fatty acid transport protein 5 (FATP5) are the major FATPs found in the liver; moreover, fatty acid translocase (FAT/CD36) or fatty acid binding proteins (FABPs), specifically fatty acid binding protein 1 (FABP1), are highly expressed in the liver (30). In this line, in a study conducted by Greco *et al.*, a

positive association was found between CD36 expression and fat liver content in humans (34). In mice, it has been observed that silencing of FABP1 and deletion of FATP5 decreased liver weight and triglyceride accumulation in this tissue (35,36). Moreover, protein expression of CD36 and FABP1 were increased in mice after feeding with a high fat diet and showing hepatic steatosis in comparison to control mice (37). However, in some cases, decreased CD36 and FATP5 expression has been observed in rats with steatosis, probably due to a compensatory mechanism (38).

ii. *De novo* lipogenesis

Another part of the hepatic pool of fatty acids arise from *de novo* lipogenesis. In fact, *de novo* lipogenesis is the second major contributor to hepatic triglycerides after plasmatic NEFAs in NAFLD. Donnelly *et al.* described that in humans 60 % of triglycerides accounting in the liver arose from plasmatic NEFAs, 26 % from *de novo* lipogenesis and 15 % from the diet (39).

In *de novo* lipogenesis long chain fatty acids are newly synthesised from acetyl-CoA, derived mainly from glucose. The rate limiting step is catalysed by acetyl-CoA carboxylase (ACC), which carboxylates acetyl-CoA to malonyl-CoA. Subsequently, fatty acid synthase (FAS) catalyses the formation of predominately palmitic acid from malonyl-CoA (**Figure 3**). Palmitic acid can be further elongated by elongases and desaturated by desaturases (30,40). *de novo* lipogenesis also needs the hydrogen donor, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), generated in the pentose phosphate pathway and in the malic enzyme reaction (41). This pathway is primarily regulated at a transcriptional level. Plasma insulin activates liver-X-receptor (LXR), which then activates sterol regulatory element binding protein-1c (SREBP-1c). This results in the upregulation of lipogenic genes. The excess plasma glucose promotes the nuclear translocation of carbohydrate-response element binding protein (ChREBP), another transcription factor that upregulates most of fatty acid biosynthetic genes (30,42). On the other hand, the ACC is inactivated via phosphorylation by AMP-activated protein kinase (AMPK), thus leading to decreased malonyl-CoA and increased fatty acid oxidation in the mitochondria (43) (**Figure 4**).

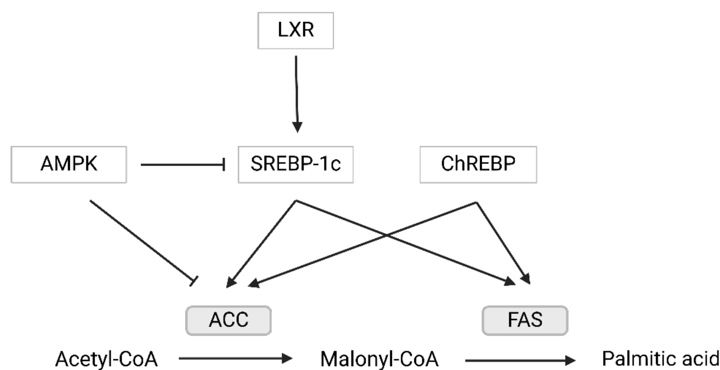


Figure 3. *de novo* synthesis of fatty acids and its transcriptional regulation. ACC: acetyl-CoA carboxylase, AMPK: AMP-activated protein kinase, ChREBP: carbohydrate-response element binding protein, FAS: fatty acid synthase, LXR: liver-X-receptor, SREBP-1c: sterol regulatory element binding protein-1c. Arrows indicate activation and T-shape lines indicate inhibition.

In several studies, carried out using isotope labelling, it is has been observed that subjects with NAFLD showed increased *de novo* lipogenesis than control subjects (44,45). Furthermore, in studies performed in animal models of hepatic steatosis, increased markers of *de novo* lipogenesis have been found. For example, in a leptin deficient mice (Ob/Ob) model, gene and protein expression of ACC, FAS, SREBP-1c and desaturases were increased compared to wild type mice (46) and in a diet-induced rat model of hepatic steatosis, activity of FAS found to be increased in rats fed with a high-fat high-fructose diet in comparison to rats fed a standard diet (38).

iii. Triglyceride assembly

Triglyceride assembly constitutes the principal means by which the liver stores fatty acids (30). It consists of the esterification of glycerol-3-phospahte (G3P) with a combination of NEFAs to generate triglycerides. The NEFAs that are incorporated into triglycerides can derive from the plasma or from *de novo* lipogenesis, whereas G3P is produced either via glycolysis or through phosphorylation of glycerol released from adipose tissue derived lipolysis (41).

The first and rate-limiting step in triglyceride synthesis is mediated by glycerol-3-phospahte acyltransferase (GPAT), which consists on the esterification of acyl-CoA chains to G3P. The second enzyme involved, acylglycerol-3-phosphate acyltransferase (AGPAT), leads to the formation of diacylglycerols. Finally, diacylglycerol acyltransferase (DGAT) catalyses the last reaction in the formation of triglycerides. Triglycerides may be stored in cytoplasmic lipid droplets or incorporated into VLDL particles, which are then secreted into the blood (40).

In vivo studies have reported the role of diacylglycerol acyltransferase (DGAT2) in de the development of hepatic steatosis. Gene and protein expression of DGAT2 were increased in a rat model of fructose-induced fatty liver (47) and overexpression of liver DGAT2 in mice led to increased hepatic triglyceride accumulation (48). Moreover, inhibition of DGAT2 ameliorated hepatic steatosis in mice fed a steatogenic diet, without inducing liver inflammation or fibrosis, probably because the inhibition of DGAT2 was accompanied by a reduction of *de novo* lipogenesis, which could explain why diacylglycerols did not accumulate (49).

Pathways involved in lipid clearance: fatty acid oxidation and triglyceride secretion

The liver can clear triglycerides mainly through mitochondrial fatty acid oxidation or through their secretion in the form of VLDLs (11).

i. Fatty acid oxidation

Fatty acids derived from hydrolysis of hepatic triglyceride stores, circulating lipids or *de novo* synthetized fatty acids can be oxidized through β-oxidation, which mainly takes place in mitochondria, but also in peroxisomes, although to a lesser extent. Peroxisomal β-oxidation involves the shortening of very long- and branched chain fatty acids, which are subsequently oxidised in mitochondria (30,31).

Mitochondrial β-oxidation is the primary route for the oxidation of short-, medium-, and long-chain fatty acids (30), and takes place in the mitochondrial matrix. Fatty acids are progressively shortened into acetyl-CoA subunits, which can be completely oxidized in the Krebs cycle or used to produce ketone bodies (31).

Before entering the β-oxidation, fatty acids are converted into acyl-CoA by the cytosolic enzyme acyl-CoA synthetase (ACS), and then, this molecule needs to be transferred into mitochondria, which is mediated by the carnitine shuttle. First, carnitine palmitoyltransferase-1 (CPT-1), located in the outer mitochondrial membrane, promotes the conversion of acyl-CoA to acyl-carnitine and its transport across the outer mitochondrial membrane. This is the rate-limiting step and an important regulation point of β-oxidation (50). This process is negatively regulated by malonyl-CoA, the product of the first step of *de novo* lipogenesis (see *de novo* lipogenesis) which is an allosteric inhibitor of CPT-1 (31). Short- and medium-chain fatty acids pass through the mitochondrial membranes and are activated by ACS within the mitochondrial matrix, thus their oxidation is not controlled by CPT-1 (41). Then, carnitine palmitoyltransferase-2 (CPT-2), located in the inner mitochondrial membrane, catalyses the transport of acyl-carnitine into the mitochondrial matrix, releasing carnitine and regenerating of fatty acyl-CoA that enters the β-oxidation (**Figure 4**). This process consists of the repeated elimination of two-carbon fragments, through oxidation that will give rise to acetyl-CoA. In each round, four enzymes participate sequentially, releasing an acetyl-CoA residue, while the acyl-CoA is shortened (31).

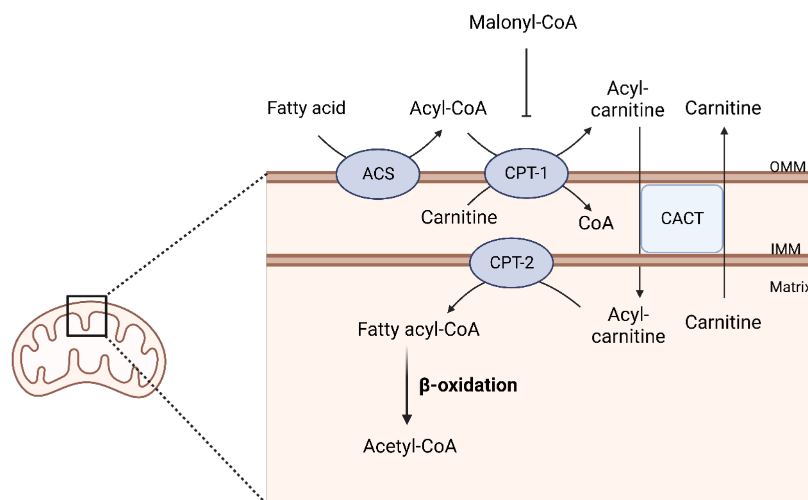


Figure 4. Fatty acid transport in mitochondria thought CPT system. ACS: acyl-CoA synthetase, CACT: carnitine-acylcarnitine translocase, CPT-1/CPT-2: carnitine palmitoyltransferase-1/2, IMM: inner mitochondrial membrane, OMM: outer mitochondrial membrane. T-shape line indicates inhibition.

The regulation of gene expression related to fatty acid oxidation is primarily regulated by peroxisome proliferator-activated receptor α (PPARα). Moreover, the sirtuin family member sirtuin 1 (SIRT1) plays an important role regulating fatty acid oxidation. SIRT1 deacetylates and activates peroxisome

proliferator-activated receptor-gamma coactivator 1 α (PGC1α), which activates PPARα, promoting fatty acid oxidation (19,51).

In a recent study, reduced hepatic mitochondrial fatty acid oxidation, along with increased dysfunctional mitochondria were observed in patients with NAFLD (52). Kohjima *et al.* observed that, in a group of subjects with NAFLD, the gene expression of *Cpt-1* and *Ppara* was decreased as compared with healthy livers (53) and SIRT1, among other sirtuins, was observed to be down-regulated in NAFLD subjects in comparison to subjects without this disease (54). In mice fed a high-fat diet, which developed steatosis, Nie *et al.*, described reduced hepatic CPT-1, PPARα, PGC1α and SIRT1 protein expression in comparison to control mice (37). However, conflicting results have been reported showing increased fatty oxidation in NAFLD. This could be explained as a compensatory mechanism activated during the early stages of NAFLD in response to the excessive fatty acids within the liver (55).

ii. Triglyceride-rich lipoprotein secretion

Under normal conditions, the liver stores small amount of triglycerides, and exports considerable amounts as VLDL particles, that deliver fatty acids to the muscle for oxidation or to adipose tissue for storage, depending in the nutritional status (30).

VLDL is a triglyceride-rich lipoprotein composed of a single molecule of apolipoprotein B-100 (apoB-100) that is lipidated by the incorporation of triglycerides facilitated by microsomal triglyceride transfer protein (MTP). The process occurs within the lumen of endoplasmic reticulum and during the maturation of VLDL particles, they are translocated across the endoplasmic reticulum membrane and they finally reach the Golgi apparatus (30,56).

Insulin exerts its anti-lipolytic action by suppressing VLDL production, probably due to the reduction in MTP activity. However, in NAFLD due to the increase in lipid availability and hepatic insulin resistance, MTP activity is increased, subsequently increasing the production of VLDL. Consequently, hypertriglyceridemia is commonly found in NAFLD patients. Although triglyceride-rich VLDL secretion rate is increased in hepatic steatosis (57), it is not enough to match the increased triglyceride availability in the liver, which leads to hepatic steatosis. In fact, it has been observed that when hepatic triglyceride infiltration exceeds 10 % (hepatic steatosis), it is no possible to further increase VLDL secretion (31,56). As shown in a study conducted by Fujita *et al.*, NASH patients showed lower VLDL secretion than patients with simple steatosis (58).

1.1.3. Pathogenic events leading to de development of non-alcoholic steatohepatitis

Progression from simple steatosis to NASH involves hepatocyte injury that can occur through different mechanisms. Moreover, apart from hepatocytes, hepatic non-parenchymal cells also contribute to liver damage, inflammation and development of fibrosis.

In previous sections, it has been explained how different mechanisms drive to the excessive accumulation of fat in the liver. It is known that the process of liver cell injury is influenced not only by the amount of accumulated lipids, but also by the specific type of lipid molecule. In this regard, triglycerides account for the major form of intrahepatic lipids accumulated in NAFLD; however, it seems that they play rather a protective role against lipotoxicity. This term refers to the process of cell injury caused by toxic lipid species. Excessive accumulation of NEFAs that cannot be incorporated into triglycerides cause lipotoxicity within the hepatocytes. Furthermore, based on their saturation NEFAs also have a different impact; while unsaturated fatty acids are associated with positive effect, saturated fatty acids are more prone to induce lipotoxicity-associated cell injury. Ultimately, lipotoxicity leads to organelle dysfunction, for instance, endoplasmic reticulum stress and mitochondrial dysfunction, cell injury and death, promoting the progression to NASH (24,59).

Another event that is of major importance in the progression from simple steatosis to more advanced stages is oxidative stress. In fact, this alteration is considered fundamental in the progression from simple steatosis to NASH (60). Oxidative stress can be defined as the disruption of the pro-oxidant/anti-oxidant balance, due to the increased production of reactive oxygen species (ROS) and/or dysfunction of the antioxidant system, causing tissue damage (10). Under healthy physiological conditions, generation of ROS is balanced by the antioxidant system, maintaining basal levels. ROS can be classified as free radicals, among which the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$) are physiologically the most relevant ones; and non-radical species, highlighting hydrogen peroxide (H_2O_2). On the other hand, the antioxidant system consists of enzymatic and non-enzymatic antioxidant mechanisms. The most relevant antioxidant enzymes include superoxide dismutase (SOD), which converts $O_2^{\bullet-}$ into H_2O_2, catalase, which neutralizes H_2O_2 into H_2O, and glutathione peroxidase (GPx), which also catalyses the reduction of H_2O_2 (**Figure 5**). The major constituent of the non-enzymatic antioxidant defence is glutathione, although other molecules such as ascorbic acid (vitamin C), retinol (vitamin A) or tocopherol (vitamin E) also act as electron receptors protecting other biomolecules and cells structures against ROS damage (55).

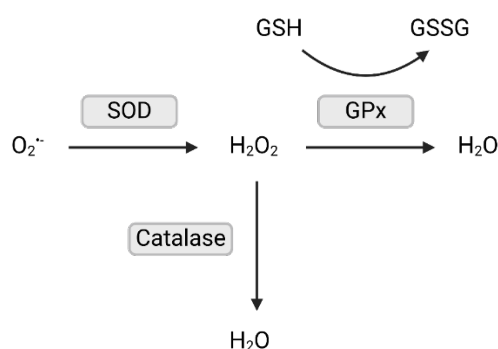


Figure 5. Activity of main antioxidant enzymes. GPx: glutathione peroxidase, GSH: reduced glutathione, GSSG: oxidized glutathione, SOD: superoxide dismutase.

Mitochondria are considered the main source of ROS. At initial stages of NAFLD, due to NEFA overload, hepatic mitochondrial oxidation increases as an adaptive mechanism. However, in a state of chronic overload, this compensatory mechanism fails and becomes a source of ROS, as a result of incomplete mitochondrial oxidation. This occurs, because the increased acetyl-CoA production as a consequence of β-oxidation, can uncouple the Krebs cycle from electron transport chain, leading to an impaired ATP and ROS synthesis (24,55,61). The decrease in electron transport chain activity results in the release of cytochrome c to the cytosol, inducing the apoptotic pathway dependent on caspases (62). In addition, due to incomplete β-oxidation, toxic lipid species that can exacerbate lipotoxicity, such as ceramides, are generated (24).

Endoplasmic reticulum is also a site where ROS production occurs triggered by excess NEFAs. When this stimulus is sustained, endoplasmic reticulum stress leads to ROS synthesis that can also promote hepatic inflammation by increasing the secretion of TNF-α. Endoplasmic reticulum stress can also favour hepatic inflammation via the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK) pathways. In addition, endoplasmic reticulum stress can induce hepatocyte apoptosis (24).

Together all these mechanisms increase ROS production in hepatocytes and the dysfunction of the antioxidant system. ROS can also lead to lipid peroxidation producing malondialdehyde (MDA), resulting in the disruption of cell membranes and other cellular structures and the production of reactive metabolites. The increase in oxidative stress causes inflammation and hepatocyte apoptosis, leading to the development of fibrosis (62,63).

Besides hepatocytes, hepatic non-parenchymal cells, among which Kupffer cells and hepatic stellate cells can be found, also participate in the progression of the disease (**Figure 2**). Oxidative stress activates Kupffer cells, the liver-resident macrophages. Under oxidative stress, polarization of Kupffer cells towards a pro-inflammatory (M1) phenotype occurs, leading to an increased release of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β. Moreover, as a consequence of oxidative stress and the activation of the inflammatory process, hepatic stellate cells are stimulated, promoting their fibrogenic phenotype, leading to the production of extracellular matrix and development of fibrosis (2,55).

In addition to the alterations that occur in the liver, the role of dysfunctional adipose tissue in the progression to NASH cannot be ignored. Adipose tissue not only plays an important role in the development of steatosis, as described above, but it is also involved in its progression to more severe states. In obesity, adipose tissue dysfunction is not only related with the development of inflammation within that tissue, but also, contributes to chronic low-grade systemic inflammation that triggers hepatic inflammation. This situation increases insulin resistance, which together with inflammation, operate in a detrimental cycle increasing liver damage (10,11).

1.2. Algae

Algae are heterogeneous photosynthetic organisms found in almost all aquatic environments, in both fresh and saltwater. Algae are classified into microalgae and macroalgae. Microalgae are small single-celled organisms, although they can also grow in colonies or in filaments, and they are branched into two major groups: prokaryotic and eukaryotic. The prokaryotic microalgae include *Cyanophyta*, also known as cyanobacteria or blue-green algae, and *Prochlorophyta*. Eukaryotic microalgae include several divisions, such as *Chlorophyta*, *Rhodophyta*, *Cryptophyta*, *Ochrophyta*, *Haptophyta, Heterokontophyta* and *Dinophyta* among others. It must be stated that algae classification is quite complex and that it continuously changes as new differentiators are discovered (64,65). Macroalgae are multicellular organisms found primarily in marine environments. In this case, they are also known as seaweeds (66) and are commonly classified based on their pigmentation: brown seaweeds (*Phaeophyta*), red seaweeds (*Rhodophyta*) and green seaweeds (*Chlorophyta*) (67).

For thousands of years, algae have been an important part of the human diet. Regarding consumption of microalgae, including cyanobacteria, there is evidence that they were consumed centuries ago in China, Mongolia, South America or Chad. *Arthrospira* (also known as Spirulina) was extensively harvested by local populations in different regions. However, in Europe the consumption of microalgae is more recent (68).

As for seaweeds, they have been historically consumed as food due to their nutritional and health benefits and organoleptic properties, especially in Asian countries such as Japan and Korea. In Europe, the traditional consumption of macroalgae was not so widespread, although in coastal countries like Portugal, Spain, France, United Kingdom and Ireland among others, seaweed have been harvested for human consumption for centuries, as well as for fertilizers and cattle fed (68). Although seaweed consumption in Europe is currently increasing, it is still far from the quantities consumed in Asian countries. While average daily intake of dried seaweed accounts for 4, 5.2 and 8.5 g/per capita in Japan, China and South Korea, respectively (69), a recent study carried out in France showed that the average seaweed consumption in the general population was equal to 293 mg/day (70).

Nowadays, algae are gaining attention because they are considered good candidates as a sustainable alternative to traditional crops to face the increase in population and the increased demand for food that it entails. In fact, in terms of sustainability, algae production does not require as much arable land as traditional crops, and can grow with minimal nutrients (68). Beyond this, algae are also attracting more attention due to their high value nutritional ingredients and health promoting properties (71). In that regard, algae are a rich source of micro- and macronutrients including, carbohydrates, polyunsaturated fatty acids (PUFAs), proteins, minerals and vitamins that increase the nutritional value of the diet. Regarding algae proteins, algae are considered a good protein alternative source since the amount of protein is significantly higher compared to other sources, and they exhibit

an excellent amino acid composition (72). Besides their nutritional components, algae are rich in several secondary bioactive metabolites (**Figure 6**), such as phenolic compounds, pigments, polysaccharides, fatty acids or peptides with therapeutic potential/health benefit activities (e.g. antioxidant, anti-inflammatory, anti-hyperlipidemic, antimicrobial, anticancer) that have not yet been fully exploited (65,67).

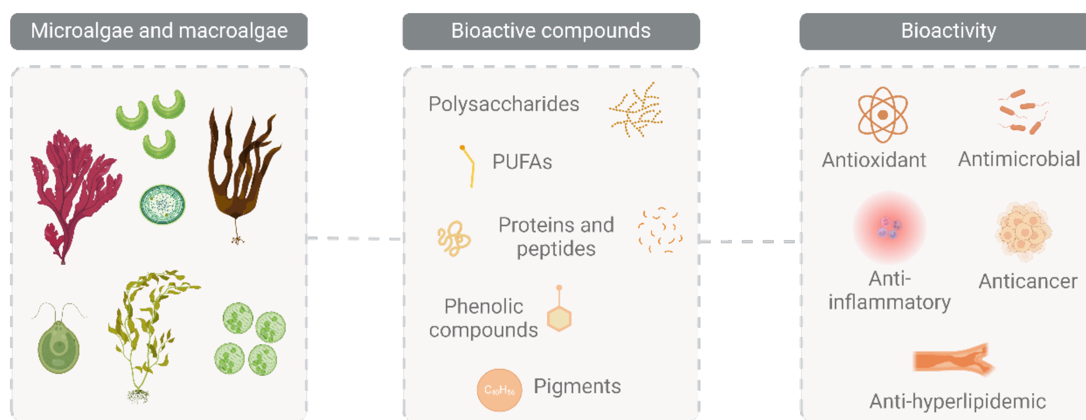


Figure 6. Representation of some of the most common bioactive compounds found in micro- and macro-algae and some of their biological activities. PUFAs: polyunsaturated fatty acids.

1.2.1. European algae production

In the past 30 years, global algae production sector has substantially grown, specifically from 0.56 million tonnes in 1950 to 35.82 million tonnes in 2019. The 97 % of global algae production is set in Asia, and macroalgae account for 99 % of global production. Regarding the European algae sector, it is still an emerging sector as it has not experienced such significant growth, however it is recognised as having substantial growth potential (66).

Microalgae production in Europe is predominantly land-based occurring in photobioreactors in closed and controlled conditions (68). Ponds and fermenters are also used although in smaller proportions, with the exception of *Spirulina*, which is predominantly produced in ponds. In terms of number of companies, Spain, Germany, France and Italy lead the European microalgae sector. Considering the production of dry weight biomass, *Chlorella* sp., *Haematococcus pluvialis* and *Nannochloropsis* sp. are the most cultivated species, and extending to global production, *Chlorella* and *Spirulina* are the two most widely produced microalgae. One of the main microalgae applications is in the food supplement and nutraceutical industry, followed by the cosmetic and well-being sector and animal fed industry (66).

The vast majority of European alga production accounts for macroalgae biomass (99 %), in comparison with microalgae, and it relies on wild harvesting as the main production method. Regarding aquaculture, the majority are located in sea installations, while a smaller percentage is land-

based. Most of companies are located in France, followed by Ireland and Spain. The main commercially exploited species are brown algae, such as *Laminaria* sp., the most important genus used for aquaculture and wild harvesting. Regarding aquaculture companies, most of them cultivate *Saccharina latissima*, followed by *Alaria esculenta* and *Ulva* sp. Main seaweed production accounts for human consumption, with a smaller proportion used with food-related purposes, such as, food supplements, thickening agents and preservatives. Macroalgae are also used for feed production and cosmetic and well-being market (66).

1.2.2. European Union legislation and Novel Foods

Algae, as food products in the European Union (EU), are subjected to the General Food Law Regulation (EU) No. 178/2002 (73) which is implemented in all member countries. Moreover, algae are considered "Novel Foods" meaning that, the entry of novel algae species into the EU market need prior authorization to ensure safety for human consumption. According to the European Commission, Novel Food is defined as food that had not been consumed to a significant degree by humans in the EU before 15 may 1997, when the first Regulation on Novel Foods (EC No. 258/97) (74) came into force. This Novel Food regulation establishes that any Novel Food requires a premarket authorization before it can be placed in the EU market (75) and nowadays, it is regulated by Novel Food Regulation (EU) No. 2015/2283 (76). Furthermore, this regulation specifies that species that have not been consumed in the EU but that are considered traditional food from a third country based on a history of safe food use can benefit from a notification system to make easer the route to the EU market.

1.2.3. Effects of algae and their bioactive compounds on non-alcoholic fatty liver disease

Several preclinical studies have shown that different algae species, both micro- and macroalgae are able to exert beneficial effects regarding hepatic fat accumulation (77). Although intervention studies in human are almost non-existent, a study found a negative association between algae consumption and NALFD (78). As mentioned before, algae are a rich source of underutilized bioactive compounds with the potential use in the prevention and treatment of several diseases. In this sense, the following bioactive compounds stand out: polysaccharides, PUFAs, proteins and peptides, polyphenols and pigments.

Polysaccharides are an important component of algae, specially found in the cell wall and they include agar, carrageenan, fucoidan, laminarin and alginate among others. Polysaccharide rich-fraction of different algae species have shown positive effects concerning hepatic lipid accumulation (79–82). Moreover, apart from polysaccharides, algae also contain lipids, which are found in higher proportion in microalgae than in macroalgae. The long chain PUFAs are of special interest due to their bioactivity. In fact, studies conducted with algae extracts rich in PUFAs have shown the potential to ameliorate hepatic steatosis (83,84). As already mentioned, algae are an interesting source of protein,

but besides the nutritional value, algal origin protein lysates and peptide extracts can exert a beneficial effect attenuating hepatic steatosis (85,86). Other bioactive compounds found in algae with promising effects are polyphenols (87–89) and pigments, such as, fucoxanthin (90,91). Furthermore, carotenoid-rich algae extracts are able to ameliorate hepatic fibrosis (92,93).

1.2.4. Algae safety

Despite their benefits, already mentioned above, algae consumption can also be harmful for human health. Some algae and cyanobacteria, can produce toxins that can make people and animals sick and affect the environment. For example, anatoxin, produced by some cyanobacteria can damage the liver or the nervous system. Moreover, several toxins produced by microscopic algae end up contaminating other commercial species (68).

Nevertheless, the presence of heavy metals such as, arsenic, lead, cadmium or mercury and an excess of certain nutrients such as iodine is a major concern regarding algae safety and the risk is associated with the ability of algae to accumulate minerals and trace elements from surrounding areas. This accumulation depends on various factors, such as, the algae species, the seasonality or environmental conditions like temperature, salinity, pH, or light conditions (68). In this line, for example, brown algae have shown a high concentration potential in comparison to other algae divisions due to their specific characteristic of the cell wall (94). Near the industrial areas, higher concentrations of heavy metals can be found in the water and substrates, which end up accumulating in algae (95). In this regard, algae are widely used in bioremediation to remove heavy metals and other pollutants from wastewaters (96,97). As a consequence, heavy metals contained in algae can also enter the food chain when eaten by organisms in higher trophic levels, which poses a risk of toxicity for humans as well (98). Even if some algae do not surpass the legal limits regarding heavy metals, the frequent and excessive consumption can lead to harmful effects associated with the bioaccumulation of these compounds in human organism (68). According to the "Report of results of the prospective study for the determination of heavy metals and iodine in marine algae" published in 2019 by the Spanish Agency for Food Safety and Nutrition (AESAN) (99), among different heavy metals studied, the result of the risk assessment for mercury showed that 80 % of the results exceeding the maximum residue limit corresponded to Kombu (*Laminaria japonica, Saccharina japónica*), while the 100 % corresponded to Hiziki (*Hizikia fusiforme*). This last species also stood out for its high content in inorganic arsenic and for that reason, its intake is discouraged.

Concerning iodine, despite being essential to humans (its deficiency can cause serious health issues), an excessive intake can have deleterious effects (e.g. thyroid dysfunctions). In this regard, special attention must be paid by high-risk population subgroups (pregnant women, children, individuals with thyroid dysfunction, heart disease or renal insufficiency) (68,95). The aforementioned report (99) concluded that the average value of the species included in the study corresponded to a high

exposure value, especially regarding the Kombu seaweed, whose iodine levels are specially high. Consequently, it is recommended to restrict its consumption.

Finally, regarding algae-related food allergies, they are quite uncommon and they are usually caused by small crustaceans that inhabit the seaweed that are cultivated and harvested in the open ocean, while other times are caused by algae components. However, these components are not well known (100).

1.2.5. Algae species used in the present Doctoral Thesis

This section includes a brief description comprising the algae species employed in the present Doctoral Thesis.

Chlorella vulgaris

Chlorella vulgaris (*Chlorophyta*) is a microscopic spherical unicellular alga with a diameter of 2-10 µm. Besides its small size, it contains many structures similar to those found in superior plants, such as cell wall, mitochondria or vacuoles and a single chloroplast. This microalga grows in fresh water and can be found either isolated or in clusters forming colonies containing a maximum of 64 cells. *C. vulgaris* is a non-motile organism that reproduces asexually by self-sporulation in which four new daughter cells are formed within the cell wall of the mother cell from which are released after their maturation. *C. vulgaris* grows in freshwater and can easily adapt to different conditions, which together with a high growth rate, makes it ideal for production. Furthermore, it is capable of growing in autotrophic, heterotrophic and mixotrophic (by performing photosynthesis as well as ingesting organic materials) conditions (101,102). According to the EU Novel Food status Catalogue (103), *C. vulgaris* is not considered a Novel Food, meaning that its access to the market is not subject to the pre-market authorisation in accordance with Regulation (EU) 2015/2283.

Nannochloropsis gaditana

Nannochloropsis gaditana (*Ochrophyta*) is a microalga species that was first isolated by Lubian *et al.* in the Bay of Cadiz (Spain). When *N. gaditana* cells are in an active growth phase, they have an ellipsoidal shape of 3.5-4 x 2.5-3 µm. Under this condition *N. gaditana* can be distinguished from *N. oculata* and *N. salina*. They are non-motile, devoid of flagella and possess a single chloroplast that occupies a significant portion of their cell. *N. gaditana* possesses a thicker and more resistant cell wall than the other two species. It is an autotrophic species and it reproduces exclusively by binary fission of cells (asexual reproduction). *Nannochloropsis* sp. primarily grow in marine environments although they can also be found in fresh and brackish waters. In this line, it has been observed that *N. gaditana* tolerates a wide range of salinities, although it is less stable at high light intensities compared to other algae, probably due to the presence of a single chlorophyll (104,105).

Gracilaria vermiculophylla

Gracilaria vermiculophylla is a cartilaginous red macroalga (*Rhodophyta*) that originates from a disc-shaped holdfast. Its thallus is densely and irregularly branched, with cylindrical branches that are slightly constricted at the base. The colour of this macroalga can range from dark-red to reddish brown, occasionally appearing greenish or black. This colour variation sets it apart from other species from the same genus, such as *Gracilaria gracilis*, which displays an intense red color. *G. vermiculophylla* is predominantly found in estuaries and intertidal habitats, particularly in shallow waters within sheltered areas influenced by freshwater. It exhibits tolerance to a wide range of temperature, light, salinity, and nutrient levels. Typically, it grows without attachment, forming loose mats on mud and sand. It is usually found in vegetative state, with few reproductive structures (106,107).

1.3. Contribution of the present Doctoral Thesis to the Sustainable Development Goals

With regard to the Sustainable Development Goals (SDGs), this Doctoral Thesis is aligned with SDG 3 "Good Health and Well-being" and SDG 12 "Responsible Consumption and Production".

SDG 3 "Good Health and Well-being"

One of the targets of goal 3 refers to "reduce by one third premature mortality from non-communicable diseases through prevention and treatment and promote mental health and well-being". In line with this target, this Doctoral Thesis aims to respond to the lack of treatment for NAFLD, a condition with a high prevalence that is present in increasingly younger individuals and for which there are no effective tools available yet. The preclinical studies carried out in the present Doctoral Thesis allow the scientific community to gain knowledge and evidence on the potential usefulness of algae and algae extract in the prevention and treatment of NAFLD.

SDG 12 "Responsible Consumption and Production"

It is known that consumption and production depend on the use of the natural environment and its resources, which is often accompanied by environmental degradation, endangering the system that our future development relies on. Algae serve as habitat for several species; they play a significant role in the marine carbon cycle (as they act as carbon stores) and are recognised as a renewable but underutilised resource of nutrients and bioactive compounds. Furthermore, the algae sector has been recognised as the most notable sector in of the EU Blue Bioeconomy. In fact, the 2022 edition of the EU Blue Bioeconomy report is focused on algae and seaweeds (108). Blue Bioeconomy includes any economic activity associated with the use of renewable aquatic biological resources to make products. Therefore, finding new applications for algae can contribute to increase their production.

2. Methodology

First, before the experimental part, a literature search was carried out. Then, the experimental methodology of the present doctoral thesis was developed. This last part was divided into two phases: a first phase that comprises *in vitro* studies and a second phase carried out in an *in vivo* model.

2.1. Literature search

A literature search was conducted in PubMed database to gather preclinical and clinical studies where extracts of microalgae and macroalgae were used to study their effects on NAFLD. Studies performed using isolated compounds were not taken into account.

The search strategy was conducted as follows:

(Fatty liver OR steatosis OR liver) AND (microalga OR macroalga OR seaweed OR alga)

The search was carried out taking into account the title and the abstract of original articles published in English until February 2021. After the screening of the articles (**Figure 7**), the studies that met the inclusion criteria were divided in animal and human studies, and the former, were divided in studies conducted with microalgae and macroalgae. Studies that used macroalgae were then subdivided in studies conducted with green, red and brown macroalgae. This literature revision resulted in Manuscript 1 (see Chapter 3).

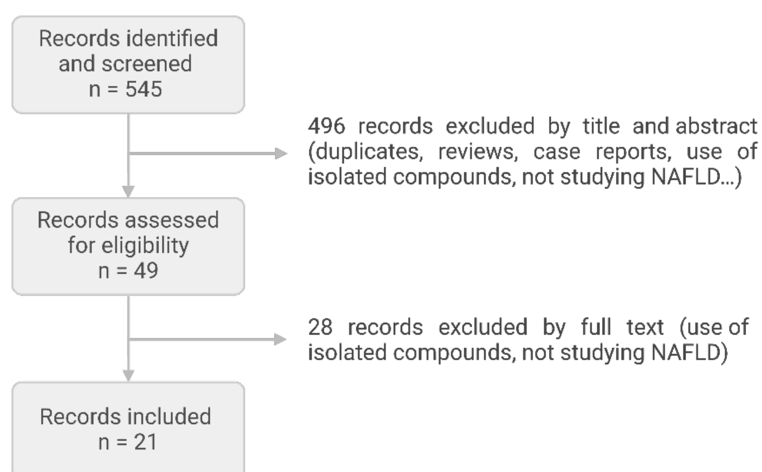


Figure 7. Flow diagram of study selection process. NAFLD: non-alcoholic fatty liver disease.

2.2. *In vitro* studies

In vitro studies were carried out in two different murine cell lines: AML12 (hepatocytes) and 3T3-L1 (adipocytes). Cells were treated with algae extracts obtained from the microalgae *Chlorella vulgaris* and

Nannochloropsis gaditana and the macroalga *Gracilaria vermiculophylla,* at four different concentrations: 10, 25, 50 or 150 µg/mL. The three algae extracts were provided by AZTI Scientific and Technological Centre (Derio, Spain) and their composition is shown in **Table 1**:

Table 1. Proximal composition and total polyphenol content of the algae extracts used in the present study.

	Chlorella vulgaris	*Nannochloropsis gaditana*	*Gracilaria vermiculophylla*
Protein (%)	48.0 ± 0.2	42.0 ± 9.2	41.4 ± 0.7
Fat (%)	11.8 ± 2.0	5.6 ± 0.1	3.5 ± 0.1
Ash (%)	20.5 ± 0.6	42.6 ± 4.2	22.1 ± 3.2
Carbohydrates (%)	21.1 ± 0.6	9.9 ± 4.4	33.4 ± 3.6
Total polyphenols (mg GAE/g DW)	12.27 ± 0.39	9.08 ± 1.56	9.08 ± 1.56

% of dry weight (DW), GAE: gallic acid equivalents. Carbohydrates were calculated by difference.

On the other hand, the characterization of the protein fraction was carried out by CIAL Food Science Research Institute (Madrid, Spain) (**Figure 8**).

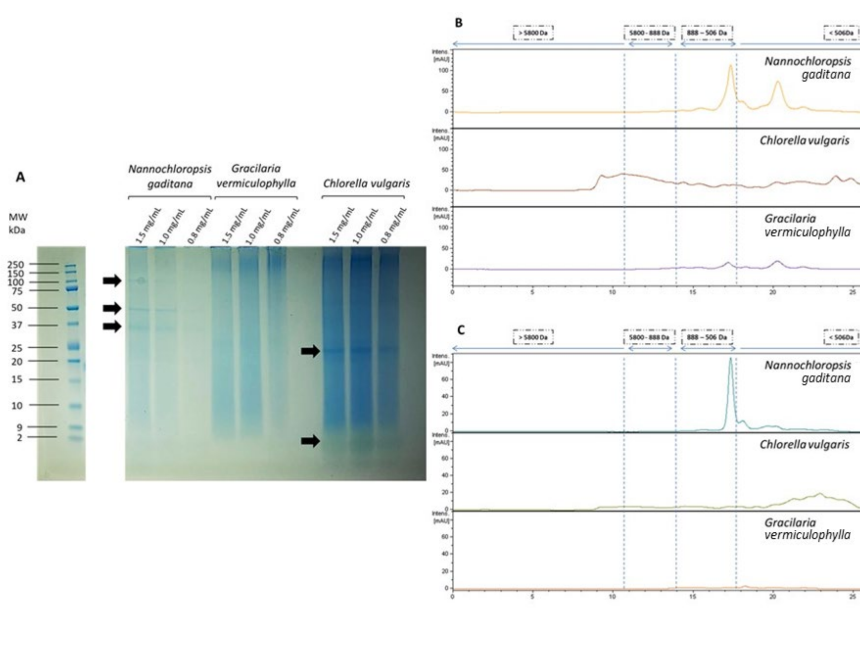


Figure 8. (**A**) SDS-PAGE protein profiles of the algae extracts at different protein concentrations. The arrows point towards the most intense bands. (**B, C**) HPLC-SEC chromatograms of the algae extracts detected at wavelengths of 214 nm (**B**) and 280 nm (**C**). The chromatograms for the three samples are shown at the same intensity scale.

The lyophilized algae extracts were dissolved in distilled water, which were first sonicated in an ultrasonic bath and subsequently filtered using syringe filters. From this stock solution, the day of each treatment, the different aliquots were prepared to achieve the final concentrations in the media (10, 25, 50 or 150 µg/mL). This same procedure was followed in all *in vitro* experiments.

2.2.1. *In vitro* study in a model of hepatic steatosis

The first study was conducted in alpha mouse liver 12 cells obtained from American Type Culture Collection (AML12, ATCC CRL-2254) (**Figure 9**). The cells were cultured in Dulbecco's Modified Eagle Medium F12 (DMEM F12) GlutaMAX™ containing 10 % fetal bovine serum (FBS), 1 % penicillin/streptomycin, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium and 40 ng/mL dexamethasone (DEXA). To create an *in vitro* model that mimics the situation of hepatocytes in fatty liver, triglyceride accumulation was induced by incubating the AML12 hepatocytes with palmitic acid (0.3 mM) for 18 hours.

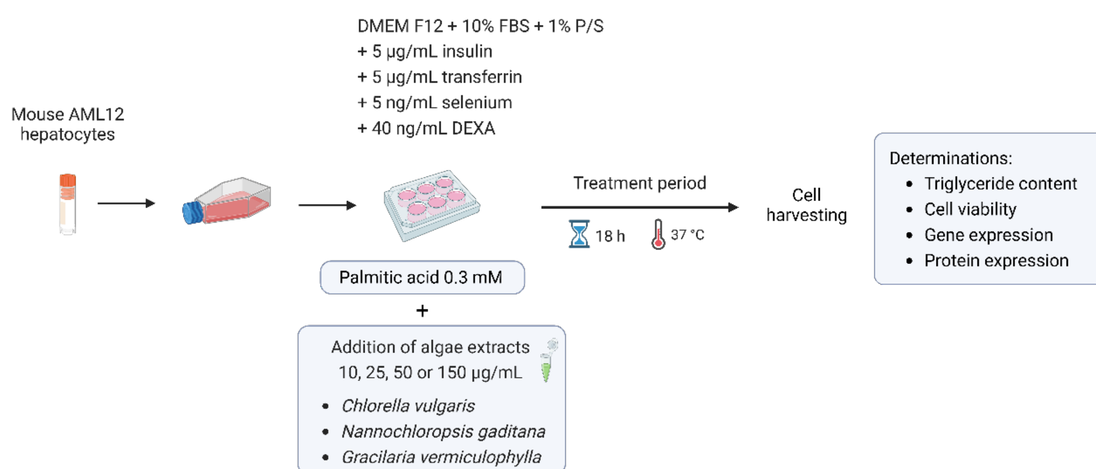


Figure 9. General scheme of the experimental design for the study carried out in AML12 hepatocytes. DEXA: dexamethasone, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, P/S: penicillin/streptomycin.

In a first experiment, cells were co-incubated (together with palmitic acid) without or with different concentrations of the three algae extracts. After 18 hours, cells were harvested for triglyceride quantification, by using a colorimetric commercial kit, and cell viability analysis, using the crystal violet assay. In a second experiment, the dose of each extract inducing the greatest reduction in triglyceride content was selected, and cells were treated following the same methodology as in the first experiment. After the treatment, incubation medium was collected and alanine aminotransferase (ALT) levels were measured by using a colorimetric commercial kit. Cells were harvested and gene and protein expression of enzymes involved in lipid metabolism were measured by Real-Time PCR and Western Blot respectively.

The detailed protocols are included in Manuscript 2 (see Chapter 3).

2.2.2. *In vitro* study in model of obesity

For the second *in vitro* study, 3T3-L1 Mouse Embryonic Fibroblasts obtained from American Type Culture Collection (ATCC CL-173) were used.

Cells were cultured in expansion medium consisting of DMEM (4.5 g/L glucose), 10 % FBS and 1 % penicillin/streptomycin. Two days after confluence (designated day 0), cells were stimulated with differentiation medium consisting of DMEM containing 10 % FBS, 10 µg/mL insulin, 0.5 mM isobutyl methylxanthine (IBMX) and 1 µM DEXA. On day 2, cells were cultured for another 2 days in adipocyte maintenance medium containing DMEM, 10 % FBS and 10 µg/mL of insulin. Thereafter, maintenance medium (DMEM containing 10 % FBS and 0.2 µg/mL insulin) was changed every two days until cells were harvested (day 8). Differentiation and maintenance mediums also contained 1 % (v/v) of penicillin/streptomycin, biotin and pantothenic acid.

In a first experiment, conducted to study the effects of the extracts on the adipogenic process, the cells were incubated with algae extracts during the 8 days of the differentiation period (**Figure 10**). Cells were differentiated in the presence or absence of 10, 25, 50 or 150 µg/mL of each algae extract. The corresponding medium was added every two days and on day 8, cells were harvested for determination of triglyceride content by using a colorimetric commercial kit and cell viability assay using the crystal violet assay. A subsequent experiment was carried out using only the doses showing the biggest reduction in triglyceride content. At the end of the experimental period (8 days after induction of differentiation), adipogenic transcription factors and mature adipocyte markers were analyzed measuring gene expression by Real-Time PCR and protein expression by Western Blot.

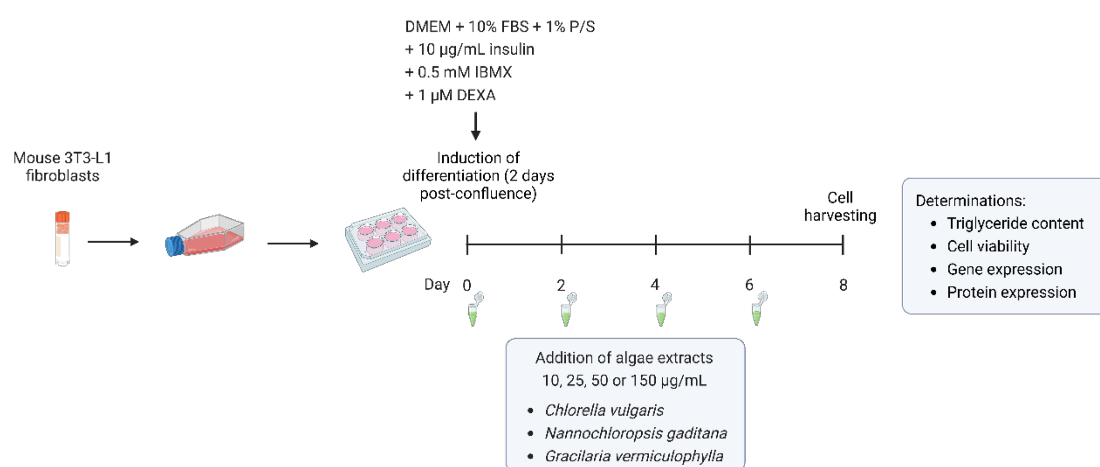


Figure 10. General scheme of the experimental design carried out in 3T3-L1 pre-adipocytes. DEXA: dexamethasone, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, IBMX: isobutyl methylxanthine, P/S: penicillin/streptomycin.

To assess the effect of algae extracts on mature adipocytes, 3T3-L1 fibroblasts were differentiated following the same protocol as in the previous experiment for 12 days. On day 12, different concentrations of each algae extract were added to the medium. After 24 hours (day 13) cells were harvested for triglyceride quantification and cell viability assay as previously described (**Figure 11**).

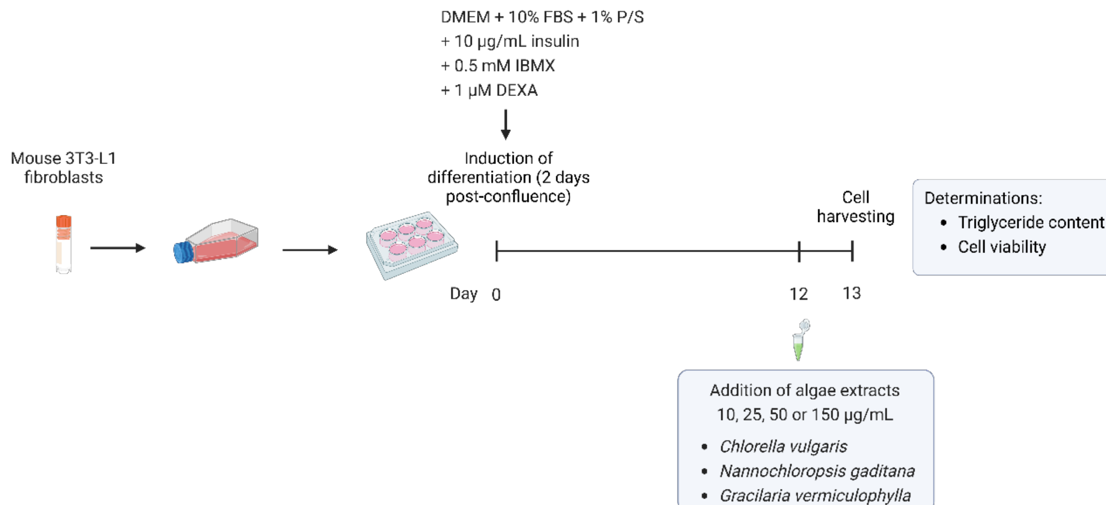


Figure 11. General scheme of the experimental design carried out in 3T3-L1 mature adipocytes. DEXA: dexamethasone, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, IBMX: isobutyl methylxanthine, P/S: penicillin/streptomycin.

The detailed protocols are included in Manuscript 3 (see Chapter 3).

2.3. *In vivo* study

This study was conducted in obese Zucker rats, a spontaneous genetic model of metabolic syndrome. The animals present an autosomal recessive mutation in the leptin receptor (*fa* allele), which is the molecular base of their phenotype. Animals that are homozygous for the *fa* allele (*fa/fa* Zucker rats) develop noticeable obesity within the first 3-5 weeks of life, preceded by hyperphagia and hyperinsulinemia, but do not become hyperglycemic. In addition, obese Zucker rats exhibit hyperlipidemia and hepatic steatosis (109–111).

These rats were supplemented or not with *Gracilaria vermiculophylla* macroalga that was collected manually from the northwest Iberian coast by Porto Muiños S.L. (Cambre, Spain). Its composition was analysed by Laber Laboratories (Corporación Laber, Laboratorio y Consultoría, S.L., Santiago de Compostela, Spain) (**Table 2**).

Table 2. Composition of *Gracilaria vermiculophylla*.

	Gracilaria vermiculophylla
Energy (kcal/100 g)	182
Total lipids (g/100 g)	1.40
Saturated fatty acids (g/100 g)	0.98
Total carbohydrates (g/100 g)	6.40
Simple carbohydrates (g/100 g)	< 0.50
Proteins (g/100 g)	22.10
Fiber (g/100 g)	27.90
Ashes (g/100 g)	28.8
Moisture (g/100 g)	13.4
Total polyphenol content (mg/kg)	1180

The experiment was conducted on 36 male obese Zucker (*fa/fa*) rats and 9 male lean Zucker (*Fa/?)* rats, aged 8 weeks, that were purchased from Charles River Laboratories (Lyon, France) **(Figure 12**). The experiment was developed according to the protocol adopted by the Ethics Committee for Animal Experimentation of the University of the Basque Country (M20_2021_214). After a six-day adaptation period, initially, obese rats were distributed into three experimental groups: an obese control group (OC), an obese group supplemented with 2.5 % of dried *Gracilaria vermiculophylla* (LGV) and an obese group supplemented with 5 % of dried *Gracilaria vermiculophylla* (HGV), mixed in food. Shortly after the beginning of the experiment, HGV group showed a reduction in food intake, thus, a pair-fed (PF) group was introduced using the obese rats that were still not assigned to any of the experimental groups. The amount of diet fed to the PF group was based on the mean consumption by the HGV group on previous day. The lean Zucker rats (*Fa/?*) were used as healthy control (LC). All the groups received the same standard diet, which provided 4 % lipids, 14.5 % protein and 48 % carbohydrates (2014 Global diet, Envigo-Mucedola SRL, Milan, Italy). Animals were housed in conventional polycarbonate cages (two rats per cage) in an air-conditioned room (22 ± 2 °C) with a 12 h light-dark cycle. Water and food were provided *ad libitum* during the 6 weeks of the experimental period, except for the PF group. Body weight and food intake were measured every day during the experimental period.

After 6 weeks of experimental period and 12 hours of fasting, animals were sacrificed under anesthesia (chloral hydrate) by cardiac exsanguination. Serum was extracted from blood samples following centrifugation (1000 *g*, 10 minutes, 4 ºC), and livers were dissected, weighed and immediately frozen in liquid nitrogen for further analysis. A piece of the largest lobule of the liver of each animal was split for the quantification of total lipid content.

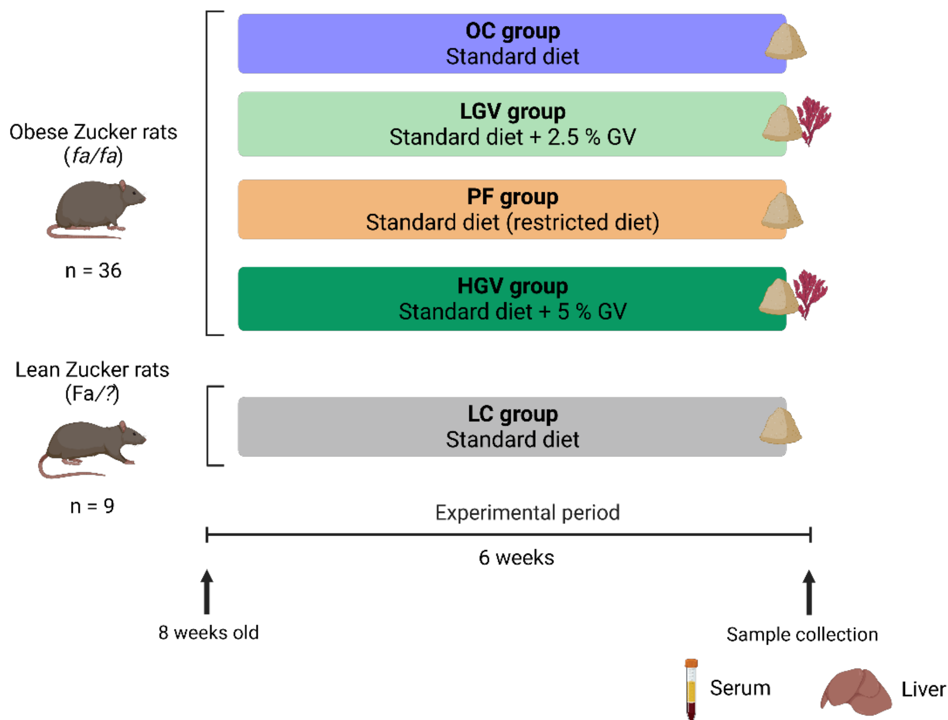


Figure 12. General scheme of the experimental design for the *in vivo* study. GV: *Gracilaria vermiculophylla*, HGV: *Gracilaria vermiculophylla* 5 % of diet, LGV: *Gracilaria vermiculophylla* 2.5 % of diet, LC: lean control, OC: obese control, PF: pair-fed.

Serum biochemical parameters, hepatic triglycerides, hepatic NEFAs, activities of enzymes involved hepatic lipid metabolism and hepatic oxidative stress markers were measured by spectrophotometric methods. Hepatic total lipids were quantified by Oil Red O staining. In liver, expression of enzymes and proteins involved in lipid metabolism and mitochondrial biogenesis were measured by Western Blot. Gene expression of inflammation related markers and fibrogenic markers were measured by Real Time PCR.

The detailed protocols can be found in Manuscript 4 (see Chapter 3).

2.4. Statistical analyses

Experimental data was analyzed with IBM® SPSS® Statistics 24.0 software. The normal distribution of data was assessed by Shapiro-Wilks test. In the case of *in vitro* studies, Student's t test was used to detect significant differences between two groups, and in the case of the *in vivo* study, ANOVA was used to perform multiple comparisons, followed by Newman-Keuls post-hoc test. Data are expressed as mean ± standard error mean (SEM). Significance was assessed at the $p < 0.05$ level.

3. Hypothesis and objectives

As it has been reported in the introduction section of the present Doctoral Thesis, NAFLD is a highly prevalent pathology. The basis of NAFLD treatment consists of energy restriction and physical activity to achieve appropriate body weight, along with the management of the comorbidities associated with this pathology. However due to the fact that, in several cases, these treatments are not efficient enough, and that there are no effective drugs approved specifically to manage this disease, it brings out the need to delve in the search of new tools. Consequently, scientific community is reaching for new compounds that could be useful in the prevention and treatment of NAFLD. Along these circumstances, algae have emerged as an important source of valuable compounds with biological activity and health promoting properties that have not been fully exploited yet.

In this context, we propose the hypothesis that the algae *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* will be useful in the management of NAFLD. In order to demonstrate or discard this hypothesis, the aim of the present Doctoral Thesis was to study the potential usefulness of two microalgae (*Chlorella vulgaris* and *Nannochloropsis gaditana*) and one macroalga (*Gracilaria vermiculophylla*) in the prevention and treatment of NAFLD.

For this purpose, four different approaches were proposed. The initial approach entailed conducting a bibliographic search to determine the potential effectiveness of algae in the treatment and/or prevention of NAFLD. The subsequent two approaches involved using *in vitro* models (hepatocytes and adipocytes) to study steatosis and related comorbidities. Lastly, the fourth approach was carried out in an *in vivo* model of genetic steatosis.

To achieve the aim of this Doctoral Thesis, the following specific objectives were proposed:

Literature search

1. To gather scientific evidence regarding the beneficial effect of microalgae and macroalgae extracts on NAFLD as well as the mechanisms of action involved in these effects (Manuscript 1).

***In vitro* study in AML12 murine hepatocytes**

2. To examine the effect of the extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana* and the macroalga *Gracilaria vermiculophylla* in lipid accumulation in AML12 murine hepatocytes, and to identify the potential mechanisms of action (Manuscript 2).

***In vitro* study in 3T3-L1 murine adipocytes**

3. To assess the effect of the extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana,* and from the macroalga *Gracilaria vermiculophylla* in the lipid

accumulation in mature 3T3-L1 murine adipocytes, as well as the potential mechanisms of action underlying this effect (Manuscript 3).

4. To determine the effect of the extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana,* and from the macroalga *Gracilaria vermiculophylla* in the lipid accumulation in maturing 3T3-L1 adipocytes, as well as the potential mechanisms of action underlying this effect (Manuscript 3).

***In vivo* study in a model of metabolic syndrome (Zucker *fa/fa* rats)**

5. To evaluate the effect of *Gracilaria vermiculophylla* on hepatic lipid accumulation, as well as the potential mechanism of action underlying this effect (Manuscript 4).
6. To analyse the effect of *Gracilaria vermiculophylla* on hepatic oxidative stress (Manuscript 4).
7. To assess the possible role of *Gracilaria vermiculophylla* on hepatic inflammation and fibrosis (Manuscript 4).

4. Results and discussion

As previously mentioned in the Introduction section of this Doctoral Thesis, the increasing prevalence and the lack of effective treatments for NAFLD leads to scientific community to look for new molecules, extracts or natural products to prevent and/or treat this disease.

In this line, the Research Group Nutrition and Obesity, leaded by Professor María Puy Portillo, has extensive experience in studying the effects of different dietary components on NAFLD and obesity. The present Doctoral Thesis is included in this research line.

In the present work, four different approaches have been carried out. First, a thorough review of the literature was conducted to determine whether algae extracts could be effective to treat or to prevent NAFLD. Then, two *in vitro* studies were conducted to explore the impact of algae extracts on lipid accumulation in both murine hepatocytes and adipocytes. These studies were carried out in collaboration with AZTI scientific and technological centre, as part of the ALGABERRI project, funded by the Basque Government. Additionally, the research group led by Dr. Isidra Recio from the CIAL research institute also contributed to this section of the Doctoral Thesis by analysing the protein fraction of the extracts. Finally, the last part involved examining the effect of a whole alga on hepatic steatosis in a rat model of metabolic syndrome. A part of this section was carried out in collaboration with the team led by Dr. Malu Martínez-Chantar from CICbioGUNE.

In order to obtain the "International Doctorate" mention, it is mandatory to spend a period of at least three months in a higher education institution or research centre abroad. In this case, the institution chosen was the Institute for Diabetes and Obesity, at Helmholtz Diabetes Center, Helmholtz Zentrum München, German Research Center for Environmental Health GmbH, in Munich (Germany) because its activity is focused on the study of the cell function on obesity and its metabolic complications. This stay allowed me to deepen my knowledge in the study of obesity and associated metabolic alterations such as diabetes, while also gaining experience in handling primary adipocyte cell cultures, along with various histology and molecular biology techniques. Although these specific techniques have not been directly applied into this Doctoral Thesis, nor in the Nutrition and Obesity research group, it is hoped that it can be done in the near future. However, all this experience has undoubtedly contributed to my research career.

As already mentioned, this Doctoral Thesis arose as part of the ALGABERRI project entitled "Obtaining new ingredients and active principles from algae with health applications", funded by the Basque Government (ELKARTEK program). In this project, the commitment of the Nutrition and Obesity group was to study the biological activity of one macroalga and two microalgae extracts on NAFLD and obesity. In the first phase of the project, AZTI developed protein/peptide extraction protocols using the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana,* and the seaweed *Gracilaria*

vermiculophylla. The freeze-dried extracts were then used for *in vitro* study of their bioactivity. We selected four different concentrations between 10 and 150 µg/mL to carry out the *in vitro* experiments, which is the most common range of concentrations used when studying effects of extracts derived from algae (112–116). Based on the results obtained after performing the *in vitro* assays, *Gracilaria vermiculophylla* macroalga was selected to perform the *in vivo* study in obese Zucker (*fa/fa*) rats, but in this case using the whole alga instead of an extract.

4.1. Literature search

Algae are an underexplored source of bioactive molecules with potential effects to modulate lipid metabolism (117–120). Given these circumstances and considering the major health issue that NAFLD represents along with its close relationship with alterations in lipid metabolism, I first performed a literature review on the effects of micro- and macro-algae extracts on hepatic steatosis to find out if they could be an effective tool at hepatic level.

After screening, duplicated articles, literature reviews, studies using isolated compounds and studies that did not directly assessed the effects on NAFLD were excluded, and at the end, 21 studies were selected to be included in the literature review. No studies conducted *in vitro* were found.

All animal studies were conducted using diet-induced models, predominantly based on high-fat/high-carbohydrate diets, in both mice and rats, with the exception of one study that employed a lipopolysaccharide-induced model. Furthermore, the majority of the studies analysed the effect of the alga supplementation at a preventive level, because the alga was administered together with the diet that induced NAFLD.

Four studies analysed the effects of microalgae supplementation and each of them was conducted using a different species of microalgae: *Scenedesmus dimorphus* and *Schroederiella apiculate* mixture, *Euglena gracilis*, *Spirulina platensis* and *Tisochrysis lutea*. Among these studies, only two reported a reduction in liver triglyceride content, and none of them studied the mechanism of action involved in this effect. Apart from hepatic fat content, the study of other alterations related to NAFLD were also included in these four studies. As far as hepatic inflammation is concerned, the three studies analysing parameters related to this alteration reported beneficial effects, by measuring infiltration of inflammatory cells, markers of pro-inflammatory macrophages or the expression of pro-inflammatory cytokines. Moreover, two studies analysed the effect on hepatic fibrosis, but only one of them observed a positive effect after microalga supplementation, as indicated by the reduction in collagen deposition. Three studies were conducted at a preventive level, as the microalga was combined with the diet inducing steatosis during the whole experimental period. However, in one study, a different approach was employed, where the steatogenic diet was administered throughout the entire experimental period, while the microalga was introduced during the second half of it.

Regarding the effects of macroalgae supplementation on NAFLD, fifteen studies were found, three of them using green macroalgae (*Caulerpa lentillifera* and *Ulva prolifera*), six using red algae (*Plocamium telfairiae*, *Palmaria mollis*, *Sarconema filiforme*, *Grateloupia elliptica* and *Gromphadorhina oblongata*) and six using brown macroalgae (*Undaria pinnatifida*, *Fucus vesiculosus* and *Ascophyllum nodosum* mixture, *Sargassum thunbergii* and *Sargassum horneri*). In this case, unlike microalgae, the effect of the same macroalga species was analysed in more than one study. In all the published studies, macroalgae showed their ability to prevent the development of steatosis induced by the diet. However, most of the studies did not addressed the potential mechanisms of action. In the studies that did analyse the mechanism underlying the reduction of fat accumulation, the authors pointed towards a reduction in lipogenesis and an increase in fatty acid oxidation. As in the case of microalgae, supplementation, macroalgae also improved hepatic markers related to inflammation.

In the case of studies performed with macroalgae, in thirteen pieces of research the macroalga was administered during the entire duration of the experiment, together with the diet; thus their effect was analysed at a preventive level. On the other hand, two studies used a mixed intervention model between prevention and treatment, because the animals were fed the same diet during the whole experimental period, but they only received the macroalga during the second half of it. None of the studies included in the review was designed to analyse the effects of algae supplementation only as a treatment.

It must be pointed out that important differences exist among the studies included in the literature review. One of these aspects has just been discussed, regarding the type of intervention (prevention or mixed model). Concerning the dose, the aforementioned beneficial effects were observed using significantly different doses of algae extracts, ranging from 7.5 to 3000 mg/kg body weight/day or from 1 to 12 % in the diet. Similarly, the experimental periods also varied significantly, between 3 and 16 weeks. Furthermore, it should be taken into consideration that the composition of algae can differ due to various factors, such as nutrient availability, temperature, light intensity, pH and salinity, as well as on genetic variations among species (121). In view of this situation, it is difficult to determine the conditions in which algae and algae extracts exert their beneficial effect.

Despite the number of studies carried out in animal models, clinical studies are still very limited and do not encompass the assessment of potential mechanisms of action. In this review, two human studies were included and each of them followed a very different approach. While Ebrahimi-Mameghani *et al.* conducted a double-blind, placebo controlled, randomised clinical trial where the intervention group that received a daily dose of 1200 mg of *Chlorella vulgaris* for eight weeks showed improved serum parameters (122), Li *et al.* performed an observational study in Chinese population where seaweed consumption was negatively associated with NAFLD, especially in non-obese subjects (78).

Following the publication of the present review, new studies have been published subsequently. In this regard, studies using microalgae extracts have reported positive effects attenuating lipid accumulation in HepG2 cells as well as reducing hepatic lipid accumulation in murine models of NAFLD (91,123). Numerous studies devoted to examine the impact of macroalgae have also been published. They show the potential of seaweeds to ameliorate hepatic steatosis and to improve serum liver function tests (ALT, aspartate aminotransferase (AST) and alkaline phosphatase (ALP)), both in mice and rats (124–129). As occurred in our review, not all the aforementioned studies examined the mechanisms responsible for the reduction observed in hepatic fat accumulation, but those studies that investigated them, observed that the macroalgae could act by modulating hepatic genes, particularly those associated with lipogenic processes. In addition, one systematic review reported that seaweed supplementation can improve liver injury in patients with NAFLD (130).

In summary, after reviewing the literature concerning the effects of both microalgae and macroalgae extracts on NAFLD, it can be stated that the results reported are encouraging. However, the number of studies are still very scarce; for the majority of algae species studied, a single publication has been documented, the mechanisms of action have not been extensively studied yet and most of the studies were carried out only in rodent models at preventive level. Human studies are still needed in order to confirm these results. In this scenario, further research is needed in order to assess the effects of algae on NAFLD.

4.2. *In vitro* study in AML12 murine hepatocytes

In this first *in vitro* experiment, the effect of three extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana*, and the macroalga *Gracilaria vermiculophylla* in the prevention of lipid accumulation in AML12 hepatocytes was determined. These cells were incubated with palmitic acid in order to create a model that mimics hepatic steatosis. The concentrations tested were 10, 25, 50 and 150 µg/mL. In addition, the mechanisms that could explain the effects observed on triglyceride accumulation were analysed.

The characterisation of the algae extracts revealed protein percentages between 41 % and 48 %. Furthermore, when the molecular weight distribution of proteins and peptides was analysed, clear bands could be observed in the case of microalgae extracts. As for *Chlorella vulgaris*, the most intense band was detected within the range of 20-25 kDa. Interestingly, coloured bands below 2 kDa were also detected, probably due to the interaction with pigments. Regarding *Nannochloropsis gaditana,* bands corresponding to 37, 50 and 100 kDa were detected. In the case of *Gracilaria vermiculophylla*, it was not possible to detect any protein band. This could be due to the lower solubility of the extract and its higher polysaccharide concentration. In view of these results, we hypothesize that the positive effect observed in AML12 hepatocytes could be attributed to proteins and peptides present in the extracts.

In particular, *Chlorella vulgaris* and *Nannochloropsis* sp. derived peptides have been recognised to possess biological activity (131,132).

Apart from bioactive proteins and peptides, the extracts used in the present study also contain phenolic compounds. It has been described that polyphenols present high attraction towards proteins, resulting in their co-extraction, which may improve their beneficial effect (72).

After the co-incubation of the extracts with the palmitic acid, we observed that none of the treatments compromised cell viability, indicating that the extracts were not cytotoxic, at least at the tested doses. Regarding lipid accumulation, the three algae extracts were able to reduce triglyceride accumulation. In the case of *Chlorella vulgaris* extract, the reduction in triglyceride content was observed after the treatment with the two highest doses (50 and 150 µg/mL). On the contrary, *Nannochloropsis gaditana* exerted its anti-steatotic effect in cells treated with the two lowest doses (10 and 25 µg/mL). As for *Gracilaria vermiculophylla* extract, the reduction in triglyceride content was observed in hepatocytes incubated with 25, 50 and 150 µg/mL following a dose-response pattern. These results show that *Gracilaria vermiculophylla* was effective at more doses than the microalgae.

In order to explain the reduction in triglyceride accumulation, different pathways involved in lipid metabolism were examined. In this case, only the dose of each algae extract that induced higher triglyceride-lowering effect was used: 150 µg/mL in the case of *Chlorella vulgaris* and *Gracilaria vermiculophylla* and 25 µg/mL in the case of *Nannochloropsis gaditana*.

The liver is a highly lipogenic organ, thus, gene expression of the main lipogenic enzymes (ACC and FAS) was measured. None of the treatments modified their gene expression, which suggests that *de novo* lipogenesis was not involved in triglyceride reduction in hepatocytes. The palmitic acid added in the culture media contributed to triglyceride synthesis. In this sense, protein expression of FATP2 was measured and it remained unchanged after treatment with algae extracts. In order to form triglycerides, fatty acids are esterified to G3P. DGAT2 is the enzyme involved in the last step of triglyceride assembly. When measuring its gene expression no changes were observed after the treatment with algae extracts. These results indicate that the algae extracts used in the present study did not exert their anti-steatotic effect by modulating pathways that favour triglyceride accumulation. Thereupon, metabolic routes involved in the reduction of triglyceride content and fatty acid oxidation were also measured.

Hepatic triglycerides can be secreted in VLDL particles. MTP is an enzyme that takes part in the formation of these particles. Among the different treatments, only *Chlorella vulgaris* upregulated protein expression of MTP, thus an increase in triglyceride secretion could in part explain the lipid reduction observed in cells subjected to this treatment.

Regarding fatty acid oxidation, CPT-1a the rate-limiting enzyme that controls mitochondrial uptake of long-chain acyl-CoAs for β-oxidation, and acyl-CoA dehydrogenase (ACADL), involved in the

first step of β-oxidation were measured. In this study, the three treatments were able to increase *Cpt-1a* gene expression but only *Gracilaria vermiculophylla* gave rise to *Acadl* mRNA levels. These results indicate that the decrease in triglyceride content may be due, at least in part, to an increase in mitochondrial fatty acid oxidation. After the positive results observed regarding fatty acid oxidation, we wanted to address if this effect was related to an enhanced mitochondrial biogenesis, thus, gene expression of mitochondrial transcription factor A (*Tfam*) and citrate synthase (*Cs*) were analysed, as markers of mitochondriogenesis and mitochondrial density, respectively. Although a non-significant increase of 145 % could be observed in cells treated with *Gracilaria vermiculophylla*, none of the treatments induced significant changes regarding *Tfam* gene expression.

Finally, ALT concentration was measured in cultured media at the end of the treatments, as it is regarded as a specific marker for liver dysfunction (133). *Chlorella vulgaris* and *Gracilaria vermiculophylla* reduced ALT levels by 69 % and 54 % respectively, suggesting that these extracts could exert a protective effect in hepatocytes by lowering ALT levels.

Taken together, the results show that the anti-steatotic capacity of the algae extracts and the pathways involved, differ within the species. *Chlorella vulgaris* exerted its anti-steatotic effect when used at 50 and 150 μg/mL, being the reduction in triglyceride content similar in both cases (22 % and 24 % respectively). This microalga seems to act by enhancing triglyceride secretion from hepatocytes and by increasing fatty acid oxidation. On the contrary, in the case of *Nannochloropsis gaditana* the doses of 10 and 25 μg/mL reduced hepatocyte triglyceride content by 27 % and 34 % respectively. This phenomenon, where lower doses show greater bioactivity than higher doses, is commonly seen in studies conducted with phytochemicals such as phenolic compounds in which a hormetic effect is observed (134–136). *Nannochloropsis gaditana* exerted the delipidating effect by increasing fatty acid oxidation. As far as *Gracilaria vermiculophylla* is concerned, a reduction in triglyceride content was observed when using the doses of 25, 50 and 150 μg/mL, with a dose-response pattern. The reduction percentages in this case were 18 %, 22 % and 24 % respectively. Apparently, this macroalga also acted by stimulating fatty acid oxidation.

In general terms, it can be concluded that under our experimental conditions, the algae extracts used are able to partially prevent palmitic acid induced lipid accumulation in cultured hepatocytes, mainly through the stimulation of fatty acid oxidation. However, additional studies in animal models are required to authenticate these findings.

4.3. *In vitro* study in 3T3-L1 murine adipocytes

Considering the strong association between obesity and an increased susceptibility to NAFLD, we performed a second *in vitro* experiment to assess whether the same three extracts, at the same concentrations (10, 25, 50 and 150 μg/mL) tested in hepatocytes, were able to reduce fat

accumulation on adipocytes. Given the role that both hyperplasia and hypertrophy have in the expansion of adipose tissue, algae extracts were tested on 3T3-L1 maturing pre-adipocytes and mature adipocytes.

As occurred in hepatocytes, cell viability was not compromised after culturing both maturing pre-adipocytes and mature adipocytes with the algae extracts at any of the doses used. Regarding adipogenesis, the three extracts induced significant reductions in triglyceride accumulation when they were administered during the eight days of the adipogenic process. *Chlorella vulgaris* induced a reduction in triglyceride content with all the tested concentrations, following a clear dose-response pattern. The reduction percentages were as follows: 24 %, 32 %, 43 % and 50 %. As far as *Nannochloropsis gaditana* is concerned, only the highest dose was able to decrease triglyceride content (-29 %) in maturing pre-adipocytes. Finally, in the case of *Gracilaria vermiculophylla*, treatments with 25, 50 and 150 μg/mL decreased triglyceride content by 26 %, 37 % and 70 % respectively (dose-response pattern). These reductions in triglyceride content during adipocyte differentiation indicate that the extracts were able to partially inhibit adipogenesis.

In order to gain more insight concerning the effects of the algae extracts on adipogenesis, we analysed potential mechanism of action, using the concentration that proved to be the most effective, which in the three cases was the dose of 150 μg/mL.

Adipogenesis is regulated by an intricate network of transcription factors. Briefly, after the induction of differentiation, the primary transcription factor involved in this process is CCAAT-enhancer-binding protein β (C/EBPβ). SREBP-1c is another transcription factor that is induced early during the adipogenic process and together with C/EBPβ, give rise to peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα), which co-regulate each-other's expression and are considered the master regulators of adipogenesis. Their expression activates a transcriptional cascade that induces the expression of characteristic genes of mature adipocytes, such as adipokines or lipogenic and lipolytic enzymes (137,138). In this regard, although treatment with *Chlorella vulgaris* increased gene expression of *Pparg*, this change was not translated into changes in protein expression. This treatment reduced gene and protein expression of C/EBPα. As for *Nannochloropsis gaditan*a extract, it reduced *Srebp1*c and *Pparg* gene expressions and tended to lessen *Cebpb* expression. Although C/EBPβ and PPARγ protein expression remained unmodified after the treatment, expression of C/EBPα was found to be decreased. Regarding *Gracilaria vermiculophylla*, this treatment resulted in the increase of C/EBPβ gene and protein expression. However, it decreased *Srebp1c, Pparg* and *Cebpa* gene expressions. These changes were only partially translated into protein expression changes, since a significant decrease was only noted in C/EBPα. It must be stated that, although the decline in PPARγ protein expression did not reach statistical significance, it decreased by 33 %, which we can be considered a relevant result. These results show that, although the algae extracts did not act throughout the whole adipogenic process, and that each of them regulated

different transcription factors, it seems that a common course of action is via the decrease in the expression of C/EBPα.

Additionally, we also carried out the analysis of the specific genes and proteins of the mature adipocyte phenotype, among which we studied ACC and adiponectin. *Chlorella vulgaris* extract induced a reduction in ACC gene and protein expression. Despite finding higher adiponectin mRNA levels after this treatment, its protein expression remained unchanged. Although *Nannochloropsis gaditana* extract increased *Acc* gene expression, protein expression was significantly down-regulated. Regarding adiponectin, both gene and protein expressions were decreased after the treatment with *Nannochloropsis gaditana*. Finally, *Gracilaria vermiculophyll*a extract was able to decrease gene and protein expression of ACC as well as of adiponectin. Taking together, these results show that the extract used in the present study successfully reduce mature adipocyte markers when the treatment is added during the eight days of the differentiation period, which is in good accordance with the reduction of triglyceride levels. These results are in line with those obtained after studying the expression of adipogenic transcription factors, and show the ability of the extracts to hinder the differentiation of pre-adipocytes into mature adipocytes.

As far as we know, this is the first study showing the anti-adipogenic effect of *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts. However, numerous studies can be found in the scientific literature that show the anti-adipogenic capacity of algae extracts obtained from different micro- and macroalgae species (119,120,128,139,140).

As it has already been discussed in the previous section concerning the effects on hepatocytes, the positive effects described in the present study can also be attributed to the proteins and peptides found in these extracts, as well as to the phenolic compounds.

When the extracts were tested on mature adipocytes (day 12 after the induction of differentiation), no reduction in triglyceride content was observed with any of the doses. Due to the lack of benefits, the effects of the extracts on lipid metabolism in mature adipocytes was not studied.

The increase in size of mature adipocytes is known as hypertrophic expansion. Unlike hyperplasia (increase in fat cell number), adipocyte hypertrophy is associated with negative metabolic consequences. In this line, in case of obesity, adipose tissue growth via hyperplasia has been proposed as a protective mechanism against adipose tissue dysfunction and its complications (141,142). However, it must be taken into account that if the excess lipids cannot be stored in adipose tissue (either due to reduced adipogenesis or because hypertrophic adipocytes have reached their storage capacity), it could lead to ectopic fat accumulation (29). Regarding this last scenario, the algae extracts used in the present Doctoral Thesis, have shown the ability to reduce lipid accumulation in hepatocytes (143), suggesting that they might protect the liver against fat accumulation. Nonetheless,

more studies are required, especially in animal models, in order to better elucidate the effects of algae components and their underlying mechanisms of action.

According to these findings, it can be concluded that despite not having the ability to reduce lipid accumulation in mature adipocytes, the algae extracts used in the present study show a potential anti-adipogenic effect. This indicates that they could serve as a valuable tool in the prevention of obesity and thus, in related co-morbidities including NAFLD.

4.4. *In vivo* study in a model of metabolic syndrome (Zucker *fa/fa* rats)

The final part of the Doctoral Thesis encompassed an *in vivo* experiment. By conducting the *in vitro* studies, already described previously, we were able to examine the primary effect of the three algae extracts on lipid accumulation in hepatocytes and adipocytes. Nevertheless, it is important to note that the efficacy of the extracts is partially dependent on their bioavailability (144). Consequently, animal models are needed before delving into clinical interventional studies to investigate their effects. In this line, based on the outcomes obtained from the *in vitro* assays, we selected one of the algae species to perform a study on an animal model of hepatic steatosis.

The *in vitro* assays showed that although *Nannochloropsis gaditana* was the extract that exerted the greatest effect in AML12 hepatocytes, in terms of its magnitude, and with one of the lowest concentrations, only one dose resulted effective in 3T3-L1 pre-adipocytes. In contrast, *Gracilaria vermiculophylla* showed effectiveness in both cell lines when added at the three highest doses. Consequently, based on these results, the macroalga *Gracilaria vermiculophylla* was chosen for the *in vivo* study.

In order to conduct the current experiment, we decided to use the whole alga instead of an extract. This approach was chosen because if it proved to be effective, it would eliminate the need for extractions, which is not only economically advantageous but also aligns with sustainability principles. Moreover, the whole algae contains additional nutrients and components that would not be found in the extracts, which could contribute to additional positive effects. *Gracilaria vermiculophylla* used in the present study was composed of 6.4 % carbohydrates, 1.4 % lipids, 22.1 % proteins and 27.9 % fibre. The proportion of ashes (28.8 %) revealed a high mineral content. Moreover, the total polyphenol content was 1180 mg/kg.

There are several animal models that replicate both histopathology and pathophysiology of each stage of human NAFLD. Among them, the Zucker (*fa/fa*) rat is one of best-known rat models of genetic steatosis and obesity (109,145). In fact, this particular model is considered a representative one for studying the metabolic syndrome, as it closely resembles the observations in humans with hepatic steatosis and obesity (146). Consequently, it allows to investigate the interplays between the different components of the metabolic syndrome. Taking this into consideration, the obese Zucker (*fa/fa*) rat

was chosen to study the effect of *Gracilaria vermiculophylla* on hepatic steatosis, allowing us to analyse its impact at treatment level.

For this current research, two different doses of the macroalga were used, taking into account the range of doses used in similar studies where the effects of dietary supplementation of algae are studied in animal models. Based on that range, we selected an intermediate (5 %) and a low dose (2.5 %).

Male Zucker (*fa/fa*) rats (aged eight weeks) were fed a standard diet supplemented either with 2.5 % or 5 % of *Gracilaria vermiculop*hylla for six weeks. Rats that did not receive the macroalga served as the obese control group. Additionally, a pair-fed (PF) group was required due to the reduction in food intake observed in rats administered the high dose of the alga when compared to the obese control rats. The PF group was offered an equivalent amount of food as the group fed with the high dose of the alga on the previous day. This allowed us to determine whether the effect was directly attributed to the alga, or whether it was a result of reduced food intake. No variations regarding food intake were observed in the group receiving the low dose of the macroalga. Therefore, the effects could solely be attributed to the macroalga.

As anticipated, all the obese Zucker rats exhibited an increase in body weight gain in comparison with the lean rats. Although the low dose of the alga did not induce any change in this parameter, the high dose managed to reduce it. Nevertheless, this effect may be attributed to a decreased food consumption, as a similar decline in body weight gain was observed in the PF group, which was subjected to a restricted food intake.

As for serum parameters associated with the glycemic control, serum glucose level in rats from the obese control group was 100 mg/dL. In contrast, rats receiving either the low dose or the high dose of the macroalga, showed normal glucose levels (76 and 78 mg/dL respectively). Moreover, rats treated with the high dose had a greater improvement of insulin function compared to those treated with the low dose, as their insulin levels were significantly reduced. This was supported by the homeostatic model assessment for insulin resistance (HOMA-IR) index. Additionally, considering that obese rats had elevated serum NEFAs, which is often associated with impaired insulin sensitivity, the revised quantitative insulin sensitivity check index (R-QUICKI) index was also evaluated. The results were in accordance with those obtained from the HOMA-IR assessment, indicating an improvement in insulin sensitivity as a result of the supplementation with the high dose of *Gracilaria vermiculophylla*. It must be pointed out that these positive effects exerted by the high dose are attributed to the reduction in food intake, as a similar response was observed in the PF group.

In relation to serum triglyceride levels, treatment with the low dose tended to reduce it levels nearly to physiological levels (151 mg/dL). Conversely, the administration of the high dose did not modify triglyceride levels, resulting in measurements above the threshold for hypertriglyceridemia (200 mg/dL).

Regarding liver weight, similar outcomes could be observed. While the low dose did not lead to a reduction in liver weight, supplementation with the high dose resulted in a significant decrease. In the case of this parameter, it is not completely clear if this effect was directly mediated by the macroalga, or if it was a result of reduced food intake, as the values of the PF group fall within the range of obese control rats and rats fed the high dose.

When hepatic lipids were analysed, none of the macroalga treated groups showed a decrease in total lipids, nor in triglycerides. These findings are consistent with the absence of effects in liver weight. These results indicate that, under our experimental conditions, *Gracilaria vermiculophylla* was not able to reduce hepatic steatosis. To the best of our knowledge, this is the first study devoted to analyse the effect of *Gracilaria vermiculophylla* on hepatic steatosis, although studies using different *Gracilaria* species can be found in the literature. Chan *et al.* reported beneficial effects regarding hepatic steatosis after the supplementation with 5 % and 10 % *Gracilaria changii* powder (147). The discrepancies between the two studies may primarily arise from the utilization of distinct *Gracilaria* species, different animal models and/or differing lengths of treatment period.

Interestingly, hepatic NEFAs were found to be reduced in both groups receiving *Gracilaria vermiculophylla*. In the case of the high dose of the alga, the reduction observed was partially due to the decrease in food intake. It is known that an excessive accumulation of NEFAs, contributes to hepatocyte injury primarily by generating harmful lipid species such as ceramides, diacylglycerols and lysophosphatidylcholine, which favour ROS production (148). In view of these results, it could be suggested that *Gracilaria vermiculophylla* could avoid the progression from simple steatosis to more advanced stages.

The liver in obese Zucker rat exhibits an elevated rate of *de novo* lipogenesis (149). Additionally, plasma NEFAs are elevated as a result of increased adipose tissue mass and peripheral insulin resistance. These lipids can enter the hepatocyte, where they can undergo either oxidation or esterification (27). In order to find the potential mechanisms underlying the reduction in NEFAs, several determinations related to lipid metabolism were carried out.

Regarding *de novo* lipogenesis, protein pACC/ACC ratio, FAS protein expression and FAS activity were analysed. Macroalga supplementation did not induce any change in the activation levels of ACC and, surprisingly, the high dose increased FAS protein expression. This effect resulted from the decrease in food intake, as revealed the PF group. On the contrary, activity of FAS was reduced by both doses of the macroalga, indicating a modulatory effect at a posttranslational level. This result suggests that *de novo* lipogenesis is in part contributing to hepatic NEFA reduction.

In relation to fatty acid oxidation, the activities of CPT-1a and acyl-CoA oxidase (ACO), which are key enzymes involved in the oxidation of fatty acids in the mitochondria and peroxisomes respectively, were diminished following the administration of a high dosage of the macroalga.

Nevertheless, it is important to highlight that this outcome was a result of the decrease in food consumption.

Mitochondrial biogenesis can lead to an enhanced fatty acid oxidation. In the present experiment, an increase in nuclear respiratory factor 1 (NRF1) and TFAM, two markers of mitochondriogenesis, protein expression levels was observed in rats receiving the low dose. Although the high dose was able to increase NRF1, it did not have the same effect on TFAM protein levels. The effect of the high dose can be attributed to a decrease in food consumption. These findings suggest that, for the low dose, an increase in mitochondriogenesis may also play a role in reducing liver NEFAs.

The increase in fatty acid oxidation can result in a greater production of ROS within the mitochondria contributing to the development of oxidative stress (150). On the other hand, the decrease in hepatic NEFA levels may suggest a reduction in lipid peroxidation (12). In order to address this situation, several markers related to oxidative stress we analysed. Under our experimental conditions, supplementation with the low dose of the alga was able to ameliorate oxidative damage, shown by the slight rise in reduced glutathione (GSH) concentration, which serves as an indicator of the non-enzymatic antioxidant defence, as well as the increase in the total antioxidant capacity (TROLOX). On the contrary, the high dose of the macroalga seemed to exacerbate the oxidative stress, as evidenced by the increase in lipid peroxidation, measured by MDA levels, and the decline in the activity of the antioxidant enzyme GPx. However, the latter was not a direct effect of the alga, but rather a result of lower food intake. In this regard, the study by Chan *et al.* reported an improvement in lipid peroxidation (MDA levels), catalase and GPx in rats supplemented with either 5 % or 10 % of *Gracilaria changii* (147), however, the disparities between the two studies must be taken into consideration.

It has been widely described that Zucker rats do not naturally develop NASH unless they are exposed to an external stimulus, such as a high-fat diet (151). Despite that, it is still worthwhile to examine potential changes in early biomarkers of inflammation and fibrosis. In terms of inflammation, in both group supplemented with *Gracilaria vermiculophylla* it was observed a reduction in adhesion G protein-coupled receptor E1 (*F4/80*) gene expression, a marker of pro-inflammatory (M1) Kupffer cells, the liver resident macrophages. This result suggests that this macroalga may possess an anti-inflammatory effect.

Finally, as far as fibrosis development is concerned, in general terms, it should be noted that *Gracilaria vermiculophylla* supplementation did not have any impact on the markers studied such as collagen type I alpha 1 chain (*Col1a1*), transforming growth factor beta 1 (*Tgfb1*) and TIMP metallopeptidase inhibitor 1 (*Timp1*), except for actin alpha 2 smooth muscle (*Acta2*) in the case of the high dose of the macroalga. This particular marker showed an increase in gene expression. Nevertheless, it is important to consider that this effect is partially influenced by reduced food intake. However, when we analysed matrix metallopeptidase 9 (*Mmp9*), its gene expression was increased in rats

supplemented with the high dose. This result can be interpreted as an adaptive mechanism against the accumulation of extracellular matrix components (152).

Taking together the results presented in this study, it can be suggested that *Gracilaria vermiculophylla* does not decrease steatosis in the liver. However, it could provide certain advantages by reducing hepatic NEFAs, oxidative stress and inflammatory markers. Nonetheless, these benefits are only achieved with the low dose (2.5 %).

1. Artearen egoera

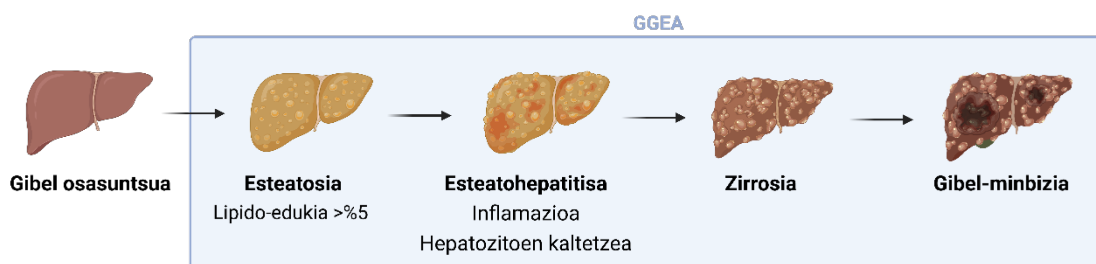
1.1. Gibel gantzatsu ez-alkoholikoa

Gibel gantzatsu ez-alkoholikoa (GGEA) gibeleko gaixotasuna da eta gehiegizko triglizerido-metaketa du bereizgarri. Alkoholik ez edo alkohol oso gutxi (< 20 g/egun emakumezkoetan, < 30 g/egun gizonezkoetan) kontsumitzen duten banakoetan (1) ematen dena eta sindrome metabolikoaren adierazpen hepatikotzat hartzen da. GGEA terminoa hamarkadetan zehar erabilia izan bada ere, "ez-alkoholiko" terminoak alkohol-kontsumoaren gabezia diagnostikorako irizpide gisa gehiegi nabarmentzen duela onartu da. Horrez gain, nomenklatura horrek ez du gibel-asaldura horren azpian dagoen disfuntzio metabolikoa behar bezala islatzen. Hori dela eta, 2020an disfuntzio metabolikoari lotutako gibel gantzatsua (DMGG) deritzon izendapen berria proposatu zen, gibel gantzatsuaren garapenarekin erlazionatzen diren arrisku-faktoreen heterogeneotasuna hobeto islatzeko helburuarekin (2). Alabaina, batzuen ustez "gantzatsu" terminoa nahiko estigmatizatzailea izan daiteke, eta horregatik, 2023an, beste proposamen bat egin zen hura birdefinitzeko. Proposatutako izendapen berria disfuntzio metabolikoari lotutako gibel-esteatosia izan zen (3). Hala ere, gaur egun adituen erakunde guztien arteko adostasun eza dela eta (4,5) GGEA terminoa erabiliko da Doktoretza-Tesi honetan zehar.

GGEA gibeleko gaixotasun kronikoen kausa nagusia da mundu mailan. Helduen artean gaixotasun honen prebalentzia % 30 ingurukoa da, gizonezkoen artean (% 40) emakumezkoen artean (% 26) baino altuagoa izanik. Hala ere, desberdintasunak daude herrialde eta munduko eskualde desberdinen artean. Gainera, prebalentzia bereziki altua da gaixotasun metabolikoak, hala nola, obesitatea (nagusiki zentrala) eta 2 motako diabetesa, dituzten pertsonen artean (6,7). Zehazki, obesitate larria duten pertsonetan prebalentzia-tasa % 90ekoa izatera irits daiteke eta % 75ekoa, aldiz, 2 motako diabetesa dutenetan (8). Alabaina, badago gorputz argal edo pisu normala duen banakoen azpimultzo bat zeinak GGEA garatzen duen. Pertsona hauek erraietako gantz-ehun handiagoa eta intsulinarekiko erresistentzia izan ohi dituzte asaldura metaboliko gisa. Ildo horretan, esan beharra dago, GGEAren mundu mailako tasak nabarmen hazi direla azken hiru hamarkadetan, eta joera hori jarraituz gero, tasa horiek igotzen jarraituko dutela obesitatearen eta 2 motako diabetesaren prebalentzien goranzko joerarekin batera (6,7).

GGEAk gibeleko hainbat egoera patologiko bat hartzen ditu bere baitan: esteatosi arruntetik hasi eta esteatohepatitis ez-alkoholikoa (NASH), fibrosia, zirrosia eta gibel-minbizia izateraino irits daitekeena (8) (**1. irudia**). Esteatosi arrunta edo gibel esteatosia, bi modutan definitu daiteke: batetik, triglizeridoen pilaketa gibelaren pisuaren > % 5 denean, edo hepatozitoen triglizerido-edukia ≥ % 5 denean (9). Gibel esteatosia gaixotasunaren agerpenik ohikoena da eta aipatutako egoera patologikoen artean onberena (8). Triglizerido-pilaketa berez ez da kaltegarria, hala ere, gehiegizko metaketak esteatohepatitisa garatzeko arriskua handitzen du, triglizeridoak esterifikatu gabeko gantz-azidoetan

(EGGA) hidrolizatu daitezkeelako eta gibelak horiek metabolizatzeko duen gaitasuna gainditu daitekeelako, hepatozitoetan metatzen direlarik (10,11). Gehiegizko EGGAek efektu toxikoa duten lipido-espezieen eraketarako substratu gisa dihardute, zeramidak eta diazilglizerolak kasu (12). Esteatohepatitisa gaixotasunaren egoera larriagoa da, non gehiegizko triglizerido-metaketaz gain, inflamazioa eta hepatozitoen puxika erako endekapena ere agertzen diren, hepatozitoen kaltetzea eraginez (10). Gibel-esteatosia duten paziente gehienek ez dute esteatohepatitisa garatzen, izan ere, gibel-esteatosiarekin kaltetutako banakoen % 10-20k garatzen du esteatohepatitisa (8). Bestalde, gibel-esteatositik estatohepatitiserako bilakaera ez da lineala, egonkortasun eta erregresio aldiak egonik (13). Hala, zenbait paziente esteatosiaren eta esteatohepatitisaren artean egon daitezke, egoera batetik bestea pasatuz. Hala ere, esan beharra dago, esteatohepatitisaren bilakaera hilkortasun handiagoarekin erlazionatzen dela, gibel-minbiziarekin eta arazo kardiobaskularrekin lotutako hilkortasuna dela eta (10). Hepatozitoen kaltetzea denbora luzean mantentzen bada, fibrosi erako lesioak agertuko dira, ondoren zirrosia, eta azkenik, gibeleko minbizia agertu daitekeelarik (11).



1. irudia. Gibel gantzatsu ez-alkoholikoaren (GGEA) bilakaera.

Urte askotan zehar, "talka-biko" teoria erabili izan da GGEAren patofisiologia azaltzeko. Teoria honen arabera, "lehenengo talka" intsulinarekiko erresistentziak, bizimodu sedentarioak eta dieta hiperkalorikoek sustatutako gibeleko lipido-metaketari dagokio. Egoera honek gibela sentikorrago bihurtzen du beste faktore kaltegarri batzuekiko, "bigarren talka" bezala ezagutzen direnak, oxidazio estresa kasu, zeinak inflamazioaren garapena faboratzen duen (14). Hala ere, teoria honek ez ditu gaixotasunaren garapenaren eta bilakaeraren konplexutasuna eta heterogeneotasuna atzematen. Termino horren ordez, "talka-anitzeko" teoriak GGEAren patogenesiaren deskribapen zehatzagoa ematen du. "Talka" hauen artean intsulinarekiko erresistentzia, adipokinen ekoizpen asaldatua, dietako osagaiak, mitokondrioetako disfuntzioa, oxidazio estresa, hesteko mikrobiota eta faktore genetiko zein epigenetikoak daude (15–17).

GGEA modu asintomatikoan ager daiteke, diagnostikoa eta gaixotasunaren benetako prebalentzia ezagutzea zail bihurtzen duelarik. Gaur egun, gibel-biopsia eta azterketa histologikoa dira da GGEAren diagnostikorako teknikarik egokienak, izan ere, gaixotasunaren egoera sailkatzeko aukera ematen dute. "NAFLD activity score" (NAS) eta "Steatosis, Activity, Fibrosis" (SAF) algoritmoa dira azterketa histologikoan oinarrituta GGEAren sailkapena egiteko gehien erabiltzen diren puntuazio

sistemak. Biek histologikoki esteatosia, lobuluen inflamazioa eta puxika erako endekapena ebaluatzen dituzte, eta nahiko korrelazio estua erakusten dute esteatohepatitisaren garapena nabarmena denean; hala ere, esteatohepatitisaren diagnostikoaren mugan dauden kasuetan, bi sistemek emaitza desberdinak iradokitzen dituzte (18). Horrez gain, analisi histologikoan oinarritutako sistema horiek erabiltzeak beste muga batzuk ere baditu, izan ere, teknika inbasiboa den aldetik, konplikazioak sor ditzake, bestetik behatzaileen artean aldakortasuna egon daiteke ezaugarri patologiak identifikatzeko orduan, eta gainera, kostu handia suposatzen teknika da. Horregatik, egoera zehatzetan bakarrik erabiltzen da. Gaur egun, ekografia abdominala (irudi-froga mota bat), erabiltzen da gibel-esteatosia ebaluatzeko lehen mailako frogatzat, alabaina, sentikortasun baxua erakusten du esteatosi arina detektatzeko orduan. Fibrosia hilkortasunaren iragarle nagusienetarikoa izanik, esteatohepatitisa eta fibrosi goiztiarra bereizteko aukera ematen duten teknikak erabiltzea ezinbestekoa da. Erresonantzia magnetiko bidezko elastografiak eta trantsiziozko elastografiak (Fibroscan) gibel ehunaren zurruntasuna neurtzen dute, hortaz, haien erabilerak fibrosiaren egoera neurtzeko balio du, teknika inbasioak izan gabe. Hala ere, teknika hauek zehaztasun baxuagoa erakusten dute obesitatea duten pertsonetan, eta nahiko garestiak dira. Egoera horren aurrean, gero eta interes handiagoa dago esteatosi arruntaren, esteatohepatitisaren eta fibrosiaren artean bereiztea ahalbidetzen duten puntuazio-sistema fidagarri eta ez-inbasiboak garatzeko. Sistema horien artean "Fibrosis-4" (FIB-4), "NAFLD Fibrosis Score" (NFS), "Hepamet Fibrosis Score" (HFS) and "Platelet Ratio Index" (APRI) izenekoak aurki daitezke, hala ere, haien sentikortasuna nahiko xumea da eta lortutako emaitzetan desadostasunak egon daitezke (19). Ildo horretan, RNA ez kodifikatzaileak gailentzen ari dira esteatosiaren, esteatohepatitisaren eta fibrosiaren artean bereiztea ahalbidetzen duten diagnostikoarako biomarkatzaile modura (19,20).

GGEAren tratamenduari dagokionez, osasuntsuak ez diren ohitura dietetikoek, obesitateak edo/eta intsulinarekiko erresistentziak GGEArekin duen lotura estua dela eta, bizi-ohituren aldaketa gaixotasunaren kudeaketarako lehen aukera terapeutikotzat hartzen da, dieta-ohituren aldaketaren bidez eta jarduera fisikoa areagotuz, gorputz-pisu egokia lortzeko (21). Zehazki, ikusi da, dieta hipokalorikoa eta jarduera fisikoa konbinatuz lortutako gorputz-pisuaren > % 10 jaitsierak, NAS indizea murrizten duela (22). Alabaina, estrategia hau ez da baliagarria kasu guztietan, izan ere, pazienteek tratamenduarekiko duten atxikidura baxua izaten da, batez ere, epe luzean, hori izanik estrategia horren muga nagusienetarikoa.

Tratamendu farmakologikoari dagokionez, nahiz eta hainbat entsegu kliniko abian dauden, gaur egun oraindik ez dago GGEArentzat guztiz espezifikoa eta eraginkorra den farmakoterapiarik. Izan ere, erabiltzen diren farmakoak gorputz-pisuaren galera eragitera (adib. lipasa inhibitzaileak), diabetesa tratatzera (adib. sodio-glukosa 2 garraiatzailearen inhibitzaileak (SGLT-2), glukagoaiaren antzeko 1 peptidoaren (GLP-1) hartzailearen agonistak, peroxisomen ugalketak aktibatutako hartzaileen (PPAR) agonistak), antioxidatzaileak (adib. E bitamina) eta fibrosiaren kontrako agenteak (adib. apoptosiaren seinalearen erregulatzailearen 1 kinasa inhibitzaileak) (8,23). Testuinguru honetan,

komunitate zientifikoa GGEA kudeatzeko terapia alternatibo edo osagarri gisa balio dezaketen molekula berrien bila ari da.

1.1.1. Esteatosiaren garapena: intsulinarekiko erresistentziaren eta obesitatearen parte-hartzea

Intsulinarekiko erresistentzia eta gibeleko gantz-metaketa estuki lotuta daude. Izan ere, intsulinarekiko erresistentzia gibel-esteatosiaren garapena sustatzen duen gertaera nagusitzat hartzen da. Egoera normaletan, intsulinak glukosa zeluletara barneratzea eta triglizeridoen sintesia sustatzen ditu, eta aldi berean, triglizeridoen hidrolisia inhibitzen du. Hala ere, plasmako glukosa-mailak etengabe balio altuetan mantentzen direnean, ehunek intsulinarekiko erresistentzia garatzen dute, intsulinaren eraginean asaldurak emanez. Gantz-ehunari dagokionez, intsulinarekiko erresistentzia dagoenean, intsulinak lipolisiaren aurka duen efektua murriztu egiten da. Horren ondorioz, triglizeridoen degradazioa handitu egiten da eta zirkulaziora askatzen diten EGGA kopurua (glizerolarekin batera) handitu egiten da, gibelean sartuko direnak (24). Erraietako gantz-ehunak (sabelaldekoak) arreta berezia merezi du, izan ere, eragin nabarmena dauka intsulinarekiko erresistentziaren garapenean (25). Gibelari dagokionez, intsulinarekiko erresistentziak eta horrekin batera agertu ohi den hiperintsulinemiak, gibeleko lipidoen metabolismoan eragina dute, gantz-azidoen sintesia estimulatuz (24). Azken hori, *de novo* lipogenesia atalean zehatzago azalduko da. Gainera, egoera honetan intsulina ez da gai gibeleko glukoneogenesia inhibitzeko, ondorioz, gibelak glukosa sintetizatzen jarraitzen du (26), *de novo* lipogenesirako substratua gehituz.

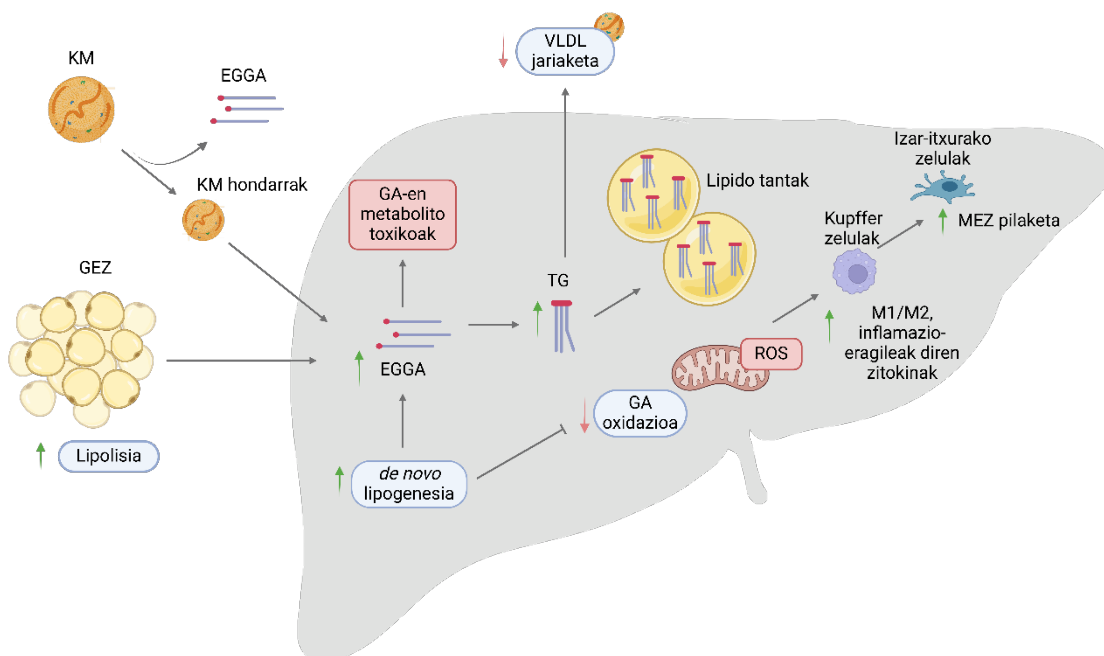
Esteatosiaren garapenarekin erlazionatutako beste egoera patologiko bat obesitatea da. Obesitatea gantz-metaketak eragindako gehiegizko gantz-ehun zuriaren handipena bezala definitzen da. Horren ondorioz, plasmako EGGAen kontzentrazioa handitu egiten da, gibelarentzat kantitate handiagoak eskuragarri daudelarik. Gainera, aipatu beharra dago, gantz-ehunaren lokalizazio-anatomiko desberdinek modu desberdinean eragiten diotela zirkulazioko EGGAen kontzentrazioari. Hori horrela, erraietako gantz-ehunak larruazalpeko gantz-ehunak baino lipolisi-tasa handiagoa dauka, ehun horrek intsulina-hartzaile gutxiago baititu. Hori dela eta, erraietako gantz-ehunak larruazalpeko gantz-ehunak baino gaitasun handiagoa dauka EGGAk askatzeko. Bestetik, erraietako gantz-ehunetik isuritako EGGAk porta benatik zuzenean iristen dira gibelera, triglizeridoen sintesia faboratuz (27).

Obesitatea eta intsulinarekiko erresistentzia, askotan aldi berean agertzen dira, gantz-ehunaren disfuntzioa dela eta. Obesitatearen garapenean zehar, gantz-ehun zuriaren masa bi mekanismoren bitartez handitu daiteke, adipozitoen tamaina handituz (hipertrofia) eta adipozitoen kopurua handituz, hau da, adipozitoen zelula-aitzindariak adipozito helduetan diferentziatuz (hiperplasia). Azken hori adipogenesi bezala ere ezagutzen da. Gantz-ehun masa handitzeko moduak eragin nabarmena dauka osasun metabolikoan, izan ere, hiperplasia ematen denean, gantz-ehunaren funtzioa mantentzen den bitartean, gehiegizko hipertrofia dela eta, adipozitoek hipoxia-egoera pairatzen dute, matrize estrazelularraren birmoldaketa desegokiaren ondorioz. Egoera horretan, inflamazio-eragileak

diren zitokinak askatu egiten dira, α tumore-nekrosiaren faktorea (TNF-α), 6 interleukina (IL-6), 1β interleukina (IL-1β) edo 1 proteina monozito-kimioerakarlea (MCP-1) bezalakoak, zelula immuneen infiltrazioa sustatzen delarik. Gainera, adipokinen sekrezioan asaldurak ematen dira (adiponektinaren murrizketa eta leptinaren igoera) (27,28). Mekanismo horien ondorioz, gantz-ehunaren disfuntzioa gertatzen da, intsulinarekiko erresistentzia sistemikoaren garapenarekin erlazionatzen dena. Gainera, gorago aipatu den bezala, hipertrofiatutako adipozitoetan ematen den lipolisiaren areagotzea dela eta, porta benatik EGGAen fluxu handiagoa bideratzen da gibelera, ehun horretan gantz-metaketa ektopikoa eraginez. Gertaera horrek intsulinarekiko erresistentzia areagotzen du (16,29).

1.1.2. Gibeleko lipidoen metabolismo fisiologikoa eta gibel gantzatsu ez-alkoholikoan ematen diren asaldurak

Gibela funtsezkoa da lipidoen metabolismoan. Egoera fisiologiko normal batean, gibelak gantz-azido kantitate handiak prozesatzen ditu, eta triglizerido kantitate erlatiboki txikiak (% 5 baino gutxiago) baino ez dira biltegiratzen zitoplasmako lipido tantetan (30). Gibel-esteatosia gibeleko lipidoen metabolismoaren asalduren ondorioa da. Zehazki, lipidoen eskuratzearen (gantz-azidoen barneraketa, *de novo* lipogenesia eta lipidoen sintesia) eta deuseztatzearen (gantz-azidoen oxidazioa, dentsitate oso baxuko lipoproteinen (VLDL) jariaketa) arteko desorekaren ondorioa da. Lipidoen metabolismoan parte hartzen duten bide metabolikoen asaldurak gehiegizko lipidoen metaketa dakar, GGEAren ezaugarri berezigarria dena (10,31) (**2. irudia**).



2. irudia. Lipidoen metabolismoan ematen diren asaldurak eta GGEAren bilakaeran inplikatutako beste bide patogeniko batzuk. KM: kilomikroiak, GA: gantz-azidoak, EGGA: esterifikatu gabeko gantz-azidoak, MEZ: matrize estrazelularra, TG: triglizeridoak, ROS: oxigenoaren espezie erreaktiboak, VLDL: dentsitate oso baxuko lipoproteina, GEZ: gantz-ehun zuria. Gezi txiki berde eta laranjek "handituta" eta "murriztuta" esan nahi dute, hurrenez hurren. Gezi grisek aktibazioa adierazten dute, eta T formako lerro grisek, inhibizioa.

Gibeleko lipidoen metaketaren aldeko bide metabolikoak: gantz-azidoen barneraketa, gantz-azidoen sintesia eta triglizeridoen esterifikazioa

Gibeleko triglizeridoen metaketa faboratzen duten gantz-azidoek jatorri ezberdina izan dezakete: lipoproteina hondarrek garraiatutako dietako lipidoak, gantz-ehunak jariatutako EGGAk eta *de novo* lipogenesiaren bidez sintetizatutakoak (32).

Dietako gantz-azidoak gainezkatuta dauden kilomikroietatik edo kilomikroi hondarretatik eratorriak izan daitezke. Gibelera iristen diren EEGAk ere gantz-ehunak jariatutakoak izan daitezke. Obesitatea dagoenean, gantz-ehunaren tamaina handitu egiten denez, ehun horretatik isuritako EGGAen fluxua areagotu egiten da (33). Intsulinarekiko erresistentzia dagoenean, gantz-ehuneko lipolisiaren gaineko efektua ez dago oztopatuta, beraz, horrek ere areagotu egiten du gibelera, eta beste ehun batzuetara, iritsiko diren plasmako EGGAn jarioa (31).

i. Gantz-azidoen barneraketa hepatozitoetan

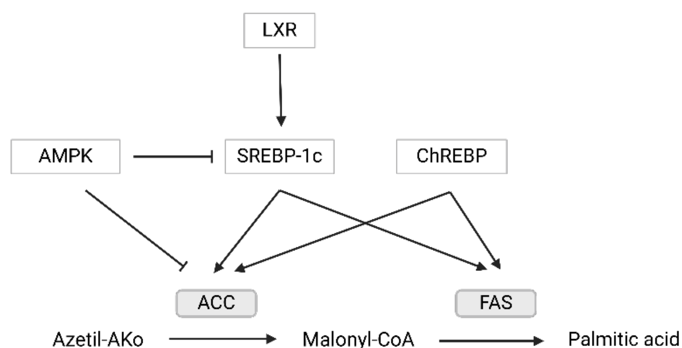
Plasmako EGGAk hepatozitoen barnealdera garraiatuak izan daitezke garraiatzaile espezifikoen bidez, hala nola, gantz-azido proteina garraiatzaileen (FATP) bidez. Zehazki, 2 gantz-azido proteina garraiatzailea (FATP2) eta 5 gantz-azido proteina garraiatzailea (FATP5) dira gibelean aurkitzen diren FATP nagusiak. Horiez gain, gantz-azido translokasa (FAT/CD36) edo gantz-azidoei lotutako proteinak (FABP), bereziki 1 gantz-azidoei lotutako proteina (FABP1), nahiko espresatzen dira gibelean (30). Ildo horretan, Grecok eta kolaboratzaileek gizakietan egindako ikerlan batean, erlazio positiboa ikusi zuten CD36ren espresioaren eta gibeleko gantz kantitatearen artean (34). Saguetan egindako ikerketetan ikusi izan da FABP1 isilarazten denean eta FATP5-aren delezioa ematen denean, gibelaren pisua eta triglizerido-metaketa murriztu egiten direla (35,36). Beste ikerlan batean ikusi zen gantzetan aberatsa zen dieta batekin elikatutako eta gibel esteatosia garatu zuten saguetan CD36-ren eta FABP1-en espresioak handitzen zirela, sagu kontrolekin alderatuta (37). Hala ere, kasu batzuetan, CD36-ren eta FATP5-aren espresioaren murrizketa ikusi izan da esteatosia duten arratoietan, ziurrenik konpentsazio-mekanismo baten ondorioz (38).

ii. *De novo* lipogenesia

Gibeleko gantz-azidoen *pool*-aren beste zati bat *de novo* lipogenesitik dator. Prozesu hau gibeleko triglizeridoen *pool*-a hornitzen duen bigarren prozesu nagusia da, plasmatik eratorritako EGGAen ondoren. Donnellyk eta kolaboratzaileek deskribatutakoaren arabera, gibeleko triglizeridoen % 60 plasmako EGGAetatik eratorria da, % 26 *de novo* lipogenesitik eta % 15 dietako gantzetatik (39).

De novo lipogenesian kate luzeko gantz-azidoak sintetizatzen dira azetil-Ako-tik abiatuta, zeina glukosatik eratorria den batez ere. Prozesu honetako entzima mugatzailea azetil-AKo karboxilasa (ACC) da eta azetil-Ako-ren karboxilazioa katalizatzen du malonil-Ako lortuz. Jarraian, gantz-azido sintasak (FAS) azido palmitikoaren (gehienbat) eraketa katalizatzen du malonil-Ako-tik hasita (**3. irudia**). Azido palmitikoaren molekula luzatu egin daiteke elongasei esker, edota lotura bikoitzak

gehitu dakizkioke, desaturasei esker (30,40). *De novo* lipogenesia eman dadin nikotinamida adenina dinukleotido fosfatoa (NADPH) ere behar da, hidrogeno emailea izango dena. Molekula hau pentosa fosfatoen bidetik eta entzima malikoaren erreakziotik lortzen da (41). Bide metaboliko honen erregulazioa batez ere transkripzio-mailan ematen da. Plasmako intsulinak gibeleko X hartzailea (LXR) aktibatzen du, zeinak esterola doitzen duen elementura loturiko 1 proteina (SREBP-1c) aktibatzen duen. Horren ondorioz, lipogenesian parte hartzen duten entzimen gain-erregulazioa ematen da. Plasmako gehiegizko glukosak karbohidratoen elementu erantzuleari lotzen zaion proteina (ChREBP) aktibatzen du, gantz-azidoen biosintesian parten duten entzima gehienen erregulazioan parte hartzen duen beste transkripzio-faktoreetako bat (30,42). Bestetik, ACC inaktibatu egin daiteke, AMPk aktibaturiko proteina kinasaren (AMPK) fosforilazioz. Ondorioz, malonil-Ako-ren murrizketa emango da eta gantz-azidoen oxidazioa areagotuko da mitokondrioetan (43) (**4. irudia**).



3. irudia. Gantz-azidoen *de novo* sintesia eta haren erregulazioa transkripzio-mailan. ACC: azetil-AKo karboxilasa, AMPK: AMP-k aktibaturiko proteina kinasa, ChREBP: karbohidratoen elementu erantzuleari lotzen zaion proteina, FAS: gantz-azido sintasa, LXR: gibeleko X hartzailea, SREBP-1c: esterola doitzen duen elementura loturiko 1 proteina. Geziek aktibazioa adierazten dute, eta T formako lerro grisek, inhibizioa.

Markaketa isotopikoaren bidez egindako zenbait ikerketetan, GGEA duten banakoetan *de novo* lipogenesia handituta dagoela ikusi da, banako osasuntsuekin alderatuta (44,45). Gibel-esteatosia duten animalia-ereduetan egindako beste ikerlan batzuetan, *de novo* lipogenesiaren markatzaileak handituta daudela ikusi da. Adibidez, leptina urritasuna duen sagu-eredu (Ob/Ob) batean, ACC, FAS, SREBP-1c eta desaturasen gene- eta proteina-espresioak handituta zeudela ikusi zen, sagu kontrolekin alderatuta (46). Dietak eragindako gibel-esteatosiaren arratoi eredu batean berriz, FAS-ren jarduera handituta zegoela ikusi zen gantz eta fruktosa ugariko dieta batekin elikatutako arratoietan, dieta estandar baten bidez elikatutakoekin konparatuz (38).

iii. Triglizeridoen eraketa

Gibelak gantz-azidoak metatzeko duen modu nagusia triglizeridoen eraketaren bidez da (30). Triglizeridoak eratzeko glizerol-3-fosfatoa (G3P) EGGAekin konbinatzen da. EGGA horiek

plasmatik edo *de novo* lipogenesitik eratorri daitezke, eta G3Pa aldiz, glikolisitik edo gantz-ehunaren lipolisitik askatutako glizerolaren fosforilaziotik eratorria izan daiteke (41).

Triglizeridoen sintesiaren lehen urratsa, prozesuaren mugatzailea dena, glizerol-3-fosfato aziltransferasa (GPAT) entzimak bideratzen du. Pauso honetan azil-Ako-ren eta G3Paren arteko esterifikazioa ematen da. Prozesuan inplikatutako bigarren entzimak, azilglizerol-3-fosfato aziltransferasak (AGPAT), diazilglizerol molekulak eratzen ditu. Azkenik, diazilglizerol aziltransferasak (DGAT) triglizeridoen eraketarako azken erreakzioa katalizatzen du. Triglizeridoak zitoplasmako lipido tantetan biltegiratu daitezke, edo VLDL partikuletan erantsiak izan daitezke, ondoren odol-zirkulaziora jariatuko direnak (40).

In vivo egindako ikerlanetan, 2 diazilglizerol aziltransferasak (DGAT2) gibel-esteatosiaren garapenean duen eragina ikusi da. Adibidez, DGAT2-ren gene- eta proteina-espresioak handitu egin zirela ikusi zen fruktosak eragindako gibel-esteatosiaren arratoi eredu batean (47). Saguetan ere ikusi izan da DGAT2-ren gain-espresioak gibeleko triglizeridoen metaketa areagotzen duela (48). Aitzitik, DGAT2-ren inhibizioak gibel-esteatosia hobetu zuela ikusi zen esteatosia sorrarazteko dietarekin elikatutako saguetan. Gainera, ez zen inflamazio edo fibrosiaren garapenik eman, ziurrenik DGAT2-ren inhibizioarekin batera, *de novo* lipogenesiaren murrizketa eman zelako. Azken horrek azal zezakeen diglizeridoen metaketarik ez gertatzea (49).

Lipidoen murrizketan parte hartzen duten bide metabolikoak: gantz-azidoen oxidazioa eta triglizeridoen jariapena

Gibelak triglizeridoak deuseztatu ditzake mitokondrioetako gantz-azidoen oxidazioaren bitartez edo VLDL gisa odolera jariatuta.

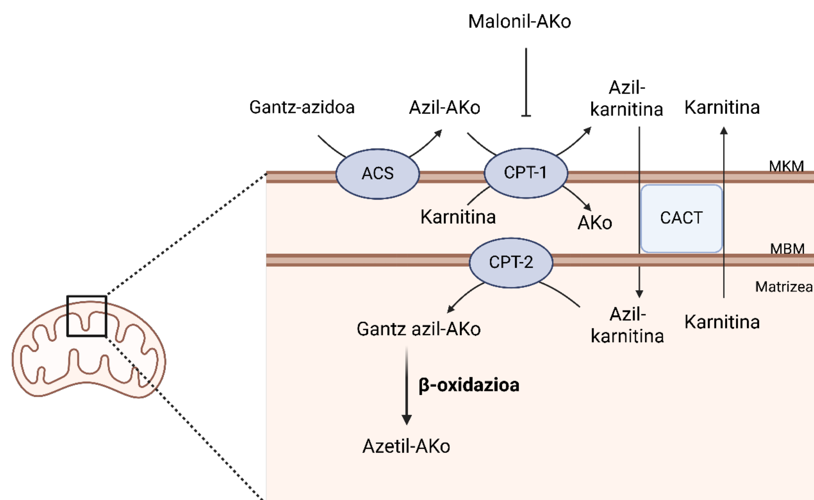
i. Gantz-azidoen oxidazioa

Gibeleko triglizerido-gordailuetatik eratorritako gantz-azidoak, zirkulazioko lipidoetatik eratorritakoak edota *de novo* sintetizatutako gantz-azidoak β-oxidazioaren bidez oxidatu daitezke. Prozesu hau, batez ere, mitokondrioetan ematen da, baina peroxisoma ere eman daiteke, neurri txikiagoan bada ere. Azken organulu horretan, kate oso luzeko gantz-azidoak eta gantz-azido adarkatuak laburtzen dira, ondoren, oxidazioak mitokondrioetan jarraituko duelarik (30,31).

Mitokondrioetako β-oxidazioa kate laburreko, ertaineko eta luzeko gantz-azidoen oxidaziorako bide nagusia da (30), eta organulu horien matrizean ematen da. Prozesuan zehar, gantz-azidoak laburtzen joaten dira progresiboki, azetil-Ako molekuak askatuz, azido trikarboxilikoen zikloan guztiz oxidatu daitezkeenak edo gorputz zetonikoen sintesirako erabili daitezkeenak (31).

β-oxidazioa eman baino lehen, gantz-azidoak azil-Ako bihurtzen dira azil-Ako sintetasa (ACS) entzimari esker. Ondoren, azil-Ako molekula hori mitokondrioen matrizera garraiatu behar da, karnitina-anezkaren bitartez. Horretarako, mitokondrioetako kanpo-mintzean kokatuta dagoen 1

karnitina palmitoiltransferasa (CPT-1) entzimak, azil-Ako-tik hasita azil-karnitina eratuko du, aldi berean, molekula hori mitokondrioaren kanpo-mintzean zehar garraiatzen delarik. Erreakzio hau gantz-azidoen oxidazioaren pauso mugatzailea izateaz gain, prozesuaren erregulazio-puntu garrantzitsua da (50). Prozesu hau negatiboki erregulatua izan daiteke malonil-Ako molekularen eraginez, *de novo* lipogenesiko lehenengo erreakzioaren produktua den horrek (ikusi *de novo* lipogenesia atala) CPT-1 entzimaren inhibitzaile alosteriko moduan jokatzen baitu (31). Kate labur eta ertaineko gantz-azidoek zuzenean zeharkatu ditzakete mitokondrioaren bi mintzak, behin mitokondrioaren matrizean daudela, ACSk aktibatzen ditu. Beraz, gantz-azido horien oxidazioa ez dago CPT-1-en kontrolpean (41). Prozesuaren azalpenarekin jarraituz, mitokondrioen barne-mintzean dagoen 2 karnitina palmitoiltransferasak (CPT-2), azil-karnitina mitokondrioen matrizera garraiatzen du, berriz ere gantz-azil-Ako molekula sortuz eta karnitina askatuz. Behin gantz-azidoa mitokondrioaren matrizean dagoela, β-oxidazioa emango da (**4. irudia**) eta bi karbonoko zatiak askatuz joango dira, azetil-Ako molekulak alegia. Erronda bakoitzean, hau da, azetil-Ako molekula bakoitza askatzeko prozesu bakoitzean, lau entzimak modu jarraian hartzen dute parte, gantz-azil-Ako bakoitza laburtzen den heinean (31).



4. irudia. Gantz-azidoen garraioa mitokondrioaren matrizera CTP sistemaren bitartez. ACS: azil-Ako sintetasa, CACT: karnitina-azilkarnitina translokasa, CPT-1/CPT-2: 1/2 karnitina palmitoiltransferasa, MBM: mitokondrioaren barne-mintza, MKM: mitokondrioaren kanpo-mintza. T formako lerroak inhibizioa adierazten du.

Gantz-azidoen oxidazioarekin lotutako gene-espresioaren erregulazioa peroxisomen ugalketak aktibatutako α hartzaileak (PPARα) erregulatzen du batik bat. Horrez gain, sirtuinen familiako kidea den 1 sirtuinak (SIRT1) paper garrantzitsua jokatzen du gantz-azidoen oxidazioaren erregulazioan. SIRT1-ek peroxisomen ugalketak aktibatutako gamma hartzailearen 1 α koaktibatzailea (PGC1α) desazetilatuz molekula hori aktibatzen du. Horrek PPARα-ren aktibazioa eragingo du gantz-azidoen oxidazioa sustatuz (19,51).

Duela gutxi argitaratutako ikerketa batean, GGEA zuten pazienteetan mitokondrioetako gantz-azidoen oxidazioa murriztuta zegoela ikusi zen. Aldi berean, mitokondrioen disfuntzioa handiagoa zela ikusi zen (52). Kohjimak eta kolaboratzaileek, GGEA zuen taldean *Cpt-1* eta *Ppara* entzimen gene-espresioak murriztuta zeudela ikusi zuten (53), gibel osasuntsua zuen taldearekin alderatuta. Beste ikerketa baten arabera, GGEA zuten pazienteetan SIRT1-en espresioa, beste sirtuina batzuen artean, murriztuta zegoela ikusi zuten gaixotasunik gabeko banakoekin konparatuta (54). Saguetan burututako ikerlan batean, animaliak gantz ugariko dieta batekin elikatu ostean (esteatosia garatu zutenak), gibeleko CPT1, PPARα, PGC1α eta SIRT1 proteinen espresioa murriztuta zegoela ikusi zen, kontrol taldeko saguekin alderatuta (37). Hala ere, ikerlan guztietako emaitzak ez datoz bat, izan ere, zenbait kasutan gantz-azidoen oxidazioaren areagotzea ere ikusi izan da GGEAn. Efektu hori GGEAren garapenaren hasierako faseetan gibeleko gehiegizko gantz-azidoen edukiari aurre egiteko konpentsazio-mekanismo bezala azal daiteke (55).

ii. Triglizeridoetan aberatsak diren lipoproteinen jariaketa

Baldintza fisiologikoetan, gibelak triglizerido kantitate txikiak biltegiratzen ditu, eta VLDL partikula gisa triglizerido kantitate handiak esportatzen ditu, modu horretan, nutrizio-egoeraren arabera, gantz-azidoak muskuluan oxidatu edo gantz-ehunean biltegiratu daitezke (30).

VLDL triglizeridotan aberatsa den lipoproteina bat da, B-100 apolipoproteina (apo-B100) molekula bakar batez osatuta dagoena eta triglizeridoen proteina garraiatzaile mikrosomalak (MTP) erantsitako triglizeridoak gehituz eratzen da. Prozesu hau erretikulu endoplasmikoko argian gertatzen da. VLDL partikulak ontzen doazen heinean, hau da, triglizerido molekula gehiago eransten zaizkion heinean, erretikulu endoplamikoaren mintzean zehar translokatzen dira Golgi-ren aparatura heltzen diren arte (30,56).

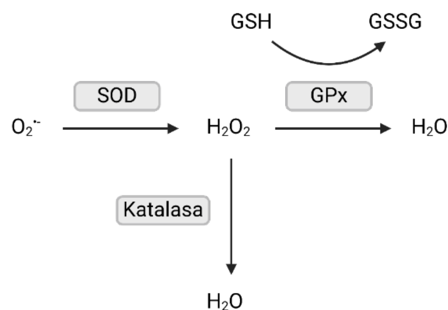
Intsulinak lipolisiaren aurka duen efektua VLDL-en ekoizpena gutxituz gauzatzen du, ziurrenik MTP-ren aktibitatea murriztuz. Hala ere, GGEAn ematen lipidoen eskuragarritasun handiagoaren eta gibelak intsulinarekiko duen erresistentzia handiagoaren ondorioz, MTP-ren aktibitatea areagotu egiten da, VLDL-en sintesia handitzen delarik. Nahiz eta gibel-esteatosian VLDL lipoproteinen jariaketa handitu egiten den (57), ez da nahikoa izaten triglizeridoen gehiegizko eskuragarritasunari aurre egiteko, eta ondorioz, gibel-esteatosia garatzen da. Gibeleko triglizeridoen infiltrazioak % 10 gainditzen duenean (gibel-esteatosia), gibela ez VLDL-en jariaketa handitzeko gai (31,56). Fujitak eta kolaboratzaileek burututako ikerlan baten arabera, estatopehatitisa zuten pazieteetan VLDL jariaketa esteatosi arrunta zuten pazienteetan baino baxuagoa zen (58).

1.1.3. Esteatohepatitis ez-alkoholikoaren garapena eragiten duten gertaera patogenikoak

Esteatosi arruntetik esteatohepatitiserako bilakaerak hepatozitoen kaltetzea dakar, hainbat mekanismoren bitartez gerta daitekeena. Gainera, hepatozitoez gain, gibeleko zelula ez-parenkimalek gibelaren kaltetzean eta inflamazioaren eta fibrosiaren garapen prozesuetan ere parte hartzen dute.

Aurreko ataletan gibelean gehiegizko lipidoen metaketa eragiten duten mekanismoak azaldu dira. Jakina da gibelaren kaltetze prozesua ez dela soilik metatutako lipido kantitatearen araberakoa, metatzen diren lipido-molekula motaren araberakoa ere bada. Zentzu horretan, esan beharra dago GGEAn lipidoak triglizerido moduan metatzen direla batez ere; baina hala ere, dirudienez, hauek ez dira lipotoxizitatearen eragile. Lipotoxizitate termino horrek erreferentzia egiten dio lipidoen espezie toxikoek eragindako zelulen kaltetzeari. Triglizeridoetan txertatu ezin daitezkeen gehiegizko EGGAen metaketak efektu lipotoxikoa dauka hepatozitoetan. Gainera, EGGAen asetasunaren arabera, eragina desberdina izango da, adibidez, gantz-azido asegabeak efektu positibo batekin erlazionatzen diren bitartean, gantz-azido aseek lipotoxizitatearekin lotutako lesio zelularrak eragiteko joera handiagoa dute. Azken batean, lipotoxizitateak organuluen disfuntzioa eragiten du, esaterako, erretikulu endoplasmikoarena edota mitokondrioena; zelulen lesioa eta heriotza eraginez, eta esteatohepatitiseranzko progresioa sustatuz (24,59).

Esteatosi arruntetik gaixotasunaren etapa aurreratuago baterako igarotzean garrantzi handia duen beste gertaera bat oxidazio estresa da, izan ere, funtsezkotzat hartzen da gibel-esteatositik esteatohepatitiserako bilakaeran (60). Oxidazio estres egoeran zelulako molekula oxidatzaileen eta antioxidatzaileen arteko oreka apurtu egiten da, oxigenoaren espezie erreaktiboen (ROS) gehiegizko eraketaren edo/eta sistema antioxidatzailearen disfuntzioaren ondorioz, ehunaren kaltetzea eragiten duena (10). Baldintza osasuntsuetan, ROS-en sorrera sistema antioxidatzaileak orekatu dezake, molekula erreaktiboen maila basalak mantenduz. ROS-ak erradikal aske gisa sailkatu daitezke; horien artean anioi superoxidoa ($O_2^{\bullet -}$) eta hidroxilo erradikala ($^{\bullet}OH$) dira garrantzitsuenak fisiologikoki. Bestetik, espezie ez-erradikalak ere badaude, hidrogeno peroxidoa (H_2O_2) nabarmentzen delarik. Sistema antioxidatzaileari dagokionez, antioxidatzaile entzimatikoek eta ez-entzimatikoek osatzen dute. Entzima antioxidatzaile garrantzitsuenen artean superoxido dismutasa (SOD), katalasa eta glutatioi peroxidasa (GPx) aurki daitezke. Lehenengoak $O_2^{\bullet -}$ H_2O_2 bihurtzen du, eta beste biek, H_2O_2 H_2O-ra neutralizatzen dute (**5. irudia**). Sistema antioxidatzaile ez-entzimatikoaren osagai nagusia glutatioia da, hala ere, beste molekula batzuek ere jardun dezakete elektroi hartzaile gisa beste biomolekula batzuk eta zelula-egiturak ROS-en kaltetik babestuz. Molekula antioxidatzaile horien artean azido askorbikoa (C bitamina), erretinola (A bitamina) edo tokoferola (E bitamina) aurki daitezke (55).



5. irudia. Entzima antioxidatzaile nagusien jarduera. GPx: glutatioi peroxidasa, GSH: erreduzitutako glutatioia, GSSG: oxidatutako glutatioia, SOD: superoxido dismutasa.

ROS-en iturri nagusia mitokondrioak dira. GGEAren hasierako etapetan, gehiegizko EGGAen ondorioz mitokondrioetako oxidazioa areagotu egiten da, mekanismo egokitzaile moduan. Hala ere, egoera hori kroniko bihurtzen denean, konpentsazio-mekanismo horrek huts egiten du eta ROS-en eraketaren iturri bihurtzen da, mitokondrioetan gantz-azidoen oxidazio partziala dela eta. Izan ere, β-oxidazioaren ondorioz sortzen den gehiegizko azetil-Ako-ren ekoizpenaren ondorioz, azido trikarboxilikoen zikloa elektroien garraio-katetik banandu egiten da, eta horrek ATP-aren eta ROS-en ekoizpenean asaldurak eragiten ditu (24,55,61). Elektroien garraio-katearen aktibitatea murriztearen ondorioz, c zitokromoa zitosolera askatzen da, eta horrek kaspasen menpeko apoptosi bidea induzitzen du (62). Horrez gain, osatu gabeko gantz-azidoen oxidazioaren ondorioz lipido-espezie toxikoak sortzen dira, hala nola, zeramidak, lipotoxikotasuna areagotu dezaketenak (24).

Erretikulu endoplasmikoa, halaber, ROS-en ekoizpena ematen den beste gune garrantzitsu bat da, gehiegizko EGGAek sustatua. Estimulu hori luzaroan mantentzen denean, erretikulu endoplasmikoko estresak ROS-en sintesia estimulatzen du, eta horrek, gibeleko inflamazioa sustatzen du TNF-α-ren sekrezioa handituz. Erretikulu endoplasmikoaren estresari lotutako inflamazioa ere beste bide batzuetatik eman daiteke, hala nola, aktibatutako B zelulen kappa kate arinaren indartzailearen faktore nuklearraren (NF-κB) aktibazioaren eta c-Jun N-bukaerako kinasaren (JNK) aktibazioaren eraginez. Erretikulu endoplasmikoaren estresak ere hepatozitoen apoptosia eragin dezake (24).

Mekanismo hauek guztiek hepatozitoetako ROS-en ekoizpena handitzen dute eta sistema antioxidatzailearen disfuntzioa eragiten dute. ROS-ek lipidoen peroxidazioa eragin dezakete, malondialdehidoa (MDA) sortuz. Horrek zelulen mintzetan eta zeluletako beste egitura batzuetan asaldurak eta metabolito erreaktiboen ekoizpena eragiten ditu. Eraldaketa horien ondorioz gibeleko oxidazio estresa areagotzen da eta horrek, hepatozitoetan inflamazioa eta apoptosiaren indukzioa dakartza, ondorioz, fibrosia garatzen hasten delarik (62,63).

Hepatozitoez gain, gibeleko zelula ez-parenkimalek ere parte hartzen dute GGEAren bilakaeran, besteak beste, Kupffer zelulek eta izar-itxurako zelulek (**2. irudia**). Kupffer zelulak gibeleko

makrofagoak dira eta oxidazio estresak haien polarizazioa eragiten du inflamazioaren aldeko fenotiporantz (M1). Hori dela eta, inflamazioa eragiten duten zitokinen (TNF-α, IL-6 eta IL-1β) produkzioa areagotzen da. Gainera, oxidazio estresaren eta inflamazioaren eraginez, izar-itxurako zelulak estimulatu egiten dira eta fibrosiaren aldeko fenotipoaren agerpena sustatzen da, ondorioz, matrize estrazelularraren ekoizpena eta fibrosiaren garapena ematen direlarik (2,55).

Gibelean gertatzen diren asaldurez gain, gantz-ehunaren disfuntzioak ere esteatohepatitiserako bilakaeran paper garrantzitsua jokatzen du. Gorago, jadanik azaldu da gantz-ehunak gibel-esteatosiaren garapenean duen esku-hartzea; baina horrez gain, gaixotasunaren larritasun handiagoko egoeretarako bilakaeran ere parte hartzen du. Obesitatean, gantz-ehunaren disfuntzioak inflamazioaren garapenarekin duen zerikusiaz gain, maila baxuko inflamazio sistemiko kronikoaren garapenarekin ere dago erlazionatuta. Egoera horrek, gibelean inflamazioa garatzea eragiten du, eta aldi berean, intsulinarekiko erresistentzia areagotzen du. Bi asaldura horiek bata-bestearen eragina handitzen duen ziklo batean jarduten dute, gibeleko kaltea areagotuz (10,11).

1.2. Algak

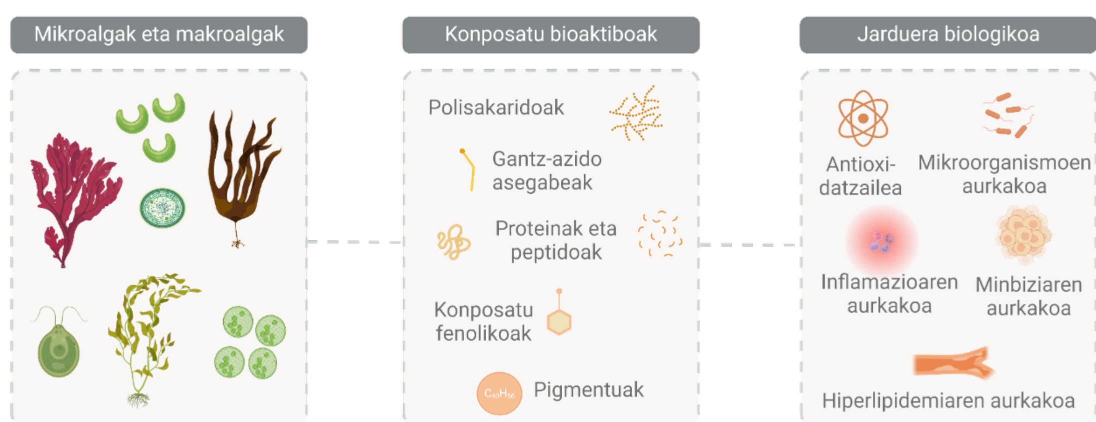
Algak oso talde heterogeneoa osatzen duten organismo fotosintetikoak dira. Ur-ingurune ia guztietan aurki daitezke, bai ur gezetan zein ur gazitan. Algak mikroalgetan eta makroalgetan sailkatzen dira. Mikroalgak organismo zelulabakar txikiak dira, eta aske edota kolonia zein harizpi-itxurako taldeetan bizi daitezke. Mikroalgak bi talde handitan sailka daitezke: prokariotoak eta eukariotoak. Lehenengoen barnean, *Cyanophyta*, zianobakterio edo alga urdin-berde izena ere hartzen dutenak, eta *Prochlorophyta* taldeak aurki daitezke. Mikroalga eukariotoen artean hainbat dibisio aurki daitezke, hala nola, *Chlorophyta*, *Rhodophyta*, *Cryptophyta*, *Ochrophyta*, *Haptophyta, Heterokontophyta* eta *Dinophyta* besteak beste. Esan beharra dago, algen sailkapena nahiko konplexua dela eta etengabe aldatzen ari dela ezaugarri bereizle berriak aurkitzen diren heinean (64,65). Makroalgak organismo zelulanitzak dira, batez ere itsasoan bizi direnak. Kasu honetan *itsas belar* deritze (66). Makroalgak sailkatzeko gehien erabiltzen den irizpidea pigmentazioan oinarritutako da, horren arabera, alga arreak (*Phaeophyta*), alga gorriak (*Rhodophyta*) eta alga berdeak (*Chlorophyta*) bereiz daitezke (67).

Milaka urteetan zehar, algak gizakien dietaren zati garrantzitsua izan dira. Mikroalgen kontsumoari dagokionez (zianobakterioak barne) duela mende asko Txinan, Mongolian, Hego Amerikan edota Txaden kontsumitzen zirela ikusi da. *Arthrospira* (*Spirulina* bezala ere ezaguna) eskualde desberdinetako populazioek modu estentsiboan jasotzen zuten. Hala ere, algen kontsumoa Europan orain dela gutxiago eman den gertakaria da (68).

Makroalgei dagokienez, historikoki elikagai gisa kontsumitu dira, bereziki Asiako herrialdeetan, Japonian eta Korean, esaterako, nutrizioaren eta osasunaren ikuspuntutik eskaintzen dituzten onurengatik eta haien propietate organoleptikoengatik. Europan ordea, makroalgen kontsumoa ez

zegoen hain hedatuta, hala ere, kostaldeko herrialde askotan, hala nola, Portugalen, Espainian, Frantzian, Erresuma Batuan eta Irlandan, mendeetan zehar gizakion kontsumorako erabiliak izan dira, baina ongarri gisa eta abereen elikadurarako ere erabiltzen ziren (68). Europan algen kontsumoa gero eta gehiago handitzen ari den arren, oraindik urrun dago Asiako herrialdeetan kontsumitutako kantitateetatik. Batez beste, 4; 5,2 eta 8,5 g/*per capita* kontsumitzen dira eguneko Japonian, Txinan eta Hego Korean hurrenez hurren (69). Bestalde, Frantzian duela gutxi egindako azterlan baten arabera, biztanleria orokorraren batez besteko alga-kontsumoa 293 mg/egunekoa da (70).

Gaur egun, algak gero eta arreta handiago jasotzen ari dira, izan ere, labore tradizionalen ondoan, alternatiba jasangarri bezala hautagai onak direla kontsideratzen da, biztanleriaren hazkundeak eta horrek suposatzen duen elikagaien eskaera handiagoari aurre egiteko. Horrez gain, algen produkzioak ez du labore tradizionalek adina lur erabiltzea suposatzen, eta nutrienteen kantitate minimoekin haz daitezke (68). Horrez gain, algak arreta gehiago erakartzen ari dira, balio handiko osagaiak eta osasuna sustatu dezaketen propietatiak eduki ditzaketelako (71). Ildo horretan, algak mikro- eta makronutrienteen iturri garrantzitsuak dira, hala nola, dietaren balio nutrizionala hobetzen duten karbohidratoak, gantz-azido poliasegabeak, proteinak, mineralak eta bitaminak eskaintzen dituzte. Proteinei dagokienez, algak proteina-iturri alternatibo oso interesgarritzat hartzen dira, daukatzen proteina kantitatea beste iturri batzuetakoa baino handiagoa baita, aminoazidoen konposizioa bikaina izateaz gain (72). Nutrienteen konposizioaz gain, algak ere interesgarriak dira 2. mailako metabolito bioaktiboei dagokienez. Besteak beste, osasunerako onuragarriak izan daitezkeen edota ahalmen terapeutikoa izan dezaketen (adib. jarduera antioxidatzailea, inflamazioaren aurkakoa, hiperlipidemiaren aurkakoa, mikroorganismoen aurkakoa, minbiziaren aurkakoa) konposatu fenoliko, pigmentu, polisakarido, gantz-azido edo peptidoetan aberatsak dira (**6. irudia**). Hala ere, azken ikuspuntu honetatik algak ez dira oraindik guztiz ustiatu (65,67).



6. irudia. Mikro- eta makroalgetan aurki daitezkeen konposatu bioaktibo nagusietako batzuk eta horiek izan ditzaketen jarduera biologikoen zenbait adibide.

1.2.1. Europako alga ekoizpena

Azken 30 urteetan, algen ekoizpen-sektorea nabarmen hazi da mundu osoan; zehazki, 1950ean 0,56 milioi tona ekoiztetik, 2019an 35,82 milioi tona ekoiztera igaro da. Algen munduko ekoizpenaren % 97 Asian ematen da, eta mundu-mailako produkzioaren % 99 makroalgei dagokie. Europako algen sektoreari dagokionez, oraindik hazten ari den sektorea da, ez baitu hain hazkunde adierazgarririk izan, baina hurrengo urteetan nabarmen haziko dela aurreikusi da (66).

Mikroalgen Europako ekoizpena nagusiki lehorrean ematen da, itxitako fotobioerreaktoreetan eta baldintza kontrolatuetan (68). Urmaeletan eta hartzigailuetan ere ekoizten dira mikroalgak, baina proportzio txikiagoetan, *Spirulina*-ren kasuan izan ezik, zeina urmaeletan ekoizten den nagusiki. Mikroalgen ekoizpenean diharduten enpresa kopuruari dagokionez, Espainia, Alemania, Frantzia eta Italia dira Europako sektorea lideratzen duten herrialdeak. Ekoiztutako biomasa pisu-lehorrean kontuan hartuta Europan gehien ekoizten diren mikroalgak *Chlorella* sp., *Haematococcus pluvialis* eta *Nannochloropsis* sp. dira. Mundu-mailako ekoizpena kontuan hartuta ordea, *Chlorella* eta *Spirulina* dira gehien ekoizten direnak. Aplikazioei dagokienez, mikroalgak elikadura-gehigarri bezala eta industria nutrazeutikoan erabiltzen dira batez ere, jarraian kosmetika eta ongizatearen sektorea eta animalientzako elikadura industria izanik arlo garrantzitsuenak (66).

Europako algen sektorea makroalgen ekoizpenean oinarritzen da (% 99), batez ere, bilketa basatia izanik ekoizpen metodo nagusia. Akuikulturari dagokionez, instalazio gehienak itsasoan daude kokatuta, eta ehuneko txikiago batean, instalazioak lehorrean daude. Enpresa gehienak Frantzian, Irlandan eta Espainian aurki daitezke. Komertzialki ustiatzen diren espezie nagusiak alga arreenak dira. Hauen artean, aipatzekoa da *Laminaria* generoko espezieak direla ustiatuenak, bai akuikulturako instalazioetan bai bilketa basatiari dagokionez. Akuikultura-enpresei dagokienez, gehienek *Saccharina latissima* hazten dute, ondoren, *Alaria esculenta* eta *Ulva* sp. (*Chlorophyceae*) izanik espezie nagusiak. Ekoizpen osoaren ehuneko handiena giza kontsumora bideratzen da, eta proportzio txikiagoan elikadurarekin lotutako beste arlo batzuetara, hala nola, elikadura-gehigarri, lodigarri eta kontserbatzaile gisa. Makroalgak pentsuen ekoizpenerako eta kosmetikaren eta ongizatearen merkatuetan ere erabiltzen dira (66).

1.2.2. Europar Batasuneko legeria eta Elikagai Berriak

Algak, Europar Batasuneko (EB) elikagai gisa, Elikagaiei buruzko 178/2002 (EB) Lege Orokorraren Erregelamenduaren (73) menpe daude. Erregelamendu hori EBeko estatu kide guztietan aplikatzen da. Gainera, algak "Elikagai Berri"-tzat hartzen dira; horren arabera, espezie berriek EBko merkatuan sartzeko aldez aurretiko baimena lortu behar dute giza kontsumorako segurtasuna bermatzeko. Europako Batzordearen arabera, Elikagai Berriak 1997ko maiatzaren 15a baino lehen EBean kantitate adierazgarrietan kontsumitu ez diren elikagaik dira, data horretan jarri baitzen indarrean Elikagai Berriei buruzko lehen araudia, 258/97 (EE) Erreglamendua (74) alegia. Erreglamendu horren arabera,

edozein Elikagai Berrik EBean merkaturatua izateko baimena eskuratu behar du (75), eta gaur egun, 2015/2283 (EB) Erreglamenduak (76) arautzen du. Gainera, erreglamendu horren arabera, EBtik kanpo tradizionalki eta modu seguru batean kontsumitzen den elikagai batek baimen-prozedura azkarrago bat jarrai dezake, EBko merkaturako bidea erraztuz.

1.2.3. Algek eta haien konposatu bioaktiboek gibel gantzatsu ez-alkoholikoan dituzten efektuak

Zenbait ikerketa preklinikok frogatu dutenez, mikroalgen zein makroalgen espezie ezberdinek, efektu onuragarriak izan ditzakete gibeleko gantz-metaketan (76). Gizakiengan egindako azterlanak oso urriak diren arren, ikerketa batek algen kontsumoaren eta GGEAren arteko erlazio negatiboa aurkitu zuen (78). Lehen aipatu bezala, algak oraindik oso erabiliak ez diren konposatu bioaktiboen iturri aberatsa dira, hainbat gaixotasunen prebentzioan eta tratamenduan erabili daitezkeenak. Horien artean, honako konposatu bioaktibo hauek nabarmentzen dira: polisakaridoak, gantz-azido poliasegabeak, proteinak eta peptidoak, polifenolak eta pigmentuak.

Polisakaridoak algen osagai garrantzitsuenetakoak dira, batez ere zelula-paretan daude eta, haien artean agarra, karragenina, fukoidana, laminarina eta alginatoa aurki daitezke. Polisakaridoetan aberatsak diren frakzioek eragin positiboak erakutsi dituzte gibeleko lipidoen metaketaren aurka (79–82). Gainera, polisakaridoez gain, algetan ere lipidoak aurki daitezke, zeinak proportzio handiagoan dauden mikroalgetan makroalgetan baino. Kate luzeko gantz-azido poliasegabeak bereziki interesgarriak dira haien jarduera bioaktiboa dela eta. Izan ere, gantz-azido poliasegabeetan aberatsak diren alga-estraktuekin egindako zenbait ikerketek frogatu dutenaren arabera, gibel-esteatosia hobetzeko ahalmena izan dezakete (83,84). Jadanik aipatu den bezala, algak proteina-iturri interesgarriak dira, haien nutrizio-balioaz gain, alga jatorriko proteina-lisatuek eta peptido-estraktuek eragin onuragarria erakutsi dute gibel-esteatosia arinduz (85,86). Efektu interesgarriak erakutsi dituzten beste konposatu bioaktibo batzuk polifenolak (87–89) eta pigmentuak, hala nola fukoxantina, dira (90,91). Horiez gain, karotenoideetan aberatsak diren alga-estraktuek gibeleko fibrosia arintzeko ahalmena erakutsi dute (92,93).

1.2.4. Algen segurtasuna

Algen onurak gorabehera, gorago aipatu bezala, algen kontsumoa gizakion osasunerako kaltegarria izan daitekeela hartu behar da kontuan. Alga eta zianobakterio batzuek pertsonak eta animaliak gaixotu ditzaketen eta ingurumena kutsatu dezaketen toxinak sor ditzakete. Adibidez, zianobakterio batzuek ekoiztutako anatoxinak gibelean edota nerbio-sisteman kalteak eragin ditzake. Gainera, mikroalgek ekoiztutako zenbait toxinek, beste alga-espezie komertzialak kutsatu ditzakete (68).

Hala ere, metal astunen, artsenikoa, beruna, kadmioa edo merkurioa kasu, eta iodoa bezalako nutrienteen kontzentrazio altuegia da algen segurtasuna baldintzatzen duen kezka nagusia da. Algek inguruneko mineral eta aztarna-elementuak metatzeko gaitasun handia dute. Metaketa hau hainbat

faktoreren araberako da, hala nola, alga-espeziearen, urtaroaren edota giro-baldintzen (tenperatura, gazitasuna, pHa, argia) araberakoa (68). Ildo horretan, alga arreek adibidez, beste alga-dibisio batzuk baino ahalmen handiagoa erakutsi dute inguruneko elementuak kontzentratzeko, haien zelula-paretaren ezaugarri bereziak direla eta (94). Orokorrean, industria-guneetatik gertu dauden ur eta substratuetan metal astunen kontzentrazio altuagoak aurki daitezke, algetan metatu egiten direnak (95). Ezaugarri hori dela eta, algak oso erabiliak dira bioerremediaziorako, zehazki, hondakin uretatik metal astunak eta beste kutsatzaile batzuk kentzeko (96,97). Ondorioz, algetan metatzen diren kutsatzaile horiek elikadura katean sar daitezke goragoko maila trofiko batean dauden organismoek jaten dituztenean, eta azkenean horrek ere gizakiontzat ere arriskua suposatu dezake (98). Nahiz eta kasu batzuetan metal astunei dagozkien legezko mugak gainditzen ez diren, alga horien gehiegizko kontsumoak ondorio kaltegarriak izan ditzake, konposatu horiek giza organismoan metatze joaten baitira (68). Elikagaien Segurtasunerako eta Nutriziorako Espainiako Agentziak (AESAN) 2019an argitaratutako txostenaren arabera (99), aztertutako metal astunen artean merkurioaren gehiegizko mugak gainditzen zituzten emaitzen % 80 Kombu algari (*Laminaria japonica, Saccharina japonica*) zegozkion. Txosten horren arabera ere, Hiziki algaren kasuan (*Hizikia fusiforme*) aztertutako laginen %100ak gainditzen zituen gehiegizko mugak. Azken espezie honetan ere artseniko inorganikoaren kontzentrazio oso altuak aurkitu ziren, eta hori dela eta, ez da bere kontsumoa gomendatzen.

Iodoari dagokionez, gizakiontzat esentzia izan arren, haren urritasunak osasun-arazo larriak eragin baititzake, gehiegi hartzeak ere ondorio kaltegarriak eragin ditzake (adibidez, tiroide-disfuntzioa). Hori dela eta, arreta berezia jarri behar da arrisku-talde desberdinetan (haurdun dauden emakumeetan, umeetan, tiroide-disfuntzioa duten banakoetan, bihotzeko gaixotasuna dutenengan edota giltzurrun-gutxiegitasuna duten banakoetan) (68,95). Lehen aipatutako AESANen txostenak (99) ondorioztatzen zuenaren arabera, aztertutako espezieen batez besteko iodoaren balioa esposizio-balio altuari zegokion. Batez ere, Kombu alga nabarmentzen zen, iodo-maila bereziki altua erakutsi baitzuen. Hori dela eta, bere kontsumoa mugatzea aholkatzen da.

Azkenik, algen kontsumoarekin erlazionatutako alergiei dagokienez, esan beharra dago, oso ohikoak ez direla, eta normalean, itsaso zabalean hazten eta biltzen diren algetan bizi diren krustazeo txikiek eragindakoak direla. Beste batzuetan aldiz, algen osagaiek eragindakoak dira, hala ere, konposatu horiek ez dira oraindik oso ondo ezagutzen (100).

1.2.5. Doktoretza-tesi honetan erabilitako alga espezieak

Atal honetan, Doktoretza-tesi honetan erabili diren alga espezieen deskribapen laburra biltzen da.

Chlorella vulgaris

Chlorella vulgaris (*Chlorophyta*) alga zelulabakar mikroskopikoa da, forma esferikoa eta 2 eta 10 µm bitarteko diametroa duena. Nahiz eta haren tamaina oso txikia izan, goi-mailako landareen antzeko egiturak dauzka, esaterako, zelula horma, mitokondrioak, bakuoloak eta kloroplasto bakarra.

Mikroalga hau ur gezatan hazten da eta banaka edo kolonietan aurki daiteke (gehienez 64 zelulaz osatutakoak). *C. vulgaris* organismo mugiezina da, eta asexualki ugaltzen da, autoesporulazio bidez. Zehazki, lau zelula-alaba berri sortzen dira zelula-amaren hormaren barruan, eta heldu ondoren, aske geratzen dira. Ur gezatan hazten den arren, erraz egokitzen da baldintza ezberdinetara. Horrez gain, hazkunde-tasa handira du, bi ezaugarri horiek direla eta, espezie ezin hobea kontsideratzen da ekoizpenerako. Gainera, baldintza autotrofo, heterotrofo eta mixotrofoetan hazteko gai da (fotosintesi bidez zein materia organikoa erabiliz) (101,102). EBeko Elikagai Berrien katalogoaren arabera (103), *C. vulgaris* ez da Elikagai Berritzat hartzen, beraz, ez du aurretiko baimenik behar merkaturatua izateko 2015/2283 (EB) Erreglamenduaren arabera (76).

Nannochloropsis gaditana

Nannochloropsis gaditana (*Ochrophyta*) mikroalga espezie bat da, Lubinek eta kolaboratzaileek Cadizko badian (Espainia) lehen aldiz isolatu zutena. Hazkunde aktiboko fasean daudenean, *N. gaditana* zelulek 5-4 x 2,5-3 µm-ko forma elipsoidala dute. Egoera horretan daudela, *N. oculata* eta *N. salina* espezieetatik bereiz daiteke. Zelula mugiezinak dira, ez dute flagelorik eta kloroplasto bakarra dute, zelularen zatirik handiena hartzen duena. Zelula-paretari dagokionez, *N. gaditana*-k aipatutako beste bi espezieek baino pareta lodiagoa eta erresistenteagoa dauka. Espezie autotrofoa da eta zelulen fisio bitarraren bitartez ugaltzen da (ugalketa asexuala). *Nannochloropsis* sp. itsas inguruneetan hazten da batik bat, baina ur gezetan eta gazikaretan ere aurki daiteke. Ildo horretan, ikusi da *N. gaditana*-k gazitasun-maila ezberdinak onartzen dituela, baina, beste alga batzuekin alderatuta, ez dela oso egonkorra argi-intentsitate handien menpean, ziur asko klorofila mota bakarra duelako (104,105).

Gracilaria vermiculophylla

Gracilaria vermiculophylla makroalga gorri kartilaginoso bat da (*Rhodophyta)*, disko itxurako euskarri batetik abiatuta sortzen dena. Bere zurtoina lodi samarra da eta irregularki adarkatua dago, oinarrian nahiko estututa dauden adar-zilindrikoak dituelarik. Makroalga honen kolorea gorri ilunetik marroi gorrixkara alda daiteke, batzuetan berdexka edo beltza izan daitekeelarik. Kolore aldaketa horrek genero bereko beste espezie batzuetatik bereiztea ahalbidetzen du, esaterako, *Gracilaria gracilis*-etik, azken honek kolore gorri bizia baitu. *G. vermiculophylla* estuario eta marearteko habitatetan aurkitzen da, bereziki sakonera gutxiko uretan, ur gezaren eragina jasaten duten eremu babestuetan. Tenperatura-, argi-, gazitasun- eta nutriente-maila aldakorren aurrean tolerantzia nahiko zabala erakusten du. Oro har, aske hazten da, inolako atxikimendurik gabe lokatzaren eta harearen gainean. Egoera begetatiboan aurkitu ohi da, ugalketa-egitura gutxirekin (106,107).

1.3. Doktoretza-tesi honen ekarpena Garapen Jasangarrirako Helburuei

Garapen Jasangarrirako Helburuei (GJH) dagokienez, Doktoretza-tesi hau bat dator 3. GJHarekin ("Osasuna eta Ongizatea") eta 12. GJHarekin ("Ekoizpen eta Kontsumo Arduratsua").

3. GJH "Osasuna eta Ongizatea"

Garapen Jasangarrirako 3. helburuaren helmugetako bat hau da: "gaixotasun ez-kutsakorren ondoriozko hilkortasun goiztiarra heren batera murriztea prebentzioaren eta tratamenduaren bidez, eta osasun mentala eta ongizatea sustatzea". Helmuga horren ildotik, Doktoretza-tesi honen helburua GGEAren tratamendu-ezari erantzutea da. Gaixotasun horrek prebalentzia handia du gaur egun, gero eta pertsona gazteagoetan agertzen da, eta oraindik ez dago bere kudeaketarako tresna eraginkorrik. Doktoretza-tesi honetan egindako ikerketa preklinikoek GGEAren prebentziora eta tratamendura bideratutako algen eta alga-estraktuen erabilgarritasunaren inguruko ezagutza eta ebidentzia handitzen laguntzen dute.

12. GJH "Ekoizpen eta Kontsumo Arduratsuak"

Jakina da baliabideen kontsumoa eta ondasunen ekoizpena ingurune naturalaren eta bertako baliabideen erabileraren menpekoak direla, askotan ingurumenaren degradazioarekin estuki erlazionatzen dena, gure etorkizuneko garapena eusten duen sistema arriskuan jarriz. Algak hainbat espezieren habitata dira, funtzio garrantzitsua dute itsasoko karbonoaren zikloan (karbono-gordailu gisa jarduten baitute), eta mantenugaien eta konposatu bioaktiboen baliabide berriztagarri kontsideratu daitezke, oraindik nahikoa ustiatu ez direnak. Gainera, algen sektorea EBeko "Bioekonomia Urdina" deritzonaren sektore nabarmenena kontsideratzen da. Izan ere, EBeko "Bioekonomia Urdina"-ren 2022ko txostenean algak dira ardatz nagusia (108). Bioekonomia Urdina"-ren barnean produktuak ekoizteko uretako baliabide biologiko berriztagarriak erabiltzearekin lotutako edozein jarduera ekonomiko sartzen da. Beraz, algentzako aplikazio berriak aurkitzeak haien ekoizpena areagotzen lagun dezake.

2. Metodologia

Lehenik, zati esperimentala burutu baino lehen, bilaketa bibliografiko bat egin zen. Horren ostean, doktoretza tesi honen metodologia esperimentala gauzatu zen. Azken zati hau bi fasetan banatzen da: lehengo fase bat *in vitro* egindako esperimentuak biltzen dituena, eta bigarren fase bat, *in vivo* eredu batean egindakoa.

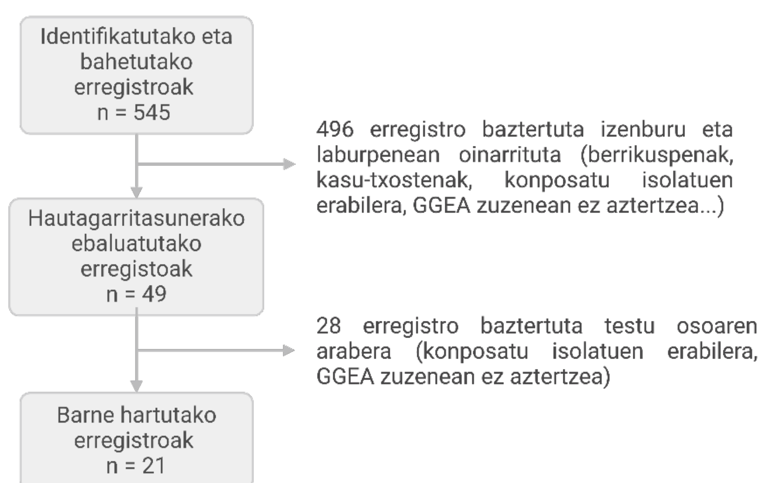
2.1. Bilaketa bibliografikoa

Literatura bilaketa bat egin zen PubMed datu basean mikroalga- eta makroalga-estraktuak erabiltzen zituzten eta haien eragina GGEAn aztertzen zuten ikerlan prekliniko eta klinikoak biltzeko. Konposatu isolatuak erabiliz egindako ikerketak ez ziren kontuan hartu.

Bilaketa-estrategia honela egin zen:

(Fatty liver OR steatosis OR liver) AND (microalga OR macroalga OR seaweed OR alga)

Bilaketan lortutako artikuluak izenburu eta laburpenaren arabera aukeratu ziren; gainera, hauek, ingelesez idatzitako artikulu originalak izan behar ziren, 2021eko otsailera arte argitaratutakoak. Artikuluen baheketa egin ondoren (**7. irudia**), aukeratutako artikuluak bi multzo nagusitan banatu ziren: animalia-ereduetan egindakoak eta gizakietan egindako ikerlanak. Animalia ereduetan egindako ikerketak mikroalgekin eta makroalgekin egindako ikerketan banatu ziren, eta azken hauek, makroalga berde, gorri eta arreekin egindako ikerketan. Berrikuspen honen emaitza 1. eskuizkribua da (ikusi 3. atala).



7. irudia. Ikerlanen hautapen-prozesuaren fluxu-diagrama. GGEA: gibel gantzatsu ez-alkoholikoa

2.2. *In vitro* egindako ikerlanak

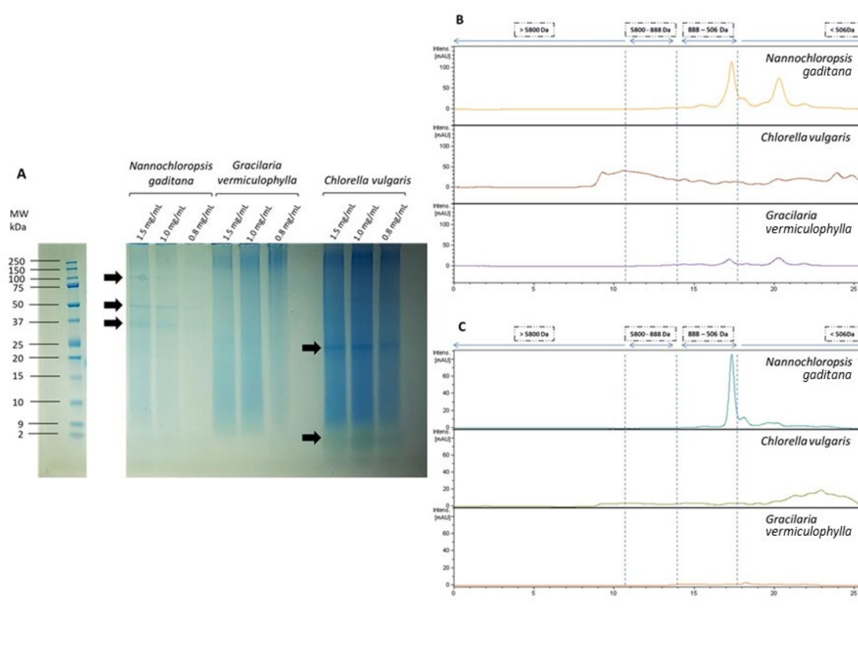
In vitro ikerketak egiteko bi zelula-lerro aukeratu ziren: AML12 (hepatozitoak) eta 3T3-L1 (adipozitoak). Zelulak *Chlorella vulgaris* eta *Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik lortutako estraktuekin tratatu ziren 10, 25, 50 or 150 µg/mL-ko kontzentrazioak erabiliz. Hiru alga-estraktuak AZTI zentro zientifiko eta teknologikoak (Derio, Espainia) eman zizkigun. Alga-estraktuen konposizioa jarraian ikus daiteke (**1. taula**):

1. Taula. Ikerlan honetan erabilitako alga-estraktuen konposizioa eta polifenolen eduki osoa.

	Chlorella vulgaris	*Nannochloropsis gaditana*	*Gracilaria vermiculophylla*
Proteinak (%)	48.0 ± 0.2	42.0 ± 9.2	41.4 ± 0.7
Lipidoak (%)	11.8 ± 2.0	5.6 ± 0.1	3.5 ± 0.1
Errautsak (%)	20.5 ± 0.6	42.6 ± 4.2	22.1 ± 3.2
Karbohidratoak (%)	21.1 ± 0.6	9.9 ± 4.4	33.4 ± 3.6
Polifenolen eduki osoa (mg GAE/g PL)	12.27 ± 0.39	9.08 ± 1.56	9.08 ± 1.56

Pisu lehorraren (PL) %, GAE: azido galikoaren baliokideak. Karbohidratoak diferentziaren arabera kalkulatu ziren.

Bestalde, proteina frakzioaren karakterizazioa CIAL "Food Science Research Institute"-k burutu zuen (Madril, Espainia) (**8. irudia**).



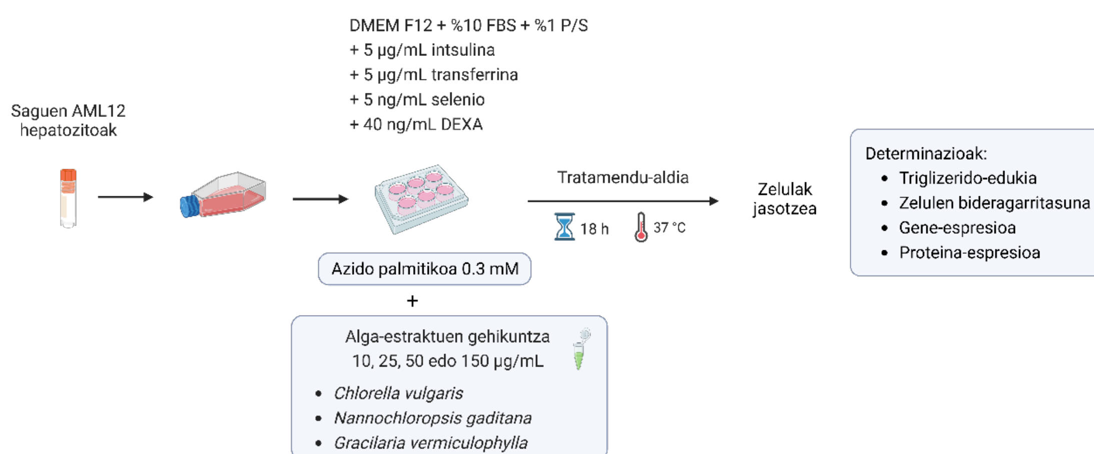
8. irudia. (**A**) SDS-PAGE bidez lortutako alga-estraktuen profilak proteina kontzentrazio desberdinak erabilita. (**B, C**) Alga-estraktuen HPLC-SEC kromatogramak 214 nm (**B**) eta 280 nm-ko (**C**) uhin luzeratan. Hiru laginen kromatogramak intentsitate eskala berean daude.

Liofilizatutako alga-estraktuak ur destilatuan disolbatu ziren. Lehenik, bainu ultrasoniko batean sonikatu ziren eta ondoren, xiringa-iragazkien bidez iragazi ziren. Disoluzio horretatik abiatuta,

tratamendu-egun bakoitzean alikuota ezberdinak prestatu ziren tratamendu bakoitzari zegokion kontzentrazioa inkubazio medioan lortzeko (10, 25, 50 edo 150 µg/mL). Prozedura bera jarraitu zen zelula-kultibo guztiekin.

2.2.1. *In vitro* ikerlana gibel-esteatosiaren eredu batean

Lehenengo ikerlan honetarako AML12 hepatozitoak erabili ziren, American Type Culture Collection-etik (AML12, ATCC, CRL-2254) eskuratuak (**9. irudia**). Zelulak GlutaMAX™ F12 Dulbecco´s Modified Eagle´s Medium-ean (DMEM F12) hazi ziren. Medio honi behi-fetuaren serumaren (FBS) % 10, penizilina/estreptomizinaren % 1, intsulinaren 5 µg/mL, transferrinaren 5 ng/mL eta dexametasonaren (DEXA) 40 ng/mL gehitu zitzaizkion. Gibel gantzatsuan hepatozitoen egoera imitatzen duen *in vitro* eredu bat sortzeko, AML12 hepatozitoak azido palmitikoarekin (0,3 mM) 18 ordutan zehar inkubatuz triglizeridoen metaketa eragin zen.



9. irudia. AML12 hepatozitoetan egindako ikerlanaren diseinu esperimentalaren eskema orokorra. DEXA: dexametasona, DMEM: Dulbecco's Modified Eagle Medium, FBS: behi-fetuaren seruma, P/S: penizilina/estreptomizina.

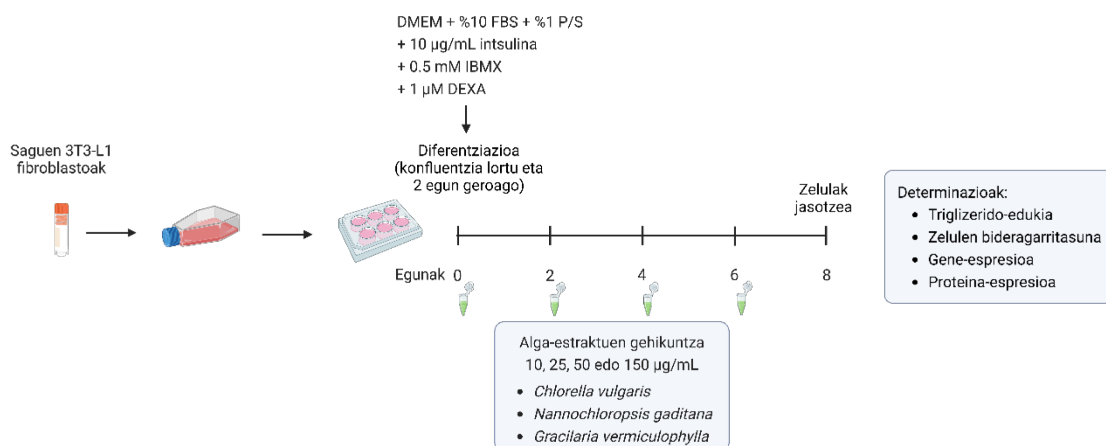
Lehen esperimentu batean, zelulak azido palmitikoarekin batera inkubatu ziren alga-estraktuen kontzentrazio desberdinak gehituta edo hauek gehitu gabe. 18 ordu eta gero, zelulak jaso eta hauek, triglizerido-edukiaren kuantifikaziorako eta zelulen bideragarritasuna aztertzeko erabili ziren. Triglizeridoen kuantifikazioa kolorimetrian oinarritutako kit komertzial baten bidez burutu zen, eta zelulen bideragarritasuna *crystal violet* metodoarekin aztertu zen. Bigarren esperimentu batean, triglizerido-edukiaren murrizketa handiena eragin zuen estraktu bakoitzaren dosia hautatu zen, eta zelulak lehen esperimentuan erabilitako metodologia bera jarraituz tratatu ziren. Tratamenduaren ostean, inkubazio medioa jaso zen eta alanina aminotransferasa (ALT/GPT) mailak neurtu ziren kolorimetrian oinarritutako kit komertzial baten bidez. Jasotako zelulak lipidoen metabolismoan parte hartzen duten gene eta proteinen espresio mailak neurtzeko erabili ziren, Real-Time PCR eta Western Blot bidez hurrenez hurren.

Erabilitako metodo eta protokolo zehatzak 2. eskuizkribuan aurki daitezke (ikusi 3. atala).

2.2.2. *In vitro* ikerlana obesitatearen eredu batean

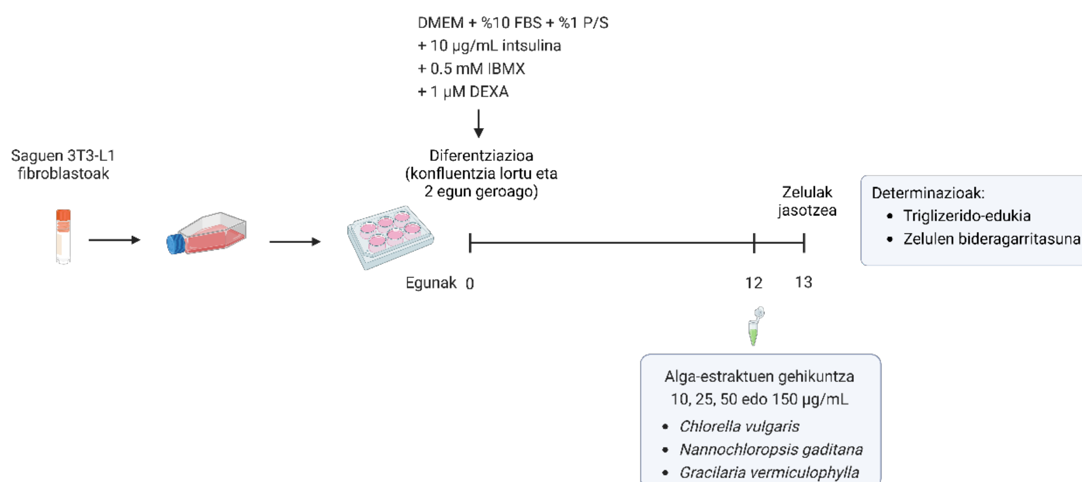
In vitro egindako 2. ikerlanean, 3T3-L1 sagu enbrioi-fibroblastoak erabili ziren American Type Culture Collection-etik (ATCC CL-173) eskuratuak. Zelulak FBS % 10 eta penizilina/estreptomizinaren % 1 zuen glukosa-maila altuko (4,5 g/L) DMEM-ean (hazkuntza-medioa) hazi ziren konfluentzia lortu arte. Konfluentzia lortu eta bi egunera (0 eguna izendatua), zelulen diferentziazioa eragin zen hazkuntza-medioa 10 µg/mL intsulinarekin, 0,5 mM isobutilmetilxantinarekin (IBMX) eta 1 µM DEXA-rekin aberastuta (diferentziazio-medioa). 2. egunean eta 48 ordutan zehar, diferentziazio-medioa mantentze-medioarekin ordezkatu zen, DMEM-a % 10 FBS-rekin eta 10 µg/mL intsulinarekin aberastuta. 4. egunetik aurrera (zelulak jaso arte), zelulak mantentze-medioan (DMEM-a % 10 FBS eta 0,2 µg/mL intsulinarekin) mantendu ziren, medioa bi egunean behin aldatuta. Diferentziazio- eta mantentze-medioei % 1 penizilina/estreptomizina, biotina eta azido pantoteniko ere gehitu zitzaien.

3T3-L1 zeluletan egindako lehenengo esperimentuan, alga-estraktuek diferentziazio-prozesuan duten eragina aztertu zen. Horretarako, zelulak alga-estraktuekin inkubatu ziren diferentziazio-prozesuak irauten duen 8 egunetan zehar alga-estraktu bakoitzaren 10, 25, 50 edo 150 µg/mL-rekin egun bakoitzean zegokion kultibo-medioarekin (**10. irudia**). Medioa bi egunean behin aldatu zen eta 8. egunean, zelulak triglizerido-edukia kuantifikatzeko eta zelulen bideragarritasuna aztertzeko erabili ziren, kolorimetrian oinarritutako kit komertzial baten bidez eta *crystal violet* metodoarekin hurrenez hurren. Ondoren, beste esperimentu bat burutu zen triglizerido-edukiaren murrizketa handiena eragin zuen dosia bakarrik erabilita. Diferentziazioa hasi eta 8. egunera, adipogenesia erregulatzen duten transkripzio-faktoreen eta adipozito helduen markatzaile gisa erabiltzen diren gene eta proteinen espresioa neurtu zen Real-Time PCR eta Western Blot bidez hurrenez hurren.



10. irudia. 3T3-L1 aurre-adipozitoetan egindako ikerlanaren diseinu esperimentalaren eskema orokorra. DEXA: dexametasona, DMEM: Dulbecco's Modified Eagle Medium, FBS: behi-fetuaren seruma, IBMX: isobutilmetilxantina P/S: penizilina/estreptomizina.

Bestetik, alga-estraktuek adipozitoen helduen lipido-metaketan zuten efektua aztertzeko, 3T3-L1 fibroblastoak, aurretik azaldutako protokolo berbera jarraituta diferentziatu ziren 12. egunera arte. 12. egunean alga-estraktuen dosi berdinak erabiliz tratatu ziren zelulak. 24 orduren ostean (13. eguna), zelulak triglizerido-edukia kuantifikatzeko eta zelulen bideragarritasuna analizatzeko jaso ziren, aurretik deskribatu den bezala (**11. irudia**).



11. irudia. 3T3-L1 adipozito helduetan egindako ikerlanaren diseinu esperimentalaren eskema orokorra. DEXA: dexametasona, DMEM: Dulbecco's Modified Eagle Medium, FBS: behi-fetuaren seruma, IBMX: isobutilmetilxantina P/S: penizilina/estreptomizina.

Erabilitako metodo eta protokolo zehatzak 3. eskuizkribuan aurki daitezke (ikusi 3. atala).

2.3. *In vivo* ikerlana

Ikerlan hau egiteko, Zucker arratoia, genetikoki sindrome metabolikoa garatzen duen animalia-eredua dena, erabili zen. Animalia hauek mutazio autosomiko azpirakor bat daukate leptinaren hartzailean (*fa* aleloa). Mutazio hau eredu honen fenotipoaren oinarri molekularra da. *fa* alelorako homozigotoak diren animaliek (*fa/fa* Zucker arratoiek) obesitate nabarmena garatzen dute bizitzako lehen 3-5 asteetan. Gertaera honen aurretik, hiperfagia eta hiperintsulinemia nabarmentzen dira, baina ez dute hipergluzemia garatzen. Horrez gain, Zucker arratoi obesoek hiperlipidemia eta gibel-esteatosia garatzen dituzte (109–111).

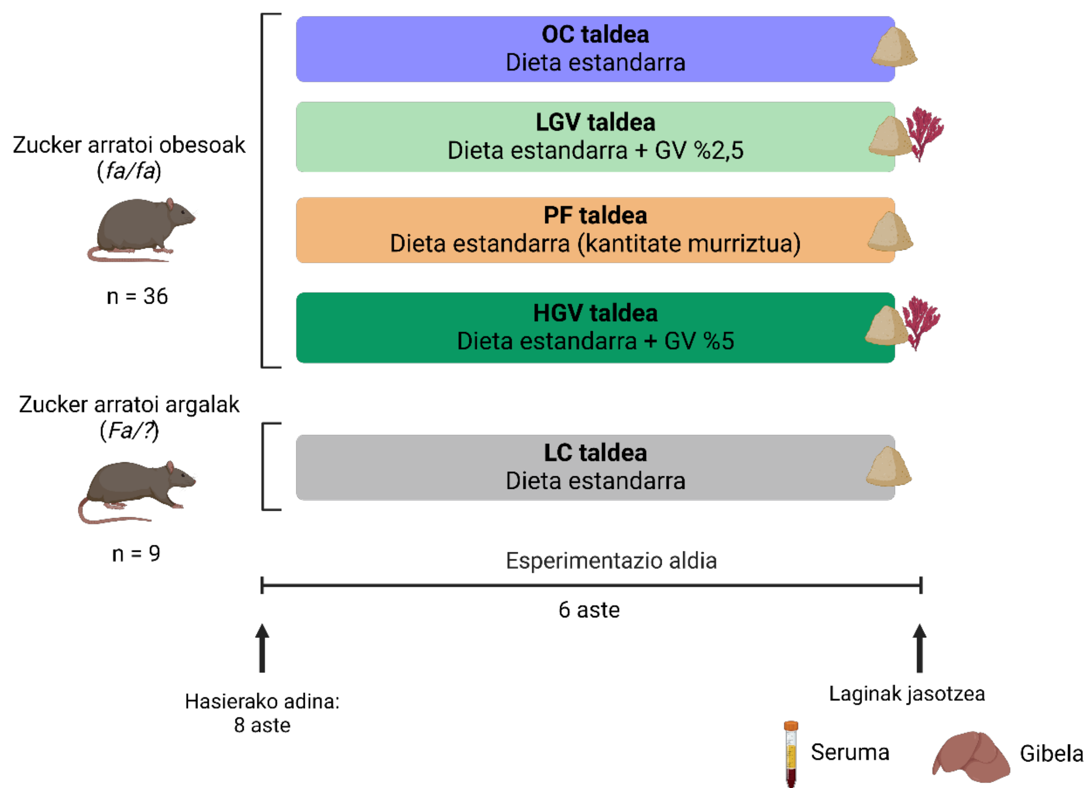
Esperimentu honetarako, arratoien dieta *Gracilaria vermiculophylla* makroalgarekin osatu zen, Porto Muiños S. M.-k (Cambre, Espainia) Iberiar kostako ipar-mendebaldean jaso zuena. Makroalgaren konposizio makroskopikoa Laber Laboratories-ek (Corporación Laber, Laboratorio y Consultoría, S.M., Santiago de Compostela, Espainia) analizatu zuen (**2. taula**).

2. taula. *Gracilaria vermiculophylla*-ren konposizioa.

	Gracilaria vermiculophylla
Energia (kcal/100 g)	182
Lipidoak (g/100 g)	1.40
Gantz-azido aseak (g/100 g)	0.98
Karbohidratoak (g/100 g)	6.40
Karbohidrato sinpleak (g/100 g)	< 0.50
Proteinak (g/100 g)	22.10
Zuntza (g/100 g)	27.90
Errautsak (g/100 g)	28.8
Hezetasuna (g/100 g)	13.4
Polifenolen eduki osoa (mg/kg)	1180

Doktoretza-tesi honen azken atal honetako diseinu esperimentalean zortzi asteko 36 Zucker (*fa/fa*) arratoi ar eta 9 Zucker (*Fa/?*) arratoi ar erabili ziren (Charles River Laboratories, Lyon, Frantzia) (**12. irudia**). Esperimentua, Euskal Herriko Unibertsitateko animalia-esperimentaziorako etika batzordeak zehaztutako protokoloa (M20_2021_214) jarraituz gauzatu zen. Sei eguneko egokitze-aldi baten ostean, hasiera batean animalia obesoak hiru esperimentazio taldetan banatu ziren: kontrol obeso taldea (OC), arratoi obesoz osatutako taldea dietan *Gracilaria vermiculophylla*-ren % 2,5 nahastuta (LGV) eta arratoi obesoz osatutako taldea dietan *Gracilaria vermiculophylla*-ren % 5 nahastuta (HGV). Esperimentua hasi eta denbora gutxira, HGV taldeak gutxiago jaten zuela behatu genuen, hori dela eta, *pair-fed* talde bat (PF) gehitu zen, oraindik talde esperimental batera esleitu gabe zeuden animaliak erabiliz. PF taldeari emandako dieta kantitatea HGV taldeak aurreko egunean jandako dieta kantitatearen arabera kalkulatu zen. Zucker arratoi argalak (*Fa/?)* kontrol osasuntsu (LC) gisa erabili ziren. Esperimentazio talde guztiak laborategiko dieta estandar berarekin elikatu ziren, makronutrienteen banaketa hurrengoa izanik: lipidoak % 4, proteinak % 14,5 eta karbohidratoak % 48 (2014 Global diet, Envigo-Mucedola SRL, Milan, Italia). Animaliak polikarbonatozko kaioletan mantendu ziren binaka, aire girotua (22 ± 2°C) eta 12 orduko argitasun/iluntasun zikloa zituen gela kontrolatu batean. Talde guztiek, PF taldeak izan ezik, nahi adina dieta jan eta ur edan zezaketen esperimentuaren iraupen osoan zehar. Animalien gorputz-pisua eta hauek jandako dieta kantitatea egunero neurtu ziren.

Esperimentazio aldi osoaren amaieran (sei aste), animalia guztiak 12 orduko barauaren ondoren hil ziren anestesiapean (kloral hidratoa) bihotzetik odolustuz. Seruma jasotako odol laginetatik lortu zen zentrifugazioaren ondoren (1000 *g*, 10 minutu, 4 ºC-tan). Gibelak atera eta pisatu ostean, nitrogeno likidotan berehala izoztu ziren. Animalia bakoitzaren gibelaren lobulu handienaren zati bat hartu zen lipido guztien kuantifikaziorako.



12. irudia. *In vivo* ikerlanaren diseinu esperimentalaren eskema orokorra. GV: *Gracilaria vermiculophylla*, HGV: *Gracilaria vermiculophylla* dietaren % 5, LGV: *Gracilaria vermiculophylla dietaren* % 2,5, LC: control osasuntsua, OC: control obesoa, PF: *pair-fed*.

Serumeko parametro biokimikoei dagokienez, gibeleko triglizeridoak, gibeleko EGGAk, gibeleko lipidoen metabolismoarekin zerikusia duten entzimen jarduerak eta oxidazio estresaren markatzaileak metodo espektrofotometrikoak erabiliz neurtu ziren. Gibeleko lipido guztien kuantifikazioa Oil Red O tindaketaren bidez burutu zen. Gibelean baita ere, lipidoen metabolismoan parte hartzen duten entzima eta proteinen adierazpena eta mitokondrioen biosintesiarekin erlazionatutako proteinen espresioa neurtu zen Western Blot bidez. Inflamazioaren eta fibrosiaren garapenarekin erlazionatutako markatzaileak Real-Time PCR bidez neurtu ziren.

Erabilitako metodo eta protokolo zehatzak 4. eskuizkribuan aurki daitezke (ikusi 3. atala).

2.4. Analisi estatistikoa

Datuen analisi estatistikoa IBM® SPSS® Statistics 24.0 softwarea erabiliz egin zen. Datuen banaketa normala Shapiro-Wilks testaren bidez ebaluatu zen. *In vitro* egindako ikerlanen kasuan, Student-en t testa erabili zen bi talderen arteko desberdintasun esanguratsuak detektatzeko; eta *in vivo* egindako ikerlanaren kasuan aldiz, ANOVA eta Newman-Keuls *post-hoc* testa erabili ziren hainbat talderen arteko konparaketak egiteko. Emaitzak batezbestekoa ± batezbestekoaren errore estandarra bezala aurkeztuta daude. Esanguratasuna $p < 0.05$ mailan balioztatu zen.

3. Hipotesia eta helburuak

Doktoretza-tesi honen sarreran azaldu den bezala, GGEAren prebalentzia nahiko altua da. Patologia honen tratamendua energia-murrizketan eta jarduera fisikoan oinarritzen da, gorputz-pisu egokia lortzeko helburuarekin. Aldi berean, patologia horri lotutako komorbilitateen kudeaketa egiten da. Hala ere, kasu gehienetan, tratamendu horiek ez dira behar bezain eraginkorrak eta gainera, gaixotasun horren tratamendura bideratutako farmako eraginkorrik ez dagoenez, tresna berrien bilaketan sakondu beharra dago. Hori dela eta, komunitate zientifikoa GGEAren prebentzio eta tratamendura bideraturako konposatu berrien bila ari da. Egoera horretan, algak jarduera biologikoa eta osasuna sustatzeko gaitasuna duten baina oraindik erabat ustiatu ez diren konposatu baliotsuen iturri garrantzitsu modura agertu dira.

Testuinguru honetan, proposatutako hipotesia hurrengoa da: *Chlorella vulgaris*, *Nannochloropsis gaditana* eta *Gracilaria vermiculophylla* algak erabilgarriak izango direla GGEAren kudeaketan. Hipotesi hori frogatzeko edo baztertzeko, Doktoretza-tesi honen helburua bi mikroalgek (*Chlorella vulgaris* eta *Nannochloropsis gaditana*) eta makroalga batek (*Gracilaria vermiculophylla*) GGEAren prebentzioan eta tratamenduan izan dezaketen erabilgarritasuna aztertzea da.

Helburu nagusi hori lortzeko, lau hurbilketa ezberdin proposatu ziren. Lehenengoa, bilaketa bibliografiko batean oinarritu zen, algek GGEAaren tratamenduan edota prebentzioan izan dezaketen eraginkortasuna ezagutzeko. Hurrengo bi hurbilketak *in vitro* ereduak erabilita (hepatozitoak eta adipozitoak) gauzatu ziren, gibel-esteatosia eta harekin erlazionatutako komorbilitateak ikertzeko. Azkenik, laugarren hurbilketa genetikoki esteatosia garatzen duen *in vivo* eredu batean gauzatu zen.

Doktoretza Tesi honen helburua lortzeko, honako helburu espezifiko hauek proposatu ziren:

Bilaketa bibliografikoa

1. Mikroalgen eta makroalgen estraktuek GGEAn duten eragin onuragarrien eta efektu horietan inplikatutako mekanismoei buruzko ebidentzia zientifikoa biltzea (1. eskuizkribua).

***In vitro* ikerlana AML12 sagu hepatozitoetan**

2. *Chlorella vulgaris* eta *Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik lortutako estraktuek AML12 sagu hepatozitoetan lipido metaketan duten efektua aztertzea eta ekintza-mekanismo potentzialak identifikatzea (2. eskuizkribua).

***In vitro* ikerlana 3T3-L1 sagu adipozitoetan**

3. *Chlorella vulgaris* eta *Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik lortutako estraktuek 3T3-L1 sagu adipozito helduetan lipido metaketarengan duten efektua aztertzea eta ekintza-mekanismo potentzialak identifikatzea (3. eskuizkribua).

4. *Chlorella vulgaris* eta *Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik lortutako estraktuek 3T3-L1 sagu aurre-adipozitoen diferentziazioan duten efektua aztertzea eta ekintza-mekanismo potentzialak identifikatzea (3. eskuizkribua).

In vivo ikerlana sindrome metabolikoaren eredu batean (Zucker _fa/fa_ arratoiak)

5. *Gracilaria vermiculophylla*-k gibeleko lipidoen metaketan duen eragina aztertzea, baita efektu horren erantzulea izan daitekeen ekintza-mekanismoa ikertzea ere (4. eskuizkribua).

6. *Gracilaria vermiculophylla*-k gibeleko oxidazio estresarengan duen efektua analizatzea (4. eskuizkribua).

7. *Gracilaria vermiculophylla*-k gibeleko inflamazioan eta fibrosian duen eragina aztertzea (4. eskuizkribua).

4. Emaitzak eta eztabaida

Doktoretza-tesi honen sarreran aipatu den bezala, GGEAren prebalentzia gero eta handiagoak eta tratamendu eraginkorren faltak, komunitate zientifikoa gaixotasun honen prebentzio edota tratamendurako eraginkorrak izan daitezkeen molekula, estraktu edo produktu natural berriak bilatzera bultzatu du.

Ildo horretan, Nutrizioa eta Obesitatea ikertaldeak, María Puy Portillo katedradunak zuzendutakoak, esperientzia handia dauka dietako konposatu desberdinek GGEAn eta obesitatean dauzkaten efektuak ikertzen. Doktoretza-tesi hau ikerketa-lerro horren barnean planteatzen da.

Doktoretza-tesi hau garatzeko lau ikerlan egin ziren. Lehenik, berrikuspen bibliografiko bat egin zen, alga-estraktuak GGEAren tratamenduan edo prebentzioan eraginkorrak izan zitezkeen jakiteko. Ondoren, *in vitro* ereduak erabiliz bi ikerketa egin ziren, alga-estraktuek hepatozito eta adipozitoetan lipido-metaketan zeukaten efektua aztertzeko helburuarekin. Bi ikerketa hauek AZTI ikerketa- eta teknologia-zentroarekin batera egin ziren, Eusko Jaurlaritzak finantzatutako ALGABERRI proiektuaren barruan. Horrez gain, Madrilgo CIAL ikerketa institutuko Isidra Recio doktoreak zuzendutako ikerketa taldeak, alga-estraktuen proteina-frakzioen karakterizazioan parte hartu zuen. Azkenik, doktoretza-tesi honen azken atala burutzeko, genetikoki sindrome metabolikoa garatzen duen arratoi-eredu bat erabili zen, alga osoaren ingestioak gibel-esteatosian izan dezakeen efektua aztertzeko. Atal honetan CICbioGUNEko Malu Martínez-Chantar doktorearen taldeak parte hartu zuen.

"Nazioarteko doktorea" aipamena lortzeko gutxienez hiru hilabeteko iraupena duen egonaldia burutu behar da Espainiar estatutik kanpoko goi-mailako irakaskuntzako erakunde batean edo ikerketa-zentro batean. Kasu honetan aukeratutako erakundea Municheko (Alemania) Helmholtz Diabetes Center ikerketa-zentroko Institute for Diabetes and Obesity izan zen, bere jarduera obesitatean zelulen funtzioa eta hari lotutako asaldura metabolikoak aztertzera zuzenduta bitago. Egonaldi honi esker, obesitatearen eta gaixotasun metabolikoen, diabetesa kasu, ikerketen inguruko ezagutza zabaltzeko aukera izan nuen, eta adipozitoen zelula primarioen kultiboekin lan egiten eta histologia eta biologia molekularreko teknikak ikasi ahal izan nituen. Teknika hauek Doktoretza-tesi honetan eta Nutrizioa eta Obesitatea ikertaldean ere oraindik zuzenean aplikatu ez badira ere, etorkizunean egitea espero da. Hala ere, egonaldi honetan jasotako esperientzia guztiak nire ikerketa-ibilbidean lagundu dit.

Aurrerako esan bezala, Doktoretza-tesi hau Eusko Jaurlaritzak finantzatutako (ELKARTEK programa) ALGABERRI proiektuaren barruan sortu zen "Obtaining new ingredients and active principles from algae with health applications" izenekoa. Proiektu honetan, Nutrizio eta Obesitatea ikertaldearen eginkizuna makroalga-estraktu baten eta bi mikroalga-estrakturen jarduera biologikoa

aztertzea zen, bai GGEAn bai eta obesitatean ere. Proiektuaren lehenengo fasean, AZTIk proteina eta peptidoak erauzteko protokoloak garatu zituen *Chlorella vulgaris Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik abiatuta. Gero, liofilizatutako estraktuak erabili ziren haien jarduera biologikoa *in vitro* aztertzeko, hepatozito zein adipozitoetan. Horretarako, lau kontzentrazio ezberdin aukeratu genituen, 10 eta 150 µg/mL artean, horixe baita alga-estraktuen efektuak aztertzeko erabiltzen den kontzentrazio-tarte ohikoena (112–116). Azkenik, *in vitro* egindako esperimentu horietatik lortutako emaitzetan oinarrituta, *Gracilaria vermiculophylla* makroalga aukeratu zen haren eragina Zucker (*fa/fa*) arratoi obesoetan aztertzeko. Kasu honetan, estraktuak erabili beharrean, deshidratatutako alga osoa erabili zen.

4.1. Bilaketa bibliografikoa

Algak oraindik gutxi aztertutako molekula bioaktiboen iturri dira, lipidoen metabolismoa modulatzeko gaitasuna izan dezaketenak (117–120). Hori kontuan izanda eta GGEAk lipidoen metabolismoaren asaldurekin lotura estua duen osasun-arazo handia dela kontuan hartuta, lehenik, bilaketa bibliografiko bat egin zen mikroalga- eta makroalga-estraktuek gibel-esteatosian duten efektuen inguruan, tresna eraginkorrak izan zitezkeen jakiteko.

Bilaketaren ostean, bikoiztutako artikuluak, berrikuspen bibliografikoak, konposatu isolatuak erabiltzen zituzten ikerlanak eta algen efektua zuzenean GGEAn ikertzen ez zituzten artikuluak baztertu egin ziren. Alga-estraktuekin *in vitro* egindako ikerketarik ez zen aurkitu.

Animaliekin egindako ikerketa guztiek dietaren bidez lortutako esteatosi-ereduak erabili zituzten, batez ere, gantz edo/eta karbohidrato ugarikoak, bai sagu zein arratoietan. Salbuespen modura, artikuluetako batek lipopolisakaridoen injekzioak induzitutako esteatosi-eredu bat erabili zuen. Horrez gain, diseinu esperimentalari dagokionez, aurkitutako artikulu gehienek prebentzio mailan ikertu zuten algekin osatutako dietaren efektua, alga GGEA induzitzeko erabili zen dietarekin batera eman baitzitzaien animaliei.

Berrikuspenean barneratutako lau artikuluk mikroalgak erabili zituzten. Artikulu bakoitzak mikroalga espezie ezberdin bat erabili zuen: *Scenedesmus dimorphus* eta *Schroederiella apiculate* arteko nahasketa, *Euglena gracilis*, *Spirulina platensis* eta *Tisochrysis lutea.* Lau artikulu hauetatik, bik bakarrik ikusi zuten triglizerido-mailaren murrizketa animalien gibelean, baina artikulu batek ere ez zuen efektu honen ekintza-mekanismorik aztertu. Gibeleko gantz edukiaz gain, lau artikulu hauek GGEArekin erlazionatutako beste asaldura batzuk ere aztertu zituzten. Hala, gibeleko inflamazioari dagokionez, hiru artikuluk inflamazio-zelulen infiltrazioa, inflamazioa sustatzen duten makrofagoen markatzaileak edo inflamazioa eragiten duten zitokinen adierazpena neurtu zuten, eta kasu guztietan efektu positiboak ikusi zituzten alga hartu zuten animalietan. Horrez gain, bi artikuluk, mikroalgen efektua aztertu zuten gibeleko fibrosian, baina bietako batek soilik ikusi zituen efektu onuragarriak,

kolagenoaren deposizio mailaren murrizketak adierazi zuen bezala. Hiru artikulutan, mikroalgen efektua gibel-esteatosiaren prebentzioan aztertu zen, mikroalga esteatosia eragiten duen dietarekin batera eman zitzaielako esperimentazio-aldi osoan zehar. Hala ere, artikuluetako batean, ikuspegi ezberdin bat erabili zen, non esteatosia eragiten zuen dieta esperimentazio-aldi osoan zehar eman zitzaien arren, mikroalga esperimentazio-aldiaren bigarren zatian soilik hartzen zuten.

Makroalgek GGEAn duten efektua ikertzen zuten hamabost artikulu aurkitu ziren, horietako hiruk makroalga berdeak erabili zituzten (*Caulerpa lentillifera* eta *Ulva prolifera*), seik makroalga gorriak (*Plocamium telfairiae*, *Palmaria mollis*, *Sarconema filiforme*, *Grateloupia elliptica* eta *Gromphadorhina oblongata*) and beste seik makroalga arreak (*Undaria pinnatifida*, *Fucus vesiculosus* eta *Ascophyllum nodosum* arteko nahasketa, *Sargassum thunbergii* eta *Sargassum horneri*). Kasu honetan, mikroalgen kasuan ez bezala, makroalga espezie berbera artikulu batean baino gehiagotan aztertu zen. Ikertutako makroalga guztiek dietaren bidez eragindako gibel-esteatosiaren garapena prebenitu zuten. Hala ere, artikulu gehienetan ez ziren aztertu gibeleko lipido-mailaren murrizketaren erantzule izan zitezkeen ekintza-mekanismoak. Mekanismo hauek aztertu zituzten ikerlanek lipogenesiaren murrizketa eta gantz-azidoen oxidazioaren handitzea ikusi zuten. Mikroalgekin egindako ikerlanekin gertatzen zen bezala, kasu honetan ere, makroalgek inflamazioarekin loturiko markatzaileak hobetu zituzten animalien gibeletan.

Makroalgen efektua aztertu zuten artikuluen artean, hamahiruk makroalgen eragina prebentzio mailan ikertu zuten, izan ere, alga esperimentuak irauten zuen aldi osoan zehar eman zitzaien animaliei. Bestalde, bi ikerlanek prebentzio eta tratamenduaren arteko interbentzio-eredu mistoa erabili zuten, non animaliek dieta berdina jaso zuten esperimentazio-aldi osoan zehar, baina makroalga bigarren erdian baino ez zuten hartu. Berrikuspenean jasotako ikerketa bakar batek ere ez zuen aztertu makroalgekin osatutako dietaren efektua gibel-esteatosiaren tratamendu mailan.

Esan beharra dago desberdintasun nabarmenak daudela berrikuspen bibliografikoan sartutako ikerlanek aukeratutako diseinu esperimentalen artean. Alderdi horietako bat jadanik eztabaidatu da, esku-hartze motari dagokiona alegia (prebentzioa edo eredu-mistoa). Animaliek jasotako algaren dosiari dagokionez, ikusitako efektu onuragarriak dosi nahiko ezberdinekin lortu ziren, 7,5 eta 3000 mg/kg gorputz-pisu/egun artekoak edo dietan % 1etik % 12ra bitartekoak. Era berean, ikerlanen esperimentazio-aldiak nahiko desberdinak ziren, zeinak 3 eta 16 aste bitartartekoak izan zitezkeen. Gainera, kontuan hartu behar da hainbat faktorek algen konposizioan eragiten dutela, hala nola, nutrienteen eskuragarritasunak, tenperaturak, argiaren intentsitateak, pH-ak, gazitasunak eta espezieen arteko aldakortasun genetikoak (121). Egoera horren aurrean, zaila da algek eta alga-estraktuek haien efektu onuragarriak zain baldintzatan eragiten dituzten determinatzea.

Animalia-ereduekin egindako ikerlanen kopurua gorabehera, algekin egindako entsegu klinikoak oso urriak dira oraindik ere, eta egindakoetan, ez dituzte ekintza-mekanismoak aztertzen. Berrikuspen honetan gizakietan egindako bi ikerlan jaso ziren, bakoitzak esku-hartze ezberdina jarraitu zuelarik.

Ebrahimi-Mameghanik eta kolaboratzaileek itsu bikoitzeko, aleatorizatutako eta plazeboarekin kontrolatutako entsegu kliniko bat burutu zuten. Zortzi astetan zehar *Chlorella vulgaris*-en 1200 mg/eguneko dosia jaso zuen interbentzio taldeak serumeko parametroen hobekuntza erakutsi zuen (122). Lik eta kolaboratzaileek ordea, ikerketa obserbazional bat burutu zuten Txinako biztanlerian, zeinean algen kontsumoa GGEArekin negatiboki erlazionatzen zen, batez ere obesitatea ez zeukaten pertsonetan (78).

Berrikuspen hau argitaratu ostean, ikerlan berriak publikatu dira. Horren harira, mikroalga-estraktuekin tratatutako HepG2 zeluletan lipidoen metaketa arintzen dela eta GGEAren karraskari-ereduetan gibeleko gantz-metaketa murrizten dela ikusi da (91,123). Makroalgen eragina aztertzen duten zenbait ikerlan ere argitaratu dira eta gibel-esteatosia hobetzeko eta gibeleko funtzioarekin erlazionatutako serum testak (ALT, aspartato aminotranferasa (AST) eta fosfatasa alkalinoa (ALP)) hobetzeko ahalmena dutela ikusi da, bai sagu zein arratoietan (124–129). Gure berrikuspenean gertatu bezala, aipatu berri diren ikerketa guzti hauek ez dituzte gibeleko gantz-metaketan ikusitako murrizketaren eragile diren ekintza-mekanismoak aztertzen. Hala ere, mekanismoak aztertzen dituzten horietan, gibeleko prozesu lipogenikoekin erlazionatutako geneen modulazioa ematen zela ikusi zen. Horrez gain, berrikuspen sistematiko batean, GGEA duten pazienteetan dieta algekin osatzeak gibeleko lesioak hobetu ditzakeela ikusi zen (130).

Laburbilduz, mikroalgek zein makroalgek GGEAn duten efektuei buruzko literatura berrikusi ondoren, esan daiteke emaitzak itxaropentsuak direla. Hala ere, oraindik ere oso ikerketa gutxi argitaratu dira, alga espezie gehienak ikerlan bakar batean baino ez dira aztertu, ekintza-mekanismoak ez dira oso sakonki aztertu eta gainera, lan gehienak karraskari ereduak erabilita egin dira (prebentzio mailan). Egoera horren aurrean, esan daiteke, gizakietan burututako ikerketa gehiago behar direla lortutako emaitzak baieztatzeko, eta orokorrean, algek GGEAn duten eragina ebaluatzeko.

4.2. *In vitro* ikerlana AML12 sagu hepatozitoetan

Lehendabiziko *in vitro* egindako esperimentu honetan, *Chlorella vulgaris* eta *Nannochloropsis gaditana* mikroalgetatik eta *Gracilaria vermiculophylla* makroalgatik lortutako estraktuek lipidoen metaketa prebenitzeko duten eragina determinatu zen AML12 hepatozitoetan. Hepatozitoak azido palmitikoarekin inkubatu ziren gibel-esteatosia imitatzen duen zelula eredua sortzeko. Zelulak tratatzeko erabilitako alga-estraktuen kontzentrazioak 10, 25, 50 eta 150 µg/mL-koak izan ziren. Gainera, triglizeridoen metaketan ikusitako aldaketen erantzule izan zitezkeen mekanismoak aztertu ziren.

Hiru alga-estraktuen karakterizazioaren arabera, haien proteina edukia % 41 eta % 48 bitartekoa zen. Gainera, proteinen eta peptidoen pisu molekularraren banaketa aztertu zenean, mikroalgen estraktuen kasuan banda oso nabarmenak ikus zitezkeen. *Chlorella vulgaris*-i dagokionez, banda intentsoena 20-

25 kDa-eko eremuan antzeman zen. 2 kDa-etik beherako kolorezko bandak ere detektatu ziren, ziurrenik proteina eta pigmentuen arteko elkarreraginaren ondorioz. *Nannochloropsis gadintana*-ri dagokionez, 37, 50 eta 100 kDa-eko bandak detektatu ziren. *Gracilaria vermiculophylla*-ren kasuan, ezin izan zen proteina-bandarik detektatu. Gertaera hau estraktuaren disolbagarritasun baxuaren ondorioz eta polisakarido eduki handiaren ondorioz izan daiteke. Emaitza hauek ikusita, AML12 hepatozitoetan hautemandako efektu positiboa estraktuetan dauden proteinei eta peptidoei egotz dakiekeela hipotetizatu liteke. Zehazki, *Chlorella vulgaris* eta *Nannokloropsis* sp-tik eratorritako peptidoek jarduera biologikoa dutela ikusi da (131,132).

Proteina eta peptido bioaktiboez gain, ikerlan honetan erabilitako estraktuek konposatu fenolikoak ere badituzte. Zenbait artikulutan deskribatu da polifenolek erakarpen handia dutela proteinekiko, eta, ondorioz, aldi berean erauzi egiten dira, haien eragin positiboa areagotu daitekeelarik (72).

Hepatozitoak alga-estraktuekin eta azido palmitikoarekin batera inkubatu ostean, ez zen zelulen bideragarritasunaren murrizketarik ikusi. Emaitza horren arabera, esan daiteke alga-estraktuak ez direla zitotoxikoak, behintzat frogatutako dosi hauetan. Lipidoen metaketari dagokionez, hiru alga estraktuek triglizeridoen metaketa murriztu ahal izan zuten. *Chlorella vulgaris* estraktuaren kasuan, triglizerido-edukiaren murrizketa ikusi zen bi dosi altuenekin (50 eta 150 µg/mL). Aitzitik, *Nannochloropsis gaditana*-k, bi dosi baxuenekin (10 eta 25 µg/mL) tratatutako hepatozitoetan izan zuen efekturik handiena. *Gracilaria vermiculophylla*-ren kasuan, triglizerido-edukiaren murrizketa 25, 50 eta 150 µg/mL-ko dosiekin inkubatutako hepatozitoetan ikusi zen, dosi-erantzun ereduari jarraituz. Hala, *Gracilaria vermiculophylla* bi mikroalgak baino dosi gehiagotan izan zen eraginkorra.

Triglizerido-edukiaren murrizketari azalpena emateko, lipidoen metabolismoko zenbait bide aztertu ziren. Determinazio hauek egiteko, triglizerido-murrizketa handiena eragin zuen alga-estraktu bakoitzaren dosia bakarrik erabili zen: 150 µg/mL *Chlorella vulgaris* eta *Gracilaria vermiculophylla*-ren kasuan eta 25 µg/mL *Nannochloropsis gaditana*-ren kasuan.

Gibela oso organo lipogenikoa da, hori dela eta, lipogenesiko entzima nagusien gene-espresioa aztertu zen (ACC eta FAS). Erabilitako tratamenduetako batek ere ez zuen gene hauen espresioa aldatu, beraz, *de novo* lipogenesia hepatozitoetan behatutako triglizerido-murrizketan inplikatuta ez dagoela adierazi daiteke. Inkubazio medioan gehitutako azido palmitikoak triglizeridoen sintesia faboratu zuen. Zentzu honetan, FATP2 proteinaren espresioa neurtu zen eta ez zen aldaketarik ikusi tratamenduen ostean. Triglizeridoen sintesi prozesuan, 3 gantz-azidoren eta G3P molekula baten arteko esterifikazioa ematen da. DGAT2 triglizeridoen esterifikazioaren azken pausuan parte hartzen duen entzima da. Bere espresio genikoa neurtu zenean, ez zen aldaketarik ikusi hepatozitoak alga-estraktuekin tratatu ondoren. Hortaz, emaitza horiek adierazten dutenez, alga-estraktuek ez zuten lipidoen murrizketa eragin triglizeridoen metaketaren alde egiten duten bide metabolikoak modulatuz. Hori dela eta, triglizerido-edukiaren murrizketarekin eta gantz-azidoen oxidazioarekin zerikusia zuten bide metabolikoak aztertu ziren.

Gibeleko triglizeridoak VLDL partikulen baitan jariatu daitezke eta MTP entzimak partikula horien eraketan parte hartzen du. Ikerlan honetan frogatutako tratamenduen artean, soilik *Chlorella vulgaris*-ekin tratatutako zeluletan ikusi zen MTP proteinaren espresioaren igoera, beraz, triglizeridoen jariapen handiago batek azaldu zezakeen tratamendu hau jaso zuten hepatozitoetan behatutako lipidoen murrizketa.

Gantz-azidoen oxidazioari dagokionez, CPT-1 mitokondrioetan β-oxidaziorako kate luzeko azil-Ako molekulak barneratzeaz arduratzen entzima mugatzailea eta azil-Ako deshidrogenasa (ACADL), β-oxidazioko lehenengo pausuan parte hartzen duen entzima, neurtu ziren. Hiru tratamenduek *Cpt-1a*-ren espresio genikoa murriztea lortu zuten bitartean, *Acadl*-ren mRNA-ren mailak *Gracilaria vermiculophylla*-k baino ez zituen murriztu. Emaitza horien arabera, triglizerido-mailen jaitsiera mitokondrioetako gantz-azidoen oxidazioa handitzearen ondorioz izan daiteke, neurri batean behintzat. Gantz-azidoen oxidazioaren inguruan lortutako emaitza positiboak zirela eta, efektu honek mitokondriogenesiarekin zerikusia zuen ala ez aztertu nahi izan zen. Horretarako, mitokondrietako A transkripzio faktorea (*Tfam*) eta zitrato sintasaren (*Cs*) espresio genikoa neurtu zen, mitokondriogenesiaren eta mitokondrioen dentsitatearen adierazle gisa, hurrenez hurren. *Gracilaria vermiculophylla*-rekin tratatutako zeluletan *Tfam*-en % 145eko igoera ez esanguratsua behatu zen arren, tratamenduetako bakar batek ere ez zuen aldaketa esanguratsurik eragin gene horren espresioan.

Azkenik, tratamendu-aldiaren bukaeran jasotako inkubazio medioan ALT kontzentrazioa neurtu zen, gibeleko disfuntzioaren markatzaile gisa erabiltzen baita (133). *Chlorella vulgaris* eta *Gracilaria vermiculophylla* espezieek % 69 eta % 54 murriztu zituzten ALT mailak, hurrenez hurren, estraktu horiek hepatozitoetan babes-efektua eragin zezaketela aditzera emanez.

Oro har, lortutako emaitzek aditzera ematen dute alga-estraktu bakoitzak hepatozitoetako lipido-metaketari aurre egiteko gaitasun desberdina duela, eta erabilitako ekintza-mekanismoak desberdinak direla. *Chlorella vulgaris*-ek 50 eta 150 µg/mL-ko kontzentrazioetan erabiltzen zenean erakutsi zuen lipidoen metaketa murrizteko gaitasuna, bi kasuetan murrizketa hau antzekoa izanik (% 22 eta % 24 hurrenez hurren). Mikroalga honek hepatozitoen triglizerido jariapena handituz eta gantz-azidoen oxidazioa areagotuz jarduten duela dirudi. Aitzitik, *Nannochloropsis gaditana*-ren kasuan, 10 eta 25 µg/mL-ko dosiek eragin zuten triglizerido metaketaren jaitsiera, % 27 eta % 34 hurrenez hurren. Fenomeno honi, non dosi txikiagoek dosi handiagoek baino aktibitate handiagoa erakusten duten, hormesia deritzo, eta adibidez, konposatu fenolikoak bezalako fitokimikoekin egindako ikerketetan ikusi ohi da (134–136). *Nannochloropsis gaditana*-k gantz-azidoen oxidazioa handituz jardun zuen. *Gracilaria vermiculophylla*-ri dagokionez, triglizerido-edukiaren murrizketa 25, 50 eta 150 µg/mL-ko kontzentrazioak erabiltzean behatu zen, dosi-erantzunaren fenomenoari jarraituz. Kasu honetan, murrizketa-ehunekoak % 18, % 22 eta % 24 izan ziren, hurrenez hurren. Antza denez, makroalga honek gantz azido oxidazioa ere areagotuz jardun zuen.

Beraz, ondoriozta daiteke, baldintza esperimental hauetan, erabilitako alga-estraktuak gai direla hepatozitoetan azido palmitikoak induzitutako lipido-metaketa partzialki prebenitzeko, batez ere, gantz-azidoen oxidazioa estimulatuz. Hala ere, oraindik ikerketa gehiago behar dira animalia-ereduetan aurkikuntza hauek egiaztatzeko.

4.3. *In vitro* ikerlana 3T3-L1 sagu adipozitoetan

Gorago azaldu den bezala, obesitateak GGEA izateko sentikortasuna areagotzen du. Hori horrela, *in vitro* egindako bigarren ikerlan honetan hepatozitoetan frogatutako estraktu eta kontzentrazio berberak (10, 25, 50 eta 150 µg/mL) erabili genituen, baina kasu honetan adipozitoetan gantz-metaketa murrizteko gaitasuna ebaluatzeko. Hiperplasiak eta hipertrofiak gantz-ehunaren hedapenean duten eginkizuna kontuan hartuta, alga-estraktuak 3T3-L1 aurre-adipozito eta adipozito helduetan frogatu ziren.

Hepatozitoetan gertatu zen bezala, zelulen bideragarritasunak ez zuen asaldurarik jasan aurre-adipozitoak zein adipozito helduak aipatutako dosi eta alga-estraktuekin tratatu ostean. Adipogenesiari dagokionez, hiru estraktuek triglizerido-metaketa nabarmen murriztu zuten aurre-adipozitoak adipogenesiaren zortzi egunetan zehar tratatu zirenean. *Chlorella vulgaris* triglizerido-kantitatea murrizteko gai izan zen frogatutako lau kontzentrazioekin, dosi-erantzun patroi argi bat jarraituta (% 24, % 32, % 43 eta % 50). *Nannochloropsis gasitana*-ri dagokionez, dosi altuena soilik izan zen gai aurre-adipozitoetan triglizerido-edukiaren jaitsiera (- % 29) eragiteko. Azkenik, *Gracilaria vermiculophylla*-ren kasuan, 25, 50 eta 150 µg/mL-ko tratamenduek % 26, % 37 eta % 70 gutxitu zuten triglizerido-edukia, hurrenez hurren (dosi-erantzun eredua). Adipozitoen desberditzapena ematen ari den bitartean ikusitako eragin honek aditzera ematen du erabilitako alga-estraktuak adipogenesia partzialki inhibitzeko gai izan zirela.

Alga estraktuek adipogenesian zehar dituzten efektuak hobeto ezagutzeko asmoarekin, ekintza-mekanismoak aztertu genituen, triglizeridoen murrizketa handiena eragin zuen kontzentrazioa erabiliz, hiru kasuetan 150 µg/mL-ko dosia izanik.

Adipogenesia transkripzio-faktore ezberdinen sare oso koordinatu batek arautzen du. Laburbilduz, diferentziazioaren indukzioaren ostean, CCAAT-ra lotzen den β proteina indartzailea (C/EBPβ) aktibatuko da, adipogenesiaren hasierako funtsezko transkripzio-faktoreetako bat. SREBP-1c adipogenesiaren fase goiztiarrean aktibatzen den beste transkripzio-faktoreetako bat da, zeinak C/EBPβ-rekin batera peroxisomen ugalketak aktibatutako γ hartzailearen (PPARγ) eta CCAAT-ra lotzen den α proteina indartzailearen (C/EBPα) espresioa eragiten duen. Bi transkripzio faktore hauek adipogenesiaren erregulatzaile nagusitzat hartzen dira, eta behin aktibatuta, batak bestearen espresioa mantentzen du. Gainera, bi faktore hauen arteko elkarlanak, adipozito helduetan

espezifikoki adierazten diren geneen aktibazioa eragiten du, adipokinak edota entzima lipogeniko eta lipolitikoak kasu (137,138).

Ikerlan honetan, *Chlorella vulgaris*-ek PPARγ-ren espresio genikoa handitu bazuen ere, aldaketa hori ez zen proteina-espresioan islatu. Tratamendu honek C/EBPα-ren espresio genikoa eta proteikoa murriztu zuen. *Nannochlororpsis gaditana* estraktuari dagokionez, *Srebp1c* eta *Pparg* geneen adierazpenak murriztu zituen eta *Cebpb*-ren adierazpena gutxitzeko joera erakutsi zuen. Nahiz eta C/EBPβ eta PPARγ-ren proteina espresioek aldaketarik jaso es zuten tratamenduaren ostean, C/EBPα-ren adierazpena murriztu egin zen. *Gracilaria vermiculophylla*-ri dagokionez, tratamendu honek C/EBPβ-ren espresio geniko eta proteikoaren handitzea eragin zuen. Aitzitik, *Srebp1c*, *Pparg* eta *Cebpa* geneen espresioak murriztu egin zituen. Aldaketa horiek ez ziren proteina-espresioaren aldaketetan guztiz gauzatu, izan ere, C/EBPα proteinaren kasuan soilik eman zen murrizketa esan-guratsu bat. Esan beharra dago, PPARγ-ren proteina-espresioaren murrizketa estatistikoki esanguratsua izan ez baren ere, hura % 33 murriztu zela, emaitza aipagarria izanik. Lortutako emaitzek aditzera ematen dute, erabilitako alga-estraktuek adipogenesi-prozesua partzialki inhibitzeko gaitasuna duten arren, ez direla gai haien eragina adipogenesi-prozesu osoan zehar gauzatzeko. Gainera, estraktu bakoitzak transkripzio-faktore desberdinak erregulatzen dituen arren, badirudi, ekintza-modu komun bat C/EBPα-ren adierazpenaren murrizketaren bidez gertatzen dela.

Adipogenesiaren erregulazioan inplikatutako transkripzio-faktoreak aztertzeaz gain, adipozito helduaren fenotipoarekiko espezifikoak diren geneen eta proteinen espresio mailaren analisia egin genuen, eta horien artean, ACC eta adiponektina aztertu ziren. *Chlorella vulgaris*-ek ACC-ren espresio geniko eta proteikoaren murrizketa eragin zuen. Tratamendu honek adiponektinaren mRNA-ren mailak handitu bazituen ere, proteinaren espresioak ez zuen aldaketarik jasan. *Nannochloropsis gaditana*-ren estraktuak *Acc* genearen espresioa handitu zuen arren, proteina espresioa nabarmen jaitsi zen. Adiponektinari dagokionez, haren gene- eta proteina-espresioak murriztu egin ziren zelulak *Nannochloropsis gaditana*-rekin tratatu ostean. Azkenik, *Gracilaria vermiculophylla* estraktuak ACC-ren zein adiponektinaren gene- eta proteina-espresioaren murrizketa eragin zuen. Oro har, emaitza horiek aditzera ematen dute ikerlan honetan erabilitako estraktuak eraginkorrak direla adipozito helduen markatzaileak murrizteko, tratamendua diferentziazio-aldiaren zortzi egunetan zehar gehitzen denean. Emaitza hauek bat datoz triglizerido-mailaren murrizketarekin eta adipogenesia erregulatzen duten transkripzio-faktoreetan ikusitako emaitzekin. Gainera, ondoriozta daiteke erabilitako estraktuek gaitasuna dutela aurre-adipozitoen desberdintzapena oztopatzeko.

Guk dakigunez, hau da *Chlorella vulgaris*, *Nannochloropsis gaditana* eta *Gracilaria vermiculophylla* estraktuen adipogenesiaren aurkako efektua erakusten duen lan bakarra. Hala ere, literatura zientifikoan hainbat ikerketa aurki daitezke mikro- eta makroalga espezie ezberdinetatik lortutako alga-estraktuen gaitasun antiadipogenikoa erakusten dutenak (119,120,128,139,140).

Hepatozitoen atalean aipatu den bezala, ikerlan honetan ikusitako ondorio positiboak erabilitako estraktuetan aurki daitezkeen proteinei, peptidoei edota konposatu fenolikoei egotzi dakizkieke.

Estraktuak adipozito helduetan frogatu zirenean (adipogenesia induzitu eta 12. egunean), erabilitako dosi bakar batekin ere ez zen triglizerido-edukiaren murrizketarik hauteman. Hortaz, oraingo honetan, ez ziren aztertu estraktuek adipozito helduen lipidoen metabolismoarengan zituzten efektuak.

Adipozito helduen tamainaren handitzeari hipertrofia deritzo. Hiperplasian (adipozitoen kopurua handitzea) ez bezala, adipozitoen hipertrofia ondorio metaboliko negatiboekin erlazionatzen da. Ildo horretan, hiperplasiaren bidezko gantz-ehunaren hedapena, gantz-ehunaren disfuntzioaren eta obesitatearen konplikazioen aurkako babes-mekanismo bezala proposatu da (141,142). Hala ere, kontuan izan behar da gehiegizko lipidoak gantz-ehunean biltegiratu ezin direnean (adipogenesia murriztu delako edo hipertrofiatutako adipozitoek lipidoak biltegiratzeko gaitasuna gainditu dutelako), gantz ektopikoaren metaketa eman daitekeela, hau da, gantza gantz-ehuna ez den beste ehun batzuetan metatzea, hala nola, gibelean, muskulu eskeletikoan, bihotzean edo pankrean (29). Azken egoera horri dagokionez, gorago aipatu den bezala, doktoretza-tesi honetan erabilitako alga-estraktuek lipido-metaketa murrizteko gaitasuna erakutsi dute hepatozitoetan (143), gibelak gantza metatzetik babestu dezaketela iradokiz. Hala ere, ikerketa gehiago egin behar dira, batez ere, animalia-ereduetan, algen osagai ezberdinen efektuak eta horien ekintza-mekanismoak zeintzuk diren hobeto argitu ahal izateko.

Ikerlan honetan egindako aurkikuntzen arabera, ondorioztatu daiteke adipozito helduetan lipidoen metaketa murrizteko gaitasunik ez izan arren, erabilitako alga-estraktuek adipogenesia oztopatzeko ahalmena dutela. Horrek aditzera ematen du, estraktu horiek tresna baliagarriak izan daitezkeela gizentasunaren prebentzioan, bai eta horrekin erlazionatutako komorbilitateak hobetzeko ere, GGEA barne.

4.4. *In vivo* ikerlana sindrome metabolikoaren eredu batean (Zucker *fa/fa* arratoiak)

Doktoretza-tesi honen azken atala *in vivo* egindako ikerlanari dagokio. Aurretik deskribatutako *in vitro* ikerlanei esker, hepatozitoetan eta adipozitoetan hiru alga estraktuek lipidoen metaketan duten eragina aztertu ahal izan genuen. Hala ere, kontuan hartu beharreko alderdi garrantzitsu bat hauxe da, estraktuen eraginkortasuna haien bioerabilgarritasunaren menpe dagoela neurri batean (144). Ondorioz, animalia ereduetan egindako ikerketa gehiago behar dira interbentziozko entsegu klinikoak aurrera eraman ahal izan baino lehen. Ildo horretan, *in vitro* ikerketetatik lortutako emaitzetan oinarrituta, alga espezieetako bat aukeratu genuen gibel-esteatosia duen animalia-eredu batean bere eragina frogatzeko.

In vitro egindako lanek erakutsi dutenez, *Nannochloropsis gaditana* izan zen AML12 hepatozitoetan eragin handiena izan zuen estraktua, efektuaren handitasunari dagokionez, eta kontzentrazio txikienetako batekin. 3T3-L1 aurre-hepatozitoetan aldiz, dosi bakar bat soilik izan zen eraginkorra. *Gracilaria vermiculophylla* ostera, eraginkorra izan zen bi zelula-lerroetan, hiru dosi altuenak erabili zirenean. Beraz, emaitza horien arabera, *Gracilaria vermiculophylla* makroalga aukeratu zen *in vivo* ikerlan hau egiteko.

Esperimentu hau egiteko, alga osoa erabiltzea erabaki genuen estraktuak erabili ordez, izan ere, eraginkorra izatekotan, ez litzateke erauzketarik egin beharko, ekonomikoki interesgarriagoa dena eta iraunkortasun-printzipioekin bat datorrena. Gainera, alga osoak estraktuetan aurki ezin daitezkeen nutriente eta konposatuak dauzka, eta horrek, ondorio positibo gehiago ekar ditzake. Ikerketa honetan erabilitako alga, *Gracilaria vermiculophylla*, % 6,4 karbohidratoz, % 1,4 lipidoz, % 22,1 proteinaz eta % 27,9 zuntzez osatuta zegoen. Errautsen proportzioak (% 28,8) mineral kantitate handia aditzera ematen zuen. Gainera, polifenolen guztizko edukia 1180 mg/kg-koa zen.

Giza GGEAren etapa bakoitzeko histopatologia eta patofisiologia erreplikatzen dituzten animalia-eredu ezberdinak aurki daitezke. Horien artean, Zucker (*fa/fa*) arratoia esteatosi eta obesitate genetikoko eredu ezagunenetariko bat da (109,145); izan ere, sindrome metabolikoa aztertzeko baliagarria den eredutzat hartzen da, gizakietan ikusten den esteatosi eta obesitatearen antz handia baitu (146). Ondorioz, sindrome metabolikoaren osagai ezberdinen arteko elkarrekintzak aztertzea baimentzen duen eredua da. Hori kontuan hartuta, Zucker (*fa/fa*) arratoi obesoa aukeratu zen *Gracilaria vermiculophylla*-ren efektua gibel-esteatosiarengan aztertzeko, konkretuki, tratamendu mailan duen eragina aztertzeko.

Ikerlan hau gauzatzeko, makroalgaren bi dosi ezberdin erabili ziren, algekin osatutako dieten efektua aztertzen duten antzeko ikerlanetan erabiltzen diren dosiak kontuak hartuta aukeratu zirenak. Literaturan erabilitako dosi tarte horretan oinarrituta, tarteko dosi bat (% 5) eta dosi txiki bat (% 2,5) aukeratu genituen.

Zortzi asteko Zucker (*fa/fa*) arratoi arrak dieta estandar batekin elikatu ziren sei astetan zehar. Dieta estandarra *Gracilaria vermiculophyla*-ren % 2,5 edo % 5-ekin osotu zen. Kontrol taldeak dieta estandar berbera jaso zuen baina algarik gabe. Talde hauez gain, "pair-fed" (PF) talde bat behar izan genuen, izan ere, algaren dosi handiarekin (% 5) tratatutako arratoiek janari gutxiago ahoratu zutela behatu zen, kontrol taldearekin alderatuta. PF taldeari aurreko egunean dosi handiarekin tratatutako taldeak ahoratu zuen dieta kantitate berdina eskaini zitzaion. Modu horretan, ikusitako efektuak zuzenean alga jatearen ala dieta kantitate gutxiago jatearen ondorio ote ziren bereizi ahal izan genuen. Algaren dosi baxua eman zitzaion taldean ez zen aldaketarik hauteman ahoratutako dieta kantitateari dagokionez, beraz, ikusitako efektuak esklusiboki makroalgari egotz dakizkioke.

Aurreikusi genuen moduan, Zucker arratoi obeso guztietan gorputz-pisuaren igoeraren handipena ikusi zen arratoi argalekin konparatuta. Algaren dosi txikiak parametro horretan aldaketarik eragin ez bazuen ere, dosi handiak gorputz-pisuaren igoeraren murrizketa eragin zuen. Hala ere, efektu hau, dietaren kontsumoaren murrizketari egotz dakioke; izan ere, PF taldean, elikagai kantitate mugatua zuen taldean, antzeko murrizketa bat ikusi zen parametro horretan.

Gluzemiaren kontrolaren serumeko parametroei dagokienez, serumeko glukosa-mailak 100 mg/dL-koak izan ziren kontrol obesoen taldeko arratoietan. Aitzitik, algaren dosi baxua eta altua jaso zuten arratoiek glukosa-maila normalak erakutsi zituzten (76 and 78 mg/dL hurrenez hurren). Are gehiago, dosi altua jaso zuten arratoietan intsulinaren funtzioaren hobekuntza ikusi zen, dosi baxua jaso zutenekin alderatuta, intsulina-mailak nabarmen murriztu baitziren. Emaitza hau bat dator intsulinaren erresistentzia balioztatzeko modelo homeostatikoarekin (HOMA-IR). Gainera, arratoi obesoek serumeko EGGAen maila altuak dituztela kontuan izanda, sarritan intsulinarekiko sentikortasunaren asaldurekin erlazionatzen dena, intsulinaren sentikortasun indize kuantitatibo berrikusia (R-QUICKI) ere aztertu zen. Lortutako emaitzak bat zetozen HOMA-IR-an ikusitako emaitzekin, intsulinarekiko sentikortasunaren hobetzearen adierazle, dieta *Gracilaria vermiculophylla*-ren dosi altuarekin osatzearen ondorioz. Esan beharra dago dosi altuak eragindako efektu positibo horiek, elikagaien kontsumoaren murriztearen ondorio direla, PF taldean antzeko erantzuna ikusi baitzen.

Serumeko triglizerido-mailei dagokienez, algaren dosi baxua hartu zuten arratoietan balio fisiologikoetaraino murrizteko joera ikusi zen (151 mg/dL). Aitzitik, dosi handia hartu zuen taldean ez zen triglizerido-mailan aldaketarik ikusi, eta hipertriglizeridemiaren atalasearen (200 mg/dL) gaineko balioak lortu ziren.

Gibel pisuen kasuan antzeko joerak behatu ziren. Dosi baxuak gibel pisuan aldaketarik eragin ez zuen bitartean, dosi handia hartu zuen taldean esanguratsuki murriztu zen parametro hau. Hala ere, ez dago guztiz argi efektu hori zuzenean makroalgak eragindakoa zen, ala ingestioa murriztearen ondorio izan zen; izan ere, PF taldeko balioak kontrol taldean behatutako balioen eta dosi altua hartu zuen taldearen balioen artean kokatzen baitira.

Gibeleko lipidoak analizatu zirenean, makroalgarekin tratatutako talde batek ere ez zuen gibeleko lipidoen murrizketarik jasa, ezta triglizerido-mailetan ere. Aurkikuntza hauek bat datoz gibel pisuan aldaketarik ez egotearekin. Emaitza hauek adierazten dute, erabilitako baldintza esperimentaletan, *Gracilaria vermiculophylla* ez zela gai izan gibel-esteatosia murrizteko. Guk dakigula, gurea da *Gracilaria vermiculophylla*-k gibel-esteatosian duen eragina aztertzen duen lehen ikerlana, nahiz eta literatura zientifikoan *Gracilaria* generoko beste espezie batzuk erabiltzen dituzten ikerketak aurkitu daitezkeen. Chanek eta kolaboratzaileek efektu onuragarriak deskribatu zituzten gibel-esteatosiari dagokionez, *Gracilaria changii*-ren % 5 eta % 10 erabilita (147). Ikerlan horren eta gurearen arteko desadostasunak, *Gracilaria* espezie desberdinak, animalia eredu desberdinak edo/eta tratamendu aldi desberdinak erabiltzearen ondorio izan litezke.

Aitzitik, *Gracilaria vermiculophylla* hartu zuten bi taldeek gibeleko EGGAen mailen murrizketa esanguratsua erakutsi zuten. Dosi altua jaso zuen taldearen kasuan, aldaketa hau, neurri batean, ingestioa murrizteari zor zaio. Jakina da gehiegizko EGGAen metaketak, hepatozitoetan kaltea eragiten duela, batez ere, lipido-espezie kaltegarriak sortuz, zeramidak, diazilglizerolak eta lisofosfatidilkolina esaterako, ROS-en ekoizpena faboratzen dutenak (148). Emaitza hauek ikusita, *Gracilaria vermiculophylla*-k esteatosi arrunta fase aurreratuagoetara igarotzea saihestu dezakeela iradoki daiteke.

Zucker arratoi obesoen gibelak *de novo* lipogenesiaren tasa altuak erakusten ditu (149). Gainera, plasmako EGGA-maila altuak ikus daitezke, gantz-ehun handiaren eta intsulinarekiko erresistentzia periferikoaren ondorioz. Lipido hauek, hepatozitoan sar daitezke, non oxidazioa edo esterifikazioa jasan dezaketen (27). EGGAen murrizketaren erantzuleak diren mekanismoak aztertzeko, lipidoen metabolismoaren inguruko hainbat determinazio egin ziren.

De novo lipogenesiari dagokionez, pACC/ACC totala ratioa, FAS-en proteina espresioa eta FAS-en aktibitatea neurtu ziren. Dietan makroalga gehitzeak ez zuen aldaketarik eragin ACC-ren aktibazio-mailan, eta harrigarriki, makroalgaren dosi altuak FAS-en proteina-espresioaren gehikuntza eragin zuen. Efektu hori ingestioaren murrizketaren ondorioz gertatu zen, PF taldeak aditzera eman zuen moduan. Aitzitik, FAS-en jarduera murriztu egin zen makroalgaren bi dosiekin, itzulpen osteko erregulazioa eman dela adieraziz. Emaitza horren arabera, *de novo* lipogenesiak gibeleko EGGAen mailak murrizten laguntzen du neurri batean gutxienez.

Gantz-azidoen oxidazioa aztertzeko, CPT-1a eta azil-Ako oxidasaren (ACO) aktibitateak neurtu ziren, mitokondrietan eta peroxisometan gertatzen den gantz-azidoen oxidazioan parte hartzen duten funtsezko entzima direnak. Bi kasuetan, entzimen aktibitatean murrizketa eman zen algaren dosi altuarekin, hala ere, azpimarratu beharra dago emaitza hori dietaren kontsumoaren murrizketaren ondorioz gertatu zela.

Mitokondrioenen biogenesiak gantz-azidoen oxidazioaren gehikuntza eragin dezake. Esperimentu honetan, 1 arnasketa faktore nuklearraren (NRF1) eta TFAM (biak mitokondriogenesiaren markatzaileak) proteina-espresioaren igoera behatu zen algaren dosi txikia jaso zuen taldean. Nahiz eta dosi altuak NRF1-en espresioa handitu zuen, ez zuen eragin bera izan TFAM-en kasuan. Dosi altuaren efektua ahorakina murriztearen ondorio izan zen. Aurkikuntza hauek iradokitzen dutenez, algaren dosi txikiak eragiten duen mitokondriogenesiaren gehikuntzak gibeleko EGGA-mailaren murrizketan eragina izan lezake.

Gantz-azidoen oxidazioaren gehikuntzak, mitokondrioenen barruan ROS gehiagoren eraketa bultza dezake, oxidazio estresaren garapenean lagunduz (150). Bestetik, gibeleko EGGA-mailaren jaitsierak lipidoen peroxidazioaren murrizketa iradoki dezake (12). Zein egoeraren aurrean gauden jakiteko, oxidazio estresarekin erlazionatutako hainbat markatzaile aztertu ziren. Gure esperimentazio-

baldintzetan, algaren dosi txikia oxidazioaren kalteak murrizteko gai izan zen, erreduzitutako glutatioiaren (GSH) igoera arinak agerian utzi zuen moduan, zeinak defentsa antioxidatzaile ez-entzimatikoaren indikatzaile gisa balio duen. Modu berean, ahalmen antioxidatzaile totalaren (TROLOX) gehikuntza ere behatu zen dosi baxua jaso zuten arratoietan. Aitzitik, algaren dosi handiak oxidazio estresa areagotzen zuela zirudien, MDA-mailaren bidez neurtutako lipidoen peroxidazioaren igoerak eta GPx entzima antioxidatzailearen aktibitatearen murrizketak erakutsi zuten moduan. Hala ere, azken hau ez zen algaren efektu zuzenaren ondorio izan, ingestioaren murrizketak eragindakoa baizik. Alde horretatik, Chanek eta kolaboratzaileek lipidoen peroxidazioaren (MDA mailak), katalasaren eta GPx-ren hobekuntza deskribatu zuten arratoien dieta *Gracilaria changii*-ren % 5 edo % 10ekin osatu ostean (147), hala ere, lan horren eta gurearen arteko desberdintasunak kontuan hartu behar dira.

Jakina da Zucker arratoiek ez dutela esteatohepatitisa espontaneoki garatzen beste estimulu baten menpe ez bada, gantz ugariko dieta kasu (151). Hori jakinda ere, interesgarria da inflamazioaren eta fibrosiaren markatzaile goiztiarretan eman litezkeen aldaketak aztertzea. Inflamazioari dagokionez, *Gracilaria vermiculophylla* jaso zuten bi taldeetan atxikidura G proteinari lotutako E1 hartzailearen (F4/80), gibelaren berezko makrofagoak diren Kupffer zelula pro-inflamatzaileen (M1) markatzailearen murrizketa ikusi zen. Emaitza honen arabera, makroalga honek inflamazioaren aurkako eragina izan lezake.

Azkenik, fibrosiaren garapenari dagokionez, oro har, esan daiteke *Gracilaria vermiculophylla*-k ez duela eraginik izan analizatutako markatzaileetan, I kolagenoa, alfa 1 katea (*Col1a1*), beta 1 hazkuntza-faktore eraldatzailea (*Tgfb1*) eta TIMP metalopeptidasaren 1 inhibitzailea (*Timp1*) kasu, aktina alfa 2 muskulu leunaren (*Acta2*) kasuan izan ezik. Markatzaile honen gene-espresioa nabarmen handitu zen, hala ere, efektu hau neurri batean, ahorakinaren murrizketaren ondorioz eman zen. Hala ere, matrizeko 9 metalopeptidasaren (*Mmp9*) gene-espresioa aztertu genuenean, haren gehikuntza ikusi genuen dosi altua jaso zuten arratoietan. Emaitza hori metabolismoaren moldaera bezala interpretatu daiteke matrize estrazelularraren osagaien metaketaren aurrean (152).

Ikerlan honetan lortutako emaitzetan oinarritua, esan daiteke *Gracilaria vermiculophylla*-k ez duela gibel-esteatosia murrizten. Hala ere, onuragarria izan daiteke gibeleko EGGA-mailak, oxidazio estresa eta inflamazio markatzaileak murrizteko. Kontuan izan behar da, efektu positibo horiek dosi baxuarekin (% 2,5) bakarrik lortu direla.

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Section 2 - Conclusions

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Conclusions

As evidenced by the results obtained, the conclusions drawn from the present Doctoral Thesis are as follows:

Literature search

1. Both microalgae and macroalgae extracts in the range of 7.5 to 3000 mg/kg body weight/day or 1 to 12 % in the diet can exert beneficial effects on hepatic steatosis in male rodent models mainly at preventive level (Manuscript 1).
2. The precise mechanism of action of the anti-steatotic effect of each algae species is yet to be stablished, due to the limited number of studies conducted on this topic (Manuscript 1).
3. More human studies are needed in order to translate the beneficial outcomes observed in rodents (Manuscript 1).

***In vitro* study in AML12 murine hepatocytes**

4. *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts, abundant in peptides/proteins, prevent lipid accumulation in cultured hepatocytes (Manuscript 2).
5. The mechanism of action underlying the anti-steatotic effect of each algae extract is not exactly the same; however, they primarily act on pathways involved in fatty acid oxidation and secretion. (Manuscript 2).

***In vitro* study in 3T3-L1 murine adipocytes**

6. *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts, abundant in peptides/proteins, do not reduce lipid accumulation in mature adipocytes cultured *in vitro* (Manuscript 3).
7. *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts, exert an anti-adipogenic effect in cultured adipocytes (Manuscript 3).
8. The anti-adipogenic effect attributed to the extracts may be explained, at least in part, by their inhibitory effect on the transcription factor C/EBPα (Manuscript 3).

***In vivo* study in a model of metabolic syndrome (Zucker *fa/fa* rats)**

9. *Gracilaria vermiculophylla* does not reduce hepatic lipid accumulation (Manuscript 4).

10. When supplemented at 2.5 % in the diet, *Gracilaria vermiculophylla* reduces hepatic NEFA content by reducing *de novo* lipogenesis and promoting mitochondriogenesis, which may result in increased fatty acid oxidation (Manuscript 4).
11. *Gracilaria vermiculophylla* is able to mitigate hepatic oxidative stress when it is included at a concentration of 2.5 % in the diet (Manuscript 4).
12. *Gracilaria vermiculophylla* improves hepatic inflammation, but not fibrosis, when included at a concentration of 2.5 % in the diet (Manuscript 4).
13. The beneficial effects in fatty liver are only observed with the concentration of 2.5 % (low dose) of *Gracilaria vermiculophylla*.

Ondorioak

Lortutako emaitzetan oinarrituta, Doktoretza-tesi honen ondorioak honako hauek dira:

Bilaketa bibliografikoa

1. Mikroalgetatik zein makroalgetatik lortutako estraktuek efektu onuragarriak dituzte gibel-esteatosia duten karraskari arretan 7,5-3000 mg/kg gorputz-pisu/eguneko edo dietan % 1-12 ematen zaienean. Efektu horiek, batez ere prebentzio mailan ematen dira (1. eskuizkribua).
2. Alga espezie bakoitzak esteatosiaren aurka duen efektuaren ekintza-mekanismo zehatza ezartzeke dago, gai honen inguruan egindako ikerketa kopuru txikia dela eta (1. eskuizkribua).
3. Gizakietan egindako ikerketa gehiago behar dira karraskarietan hautemandako emaitza onuragarriak itzuli ahal izateko (1. eskuizkribua).

***In vitro* ikerlana AML12 sagu hepatozitoetan**

4. *Chlorella vulgaris*, *Nannochloropsis gaditana* eta *Gracilaria vermiculophylla* algetatik lortutako estraktuek, peptido eta proteinetan ugariak direnak, lipido-metaketa saihesten dute hepatozitoen zelula-kultiboetan (2. eskuizkribua).
5. Alga-estraktu bakoitzak esteatosiaren aurka duen efektuaren azpiko ekintza-mekanismoa ez da guztiz berdina, hala ere, gantz-azidoen oxidazioarekin eta sekrezioarekin dute zerikusia (2. eskuizkribua).

***In vitro* ikerlana 3T3-L1 sagu adipozitoetan**

6. *Chlorella vulgaris*, *Nannochloropsis gaditana* eta *Gracilaria vermiculophylla* alga-estraktuek, peptido eta proteinetan ugariak direnak, ez dute lipidoen metaketa murrizten *in vitro* hazitako adipozito helduetan (3. eskuizkribua).
7. *Chlorella vulgaris*, *Nannochloropsis gaditana* eta *Gracilaria vermiculophylla* alga-estraktuek adipogenesiaren aurkako efektua dute adipozitoen kultiboetan (3. eskuizkribua).
8. Alga-estraktuei egozten zaien adipogenesiaren aurkako efektua C/EBPα transkripzio-faktorearengan duten eraginagatik azal daiteke, neurri batean behintzat (3. eskuizkribua).

***In vivo* ikerlana sindrome metabolikoaren eredu batean (Zucker *fa/fa* arratoiak)**

9. *Gracilaria vermiculophylla* ez da gibeleko lipido-metaketa murrizteko gai (4. eskuizkribua).

10. Dieta % 2,5 osatzen denean, *Gracilaria vermiculophylla*-k gibeleko EGGAen edukia murrizten du *de novo* lipogenesia murriztuz eta mitokondriogenesia sustatuz. Azken horrek, gantz-azidoen oxidazioa areagotzea ekar dezake (4. eskuizkribua).

11. *Gracilaria vermiculophylla* gibeleko oxidazio estresa arintzeko gai da dietan % 2,5eko kontzentrazioan gehitzen denean (4. eskuizkribua).

12. *Gracilaria vermiculophylla*-k gibeleko inflamazioa hobetzen du, baina ez fibrosia, dietan % 2,5eko kontzentrazioan gehitzen denean (4. eskuizkribua).

13. *Gracilaria vermiculophylla*-k gibel-esteatosian dituen efektu onuragarriak % 2,5eko kontzentrazioarekin (dosi txikia) bakarrik ikusten dira (4. eskuizkribua).

Section 3 - Appendix
Published works

3. atala - Eranskina
Argitaratutako lanak

Manuscript 1 / 1. eskuizkribua

Effect of Microalgae and Macroalgae Extracts on Non-Alcoholic Fatty Liver Disease

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Abstract

The present review aims to gather scientific evidence regarding the beneficial effects of microalgae and macroalgae extracts on non-alcoholic fatty liver disease (NAFLD). The described data shows that both microalgae and macroalgae improved this alteration. The majority of the reported studies analysed the preventive effects because algae were administered to animals concurrent with the diet that induced NAFLD. The positive effects were demonstrated using a wide range of doses, from 7.5 to 300 mg/kg body weight/day or from 1 to 10% in the diet, and experimental periods ranged from 3 to 16 weeks. Two important limitations on the scientific knowledge available to date are that very few studies have researched the mechanisms of action underlying the preventive effects of microalgae on NAFLD and that, for the majority of the algae studied, a single paper has been reported. For these reasons, it is not possible to establish the best conditions in order to know the beneficial effects that these algae could bring. In this scenario, further studies are needed. Moreover, the beneficial effects of algae observed in rodent need to be confirmed in humans before we can start considering these products as new tools in the fight against fatty liver disease.

Keywords: non-alcoholic fatty liver disease; liver steatosis; macroalgae; microalgae

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) covers a wide spectrum of histopathological abnormalities ranging from simple steatosis to steatohepatitis (NASH). Hepatic steatosis is the most benign and common form of NAFLD that is defined as intrahepatic fat accumulation of at least 5% of liver weight. However, this condition can evolve to more advanced stages if hepatocytes are exposed to stress, causing cell death, apoptosis, inflammation and fibrosis and leading to NASH. This NASH can result in cirrhosis and hepatocellular carcinoma (1,2). The prevalence of NAFLD shows a high variability, ranging from 6 to 35% in the general population. These rates are experiencing an upward trend due to the current epidemic of obesity (3) and type 2 diabetes (2); in fact, about 50% of NAFLD patients and 80% of patients with NASH are obese (4).

NAFLD is supposed to occur in patients with no alcohol or little alcohol consumption. Some authors have suggested that the term NAFLD overemphasizes the "non-alcoholic" aspect, and due to its association with a greater number of co-morbidities, they have proposed substituting NAFLD with metabolic associated fatty liver disease (MAFLD) (5,6).

Currently, there is no specific treatment for liver steatosis. The first step in its management consists of lifestyle intervention with caloric intake restriction and exercise. However, patients find it difficult to implement and achieve these lifestyle modifications. At present, no drugs have yet been approved, and pharmacological treatment devotes efforts to associated co-morbidities that contribute to the pathogenesis of NAFLD, such as, obesity, type 2 diabetes mellitus or dyslipidemia (4). Due to the increasing prevalence and treatment limitations of NAFLD, there is an urgent need to seek new sources of bioactive compounds with potential preventive and/or therapeutic action.

In this context, there is increasing research on algae, based on the fact that many cultures have traditionally used them as food and medicine, and that 70% of the earth's surface is covered by the ocean, which may provide additional opportunities in view of the limitation of terrestrial resources. Algae are typical of Japanese, Korean and Chinese dietary patterns, and more recently, seaweeds and seaweed-based foodstuffs, such as sushi, are commonly found in western societies. Moreover, they are an important component of the Atlantic dietary pattern. Significantly, algae represent a good source of proteins, vitamins, minerals and fiber (7–10). In addition, they are rich in a great number of bioactive compounds, such as peptides, pigments, phenolic compounds and fatty acids with potential applications in health, due to their antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic, antihypertensive, antihiperlipidaemic and antiobesity effect (11,12). Algae can also be used as ingredients to be included, dehydrated or powdered, in functional foods. Indeed, they can have a relevant role in reformulation strategies for the manufacture of technologically and sensorily viable food stuffs with health-promoting compositions due to their technological, organoleptic, and nutritional properties. An example is the reformulation of meat products (frankfurters, patties and

restructured steaks) by adding nori, wakame or sea spaghetti seaweeds, reported by Cofrades et al. (13).

To the best of our knowledge, no in vitro studies with algae extracts in cultured hepatocytes have been carried out to date. The present review aims to gather scientific evidence regarding the beneficial effect of microalgae and macroalgae extracts on hepatic steatosis in preclinical and clinical studies, as well as the potential mechanisms involved in these effects.

2. Animal Studies

The vast majority of algae extract effects have been studied in rodent models. **Figure 1** summarizes the main effects observed in the present review.

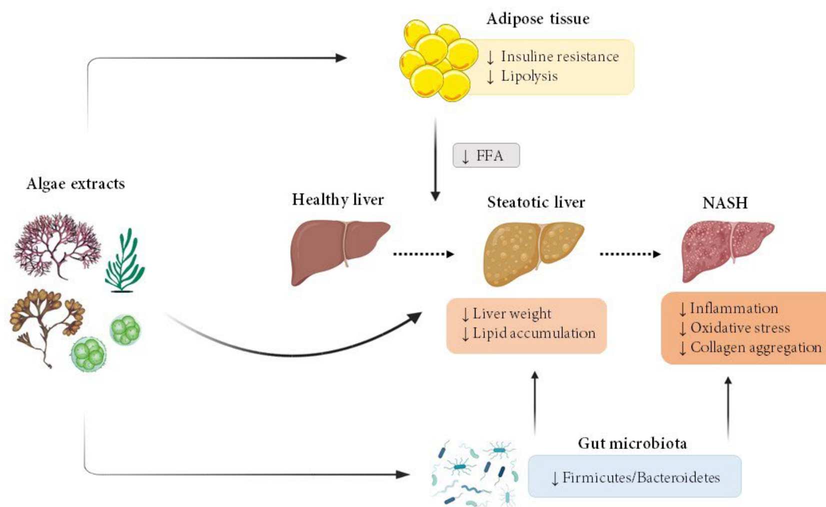

Figure 1. Effects of microalgae and macroalgae extracts on metabolic alterations leading to steatohepatitis. FFA, free fatty acids; NASH, non-alcoholic steatohepatitis.

2.1. Microalgae

As far as we know, only four studies analysing the effects of microalgae extracts on NAFLD in animal models have been published to date (**Table 1**).

Table 1. Effects of microalgae extracts in animal models.

Author	Algae species	Animal model and experimental period length	Experimental groups	Effects	Mechanisms
Murata *et al.* 1999 (27)	*Undaria pinnatifida*	Male Sprague-Dawley rats 3 weeks	Standard diet Standard diet + 0.5 % *U. pinnatifida* Standard diet + 1 % *U. pinnatifida* Standard diet + 2 % *U.pinnatifida* Standard diet + 5 % *U. pinnatifida* Standard diet + 10 % *U.pinnatifida*	↓ Liver TG content in 1, 2, 5 and 10% groups ↓ Liver TC content in 10% group	↓G6PD activity in 5 and 10% groups ↑ CPT activity in 10% group ↑ ACADs activity in 5 and 10 % groups ↑ ACO in 10% group ↑ DECR1 in 5 and 10 % groups
Murata *et al.* 2002 (28)	*Undaria pinnatifida*	Male Sprague-Dawley rats 4 weeks	Standard diet Standard diet + 19.1% *U. pinnatifida*	↓ Liver weight ↓ Hepatic TG, TC and phospholipids levels	↓ G6PD activity ↑ ACO and 3-hydroxiacil-CoA dehydrogenase activities CPT activity: NS
Li *et al.* 2020 (29)	*Undaria pinnatifida*	Male C57BL/6 mice 10 weeks	Standard diet Standard diet + 10 % *U. pinnatifida* High-fat diet High-fat diet + 10 % *U. pinnatifida*	↓ Liver steatosis ↓ Glucose levels	No information provided
Gabbia *et al.* 2020 (30)	*Fucus vesiculosus* + *Ascophyllum nodosum*	Male Wistar rats 5 weeks	High-fat diet (HFD) High-fat diet + 7.5 mg/kg BW/day of *F. vesiculosus* and *A. nodosum*	↓ Liver weight ↓ Microvesicular steatosis ↓ Plasma ALT and AST levels Lower and delayed glucose peak	No information provided
Kang *et al.* 2020 (31)	*Sargassum thunbergii*	Male C57BL/6 mice 7 weeks	Standard diet High-fat diet High-fat diet + 100 mg/kg BW/day of *S. thunbergii* High-fat diet + 300 mg/kg BW/day of *S. thunbergii*	↓ Lipid steatosis	No information provided
Murakami *et al.* 2021 (32)	*Sargassum horneri*	Male C57BL/ 6J mice 13 weeks	Standard diet High-fat diet (HF) High-fat diet + 2 % *S. horneri* (HF + ShL) High-fat diet + 6 % *S. horneri* (HF + ShH)	↓ Liver weight ↓ Liver TG content ↓ Serum glucose, insulin, ALT, AST, ALP and LAP levels ↑ Serum adiponectin ↓ Serum TNF-α	↓ Pancreatic lipase activity

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BW: body weight, Col1a1: collagen type I alpha 1 chain, F4/80: homologue in mouse to epidermal growth factor-like 1 in humans, HOMA-IR, homeostasis model assessment of insulin resistance; Il-1β: interleukin-1β, α-SMA: alpha smooth muscle actin, TG: triglycerides, TNF-α, tumor necrosis factor-α, ↑: increased, ↓: decreased, NS: no significant.

Kumar et al. (14) studied the effect of blending two microalgae, *Scenedesmus dimorphus* and *Schroederiella apiculata*, which contained 46.1% protein, 19.6% insoluble fibre and 2.8% omega-3 fatty acids. For this purpose, Wistar rats were distributed into four experimental groups: the control group was fed

with a corn starch diet (C) containing 68% carbohydrates as polysaccharides; a second group received a high-carbohydrate high-fat diet (H) where fat, fructose and sucrose represented 24%, and drinking water contained 25% fructose (total 68% carbohydrates in food and water); and the remaining two groups were fed with the corn starch diet supplemented with 5% *Scenedesmus dimorphus* and *Schroederiella apiculata* blend (CSC), or the high-carbohydrate high-fat diet supplemented with 5% *Scenedesmus dimorphus* and *Schroederiella apiculata* mixture (HSC). Although the total experimental period length was 16 weeks, the microalgae combination was only provided during the last eight weeks. As expected, and compared to the C group, the rats fed with the high-carbohydrate high-fat diet showed increased liver weight, higher presence of enlarged fat vacuoles and higher infiltration of inflammatory cells. Interestingly, these effects were totally prevented by the inclusion of the microalgae mixture into the diet. In line with these effects, and compared to the control group, the increased values observed in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the H group, which indicated liver damage, were normalized. Moreover, improved glucose tolerance and insulin sensitivity, which are closely related to steatosis development, were observed in the groups supplemented with the microalgae mixture. No effects induced by the microalgae extract were detected in the CSC group because, due to the standard diet composition provided to these rats, all liver parameters were normal.

Nakashima et al. (15) studied the effects of *Euglena gracilis* (29.4% carbohydrate, 42.3% protein and 19% fat) using STAM (Stelic Animal Model) mice, a model of non-alcoholic steatohepatitis, induced by streptozocin. Mice were fed with a high-fat diet and *Euglena gracilis* was orally administered, at a dose of 3 g/kg body weight, for 27 days. At the end of the experimental period, liver weight, liver triglycerides and plasma ALT levels were measured, and no differences were observed between the *Euglena gracilis*-treated group and the control group. Rats treated with *Euglena gracilis* showed lower hepatic fibrosis than the controls. Although fibrosis is one of the parameters involved in the calculation of the NAFLD activity score (NAS score), this index did not differ among rats receiving the microalga and the vehicle. The gene expression of the inflammation related genes interleukin 1 β (*Il-1b*), interleukin 6 (*Il-6*), tumour necrosis factor α (*Tnf-a*) and monocyte chemoattractant protein 1 (*Mcp-1*), as well as fibrogenic markers such as *a-Sma*, collagen type I alpha 2 chain (*Col1a2*) and collagen type III alpha 1 chain (*Col3a1*) remained unchanged. The authors concluded that *Euglena gracilis* may not be involved in inflammation, although it may be effective in inhibiting the activation of hepatic stellate cells, thus attenuating collagen overproduction.

Pham et al. (16) studied the antifibrotic effect of the blue-green microalga *Spirulina platensis* in a mouse model of diet-induced fibrosis. C57BL/6J mice were assigned to three groups: a group fed with a control low-fat diet (LF; 6% fat), a second fed with a highfat/high-sucrose/high-cholesterol diet (HF; 34%/38%/2%), and a third group fed with the HF diet supplemented with 2.5% *Spirulina platensis* (HF/SP) for 20 weeks. Liver weight was significantly higher in the HF and HF/SP groups than in the LF group. Although *Spirulina platensis* supplementation did not modify liver triglyceride content,

it reduced plasma ALT level at 16 weeks. Mice in HF and HF/SP groups displayed collagen accumulation in hepatocytes, data which are in accordance with mRNA levels of collagen type I alpha 1 chain (*Col1a1*). Regarding serum parameters, although *Spirulina platensis* supplementation did not prevent the increment produced by the HF diet in glucose concentrations, it improved slightly glucose tolerance, which is a positive effect taking into account that a bad glycaemic control is a risk factor for liver steatosis development.

The spleen is a secondary lymphoid organ, closely associated with the liver, via the portal vein system. It is a source of inflammatory cells that migrate to the liver upon liver injury, thus contributing to the development of liver fibrosis. Spleen weight was increased in the HF group, and *Spirulina platensis* supplementation did not prevent this effect. When splenocytes were isolated, mice receiving *Spirulina platensis* supplementation had significantly lower gene expression level of basal *Il-1b* and a trend to decreased expression of *Il-6*. When splenocytes were tested ex vivo for their lipopolysaccharide (LPS) sensitivity, the results showed that the cells from mice fed with the supplemented diet had significantly lower mRNA levels of *Il-1b* and *Tnf-a*, after LPS induction, while *Il-6* expression level remained unchanged. The authors suggested that *Spirulina platensis*, at the dose used in their study, exerted anti-inflammatory effects, although it did not prevent the fibrosis development induced by the HF diet.

Mayer et al. (17) have recently studied the preventive effects of *Tisochrysis lutea* (Tiso) on metabolic alterations associated with obesity, including NAFLD.Wistar rats were distributed into three experimental groups and were fed with a standard diet (CTRL), a high-fat high-fructose diet (HF) with 10% fructose in drinking water, or the HF diet but supplemented with 12% of *Tisochrysis lutea* (HF-Tiso), for eight weeks. At the end of the experimental period, and compared to other groups, the HF-Tiso group showed decreased values of body weight, abdominal and epididymal adipose tissues, liver triglyceride content, and plasma AST level, but no changes in ALT level were identified. Accordingly, the HF-Tiso group showed a lower AST/ALT ratio. The alga supplementation also lowered plasma glucose, insulin and the homeostatic model assessment for insulin resistance (HOMA-IR) index, meaning that insulin resistance was reduced. The pro-inflammatory cytokines TNF-α and IL-6 were increased in the HF group and *Tisochrysis lutea* supplementation significantly prevented the effect on TNF-α, without affecting IL-6.

To sum up, after reviewing the literature concerning the effects of microalgae on NAFLD, it can be pointed out that although the results reported are encouraging, the number of studies is still very scarce. In addition, the reported studies address the effects of different microalgae, and thus only one source of information is available for each one. According to the experimental design used, all the reported studies analysed the preventive effects of microalgae because these were included in the diet that induced liver alterations. Although in all of them microalgae improved several alterations, there is a lack of consensus on the specific effects observed. Thus, whereas the three studies that analysed

this parameter reported a reduction of liver inflammation, only two of these studies described a reduction of liver triglyceride content (the other two studies did not find significant changes). Moreover, two of the studies analysed the effects of fibrosis and only one observed a significant improvement. In this scenario, further research is needed in order to assess the effects of microalgae on NAFLD.

2.2. Macroalgae

Several studies have analysed the effects of green, red and brown macroalgae on NAFLD using different animal models.

2.2.1. Green Algae

Two green algae have been studied in the reported literature: *Caulerpa lentillifera* and *Ulva prolifera* (**Table 2**).

The effect of the green seaweed *Caulerpa lentillifera* in C57BL/6J mice was studied by Sharma et al. (18). Mice were distributed into three experimental groups: control group (fed with a standard diet), HFD group (fed with a diet containing 60% of energy as fat) and HFD + CL fed with the same high-fat diet and 250 mg/kg body weight of the algae, administered by oral gavage, for ten weeks. HFD increased plasma levels of free fatty acids, glucose and insulin, and the addition of the algae extract to the diet led to decreased levels of these parameters. HFD produced increases in liver weight and hepatic triglycerides. The administration of *Caulerpa lentillifera* prevented all these effects.

Du Preez et al. (19) studied the effects of the same seaweed in Wistar rats. Animals were distributed in four groups: two groups received either corn starch (C) or highcarbohydrate high-fat (H) diets for 16 weeks. The other two groups received C or H diets for the first eight weeks and after, they were fed on the same diets but supplemented with 5% dried *Caulerpa lentillifera* (44% carbohydrates, 14% lipids, 7% protein and 17.5% fibre) for the remaining eight weeks (CCL and HCL groups, respectively). Compared to the C group, liver fat deposition was higher among rats from the H group, and the addition of *Caulerpa lentillifera* to the diet partially prevented this effect. However, infiltration of inflammatory cells and plasma activities of ALT and AST did not differ among the experimental groups. The analysis of microbiota composition revealed that there were no differences in diversity and richness among the four experimental groups. However, rats fed with the diets supplemented with *Caulerpa lentillifera* showed lower *Firmicutes/Bacteroidetes* ratios than rats fed with a high-carbohydrate high-fat diet. The authors concluded that these changes in gut microbiota could be a mechanism that justified the improvement of the abovementioned parameters in the HCL group, as they found a strong correlation between bacterial community structure and oral glucose tolerance test, liver weight, retroperitoneal, epididymal and total abdominal fat.

Table 2. Effects of green macroalgae extracts in animal models.

Author	Algae species	Animal model and experimental period length	Experimental groups	Effects	Mechanisms
Sharma *et al.* 2017 (18)	*Caulerpa lentillifera*	Male C57BL/6J mice 10 weeks	Standard diet High-fat diet High-fat diet + 250 mg/kg BW/day of *C. lentillifera*	↓ Liver weight ↓ Liver TG, TC and FFA ↓ Plasma FFA, glucose and insulin	No information provided
du Preez *et al.* 2020 (19)	*Caulerpa lentillifera*	Male Wistar rats 16 weeks	Corn starch (C) Standard diet (C) High-carbohydrate high-fat (H) Standard diet + 5% *C. lentillifera* (CCL) High-carbohydrate high-fat + 5% *C. lentillifera* (HCL)	↓ Liver TG content (H vs HCL) Inflammatory cell infiltration: NS Plasma ALT, AST: NS ↓Firmicutes/Bacteroidetes ratio (H vs HCL)	No information provided
Song *et al.* 2018 (20)	*Ulva prolifera*	Male C57BL/6 mice 8 weeks	Standard diet High-fat diet High-fat diet + 2% ethanol extract of *U. prolifera* in drinking water High-fat diet + 5% ethanol extract of *U. prolifera* in drinking water	↓ Liver weight ↓ Liver TG content ↓ Serum insulin ↓ Oxidative stress	↓ Dgat1 and Dgat2 liver mRNA levels ↑ Cpt-1a, Acadm and Acox1 liver mRNA levels ↓ Serum IL-1β, IL-6 and TNF-α ↓ IL-1β, IL-6 and TNF-α liver mRNA levels ↓ Liver ROS ↑ GSH content and GSHPx activity

Acadm: medium-chain acyl-CoA dehydrogenase, Acox1: acyl-CoA oxidase 1, BW: body weight; Cpt-1a: carnitine palmitoyltransferase 1A, Dgat1: diacylglycerol O-acyltransferase 1, Dgat2: diacylglycerol O-acyltransferase 2, FFA: free fatty acids, GSH: reduced glutathione, GSH-Px: glutathione peroxidase, IL-1β: interleukin-1β, IL-6: interleukin-6, ROS: reactive oxygen species, TC: total colesterol, TG: triglycerides, TNF-α: tumor necrosis factor-α, ↑: increased, ↓: decreased. NS: not significant.

Song et al. (20) conducted a study using C57BL/6J mice. The control group was fed with a standard diet, another group was administered a high-fat diet (60% fat; HFD group) and the remaining two groups received the same diet but with 2 or 5% of an *Ulva prolifera* ethanol extract added to the drinking water (HFD2 and HFD5 groups, respectively). After eight weeks, hepatic triglyceride content, as well as serum insulin was increased following high-fat feeding. All these deleterious effects were prevented by the administration of *Ulva prolifera* (at both doses). Similarly, the impairment of glucose tolerance and the reduction in insulin sensitivity were also prevented. In order to determine some of the mechanisms of action that explain the anti-steatosis effect of *Ulva prolifera*, the authors analysed liver gene expression of diacylglycerol O-acyltransferase 1 (*Dgat1*) and diacylglycerol O-acyltransferase 2 (*Dgat2*), two enzymes involved in triglyceride assembly, and in line with the results concerning liver triglycerides, the increase induced by the high-fat diet in these parameters was prevented by both doses of the seaweed. Moreover, gene expression of enzymes involved in fatty acid oxidation, a process that consumes this lipid species avoiding its availability for triglyceride synthesis, were also analysed. In this case, and compared to the control group, mRNA levels of carnitine palmitoyltransferase 1A (*Cpt-1a*), medium-chain acyl-CoA dehydrogenase (*Acadm*) and acyl-CoA oxidase 1 (*Acox1*) were decreased in the HFD group, and yet again, this effect was prevented by both doses of *Ulva prolifera*. Concerning inflammation-related parameters, serum IL-1β, IL-6 and

TNF-α concentrations, and liver gene expression of *Il-1b*, *Il-6* and *Tnf-a* were determined. Higher values were found in the HFD group, and this effect was prevented in both the HFD2 and the HFD5 groups. With regard to oxidative stress, the negative effect induced by the high-fat diet on reactive oxygen species (ROS) content, glutathione (GSH) content and glutathione peroxidase (GSHPx) activity were arrested by both doses of *Ulva prolifera*. A dose-response pattern of reaction was not found in these effects.

In summary, as in the case of microalgae, the number of studies addressing the preventive effect of green algae on NAFLD is still scarce. The three reported works (two of them carried out with *Caulerpa lentillifera*) revealed a reduction in hepatic triglyceride accumulation when animals were fed with a high-fat diet, but only one of the studies addressed several aspects of the potential mechanisms of action (insulin resistance, fatty acid and triglyceride metabolism, oxidative stress). In one of these pieces of research, the authors analysed the effects of seaweed supplementation on gut microbiota composition, but they did not establish a clear relationship between these changes and the effects on liver steatosis, just a significant correlation.

2.2.2. Red Algae

Five red algae were studied in the reported literature: *Plocamium telfairiae, Palmaria mollis, Sarconema filiforme Grateloupia elliptica* and *Gromphadorhina oblongata* (**Table 3**).

Table 3. Effects of red macroalgae extracts in animal models.

Author	Algae species	Animal model and experimental period length	Experimental groups	Effects	Mechanisms
Kang *et al.* 2016 (21)	*Plocamium telfairiae*	Male C57BL/6 mice 14 weeks	Standard diet High-fat diet High-fat diet +100 mg/kg BW/day of *P. telfairiae*	↓ Liver steatosis ↓ Serum glucose	No information provided
Lu *et al.* 2020 (22)	*Plocamium telfairiae*	Male C57BL/6 mice 7 weeks	Standard diet High-fat diet High-fat diet +100 mg/kg BW/day of *P. telfairiae* High-fat diet +165 mg/kg BW/day of *P. telfairiae* High-fat diet + 300 mg/kg BW/day of *P. telfairiae*	↓ Hepatic steatosis (all doses)	No information provided
Nakayama *et al.* 2018 (23)	*Palmaria mollis*	Male NSY/HOS mice *4 weeks*	Standard diet High-fat diet High-fat diet + 2.5 % of *P. mollis*	↓ Liver TG content	↑ Pparα, C/ebpα and Acox1 mRNA levels ↓ Pparɣ mRNA levels Acadm and Srebf1 mRNA levels: NS
du Preez *et al.* 2020 (24)	*Sarconema filiforme*	Male Wistar rats 16 weeks	Corn starch diet Corn starch diet + 5% *S. filiforme* High-carbohydrate high-fat diet	↓ Liver steatosis and infiltration of inflammatory cells (high-carbohydrate high-fat diet 5% *S. filiforme* vs high-	No information provided

			High-carbohydrate high-fat diet + 5% *S. filiforme* (drinking water of rats fed the steatotic diet was supplemented with 25% fructose)	carbohydrate high-fat diet) Serum glucose: NS ↓ Serum ALT and AST (high-carbohydrate high-fat diet 5% *S. filiforme* vs high-carbohydrate high-fat diet) Firmicutes/Bacteroidetes: NS	
Lee *et al.* 2020 (25)	*Grateloupia elliptica*	Male C57BL/6 mice 7 weeks	Standard diet High-fat high-sucrose diet High-fat high-sucrose diet+125 mg/kg BW/day of *G. elliptica* High-fat high-sucrose diet +250 mg/kg BW/day of *G. elliptica*	↓ Hepatic steatosis	No information provided
Nabil-Adam *et al.* 2021 (26)	*Gromphadorhina oblongata*	BALB/C mice 1 week	Negative control: saline solution Induction control: 5 mg/kg BW/day of LPS Protected group: 200 mg/kg BW/day of *G. oblongata* 2 h before LPS Positive control: 200 mg/kg BW/day of *G. oblongata* without LPS	↓ Liver injury (inflammation and oxidative stress) ↑ Liver apoptosis ↓ Serum ALT, AST	No information provided

Acadm: acyl-CoA dehydrogenase medium chain, Acox1: peroxisomal acyl-CoA oxidase 1, ALT: alanine transaminase, AST: aspartate transaminase, BW: body weight, C/ebpα: CCAAT/enhancer-binding protein alpha, LPS: lipopolysaccharide, Pparα: peroxisome proliferator-activated receptor alpha, Pparγ: peroxisome proliferator-activated gamma, Srebf1: sterol regulatory element-binding protein 1, TG: triglycerides, ↑: increased, ↓: decreased, NS: not significant.

Using C57BL/6 mice, Kang et al. (21) analysed the effects of *Plocamium telfairiae*, administered for 14 weeks. Animals were distributed into three groups: the control group was fed with a standard diet, the HFD group received a high-fat diet and the HFD + PL group was administered the same high-fat diet but supplemented with 100 mg/kg body weight of *Plocamium telfairiae*. Hepatic steatosis was induced in the HFD group, and compared to the controls, this deleterious effect was partially prevented by *Plocamium telfairiae*. In serum, glucose was also increased in the HFD group, and the seaweed supplementation totally prevented these effects.

Using the same algae and the same mice model, in the study of Lu et al. (22) mice were distributed into five groups and were fed with experimental diets for seven weeks: a control group was fed with a chow diet, a high-fat diet group (HFD) received a high-fat diet (45% fat), a PTE100 group was administered the high-fat diet but supplemented with 100 mg/kg body weight/day of *Plocamium telfairiae*, a PTE165 group was fed with the high-fat diet but supplemented with 165 mg/kg body weight/day of *Plocamium telfairiae* and a PTE300 group was administered the high-fat diet but supplemented with 300 mg/kg body weight/day of *Plocamium telfairiae*. Compared to the control group, hepatic histological analysis revealed increased hepatic steatosis in HFD mice, which in turn was reduced in mice fed with the algae. The authors did not indicate if a dose-response pattern was observed.

Nakayama et al. (23) studied the potential effects of the red algae *Palmaria mollis* using NSY/HOS mice, assigned to three experimental groups for four weeks. Mice were fed with a standard diet or a high-fat diet, supplemented or not with the red alga *Palmaria mollis* (2.5% w/w). At the end of the study, and compared to the mice fed with the high-fat diet, fasting blood glucose tended to be decreased in mice treated with the alga. *Palmaria mollis* significantly reduced hepatic triglyceride accumulation induced by high-fat feeding, but the control level was not reached. To explain the delipidating effect of the seaweed, the authors measured mRNA levels of several genes involved in hepatic β-oxidation and lipid synthesis. With regard to β-oxidation, peroxisome proliferator-activated receptor alpha (*Ppara*) gene expression was higher in the mice treated with the alga than in the other groups. In the absence of *Palmaria mollis*, *Acox1* gene expression was lower among mice fed with the steatotic diet than in the other groups and *Acadm* gene expression remained unchanged among the three groups. Regarding lipid synthesis-related genes, whereas sterol regulatory element binding transcription factor 1 (*Srebf1*) gene expression remained unchanged among the three groups, peroxisome proliferator-activated receptor gamma (*Pparg*) expression, which was increased by the steatotic feeding, was completely restored by *Palmaria mollis* supplementation. Moreover, CCAAT/enhancer-binding protein alpha (*C/ebpa*) gene expression was higher in the mice treated with the alga than in the other groups. Thus, *Palmaria mollis* showed its ability to suppress hepatic lipid accumulation by the modulation of β-oxidation and of lipid synthesis.

In another study, du Preez et al. (24) analysed the effects of the red algae *Sarconema filiforme* extract that provided 34% of carbohydrates and 12% of proteins in rats. Two groups were fed with either a standard corn starch diet (C) or a high-carbohydrate, high-fat diet (H) for 16 weeks and the other two groups received the same diets but supplemented with 5% of *Sarconema filiforme* extract for the remaining eight weeks. In addition, the drinking water of the rats fed with the high-carbohydrate, high-fat diet was supplemented with 25% fructose. At the end of the experimental period, the high-carbohydrate, high-fat diet significantly increased hepatic fat deposition and infiltration of inflammatory cells, and *Sarconema filiforme* partially prevented these effects. Regarding the serum parameters, glucose levels were higher in the groups that received the high-carbohydrate, high-fat diet than in the groups fed with the control diet. What is more, the algae had no effect on these parameters. In contrast, it did reduce ALT and AST values.

When gut microbiota was analysed, no significant differences were observed in Shannon's diversity or richness of faecal samples among the experimental groups. However, bacterial community structure was affected by both the diet and the alga. Rats fed with the standard diet had lower *Firmicutes/Bacteroidetes* ratio than those fed with the highcarbohydrate, high-fat diet. Nevertheless, no differences in this ratio were observed in rats supplemented with the alga when compared with their pertinent control group. Compared to the other three groups, the abundance of bacteria from the *Bacili* class and from the *Lactobacillaceae* family was found to be lower in rats fed with the high-carbohydrate, high-fat diet with algae supplementation. One zOTU (zero-radius operational

taxonomic unit), which belongs to the *Muribaculaceae* family, was only found in rats not supplemented with *Sarconema filiforme*, whereas another zOTU belonging to the *Ruminococcaceae* and *Desulfovibrionaceae* families was only found in the groups that received the alga. In conclusion, *Sarconema filiforme* supplementation modulated gut microbiota without changing the *Firmicutes*/*Bacteroidetes* ratio. The correlations between changes in the gut microbiota and physiological changes suggest that this is likely to be one of the mechanisms that explain the beneficial effects of *Sarconema filiforme* on this experimental model.

Lee et al. (25) conducted a study aimed at researching the effects of the red algae *Grateloupia elliptica* using C57BL/6 mice. Animals were distributed into four groups: the control group was fed with a chow diet; the HFD group received a high-fat high sucrose diet; the L-GEE group was administered the same high-fat high-sucrose diet but supplemented with a low dose of *Grateloupia elliptica* (125 mg/kg body weight/day) and the H-GEE group was fed with the same high-fat high-sucrose diet but supplemented with a high dose of *Grateloupia elliptica* (250 mg/kg body weight/day). The alga extract was administered orally for seven weeks. Both doses of the *Grateloupia elliptica* significantly reduced the hepatic steatosis, although control values were not reached. The authors did not indicate if a dose-response pattern was observed.

Nabil-Adam et al. (26) have recently published a study devoted to assessing the potential of red alga *Gromphadorhina oblongata* on the prevention of LPS-induced liver inflammation and injuries. For this purpose, BALB/C mice were divided into four groups: a negative control group that received a daily intraperitoneal saline solution, an induction control group that was given 5 mg/kg body weight/day of LPS intraperitoneally; a protected group that received 200 mg/kg body weight/day of the seaweed extract for two hours before LPS treatment, and a positive control group that received 200 mg/kg body weight/day of the seaweed extract without LPS. The experimental period length was one week. At the end of this week, serum transaminases (ALT and AST) were higher in the induction control group than in the other experimental groups. Livers displayed an abnormal histopathological appearance as portal lymphoplasmacytic, inflammatory infiltrates, hydropic alterations, portal lymph plasmatic infiltrate and parenchymal hydropic alterations with apoptosis and binucleated cells were found in the induction group. The *Gromphadorhina oblongata* extract protected against LPS effects.

According to the reported studies, it can be concluded that the five red algae analysed were able to prevent liver triglyceride accumulation induced by diets rich in fat and those rich in fat and sugars. This positive effect is found in both rats and mice. The majority of the reported studies did not address the mechanisms of action involved in this effect. Interestingly, two studies that were focused on *Plocamium telfairiae* showed that the positive effects on liver fat accumulation were observed with quite different experimental period lengths (7 and 14 weeks).

2.2.3. Brown Algae

Four brown algae have been researched in the studies reported in the literature: *Undaria pinnatifida, Fucus vesiculosus, Ascophyllum nodosum Sargassum thunbergii* and *Sargassum horneri* (**Table 4**).

Table 4. Effects of brown macroalgae extracts in animal models.

Author	Algae species	Animal model and experimental period length	Experimental groups	Effects	Mechanisms
Murata *et al.* 1999 (27)	*Undaria pinnatifida*	Male Sprague-Dawley rats 3 weeks	Standard diet Standard diet + 0.5 % *U. pinnatifida* Standard diet + 1 % *U. pinnatifida* Standard diet + 2 % *U.pinnatifida* Standard diet + 5 % *U. pinnatifida* Standard diet + 10 % *U.pinnatifida*	↓ Liver TG content in 1, 2, 5 and 10% groups ↓ Liver TC content in 10% group	↓G6PD activity in 5 and 10% groups ↑ CPT activity in 10% group ↑ ACADs activity in 5 and 10 % groups ↑ ACO in 10% group ↑ DECR1 in 5 and 10 % groups
Murata *et al.* 2002 (28)	*Undaria pinnatifida*	Male Sprague-Dawley rats 4 weeks	Standard diet Standard diet + 19.1% *U. pinnatifida*	↓ Liver weight ↓ Hepatic TG, TC and phospholipids levels	↓ G6PD activity ↑ ACO and 3-hydroxiacil-CoA dehydrogenase activities CPT activity: NS
Li *et al.* 2020 (29)	*Undaria pinnatifida*	Male C57BL/6 mice 10 weeks	Standard diet Standard diet + 10 % *U. pinnatifida* High-fat diet High-fat diet + 10 % *U. pinnatifida*	↓ Liver steatosis ↓ Glucose levels	No information provided
Gabbia *et al.* 2020 (30)	*Fucus vesiculosus* + *Ascophyllum nodosum*	Male Wistar rats 5 weeks	High-fat diet (HFD) High-fat diet + 7.5 mg/kg BW/day of *F. vesiculosus* and *A. nodosum*	↓ Liver weight ↓ Microvesicular steatosis ↓ Plasma ALT and AST levels Lower and delayed glucose peak	No information provided
Kang *et al.* 2020 (31)	*Sargassum thunbergii*	Male C57BL/6 mice 7 weeks	Standard diet High-fat diet High-fat diet + 100 mg/kg BW/day of *S. thunbergii* High-fat diet + 300 mg/kg BW/day of *S. thunbergii*	↓ Lipid steatosis	No information provided
Murakami *et al.* 2021 (32)	*Sargassum horneri*	Male C57BL/ 6J mice 13 weeks	Standard diet High-fat diet (HF) High-fat diet + 2 % *S. horneri* (HF + ShL) High-fat diet + 6 % *S. horneri* (HF + ShH)	↓ Liver weight ↓ Liver TG content ↓ Serum glucose, insulin, ALT, AST, ALP and LAP levels ↑ Serum adiponectin ↓ Serum TNF-α	↓ Pancreatic lipase activity

ACADs: acyl-CoA dehydrogenases, ACO: acyl-CoA oxidase, ALT: alanine transaminase, ALP: alkaline phosphatase, AST: aspartate transaminase, CPT: carnitine palmitoyltransferase; DECR1: 2,4 dienoyl-CoA reductase, G6PD: glucose-6-phosphate dehydrogenase, LAP: leucine aminopeptidase, TC: total colesterol TG: triglycerides, TNF-α: tumor necrosis factor-α, ↑: increased, ↓: decreased, NS: not significant.

Murata et al. (27) carried out a study using Sprague-Dawley rats fed with a standard diet supplemented or not with a percentage of 0.5, 1, 2, 5 and 10 of *Undaria pinnatifida* for three weeks. There was a reduction in hepatic triglycerides in the groups supplemented with 1, 2, 5 and 10% of the seaweed. In order to understand the mechanism involved in these effects, two metabolic pathways, lipogenesis and β-oxidation, were analysed in the groups supplemented with 5 or 10% *Undaria pinnatifida*. Regarding the lipogenic pathway, Acyl-CoA dehydrogenase (ACAD) and 2,4-dienoyl-CoA reductase (DECR1) activities were increased and glucose-6-phosphate dehydrogenase (G6PD) activity was reduced in both supplemented groups. Concerning lipid oxidation, CPT and ACO were increased only in the group receiving 10% *Undaria pinnatifida*. Based on these results, the authors concluded that *Undaria pinnatifida* supplementation increased the activity of fatty acid oxidation, which is responsible for the lower levels hepatic triglycerides. They also pointed out that it was necessary to examine lipid metabolism in more detail.

A further study was carried out by the same group (28) using the same animal model, but in this case, they administered a high dose of seaweed (19.1%) *Undaria pinnatifida* for a period of four weeks. Liver weight and triglyceride content were lower in supplemented animals than in the controls. With regard to the mechanisms of action, G6PD activity was reduced, whereas ACO and 3-hydroxiacil-CoA dehydrogenase were increased in the *Undaria pinnatifida* group. Contrarily to the effects observed in the previous study, CPT activity remained unchanged, but the authors did not explain this discrepancy.

Using the same brown alga, Li et al. (29) conducted a study using C57BL/6J mice which were distributed in four groups: standard diet group (C), standard diet group with 10% of *Undaria pinnatifida* (NUP), high-fat diet group (HFD) and high-fat diet group with 10% *Undaria pinnatifida* (HUP). After ten weeks of treatment, the glycemic analysis showed higher values in the HFD group than in the C, NUP and HUP groups. The histological analysis showed lager hepatocytes size and fat vacuoles in the HFD group. The analysis of gut microbiota composition revealed that *Undaria pinnatifida* restored the instability in *Firmicutes* and *Bacteroides* induced by the high-fat diet. Related to family level, *Undaria pinnatifida* restored the abundance of *Lachnospiraceae* and *Streptococcaceae*. Furthermore, in HUP there were a higher abundance of *Bacteroidaceae* and lower abundance of *Marinifilaceae*, compared with the HFD group.

Gabbia et al. (30) used a phytocomplex extracted from the brown seaweeds *Fucus vesiculosus* and *Ascophyllum nodosum* to analyse its protective effect on rats fed with a high fat diet. Wistar rats were randomised into two experimental groups and were administered a high-fat diet (HFD) (60.3% of energy from fat). One of the groups was treated with 7.5 mg/kg body weight of the alga extract by intragastric gavage on a daily basis. Liver weight of animals supplemented with the seaweed extract was significantly lower than that of rats receiving the HFD alone. While HFD rats showed moderate microvesicular steatosis, animals treated with the seaweed extract just showed isolated steatotic

hepatocytes. This effect was in accordance with plasma ALT and AST levels, which were significantly decreased. The authors additionally measured postprandial blood glucose levels, and they observed that after a starch-simulated, high-carbohydrate meal, animals receiving the seaweed extract showed a delayed and lower blood glucose peak, indicating that the treatment improved post-prandial glucose control.

Kang et al. (31) studied the effect of the brown algae *Sargassum thunbergii* on lipid accumulation in the liver of mice which displayed steatosis induced by a high-fat diet. C57BL/6 mice were distributed into four experimental groups: the control group was fed with a chow diet, the HFD group received a high-fat diet (45% fat), the ST100 group was administered the same high-fat diet together with 100 mg/kg/d of *Sargassum thunbergii* extract and the ST300 group was fed with the same high-fat diet along with 300 mg/kg body weight/day of *Sargassum thunbergii* extract. The extract was dispensed orally for seven weeks. Hepatic histological analysis revealed that, as expected, the high-fat diet increased lipid accumulation into the liver. When compared to the HFD group, smaller lipid droplets were found in the ST100 and in ST300 groups. This reduction was found in a dose-dependent manner.

Using an alga from the same genus, *Sargassum horneri*, but administered to mice instead of rats, Murakami et al. (32) carried out a study where C57BL/6J mice were fed with a normal diet, a high-fat diet (HF) or a high-fat diet supplemented with either 2% (HF + ShL) or 6% (HF + ShH) of the seaweed. After 13 weeks of treatment, and compared to animals fed with the high-fat diet, both groups receiving the alga showed reductions in body and white adipose tissue weight, as well as in serum glucose and insulin levels. Mice in the HF group developed liver steatosis, as shown by the increase in liver weight and triglyceride content. This effect was also revealed through histological analysis. Supplementation with the seaweed avoided these effects in a dose-dependent manner. Serum ALT and AST levels were also normalised, but only after supplementation with the highest dose. Since animals receiving seaweed supplementation showed higher content of fat in faeces, the authors hypothesised that the reduction in liver triglycerides might have been due to a diminished absorption of dietary lipids.

Among the brown macroalgae, the most frequently analysed is *Undaria pinnatifida*, which has been demonstrated to be effective in both rats and mice after medium length and longer experimental periods. Nevertheless, in the three studies reported, where this type of alga has been used, the diets administered to animals were standard diets providing normal amounts of fat and sugars, the two nutrients that increase liver triglyceride accumulation. Consequently, further studies that make use of the diet that induces steatosis are needed to confirm the preventive effects of this seaweed. In the case of *Sargassum thunbergii*, this seaweed prevents the liver steatosis induced by a high-fat diet, in both mice and rats, in a dose-dependent manner.

3. Human Studies

To date, very little information aimed at analysing the effects of algae in humans has been reported. Ebrahimi-Mameghani et al. (33) carried out a double-blind, placebocontrolled, randomised clinical trial in 55 obese patients aged 20–50 years with confirmed NAFLD by ultrasonography. Individuals in the intervention group (29) received 1200 mg/day of *Chlorella vulgaris* dispensed in four tablets of 300 mg and 400 mg/day of vitamin E, whereas the placebo group (26) received 400 mg/day of vitamin E and four placebos for eight weeks. In this study, the only parameters related to the liver were serum transaminases, which did not yield differences between both experimental groups.

Li et al. (34) studied the association of algae consumption with newly diagnosed NAFLD by ultrasound in the adult population. To do so, they carried out a cross-sectional study involving 24,572 adult subjects from The Republic of China. The authors observed that algae consumption, assessed using a food frequency questionnaire, was negatively associated with the prevalence of NAFLD, especially in non-obese patients. Adjustments for several factors were implemented: age, sex, body mass index (BMI), smoking status, alcohol drinking status, socioeconomic status, physical activity, family history of disease (including cardiovascular disease, hypertension, hyperlipidaemia and diabetes), hypertension, hyperlipidaemia, diabetes and total energy intake. Additional adjustments were also applied for “fruits and sweet”, “healthy” and “animal foods” dietary pattern scores. The authors stated that to clarify the causality, more prospective studies and clinical trials were required.

4. Concluding Remarks

The reported data described in the present review show that there is scientific evidence supporting the beneficial effects of microalgae and the different types of macroalgae (green, red and brown) on liver steatosis in rodent models (**Figure 1**). The vast majority of the reported studies analysed the preventive effects, since algae were administered to animals together with the diet that induced liver steatosis. The positive effect has been demonstrated using a wide range of doses in the diet, 7.5 to 300 mg/kg body weight/day or 1 to 10% and experimental periods ranging from 3 to 16 weeks. Different ways of algae administration have been used in the published studies, included in the diet, included in the drinking water or administered by oral gavage, and all of them have been adequate to show the beneficial effects of algae.

Two important limitations of the scientific knowledge available to date are: (a) very few studies have investigated the mechanisms of action underlying the preventive effects of algae on liver steatosis, (b) for the majority of the algae studied, a single paper has been reported and thus, it is not possible to establish the best conditions so as to recognize their beneficial effects and (c) all the studies have been performed in male rodents, and consequently potential sexual dimorphism has not been addressed. In this scenario, further studies are necessary not only to clarify the mechanisms of action

of the anti-steatotic effects of algae, but also to analyse their potential effects on the management of liver steatosis when this alteration is already developed.

Moreover, the beneficial effects of algae observed in rodent need to be standardised to humans, before we can start thinking about these products as the new tools in the fight against fatty liver. To date, only two studies have addressed the effects of seaweeds on liver steatosis, a study where *Chlorella vulgaris* was administered as a nutraceutical (tablets) and another study based on the daily algae consumption of the studied population. Despite the great differences in terms of experimental design, both of them revealed positive aspects of algae consumption.

Taking into account that the susceptibility to develop NAFLD depends on genetic background, among other factors (35), it is important to address future studies devoted to analysing potential interactions of algae treatments with genetics and epigenetics, in order to established which subjects can get the most benefit, in the framework of personalized nutrition. Another factor with an important role in the development of NAFLD is gut microbiota (36). Considering that several components of algae are able to modify microbiota composition (37-39), an interesting field of future research is to establish the relationship between these modifications and the improvement of NAFLD produced by algae.

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Anti-Steatotic Effects of *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* Algae Extracts in AML-12 Hepatocytes

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is considered the most common chronic liver alteration whose prevalence is increasing in Western countries. Microalgae and macroalgae have attracted great interest due to the high content in bioactive compounds with beneficial effects on health. The aim of the present study is to assess the potential interest of extracts rich in proteins obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana* and the macroalga *Gracilaria vermiculophylla* in the prevention of lipid accumulation in AML-12 hepatocytes. Toxicity was not observed at any of the tested doses. Both microalgae and the macroalga were effective in preventing triglyceride accumulation, with *Nannochloropsis gaditana* being the most effective one. Although the three algae extracts were able to increase different catabolic pathways involved in triglyceride metabolism, the mechanisms underlying the anti-steatotic effect were different in each algae extract. In conclusion, the present study demonstrates that *Chlorella vulgaris, Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts are able to partially prevent the accumulation of triglycerides induced by palmitic acid in cultured hepatocytes, a model used to mimic the steatosis induced in liver by dietary patterns rich in saturated fat.

Keywords: microalgae; macroalgae; seaweed; extracts; non-alcoholic fatty liver disease; steatosis; proteins; lipid metabolism

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), characterised by the excess of lipid accumulation within hepatocytes (>5%) (1), is the most common chronic liver disease in Western countries, affecting approximately 25% of the adult population (2). Its prevalence continues to grow due to the increase in sedentary behaviours and westernisation of the diet, factors that contribute to the above-mentioned metabolic alteration. The development of NAFLD is closely related to obesity and insulin resistance (3). Recently, a panel of experts has proposed renaming NAFLD as metabolic dysfunction-associated fatty liver disease (MAFLD) since it more accurately reflects the heterogeneous pathogenesis and the underlying metabolic abnormalities (4).

Nowadays, there is no pharmacological treatment approved for liver steatosis. Diet and physical activity are the main common strategies used to prevent or treat this hepatic alteration (5). Consequently, there is a growing interest in the development of new therapies (2) based, for instance, on bioactive molecules present in foodstuffs and plants. In this context, many authors are focusing their attention on marine algae, which contribute to the sustainable blue growth in the European Union, a strategy that ranges from responsible food systems to decarbonisation, as well as biodiversity and coastal resilience to circularity (6). Algae are rich in several bioactive compounds such as pigments, polyunsaturated fatty acids, phenolic compounds, peptides, lipids, vitamins, polysaccharides or sterols, among others. It has been reported that they exert antioxidant, anti-inflammatory, anti-obesity, anti-diabetic, hypolipidemic, anti-hypertensive and anti-cancer activities (7–9). As a result, diets supplemented with these organisms, both micro- and macroalgae, may have positive effects on chronic diseases (10-13).

Regarding the effects of algae on hepatic steatosis, although several species of both microalgae and macroalgae have demonstrated to have a positive effect on this alteration, the reported studies are still scarce (10). The aim of the present study is to assess the potential interest of extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana* and from the macroalga *Gracilaria vermiculophylla* in the prevention of liver steatosis. To our knowledge, the activity of these algae on hepatic triglyceride accumulation has yet to be studied. Furthermore, our aim is to identify the mechanisms responsible for this effect.

2. Materials and Methods

2.1. Algae Extract Preparation

Gracilaria vermiculophylla was manually collected in September 2019 in the Bidasoa estuary (Hondarribia, Gipuzkoa, Spain). After collection and washing with marine water in the field, a second washing with fresh water was carried out in the lab to remove the impurities. After, samples were vacuum packed and frozen at −20 °C. Both microalgae (*Chlorella vulgaris* and *Nannochloropsis gaditana*)

were provided by NEOALGAE (Gijón, Asturias, Spain) in frozen fresh-paste format (20–25% total solids) and stored at −20 °C until use. Before extraction, frozen biomass was thawed at 40 °C and diluted to the desired solid content with distilled water.

Microalgae extracts were produced by adapting the methodology of Safi et al. (14,15). A suspension of microalgae at 10% dry weight and adjusted to pH 12 with NaOH 10 M was prepared. The suspension was vacuum percolated through 100 μm filters to avoid issues with the Ultra High-Pressure Homogenizer (UHPH) equipment MicroDeBee (Bee International, South Easton, USA) and stored in cold and dark atmosphere to avoid deterioration. The applied UHPH conditions were 250 MPa, 250 μm orifice and three cycles. The samples were submerged in an ice-water bath for proper preservation. After the UHPH process, samples were centrifuged at 10,000× *g* for 10 min at 4 °C (avoiding direct light). Soluble proteins were collected in the supernatant. These proteins were precipitated by adjusting the pH to 3.0 with 6 M HCl. Samples were again centrifuged at 10,000× *g* for 10 min at 4 °C, and the pellet was resuspended in 0.1 M phosphate buffer with a pH of 7.5.

Macroalga extract was prepared as follows: frozen samples were thawed in a water bath at 40 °C and crushed to a particle size between 1 and 5 mm. A suspension of the macroalga in water at 5% of dry weight solids was prepared, grinded with a homogeniser (Ultra-turrax-IKA-T25, Staufen Germany) for two minutes at 18,000 rpm and adjusted to a pH of 12 with NaOH 10 M. The suspension was then treated by ultrasounds (VibraCell 75042, Bioblock Scientific, Illkirch, France) for 1 h and 30 min, in cycles of 59 s ON and 15 s OFF. This treatment was also submerged in an ice-water bath to avoid an increase in temperature and protected from light to prevent alterations in bioactive compounds. After the extraction process, samples were handled in the same way as the microalgae suspensions after the UHPH procedure.

2.2. Algae Extract Composition

The proximate composition of the extracts was analysed according to the Association of Official Analytical Chemists (AOAC) Official Methods (16). The moisture of the samples was determined through a drying process at 100 °C to a constant weight (method 934.01). Crude protein content was determined by Kjeldahl methodology, using a conversion factor for nitrogen-to-protein of N × 6.25 and 4.59 for microalgae and macroalga, respectively (method 955.04). Crude fat content was determined by Soxhlet methodology (method 920.39). Carbohydrate content was calculated by the difference in 100 g of algae extract. The total phenolic content was measured following the procedure described by Singleton and Rossi (17).

2.3. Characterization of the Protein Fraction

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used on the algae extracts. Based on the protein content estimated by Kjeldahl, the extracts were dissolved in sample buffer, which contained Tris-HCl (0.05 M, pH 6.8, SDS (1.6% *w:v*), glycerol (8% *v:v*), β-

mercaptoethanol (2% *v:v*) and bromophenol blue indicator (0.002% *w:v*) at protein concentrations of 0.8, 1.0 and 1.5 mg/mL. The samples were heated at 95 °C for 5 min and were subsequently loaded on the 12% Bis-Tris polyacrylamide gel (Criterion XT, Bio-Rad, Richmond, CA, USA). Electrophoretic separations were carried out at 150 V using XT-MES as a running buffer (Bio-Rad) in the Criterion cell (Bio-Rad). The gels were stained with Coomassie Blue (Instant Blue, Expedeon, Swavesey, UK), and images were taken with a Molecular Imager VersaDoc™ MP 5000 system (Bio-Rad, Hercules, CA, USA).

The protein/peptide size distribution was evaluated with high-performance size-exclusion chromatography (HPLC-SEC) using an ultra-high performance liquid chromatography instrument (Waters) equipped with a bioZenTM 1.8 µm 150 × 4.6 mm column and a bioZenTM SEC-2, 4.6 mm pre-column (Phenomenex). The mobile phase was a 55:45:0.1 water:acetonitrile:TFA (*v/v/v*) solution with a flow of 100 µL/min. The algae extracts were dissolved in water at a concentration of 3 mg/mL, diluted in the mobile phase to obtain a protein concentration of 1.5 mg/mL and centrifuged for 5 min at 12,800× *g* at room temperature to separate the soluble fraction. A total of 3 µL of each sample was injected into the system and the absorbance was registered at wavelengths of 214 and 280 nm. Peptides of known molecular weight (insulin with 5800 Da and peptides VPFPGPI and VYII with 888 Da and 506 Da, respectively) were used as molecular weight markers using the same chromatographic conditions.

2.4. Cell Culture and Treatment

A mouse hepatocyte AML12 cell line (alpha mouse liver 12; ATCC® CRL-2254™), obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), was maintained in 75 cm^2 flasks in DMEM/HAM's F12 Glutamax and supplemented with 10% heat inactivated foetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone and 1% penicillin/streptomycin (10,000 U/mL) at standard cell culture conditions (37 °C, 5% CO_2). When the cell monolayer reached 75% of confluence, cells were detached with a solution of trypsin-EDTA and harvested to perform subsequent experiments.

AML12 cells were incubated with palmitic acid (PA) to create an in vitro model of steatotic hepatocytes (18). Cells were briefly exposed or not to 0.3 mM of PA to induce triglyceride accumulation and co-incubated with algae extracts for 18 h, adjusting the final concentration of each algae extract in culture media to 10, 25, 50 or 150 µg/mL. The control groups received the same amount of the vehicle. Each experiment was performed at least three times.

After 18 h, the incubation medium was collected, and cells were harvested according to the subsequent analysis. Cells used for triglyceride determination and protein immunodetection were collected in 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer through scrapping. The cell suspension was then sonicated with 5 s bursts in a

Branson Sonifier SFX550 (San Luis, Missouri, MO, USA) fitted with a microtip. Cells used for RNA extraction were collected in TRIzol® reagent.

2.5. Cell Viability Assay

Cell viability was assessed using the crystal violet assay, based on cell staining with crystal violet (19). Following treatment, AML12 cells were briefly washed with phosphate buffered saline (PBS), fixed in 3.7% formaldehyde and stained with 0.25% crystal violet in the dark for 20 min. Lastly, the resulting crystals were solubilised with 33% acetic acid, and the absorbance was registered at 590 nm in an iMark microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was expressed as the percentage of the control cells.

2.6. Determination of Triglyceride Content

Triglyceride content in cell suspension was measured using a commercial kit (Spinreact, Girona, Spain). Protein measurements were performed using the Bradford method (20). Triglyceride content values were obtained as mg triglycerides/mg protein and expressed as the percentage of the control cells.

2.7. Detection of Alanine Aminotransferase (ALT/GPT) Levels in Cell Culture Medium

To determine ALT/GPT levels in cell culture medium, a commercial kit (BioSystems, Barcelona, Spain) was used.

2.8. Analysis of Gene Expression by Real-Time PCR

RNA was extracted from cells using RNeasy Mini Spin Columns (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase treatment (Ambion, Foster City, CA, USA), the integrity of the RNA was verified and quantified using an RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). A total of 1.5 μg of total RNA in a total reaction volume of 30 μL from each sample was reverse-transcribed into complementary DNA (cDNA) using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Reactions were incubated initially at 25 °C for 5 min, subsequently at 42 °C for 30 min and finally at 85 °C for 5 min.

Adipose triglyceride lipase *(Atgl),* carnitine palmitoyltransferase I a *(Cpt1a),* citrate synthase *(Cs),* fatty acid synthase *(Fasn),* long chain acyl-CoA dehydrogenase *(Acadl),* mitochondrial transcription factor A *(Tfam)* and uncoupling protein 2 *(Ucp2)* mRNA levels were quantified. Beta actin (*Actb*) served as housekeeping for posterior normalisation. An aliquot of 4.75 μL of diluted cDNA sample was amplified by Real-Time PCR in a 12.5 μL reaction volume. The cDNA samples were amplified in an iCycler-MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in the presence of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the sense and antisense primers (300 nM each except for *Fas*, where a concentration of 600 nM was used). The primer sequences are described in Table 1. The PCR parameters were as follows: initial 2 min at 50 °C,

denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 60 °C for 30 s. For *Acadl* and *Fasn*, the annealing temperature was 61 and 62.2 °C, respectively.

Acetyl-CoA carboxylase (*Acc*) and diacylglycerol acyltransferase (*Dgat2*) were amplified using TaqMan probes. *Actb* mRNA levels were similarly measured and served as the reference gene. In total, 4.5 µL of each diluted cDNA sample was added to the PCR reagent mixture (final volume of 10 µL), which consisted of TaqMan Fast Advanced Master Mix (Applied Biosystems, Vilna, Lithuania) and TaqMan Gene Expression Assay Mix (Applied Biosystems, Foster City, Ca, USA) containing specific primers and probes (**Table 1**). The PCR parameters were as follows: initial 2 min at 50 °C, denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 3 s and combined annealing and extension at 60 °C for 30 s.

In all cases, the results were expressed as fold changes of the threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method (21).

Table 1. Primer sequences, gene accession and assay ID for quantitative Real-Time PCR amplification.

SYBR Green RT-PCR			
Gene	**Gene Accession**	**Sense Primer 5'-3'**	**Antisense Primer 5'-3'**
Acadl	NM_007381.4	TGG GGA CTT GCT CTC AAC A	GGC CTG TGC AAT TGG AGT
Actb	NM_007393.5	ACG AGG CCC AGA GCA AGA G	GGT GTG GTG CCA GAT CTT CTC
Atgl	NM_025802.3	GAG CTT CGC GTC ACC AAC	CAC ATC TCT CGG AGG ACC A
Cpt1a	NM_013495.2	CGG TTC AAG AAT GGC ATC ATC	TCA CAC CCA CCA CCA CGA T
Cs	NM_026444.4	GCC TCT GCA TGG ACT AGC AAA	TTG CCG ACT TCC TTC TGT AGC T
Fasn	NM_007988.3	AGC CCC TCA AGT GCA CAG T	TGC CAA TGT GTT TTC CCT G
Tfam	NM_009360.4	AAG CTT ATC CAT GAC AGC TAA AGG	GGC TGG CTC ACC ACA GTT
Ucp2	NM_011671.5	TAC TCT CCT GAA AGC CAA CCT C	CAA TGA CGG TGG TGC AGA AG
Taqman RT-PCR			
Gene	**Gene Accession**	**Assay ID**	
Acc	NM_133360.2	Mm01304285_m1	
Actb	NM_007393.5	Mm02619580_g1	
Dgat2	NM_026384.3	Mm00499536_m1	

Acadl: long chain acyl-CoA dehydrogenase; *Acc*: acetyl-CoA carboxylase; *Actb*: beta actin *Atgl: adipose triglyceride lipase; Cpt1a*: carnitine palmitoyltransferase I A, *Cs*: citrate *synthase; Dgat2*: diacylglycerol acyltransferase; *Fasn*: fatty acid synthase; *Tfam:* mitochondrial transcription factor A; *Ucp2*: uncoupling protein 2.

2.9. Analysis of Protein Expression by Western Blot

Solute carrier family 27 member 2 (FATP2) and microsomal triglyceride transfer protein (MTTP) were assessed by western blot. The protein concentration was determined according to the Bradford protocol (20). Protein samples (40 µg) were denaturalised at 95 °C for 3 min in Laemmli buffer (22) and loaded into 4–15% Mini-PROTEAN TGX Precast Gels (BioRad, Hercules, CA, USA). The proteins were then transferred onto PVDF membranes (Millipore, MA, USA) by electroblotting and later blocked with 5% casein and 0.5% bovine serum albumin (BSA) PBS-tween buffer for 2 h at

room temperature. Subsequently, membranes were incubated with anti-FATP2 (1:500) (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-MTTP (1:500) (Abcam, Cambridge, UK) and anti-α-tubulin (1:2000) (Cell signaling, Beverly, MA, USA) for at least 1 h at room temperature and kept at 4 °C overnight. After washing, membranes were incubated with secondary antibodies (mouse anti-goat IgG; 1:5000 and mouse anti-rabbit IgG; 1:5000) (Santa Cruz Biotech, CA, USA); the immunoreactive proteins were detected by the Forte Western HRP substrate (Millipore; Burlington, MA, USA), and the blots were imaged by scanning with the ChemiDoc™MP Imaging System (Bio-Rad, CA, USA). α-tubulin was used as housekeeping.

2.10. Statistical Analysis

The results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 26.0 (SPSS, Chicago, IL, USA). The normal distribution of the data was tested using the Shapiro-Wilk test. Data from the groups treated with algae extracts were compared with control cells or with PA cells using the Student's *t* test or Mann-Whitney's U test as appropriate. Statistical significance was established at the $p < 0.05$ level.

3. Results

3.1. Composition of Algae Extracts

The proximal composition of algae extracts is shown in **Table 2**. Dry weights were 90.27 ± 0.80 for *Chlorella vulgaris,* 90.02 ± 0.13 for *Nannochloropsis gaditana* and 91.70 ± 2.38 for *Gracilaria vermiculophylla*. The lowest fat percentage was found in *Gracilaria vermiculophylla* extract, and the highest one was found in the *Chlorella vulgaris* extract. By contrast, the percentage of protein was quite similar in the three extracts. Finally, regarding minerals, the *Nannochloropsis gaditana* extract showed a two-fold percentage compared to the other two extracts.

Table 2. Proximal composition of the algae extracts used in the present study.

	Protein (%)	Fat (%)	Ash (%)	Carbohydrates (%)
Chlorella vulgaris	48.0 ± 0.2	11.8 ± 2.0	20.5 ± 0.6	21.1 ± 0.6
Nannochloropsis gaditana	42.0 ± 9.2	5.6 ± 0.1	42.6 ± 4.2	9.9 ± 4.4
Gracilaria vermiculophylla	41.4 ± 0.7	3.5 ± 0.1	22.1 ± 3.2	33.4 ± 3.6

% of dry weight (DW). Carbohydrates were calculated by difference.

The *Chlorella vulgaris* extract contained 12.27 ± 0.39 mg of total polyphenols/dry weight, calculated as gallic acid equivalents, *Nannochloropsis gaditana* 9.08 ± 1.56 mg and *Gracilaria vermiculophylla* 2.72 ± 0.08 mg. Thus, the extract of *Chlorella vulgaris* had the highest concentration and *Gracilaria vermiculophylla* had the lowest.

The molecular weight (MW) distribution of proteins and peptides (above 10 kDa) in the algae extracts was studied through SDS-PAGE under denaturising conditions. As shown in **Figure 1A**, while some bands were clearly visible in the two microalgae extracts, it was not possible to identify protein bands in the case of the *Gracilaria* macroalga, even at the highest protein concentration tested. This was most likely caused by the lower solubility of this extract and its higher polysaccharide concentration, thus, hindering the transport and staining of the protein through the well. Regarding the *Chlorella* microalga, the most intense band was detected within the range of 20-25 kDa. Moreover, a coloured band lower than 2 kDa was also detected. In the case of the *Nannochloropsis* microalga, the main bands corresponded to 37 kDa, 50 kDa and 100 kDa.

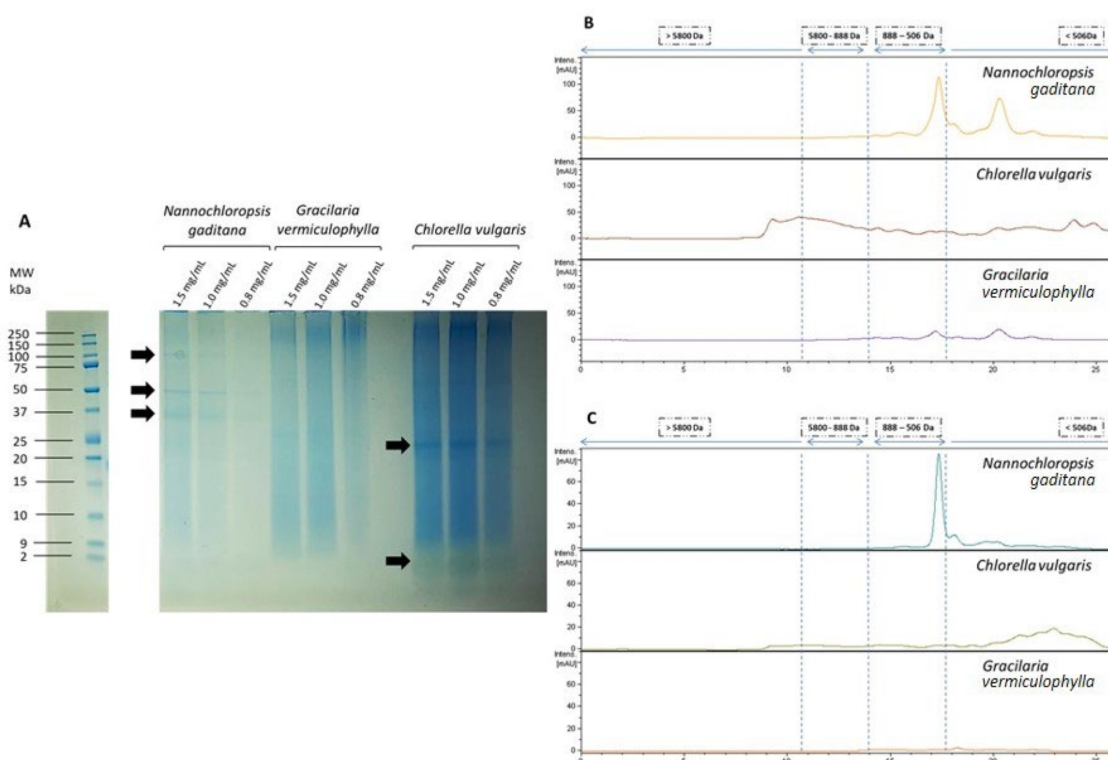

Figure 1. (**A**) SDS-PAGE protein profiles of the algae extracts at different protein concentrations. The arrows point towards the most intense bands. (**B**,**C**) HPLC-SEC chromatograms of the algae extracts detected at wavelengths of 214 nm (**B**) and 280 nm (**C**). The chromatograms for the three samples are shown at the same intensity scale.

The molecular weight distribution of the extracts was further studied by means of HPLC-SEC, and representative chromatograms obtained at wavelengths of 214 nm and 280 nm (specific for amino acid residues with aromatic rings) are shown in **Figure 1B,C**, respectively. With regards to the *Chlorella vulgaris* extract, the chromatogram at 214 nm showed a very heterogeneous size distribution, with peaks appearing in a wide range of MW, while at 280 nm, only small peptides (<500 Da) were detected. The extract from *Nannochloropsis gaditana* revealed more intense peaks, specifically within the

range of 1-0.5 kDa and <0.5 kDa. Contrarily, the extract from the macroalga *Gracilaria* showed a very low intensity, once again suggesting the low solubility of the proteins from this extract. Thus, only two small peaks corresponding to low MW compounds (<1 kDa) were visible in the chromatograms.

3.2. Cell Viability

Hepatocytes incubated with 10, 25, 50 or 150 µg/mL of each algae extract, together with PA, showed no loss of viability compared with cells incubated with PA alone (**Figure 2 A–C**). However, cell viability was decreased by 36% in the PA group with regards to the control group.

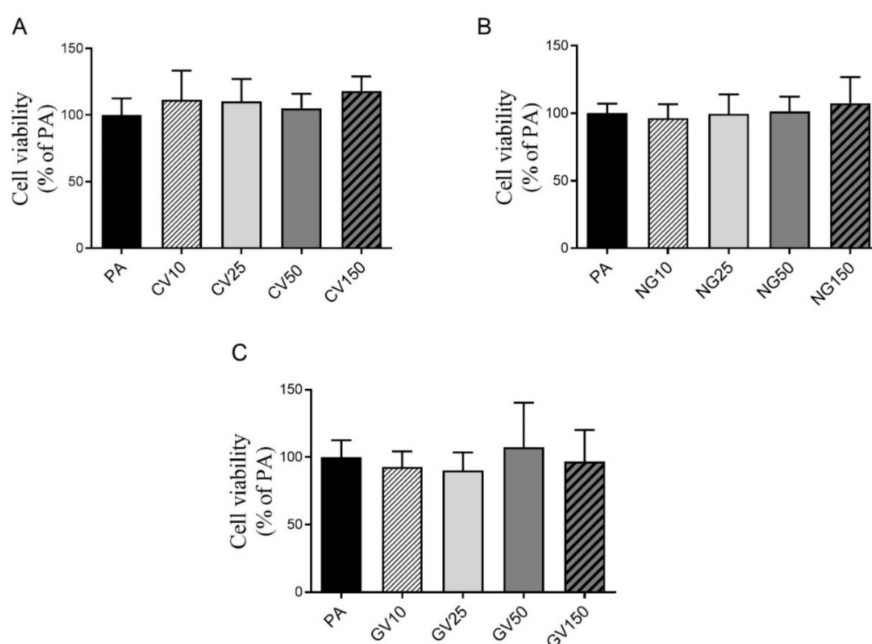

Figure 2. Cell viability in AML12 hepatocytes exposed to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) (**A**)*, Nannochloropsis gaditana* (NG) (**B**) and *Gracilaria vermiculophylla* (GV) (**C**) at 10, 25, 50 or 150 µg/mL for 18 h. Data are means ± SEM (standard error of the mean).

3.3. Effects on Triglyceride Accumulation

When cells were treated with *Chlorella vulgaris*, only those receiving 50 or 150 µg/mL of the microalga extract showed a decrease in triglyceride content (−22% and −24%, respectively) (**Figure 3A**). *Nannochloropsis gaditana* extract prevented triglyceride accumulation when cells were exposed to 10 or 25 µg/mL (−27% and −34%, respectively), but not at higher doses (**Figure 3B**). After treatment with *Gracilaria vermiculophylla*, the dose of 10 µg/mL proved ineffective, whereas the other three doses significantly avoided triglyceride accumulation induced by PA (−18%, −22% and −24%, respectively) (**Figure 3C**). In all cases, with the exception of *Nannochloropsis gaditana* at 25 µg/mL, the prevention of triglyceride accumulation was partial because values did not reach those found in the control hepatocytes.

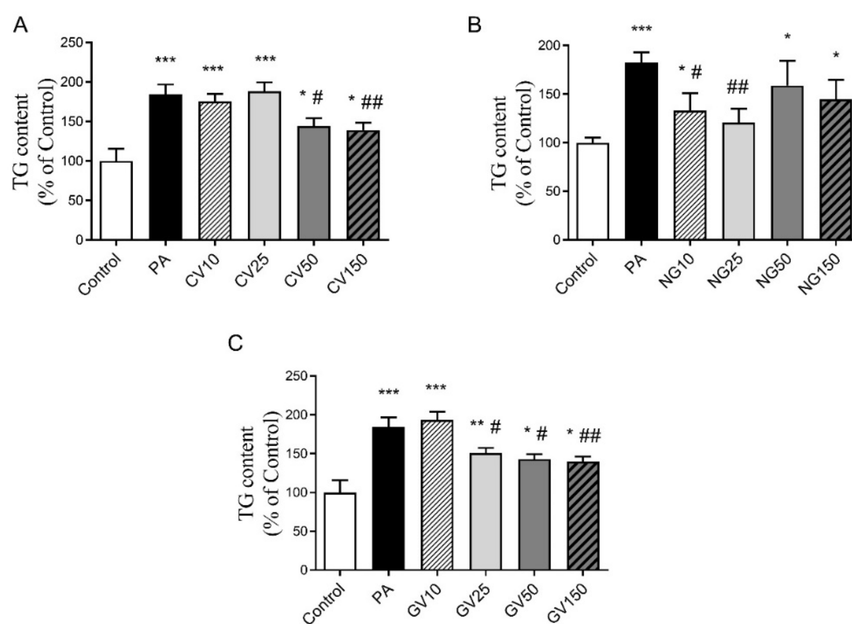

Figure 3. Triglyceride (TG) content in AML12 hepatocytes exposed or not to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) (**A**), *Nannochloropsis gaditana* (NG) (**B**), and *Gracilaria vermiculophylla* (GV) (**C**) at 10, 25, 50 or 150 µg/mL for 18 h. Data are means ± SEM (standard error of the mean). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control cells, # $p < 0.05$ and ## $p < 0.01$ vs. PA cells.

3.4. Detection of ALT/GPT Level in Cell Culture Medium

The ALT/GPT concentration was measured in cell culture medium after the treatment of hepatocytes, with the dose of each algae extract showing the greatest reduction in triglyceride content: that is, 150 µg/mL for *Chlorella vulgaris* and *Gracilaria vermiculophylla* and 25 µg/mL for *Nannochloropsis gaditana*.

In PA cells, ALT/GPT level increased significantly compared to the control cells. Regarding those treated with the algae extracts, ALT/GPT levels were significantly reduced in *Chlorella vulgaris* and *Gracilaria vermiculophylla* cells when compared to PA cells. The concentration in the incubation medium of cells treated with *Nannochloropsis gaditana* was reduced (−37%), although statistical significance was not reached (**Figure 4**).

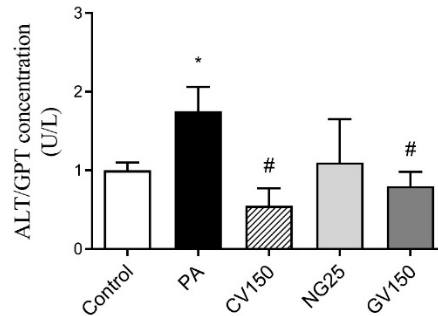

Figure 4. Alanine aminotransferase (ALT/GPT) level in cell culture medium of AML12 hepatocytes exposed or not to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) at 150 µg/mL, *Nannochloropsis gaditana* (NG) at 25 µg/mL and *Gracilaria vermiculophylla* (GV) at 150 µg/mL for 18 h. Data are means ± SEM (standard error of the mean). * $p < 0.05$ vs. control cells, # $p < 0.05$ vs. PA cells.

3.5. Effects on Genes and Proteins Involved in Triglyceride Metabolism

Gene and protein expressions were analysed in hepatocytes treated with the dose of each algae extract showing the greatest reduction in triglyceride content: that is, 150 µg/mL for *Chlorella vulgaris* and *Gracilaria vermiculophylla* and 25 µg/mL for *Nannochloropsis gaditana*.

Concerning the metabolic pathways that contribute to triglyceride accumulation in liver, the expression of genes and proteins involving fatty acid synthesis, fatty acid uptake and triglyceride assembly were analysed. Regarding de novo lipogenesis, gene expressions of *Acc* (**Figure 5A**) and *Fasn* (**Figure 5B**) were not significantly modified by any of the treatments. Gene expression of *Fatp2*, which is a transmembrane protein in charge of exogenous long chain fatty acid uptake, was not detected, and mRNA levels of *Dgat2*, involved in triglyceride assembly, remained unchanged (**Figure 5D**). In view of the fact that *Fatp2* gene expression was not detected, FATP2 protein expression was measured, and it was observed that it remained unchanged after the treatment with algae extracts. Nevertheless, it is worth mentioning that a non-statistically significant trend towards reduced levels (−50%) was appreciated in hepatocytes treated with *Gracilaria vermiculophylla* ($p = 0.084$) (**Figure 5C**) when compared to the PA group.

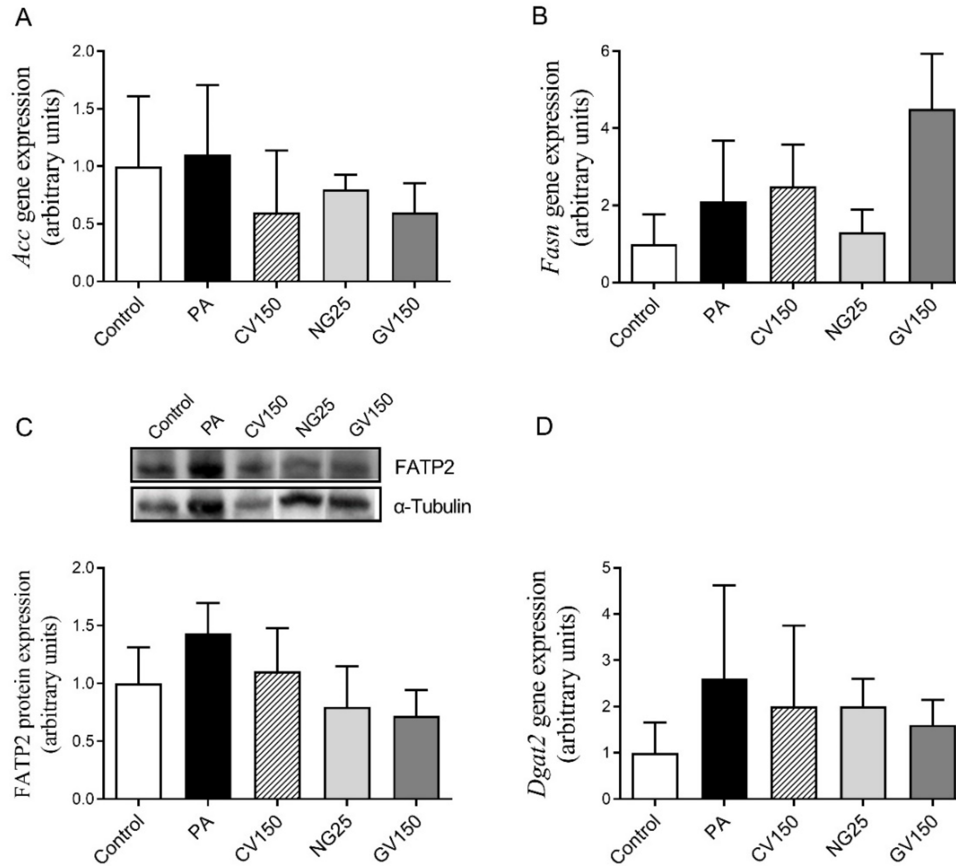

Figure 5. Gene expression of *Acc* (**A**) and *Fasn* (**B**), protein expression of FATP2 (**C**) and gene expression of *Dgat2* (**D**) in AML12 hepatocytes exposed or not to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) at 150 µg/mL, *Nannochloropsis gaditana* (NG) at 25 µg/mL and *Gracilaria vermiculophylla* (GV) at 150 µg/mL for 18 h. Data are means ± SEM (standard error of the mean). The western blot bands shown are representative of 6 samples/group. *Acc*: acetyl-CoA carboxylase; *Dgat2*: diacylglycerol acyltransferase; *Fasn*: fatty acid synthase; FATP2: fatty acid transport protein 2.

With regard to triglyceride secretion, only hepatocytes treated with *Chlorella vulgaris* showed a significant increase in MTTP protein expression; the rest of the algae treatments induced no changes (**Figure 6A**). Gene expression of *Atgl*, which is responsible for triglyceride hydrolysis, was not modified by any of the algae treatments when compared with PA incubation alone, although its expression was increased by 133% in the GV group (**Figure 6B**). Regarding fatty acid oxidation, gene expression of *Cpt1a* and *Acadl* were analysed; the first one is responsible for the transportation of long-chain fatty acids into mitochondrial matrix for beta-oxidation, and hile the second gene catalyses the first step of mitochondrial fatty acid beta-oxidation. *Cpt1a* showed a significant increase in cells treated with the three algae extracts (**Figure 6C**). Lastly, only *Gracilaria vermiculophylla* extract increased mRNA levels of *Acadl* (**Figure 6D**).

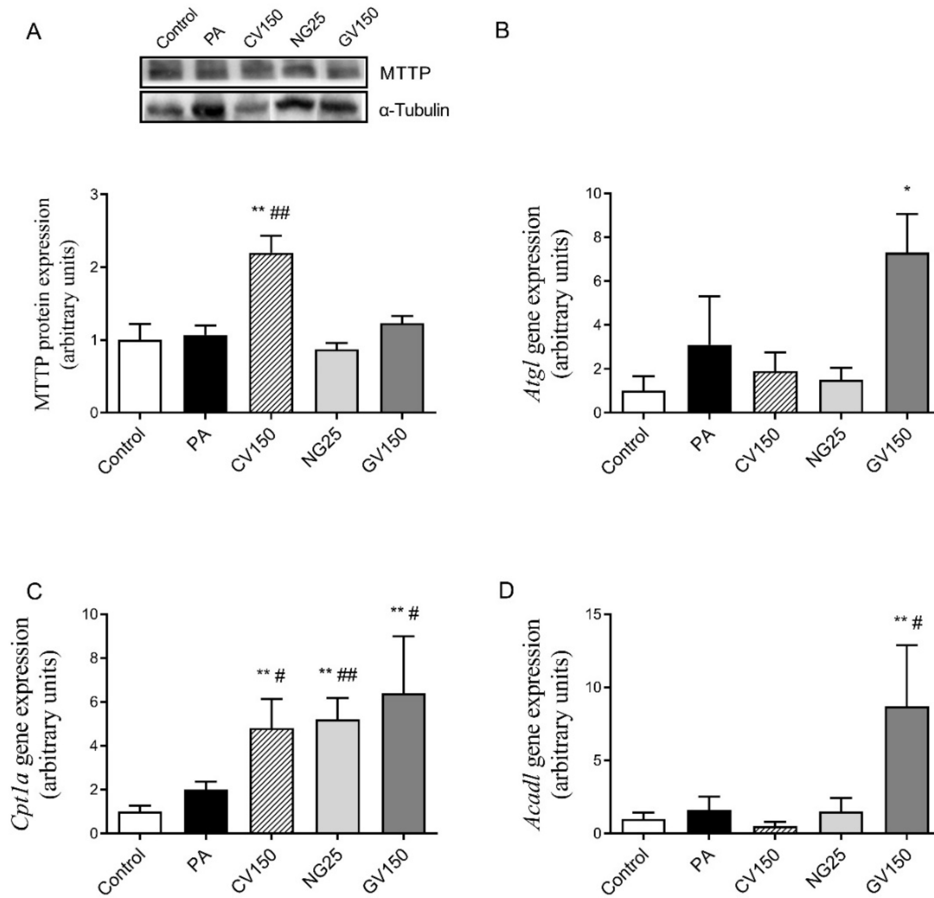

Figure 6. Protein expression of MTTP (**A**) and gene expression of *Atgl* (**B**), *Cpt1a* (**C**) and *Acadl* (**D**) in AML12 hepatocytes exposed or not to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) at 150 μg/mL, *Nannochloropsis gaditana* (NG) at 25 μg/mL and *Gracilaria vermiculophylla* (GV) at 150 μg/mL for 18 h. Data are means ± SEM (standard error of the mean). * $p < 0.05$, ** $p < 0.01$ vs. control cells. # $p < 0.05$ and ## $p < 0.01$ vs. PA cells. *Acadl*: long chain acyl-CoA dehydrogenase; *Atgl*: adipose triglyceride lipase; *Cpt1a*: carnitine palmitoyltransferase 1a; MTTP: microsomal triglyceride transfer protein.

mRNA levels of *Cs* and *Tfam*, related to mitochondrial density and mitochondriogenesis, respectively, were not modified by algae treatments in comparison with cells incubated only with PA. However, it is worth mentioning that an increase of 145% was observed in the GV group. Concerning *Ucp2*, a possible indicator of NAFLD development, the treatment with algae extracts did not modify its expression in comparison with the PA group (**Figure 7**).

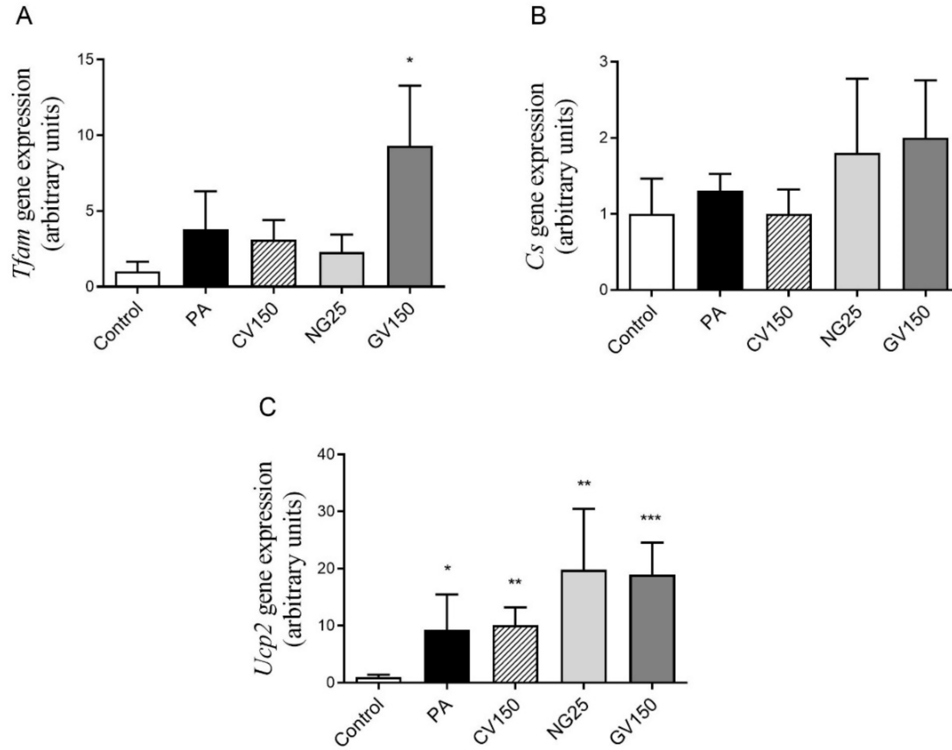

Figure 7. Gene expression of *Tfam* (**A**) and *Cs* (**B**) and *Ucp2* (**C**) in AML12 hepatocytes exposed or not to 0.3 mM of palmitic acid (PA) with or without *Chlorella vulgaris* (CV) at 150 µg/mL, *Nannochloropsis gaditana* (NG) at 25 µg/mL and *Gracilaria vermiculophylla* (GV) at 150 µg/mL for 18 h. Data are means ± SEM (standard error of the mean).* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control group. *Cs*: citrate synthase; *Tfam*: mitochondrial transcription factor A, *Ucp2*: uncoupling protein 2.

4. Discussion

The increasing prevalence of NAFLD has encouraged the interest in new strategies for its prevention and treatment. In this scenario, microalgae and macroalgae extracts have received great attention from the scientific community in recent years. Data from the literature show that, in addition to the effects attributed to the pigments present in algae, some extracts containing high percentages of protein-like compounds and carbohydrates have been reported to be able to modulate lipid metabolism (23–29). In the present study, the efficacy on liver steatosis of extracts rich in proteins and peptides, obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana* and the macroalga *Gracilaria vermiculophylla,* was assessed.

Regarding algae extract composition, in that obtained from *Chlorella vulgaris*, the most intense protein band was detected within the range of 20–25 kDa. In this line, a previous study showed that the main band for the proteins extracted from *Chlorella vulgaris* at alkaline pH was located at 30 kDa. However, an intense band corresponding to proteins of more than 670 kDa and 50–75 kDa was also observed (30). In another study carried out in a different species of *Chlorella,* protein extracts produced by

alkaline solubilisation corresponded to ~8 kDa (31). In the extract obtained from *Nannochloropsis gaditana*, the main protein bands corresponded to 37 kDa, 50 kDa and 100 kDa. In this case, only the band at 50 kDa had been previously detected for this microalga species (32). In the study reported by Vizcaíno et al. (32), molecular weight bands lower than 14 kDa were also visualised in the native microalga, but not in the present study. Moreover, in a piece of research addressed with another species of *Nannochloropsis,* proteins extracted by alkaline solubilisation showed major peaks corresponding to 10 kDa and 1.6 kDa (31). Regarding the macroalga *Gracilaria vermiculophylla*, it seems that the proteins present in the extract had very limited solubility, probably due to the formation of protein–polysaccharide complexes, thus hindering their characterisation by SDS-PAGE and HPLC-SEC. Interestingly, in the case of the *Chlorella vulgaris*, low molecular weight peptides may be interacting with pigments. The differences observed between the results obtained in the present study and those reported in the literature can be due to the extraction protocol applied. A limitation of the present study is that the proteins and peptides present in the extracts have not been identified. Thus, a more precise characterisation is an interesting aspect to be addressed in future studies.

In the present study, the concentrations of the extracts utilised for hepatocyte incubation ranged from 10 μg/mL to 150 μg/mL, a range of doses commonly used with algae extracts. In this experiment, palmitic acid was used to stimulate triglyceride accumulation into the cells; and to mimic the influx of excess free fatty acids into hepatocytes, which takes place under overfeeding conditions and obesity, and thus, to induce steatosis. Under the present experimental conditions, the data demonstrate that the three algae extracts partially prevented the accumulation of triglycerides induced by palmitic acid in cells; the percentages of prevention ranged from 18% to 34%. The microalga *Nannochloropsis gaditana* was the most effective because it induced the highest reduction in triglyceride accumulation (−34%); it was also the most powerful since it was effective at a dose lower than the other algae (10 μg/mL). This fact could be due to their high content in soluble proteins and peptides. In the case of this microalga, surprisingly, whereas 10 and 25 μg/mL were effective, higher doses, 50 and 150 μg/mL, were not. This phenomenon has also been observed in studies devoted to analysing the metabolic effects of phytochemicals such as phenolic compounds, where on occasion, a lower dose appears to be more effective than a higher one (18,33,34). Nevertheless, the reasons that justify this phenomenon have not been described to date. In contrast, the *Gracilaria vermiculophylla* extract showed a dose response pattern on triglyceride accumulation.

These beneficial effects are probably caused by the proteins and peptides present in the extracts. Moreover, in the case of *Nannochloropsis gaditana*, it has been observed that its protein and peptides are biologically active (26). In fact, it has been recently discovered that it contains UCA01, a protein of around 25 and 37 KDa with anti-tumoral, antioxidant and mitochondrial protective effects on HepG2 hepatocytes (patent number: 201930775) (35,36).

Although algae have been recognised and exploited as alternative food supplements, it has been reported that some species can produce compounds with toxic effects (6,37). In order to address this issue, hepatocyte viability was assessed, and no significant effects were observed for any algae at any of the doses tested. Moreover, a decrease in ALT levels was found when cells were treated with *Chlorella vulgaris* and *Gracilaria vermiculophylla*, indicating a protective effect on hepatocytes. In the case of the *Nannochloropsis gaditana* extract, no differences on its secretion were identified between this group and the PA group.

After confirming the positive effects of the algae extracts, the potential mechanisms underlying the anti-steatotic effects were assessed. For this purpose, the sets of cells treated with the dose of each algae extract showing the greatest reduction in triglyceride content were used (*Chlorella vulgaris* at 150 µg/mL, *Nannochloropsis gaditana* at 25 µg/mL and *Gracilaria vermiculophylla* at 150 µg/mL.) The genes and proteins analysed are related to the main metabolic pathways involved in hepatic triglyceride accumulation: (a) pathways favouring triglyceride accumulation, de novo lipogenesis and fatty acid uptake for the synthesis and triglyceride assembly, and (b) pathways avoiding triglyceride accumulation, triglyceride mobilization, fatty acid oxidation and triglyceride secretion.

In the case of *Chlorella vulgaris*, the results suggest that one mechanism involved in the prevention of triglyceride accumulation intensified triglyceride secretion from hepatocytes, mediated by a boost in MTTP protein expression. *Cpt1a* was also increased, suggesting enhanced fatty acid oxidation, and thus, a lower availability of free fatty acids for triglyceride synthesis. According to data concerning *Cpt1a* gene expression, the triglyceride lowering effect of *Nannochloropsis gaditana* was probably caused by an increase in fatty acid oxidation. In the case of *Gracilaria vermiculophylla*, the results show a trend towards reduced fatty acid uptake (FATP2) and an augmented lipolysis (*Atgl*) that can be linked to the increase in fatty acid oxidation (*Cpt1a, Acadl* and *Tfam*).

Due to the fact that a great number of compounds with potential anti-steatotic effect are present in the three algae extracts, it is not possible to state which of them is truly responsible for the positive effects induced by the algae in hepatocytes. However, the identification of the bioactive compounds or metabolites from algae extracts that induce the beneficial effects will be crucial in the upcoming years for the development of new therapies targeting fatty liver and other metabolic diseases; thus, it requires further research.

In conclusion, the present study demonstrates for the first time that *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* extracts abundant in proteins are able to partially prevent the accumulation of triglycerides induced by palmitic acid in cultured hepatocytes, a model used to mimic the steatosis induced in liver by dietary patterns rich in saturated fat. The three algae extracts analysed prevent hepatic steatosis by acting on different metabolic pathways involved in hepatic lipid metabolism. Nevertheless, further research is needed to validate these results in in vivo studies and

to test the effects of these extracts on other processes involved in the development of liver steatosis and its progression to steatohepatitis, such as oxidative stress and inflammation.

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Effects of *Chlorella vulgaris, Nannochloropsis gaditana* and *Gracilaria vermiculophylla* algae extracts on maturing and mature 3T3-L1 adipocytes

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Abstract

The prevalence of obesity is increasing worldwide and it is a risk factor for various metabolic disorders. White adipose tissue expansion depends on hyperplasia and hypertrophy of adipocytes, thus the search for new tools that target these two processes is relevant for the prevention and treatment of obesity. Algae are an interesting source of bioactive compounds and have been demonstrated to exert beneficial health effects. Therefore, this study aims at analysing the anti-adipogenic and delipidating capacity of peptide- and protein-rich extracts obtained from the microalgae *Chlorella vulgaris* and *Nannochloropsis gaditana* and the macroalga *Gracilaria vermiculophylla*. 3T3-L1 pre-adipocytes and mature adipocytes were incubated with each of the extracts at 10, 25, 50 and 150 µg/mL. The extracts were found to reduce triglyceride content in maturing pre-adipocytes, without cytotoxic effects. Although, in all cases, it seems that the extracts cannot act throughout the whole adipogenic process, they are effective in suppressing pre-adipocyte differentiation, to a greater or lesser extent. This effect is mainly mediated by the transcription factor C/EBPα. Under our experimental conditions, the extracts used in the present study are effective in inhibiting adipogenesis but they are not able to reduce triglyceride accumulation in mature adipocytes.

Keywords: microalgae, macroalgae, 3T3-L1, pre-adipocytes, mature adipocytes, adipogenesis, obesity

1. Introduction

Obesity is one of the most serious public health problems today, affecting more than one billion adults, adolescents and children affected. Worldwide, obesity has tripled since 1975 and the World Health Organization (WHO) estimates that the prevalence is still increasing (1). Moreover, obesity is a risk factor for various associated diseases, such as type 2 diabetes, cardiovascular diseases, cancer and mental health issues. Obesity is defined as excessive fat accumulation in white adipose tissue and it can be developed by increasing adipocyte number (hyperplasia) and/or size (hypertrophy) (2,3). When hyperplasia takes place, there is a stimulation of pre-adipocyte proliferation and further differentiation. This process, which promotes pre-adipocyte differentiation into mature adipocytes, is known as adipogenesis. Hypertrophy involves the accumulation of triglycerides in mature adipocytes, resulting in an increase in cell size. Both hyperplasia and hypertrophy determine the capacity of the adipocytes to store lipids (4). During childhood, fat accumulation takes place mainly via a boost in the number of adipocytes, but also by increasing cell expansion. In contrast, in adulthood the number of adipocytes tends to be more constant and white adipose tissue expansion occurs mainly through adipocyte hypertrophy, although adipogenesis, can also take place to a lesser extent under circumstances like obesity (5,6).

Whit obesity reaching epidemic proportions, scientific research is constantly searching for possible approaches and new molecules that could be useful in preventing and treating obesity. In fact, modulating adipogenesis may be one of the strategies to target obesity, as this would prevent the increase in the number of adipocytes that are then susceptible to expand via hypertrophy, which is related to insulin resistance and overall, adipocyte dysfunction. In this context, algae are rich in several bioactive compounds that have been reported to have a beneficial impact on health due to their antioxidant, anti-inflammatory, anti-obesity, anti-diabetic, hypolipidemic and antihypertensive effects (7-9). As a result, diets supplemented with both micro and macroalgae may have a positive influence on chronic diseases (10-13).

The objective of the present study is to search for new extracts with high content of peptides/proteins, obtained from microalgae and macroalgae, with potential beneficial effects on adipogenesis and adipocyte triglyceride accumulation, that have not been previously studied. For that purpose, two microalgae (*Chlorella vulgaris* and *Nannochloropsis gaditana*) and one macroalga (*Gracilaria vermiculophylla*) were selected. In a previous study carried out by our research group, these precise extracts were able to partially prevent the accumulation of triglycerides in AML-12 hepatocytes, meaning that they could probably represent useful tools in the prevention of hepatic steatosis (14). In the present work, the effects of the same algae extract on triglyceride accumulation in both pre-adipocytes and mature adipocytes are studied. Furthermore, the analysis of the mechanisms responsible for the observed effects is also addressed.

2. Materials and Methods

2.1. Algae extract preparation

Algae extracts preparation and composition were previously described (14). Briefly, *Gracilaria vermiculophylla* was collected in September 2019 in the Bidasoa estuary (Hondarribia, Gipuzkoa, Spain). After washing and removing impurities, samples were vacuum-packed prior to their storage at -20°C. Both microalgae (*Chlorella vulgaris* and *Nannochloropsis gaditana*) were provided by NEOALGAE (Gijón, Asturias, Spain) in frozen fresh-paste format (20-25% total solids) and stored at -20°C until use.

Regarding microalgae, the extracts were obtained following the methodology of Safi *et al.* (15,16). A suspension of microalgae at 10% dry weight and adjusted to pH 12 with NaOH was prepared. The suspension was percolated in order to avoid issues with the Ultra High-Pressure Homogenizer (UHPH) equipment MicroDeBee (Bee International, South Easton, USA). The UHPH conditions were 250 MPa, 250 μm orifice and three cycles. Next, samples were centrifuged at 10,000 × *g* for ten minutes at 4°C. The supernatant containing soluble proteins was collected. These proteins were precipitated by adjusting the pH to 3.0 with HCl. Samples were further centrifuged under the same conditions and the pellet was resuspended in 0.1 M phosphate buffer pH 7.5.

Regarding macroalga extract, frozen samples were crushed to a particle size of 1-5 mm and a suspension of the macroalga in water was prepared at 5% of dry weight solids, grinded with a homogeniser (Ultra-turrax-IKA-T25, Staufen Germany) for two minutes at 18,000 rpm and adjusted to pH 12 with NaOH 10 M. The suspension was then ultrasonicated (VibraCell 75042, Bioblock Scientific, Illkirch, France) for 1 h and 30 min, in cycles of 59 seconds ON and 15 seconds OFF. Finally, after the UHPH procedure, samples were handled in the same way as the microalgae suspensions. Results concerning algae extract composition are published in a previous paper (14).

2.2. Experimental design and cell treatment

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L glucose) containing 10% foetal bovine serum (FBS). When the monolayer reached 70% of confluence, cells were detached and seeded either in 6 or 96 well plates, to perform the pertinent experiments. 96 well plates were used for viability determinations and the other experiments were carried out in six well plates.

Two days after confluence (day 0), cells were induced to differentiate using DMEM, 10% foetal bovine serum (FBS), supplemented with 10 μg/mL insulin, 0.5 mM isobutyl methylxanthine (IBMX) and 1 μM dexamethasone, for two days. On day two, the differentiation medium was replaced by DMEM/FBS medium (10%) containing 10 μg/mL of insulin. From day four onward, the differentiation medium containing DMEM/FBS medium (10%) and 0.2 μg/mL insulin was changed every two days until the cells were harvested (eight days after confluence in the case of maturing pre-

adipocytes and 13 days for mature adipocytes). All media contained 1% penicillin/streptomycin (10,000 U/mL) and the differentiation media also contained 1% (v/v) of biotin and pantothenic acid. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

For experiments to determine the impact of the above-mentioned algae extracts on maturing pre-adipocytes, cells grown in 6-well and 96-well plates were differentiated in the presence or absence of 10, 25, 50 or 150 µg/mL of each alga extract during differentiation. Media was changed every two days (on day 0, day 2, day 4 and day 6). The same amount of the vehicle (water) was added to control cells. On day eight, cells were harvested for subsequent analysis. Each experiment was performed three times for viability and lipid content, and once for gene and protein expression analysis (six wells for gene and protein determinations).

For experiments in mature adipocytes, cells grown in 6-well and 96-well plates were treated with 10, 25, 50 or 150 µg/mL of each alga extract on day 12 after the induction of differentiation. The same amount of the vehicle was added to the control cells. After 24 hours of treatment (day 13), cells were harvested for subsequent analysis. Each experiment was performed three times, except for gene and protein expression.

2.3. Cell viability assay

Cell viability was assessed using the crystal violet assay, based on the cell staining with crystal violet (0.25%) as previously described (14,17). This experiment was repeated in triplicate (eight wells per experiment).

2.4. Determination of triglyceride content

After the treatment, the incubation medium was removed; cells were washed with PBS and collected in 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer through scrapping. Cell suspension was then sonicated with 5 s bursts in a Branson Sonifier SFX550 (San Luis, Missouri, MO, USA), fitted with a microtip and the glyceride content was measured by means of a commercial kit (Ref: 1001313, Spinreact, Girona, Spain). For protein measurements the Bradford method (18) was followed. Triglyceride content values were obtained as mg triglycerides/mg protein and expressed as the percentage of the control cells. This experiment was carried out in triplicate (six wells per experiment).

2.5. Analysis of gene expression by Real-Time PCR

After the treatment, cells were collected in TRIzol® reagent (Ref: 15596026, Invitrogen, CA, USA) and RNA was extracted from cells following the manufacturer's instructions. After DNase treatment (Ref: 8174G, Ambion, CA, USA), RNA was quantified using an RNA 6000 Nano Assay (Thermo Scientific, DE, USA). 1.5 µg of total RNA in a total reaction volume of 30 µL from each sample was reverse-transcribed to complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad,

CA, USA). Reactions were incubated initially at 25°C for 5 min, subsequently at 42°C for 30 min and finally at 85°C for 5 min.

Acetyl-CoA carboxylase (*Acc*), adiponectin (*Adipoq*), CCAAT/enhancer binding proteins α and β (*Cebpa* and *Cebpb*), peroxisome proliferator-activated receptor γ (*Pparg*) and sterol regulatory element binding transcription factor 1c (*Srebp1c*) mRNA levels were quantified in maturing pre-adipocytes. *Gapdh* served as housekeeping for posterior normalization. Diluted cDNA samples were amplified in an iCycler-MyiQ Real-Time PCR Detection System (Bio-Rad, CA, USA), in the presence of SYBR Green Master Mix (Applied Biosystems, CA, USA) and the sense and antisense primers (300 nM each). The primer sequences are described in **Table 1**. The PCR parameters were as follows: initial 2 min at 50ºC, denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 60ºC for 30 s and extension at 60ºC for 30 s. For *Cebpa*, the annealing temperature was 66ºC. In all cases, the results were expressed as fold changes of the threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method (19).

Table 1. Primer sequences for quantitative Real-Time PCR amplification.

Gene	Sense primer 5'-3'	Antisense primer 5'-3'
Acc	GGA CCA CTG CAT GGA ATG TTA	TGA GTG ACT GCC GAA ACA TCT
Adipoq	TGT ACG ATT GTC AGT GGA TCT G	CTC TTC AGT TGT AGT AAC GTC ATC
Cebpa	TGG ACA AGA ACA GCA ACG AG	TCA CTG GTC AAC TCC AGC AC
Cebpb	CAA GCT GAG CGA CGA GTA ACA	CAG CTG CTC CAC CTT CTT CT
Gapdh	ATG TTC CAG TAT GAC TCC ACT CAC G	GAA GAC ACC AGT AGA CTC CAC GAC A
Pparg	TCG CTG ATG CAC TGC CTA TG	GAG AGG TCC ACA GAG CTG ATT
Srebp1c	GCT GTT GGC ATC CTG CTA TC	TAG CTG GAA GTG ACG GTG GT

Acc: acetyl-CoA carboxylase; *Adipoq*: adiponectin; *Cebpa*; CCAAT/enhancer binding protein α; *Cebpb*: CCAAT/enhancer binding protein β; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *Pparg*: peroxisome proliferator-activated receptor γ; *Srebp1c*: sterol regulatory element binding transcription factor 1c.

2.6. Analysis of protein expression by Western Blot

Acetyl-CoA carboxylase (ACC), adiponectin (ADIPOQ), CCAAT/enhancer binding proteins α and β (C/EBPα and C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element binding transcription factor 1c (SREBP-1c) were assessed by western blot. Cells were harvested in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM iodoacetamide, and after sonication (5 s bursts in a Branson Sonifier SFX550) total protein concentration was determined according to the Bradford protocol (18). Protein samples (15 µg) were denaturalized at 95ºC for 3 min in Laemmli buffer (Bio-Rad, CA, USA) and then loaded into 4-15% Mini-PROTEAN TGX Precast Gels (BioRad, CA, USA). Gels were then transferred onto PVDF membranes (Millipore,

MA, USA) by electroblotting and then blocked with 5% casein and 0.5% bovine serum albumin (BSA) PBS-tween buffer for 2 h at room temperature. The membranes were then incubated overnight at 4ºC with primary antibodies: rabbit anti-ACC (4190 Cell Signalling Technology, 1:1000), rabbit anti-adiponectin (2789 Cell Signalling Technology, 1:1000), rabbit anti-C/EBPα (2295 Cell Signalling Technology, 1:500), anti-C/EBPβ (3087 Cell Signalling Technology, 1:1000), rabbit anti-PPARγ (2430 Cell Signalling Technology, 1:500), rabbit anti-SREBP1c (ab28481 Abcam, 1:1000) and rabbit anti-α-Tubulin (2125 Cell Signalling Technology, 1:1000). Following washing, proteins were detected after 2 h incubation with anti-rabbit secondary antibody (sc-2357, Santa Cruz Biotech, CA, USA) using the Forte Western HRP substrate (WBLUF0100, Millipore, MA, USA), and the blots were imaged by scanning with the ChemiDoc™MP Imaging System (Bio-Rad, CA, USA). α-tubulin was used as housekeeping.

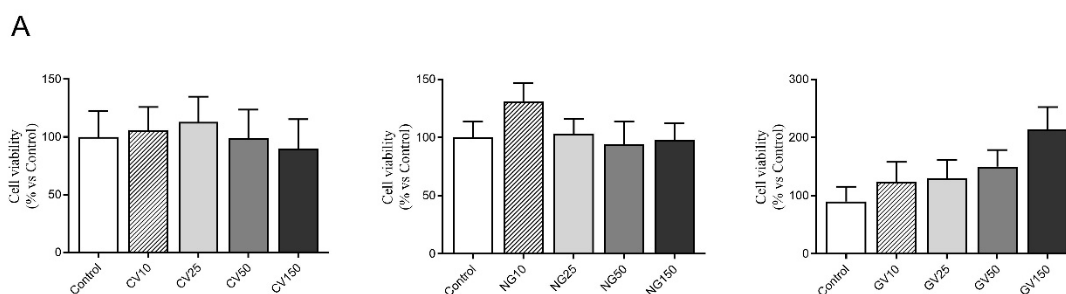
2.7. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 26.0 (SPSS, IL, USA). The normal distribution of the data was tested using the Shapiro-Wilk test. Data from each group treated with algae extracts were compared with control cells using Student's *t*-test. Statistical significance was established at $p < 0.05$ level.

3. Results

3.1. Cell viability

Treatment with 10, 25, 50 or 150 µg/mL of *Chlorella vulgaris*, *Nannochloropsis gaditana* and *Gracilaria vermiculophylla* did not produce loss of cell viability at any of the tested doses in pre-adipocytes (**Figure 1A**), nor in mature adipocytes (**Figure 1B**).



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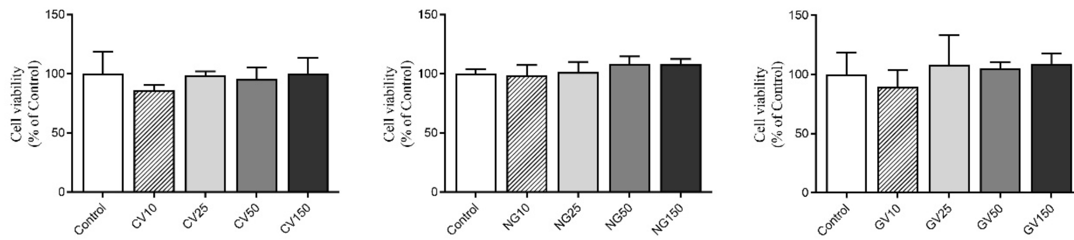


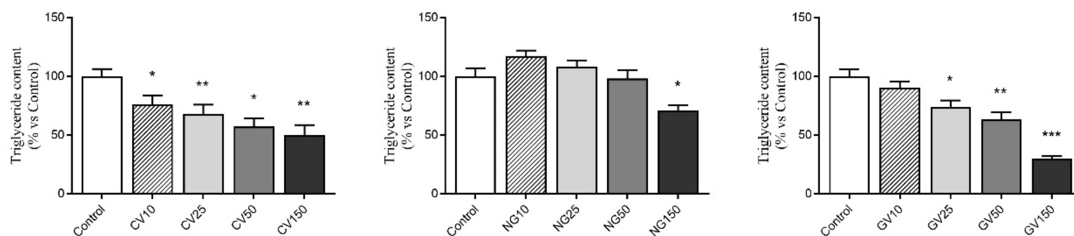
Figure 1. Cell viability of 3T3-L1 maturing pre-adipocytes (A) and mature adipocytes (B) treated with 10, 25, 50 or 150 µg/mL of *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV). Control cells were not treated with algae extracts. Data are mean ± SEM (standard error of the mean). Student's *t*-test was used for the analysis of comparisons between each treatment and the control group.

3.2. Effects of algae extracts on triglyceride accumulation during pre-adipocyte differentiation

Pre-adipocytes incubated with *Chlorella vulgaris* extract at 10, 25, 50 and 150 µg/mL from day 0 to day 8 showed a significant decrease in triglyceride content in a dose-dependent manner, with reduction percentages of 24%, 32%, 43% and 50%, respectively. *Nannochloropsis gaditana* did not promote a significant reduction in triglyceride accumulation in the range of concentration between 10 and 50 µg/mL; however, in cells incubated with the highest dose (150 µg/mL) the triglyceride content was lessened by 29%. The treatment with the macroalga (*Gracilaria vermiculophylla*) reduced triglycerides at the doses of 25 µg/mL (26%), 50 µg/mL (37%) and 150 µg/mL (70%) µg/mL, following a dose-response pattern, while the dose of 10 µg/mL did not lead to any significant change (**Figure 2A**).

In addition, the effects on lipid accumulation were also examined by optical microscopy (**Figure 2B**). Images revealed less and smaller cytoplasmic fat vacuoles after co-incubation of 3T3-L1 pre-adipocytes with each algae extract (150 µg/mL).

A



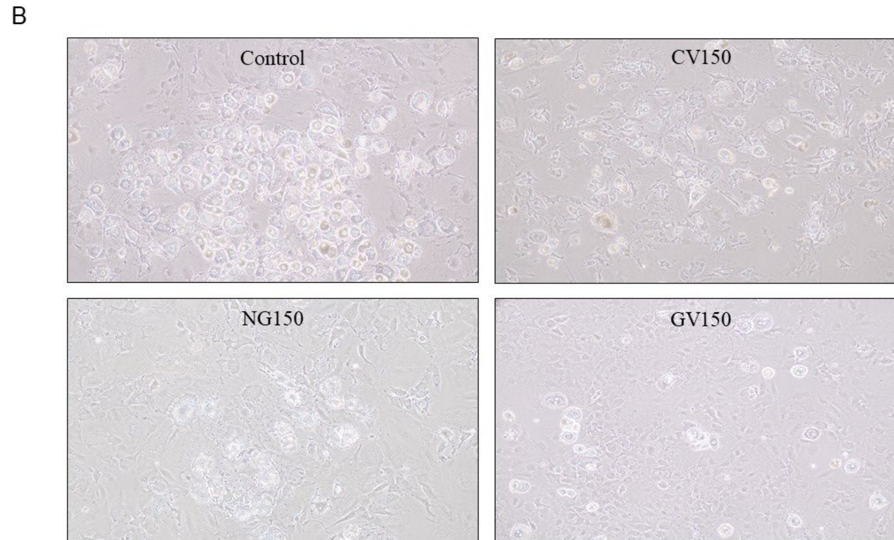


Figure 2. Triglyceride content in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 with 10, 25, 50 or 150 µg/mL of *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV) (A) and optical microscopy images showing lipid accumulation at day 8 in 3T3-L1 maturing pre-adipocytes treated with *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV) at 150 µg/mL (B). Control cells were not treated with the algae extracts. Data are mean ± SEM (standard error of the mean). Student's *t*-test was used for the analysis of comparisons between each treatment and the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.3. Effects of algae extracts on triglyceride accumulation in mature adipocytes

As shown in **Figure 3**, no significant changes in mature adipocyte triglyceride content were observed in cells treated with 10, 25, 50 or 150 µg/mL of *Chlorella vulgaris, Nannochloropsis gaditana* or *Gracilaria vermiculophylla* extracts.

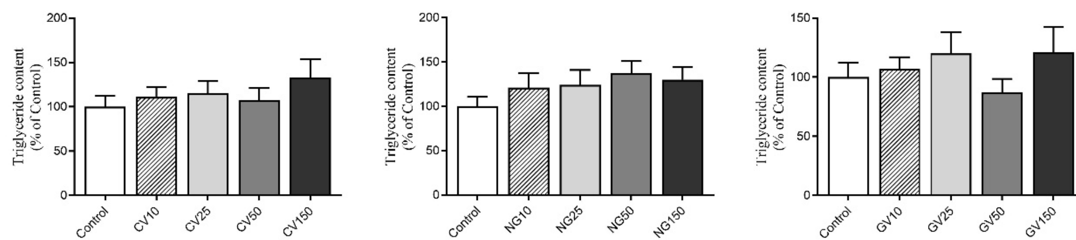


Figure 3. Triglyceride content in 3T3-L1 mature adipocytes treated on day 12 for 24 h with 10, 25, 50 or 150 µg/mL of *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV). Control cells were not treated with the algae extracts. Data are mean ± SEM (standard error of the mean). Student's *t*-test was used for the analysis of comparisons between each treatment and the control group.

3.4. Effects of algae extracts on gene expression in maturing pre-adipocytes

In order to explain the triglyceride reduction observed in maturing pre-adipocytes, gene expression of transcription factors involved in adipogenesis was measured after the treatment with the three algae extracts during the first eight days of the adipogenic process, using the concentration that induced the biggest reduction in triglyceride content (150 µg/mL for the three algae extracts) (**Figure 4**). Mature adipocyte-specific genes (late adipogenic markers) were also analysed in order to elucidate whether the mature specific phenotype development was prevented by the algae extracts.

Regarding gene expression of the transcription factors involved in adipogenesis, *Chlorella vulgaris* significantly reduced *Cebpa* gene expression, increased *Pparγ* and did not induce any change in *Cebpb* or *Srebp1c* gene expression. Regarding the markers in the late adipogenic stage, *Acc* mRNA expression decreased significantly, whereas *Adipoq* was significantly boosted. When pre-adipocytes were exposed to the *Nannochloropsis gaditana* extract, a drop in *Pparγ* gene expression was observed; *Cebpb* and *Srebp1c* tended to reduce their expression and *Cebpa* remained unchanged. Whit regard to markers of mature adipocyte, *Acc* showed increased gene expression, while *Adipoq* was reduced. The *Gracilaria vermiculophylla* extract significantly decreased mRNA levels of the adipogenic markers *Cebpa, Pparg* and *Srebp1c.* Contrarily, *Cebpb* mRNA levels showed a significant increase. Regarding gene expression of mature-adipocyte specific markers, both *Acc* and *Adipoq* were attenuated.

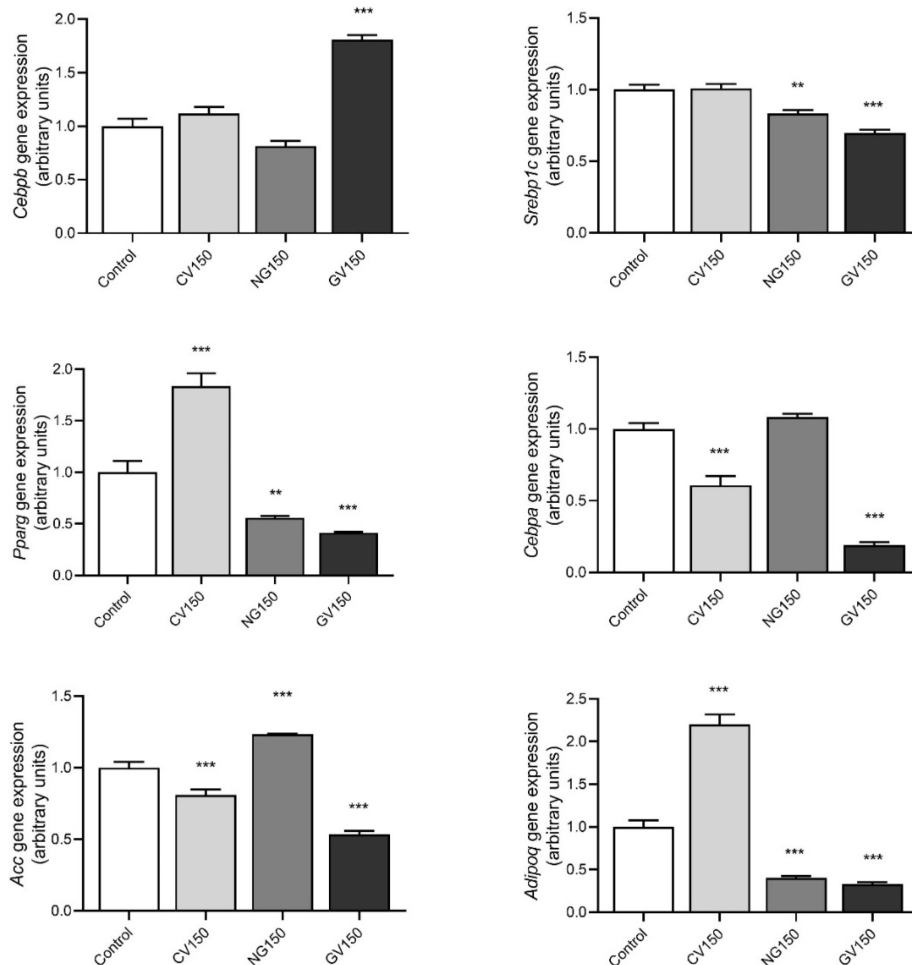


Figure 4. Gene expression of *Cebpb*, *Srebp1c*, *Pparg*, *Cebpa*, *Acc* and *Adipoq* in 3T3-L1 maturing pre-adipocytes treated with 150 µg/mL of *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV) from day 0 to day 8 (cells harvested on day 8). Control cells were not treated with the algae extracts. Data are mean ± SEM (standard error of the mean). Student's *t*-test was used for the analysis of comparisons between each treatment and the control group. $^{**}P < 0.01$, $^{***} P < 0.001$. *Acc*: acetyl-CoA carboxylase; *Adipoq*: adiponectin; *Cebpa*; CCAAT/enhancer binding protein α; *Cebpb*: CCAAT/enhancer binding protein β; *Pparg*: peroxisome proliferator-activated receptor γ; *Srebp1c:* sterol regulatory element binding transcription factor 1c.

3.5. Effects of algae extracts on protein expression in maturing pre-adipocytes

In order to better understand the metabolic pathways involved in the anti-adipogenic effect of the algae extracts, protein expression analysis of transcription factors regulating the adipogenic process was performed along with markers of mature adipocytes (**Figure 5**). Cells incubated with *Chlorella vulgaris* showed an upward trend in the expression of C/EBPβ protein levels. PPARγ remained unchanged, while C/EBPα was significantly reduced. ACC protein expression was decreased and no significant changes in ADIPOQ protein levels were observed.

Regarding the treatment with *Nannochloropsis gaditana*, C/EBPβ expression increased in treated cells, PPARγ protein levels tended to decrease and C/EBPα was significantly lowered. Protein expressions of ACC and ADIPOQ were also decreased. In cells subjected to *Gracilaria vermiculophylla* extract, a greater protein expression of C/EBPβ was observed. In contrast, C/EBPα levels were significantly reduced and PPARγ levels remained unchanged. Regarding mature adipocyte markers, both ACC and ADIPOQ showed a lowered expression. In all cases, SREBP-1c could not be detected.

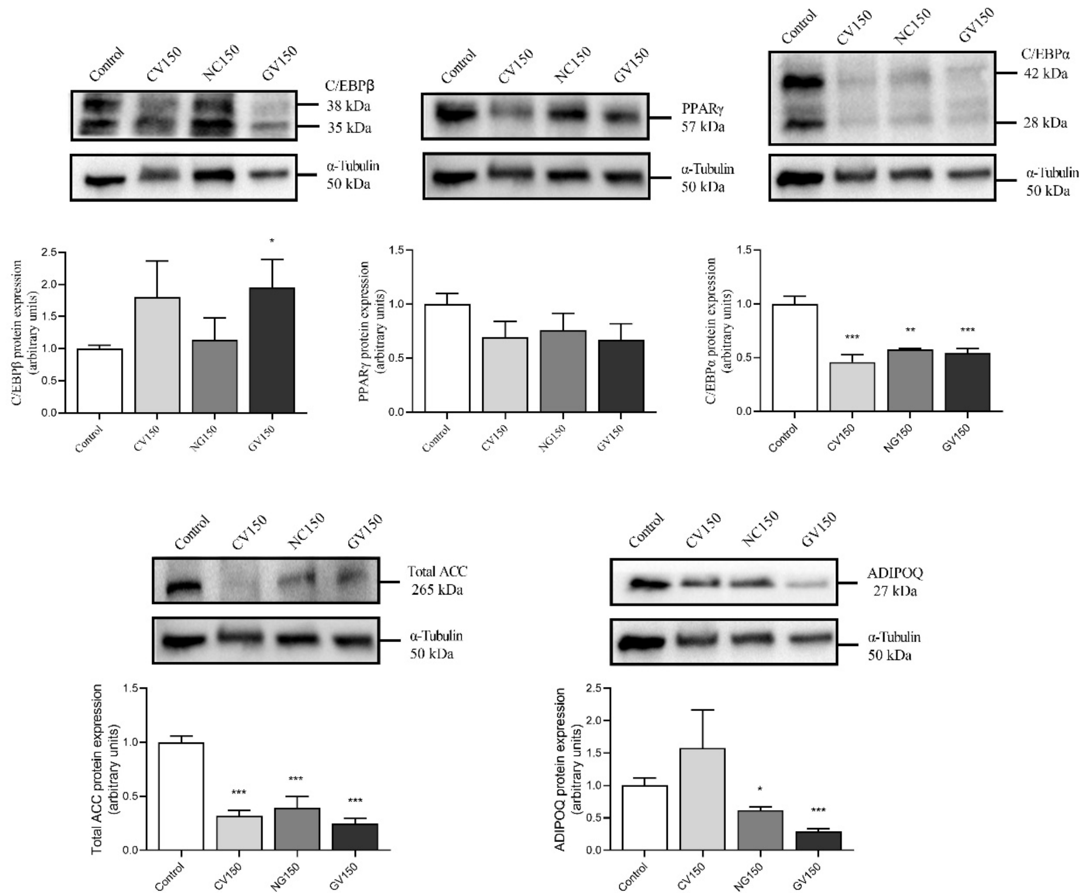


Figure 5. Protein expression of total C/EBPβ, PPARγ, C/EBPα ACC and ADIPOQ in 3T3-L1 maturing pre-adipocytes treated with 150 μg/mL of *Chlorella vulgaris* (CV), *Nannochloropsis gaditana* (NG) and *Gracilaria vermiculophylla* (GV) from day 0 to day 8 (cells harvested on day 8). Control cells were not treated with the algae extracts. Data are mean ± SEM (standard error of the mean). Student's *t*-test was used for the analysis of comparisons between each treatment and the control group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ACC: acetyl-CoA carboxylase; ADIPOQ: adiponectin; C/EBPα: CCAAT/enhancer binding protein α; C/EBPβ: CCAAT/enhancer binding protein β; PPARγ: peroxisome proliferator-activated receptor γ.

4. Discussion

White adipocytes play a crucial role in the regulation of energy balance. In this context, lipid accumulation in mature adipocytes (hypertrophy) and adipocyte differentiation (hyperplasia) are involved in the development of obesity (20). Due to the fact that both microalgae and macroalgae

may have beneficial effects on obesity (11,12), the responses of 3T3-L1 maturing pre-adipocytes and mature adipocytes to three algae extracts, *Chlorella vulgaris, Nannochloropsis gaditana* and *Gracilaria vermiculophylla*, were analysed in the present work. Nevertheless, in order to know whether these algae extracts are in fact useful anti-obesity tools, further studies are needed, first in animal models and then in humans. Interestingly, *in vitro* studies allow the researchers to test a large number of extracts simultaneously (from different algae and at different doses) and to better distinguish the difference between the effects on pre-adipocytes (and thus on adipogenesis) and mature adipocytes. Thus, this is the reason for choosing this experimental model for the present study, as a first step in the frame of a bigger study.

Regarding maturing pre-adipocytes, treatment with *Chlorella vulgaris* exhibited a lower triglyceride content at all the tested doses in a dose-dependent manner at the end of the maturation process. Taking into account that cell viability was not affected, these results indicate that this extract induced a reduction in adipogenesis. In contrast, pre-adipocytes treated with *Nannochloropshis gaditana* showed a significant reduction in adipogenesis only when incubated at the highest dose (150 µg/mL) without compromising cell viability. *Gracilaria vermiculophylla* decreased triglyceride content in the range of doses from 25 to 150 µg/mL without modifying cell viability. Although the extracts used are rich in peptides and proteins (14), considering that carbohydrates represent a non-negligible percentage in these extracts, their potential contribution to the observed effects, together with that of the peptides/proteins, cannot be discarded. To the best of our knowledge, there are no studies in the literature on the anti-adipogenic effect of these algae extracts, so no comparison can be made.

Traditionally, compounds showing the ability to reduce adipogenesis have been considered as potential anti-obesity agents (21-23). However, it is well known that adipogenesis has emerged as a possible therapeutic strategy to enhance adipose tissue health and counteract the negative metabolic consequences derived from adipocyte hypertrophy (24). In this regard, hypertrophic adipose tissue growth has been correlated with metabolic dysfunction, characterized by a pro-inflammatory profile and insulin resistance, whereas, adipose tissue growth via hyperplasia, has been postulated to be protective against the metabolic complications of obesity, because it allows the proper vascularization of the tissue, that together with the reduced number of hypertrophic adipocytes, results in higher insulin sensitivity and lower levels of pro-inflammatory cytokines (2,24). It is also important to point out that, if the excess energy cannot be stored in the adipose tissue (either because adipogenesis is reduced and/or because hypertrophic adipocytes have reached their storage capacity), this excess could be accumulated in other tissues, such as skeletal muscle or liver (ectopic fat accumulation), with the subsequent deleterious consequences on health. Another effect derived from impaired adipocyte development, is the so-called lipodystrophy, a condition resulting in an overflow of fatty acids into non-adipose tissues (25). With regard to ectopic lipid accumulation in liver, the algae extracts used in the present study were able to diminish triglyceride accumulation in cultured hepatocytes by increasing fatty acid oxidation (14). This suggests a potential beneficial effect of these algae extracts

in preventing ectopic fat accumulation in the liver, in case of a reduced storage in white adipose tissue. Taking into account the limitations inherent to *in vitro* studies, it is clear that it cannot be fully addressed whether these effects would be reproduced in an animal model, however, these results still cast some light on the existing evidence regarding the contribution of these algae extracts on metabolic health.

Another objective of the present study was to determine the mechanism by which these extracts reduced adipogenesis, and for that purpose, the expressions of genes and proteins involved in this process were measured in cells incubated with the most effective dose in decreasing triglyceride content for each extract (150 μg/mL). The differentiation of pre-adipocytes into mature adipocytes involves a complex network of transcription factors that regulate this process (**Figure 6**). Right after the initiation of pre-adipocyte differentiation, the expression of C/EBPδ and then C/EBPβ is induced, which subsequently promotes the expression of PPARγ. C/EBPβ can also prompt the expression of C/EBPα (26). PPARγ and C/EBPα co-regulate each other's expression and are considered to be the main regulators of adipogenesis (27,28) inducing the transcription of different genes encoding adipocyte-specific phenotype (26). SREBP1c is another pro-adipogenic transcription factor that regulates the expression of PPARγ and is directly regulated by C/EBP factors (29). At the end of the differentiation process, cells begin to express characteristic markers of the mature adipocyte phenotype, such as adipokines and lipogenic enzymes (26).

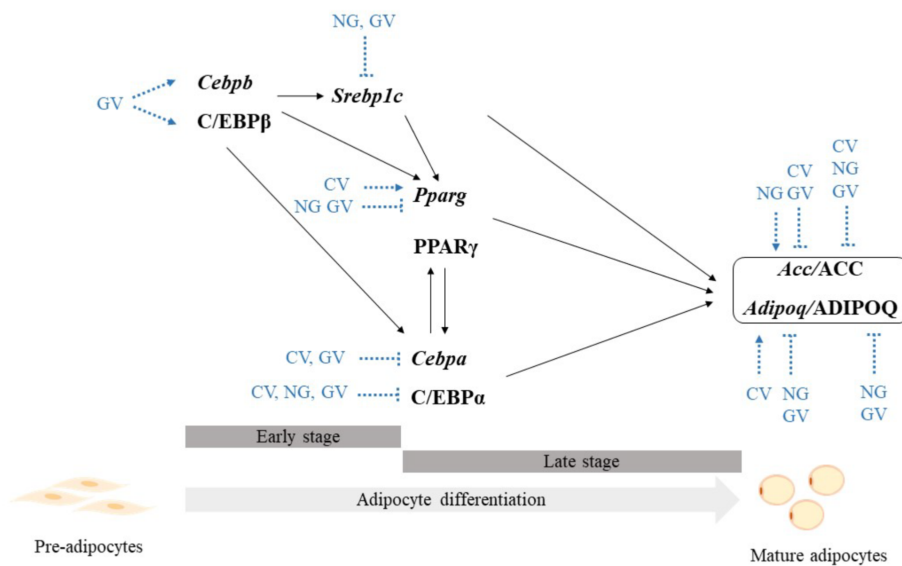


Figure 6. Effects of the different algae extracts in genes and proteins involved in adipocyte differentiation. *Acc*, ACC: acetyl-CoA carboxylase; *Adipoq, ADIPOQ*: adiponectin; *Cebpa*, C/EBPα: CCAAT/enhancer binding proteins α; *Cebpb*, C/EBPβ: CCAAT/enhancer binding proteins β; CV: *Chlorella vulgaris*; GV: *Gracilaria vermiculophylla*; NG: *Nannochloropsis gaditana*, *Pparg*, PPARγ: peroxisome proliferator activated receptor γ; *Srebp1c*: sterol regulatory element binding transcription factor 1c. Names in italics correspond to genes and in upper case correspond to proteins. Dotted lines with pointed arrow head mean stimulation and dotted lines with blunt arrow head mean inhibition.

Although *Chlorella vulgaris* extract increased *Pparg* gene expression, its protein expression was not modified. Moreover, this treatment was able to reduce *Cebpa* gene expression and to down-regulate its protein expression, which resulted in a significant decline in triglyceride accumulation (by 50%). Regarding the genes and proteins used as markers of mature adipocytes, the decrease observed in total *Acc* mRNA levels is consistent with the reduction in its protein levels. Although *Chlorella vulgaris* treatment up-regulated *Adipoq* gene expression, this increase was not translated into higher protein levels, which remained the same. Altogether, these results suggest that *Chlorella vulgaris* could exert a beneficial effect in partially preventing pre-adipocyte differentiation into mature adipocytes, mainly by modulating the transcription factor *Cebpa*.

Nannochloropsis gaditana extract reduced *Pparγ* and *Srebp1c* gene expression and tended to lessen *Cebpb* expression, but these changes were not accompanied by shifts in their protein expression. In contrast, the C/EBPα protein level was significantly reduced, meaning that, as in the case of *Chlorella vulgaris*, the extract was not able to mitigate the whole adipogenic process, as only some of the transcription factors involved in adipogenesis were modified by the treatment. With regard to mature adipocyte-specific markers, *Acc* gene expression was up-regulated, while total ACC protein levels were diminished by day 8 after the induction of differentiation. In line with these results, both the adiponectin gene and the protein expression were inhibited by the treatment with this microalgae extract, indicating the potential of *Nannochloropsis gaditana* to inhibit the differentiation process. The reduction in pre-adipocyte triglyceride content (by 30%) indicates that the effects described on genes and proteins were enough to inhibit the differentiation of these cells.

Finally, regarding *Gracilaria vermiculophylla*, in terms of gene expression, although *Cebpb* levels were increased, significant reductions were observed in *Srebp1c, Pparg* and *Cebpa*. These effects were only partially translated into changes in protein expression, since a tendency towards reduced values was observed for PPARγ (-33%) and a significant decline was only observed for C/EBPα. Taking into account that both PPARγ and C/EBPα are the key regulators of adipogenesis, the depletion of their expression can justify the decrease in pre-adipocyte triglyceride content (by 70%). Once again, it seems that this alga extract could lead to a reduction in triglyceride accumulation by the inhibition of only some stages of the adipogenic process. Furthermore, the ability of *Gracilaria vermiculophylla* in inhibiting adipogenesis can also be concluded from the results obtained from the expression of mature adipocyte-specific markers, as their expression was inhibited by eight day of the differentiation.

As it can be observed, in the present study, the changes induced in protein expression sometimes are not in good accordance with the gene expression. In general terms, it is accepted that protein levels are largely determined by transcript concentrations. However, during highly dynamic phases, such as cellular differentiation or stress response, post-transcriptional processes may lead to stronger deviations from an ideal correlation. The spatial and temporal variations of mRNAs, as well as the

local availability of resources for protein biosynthesis, strongly influence the relationship between protein levels and their coding transcripts. Consequently, the lack of correlation between protein and gene expression in the present study is not as surprising as thought. Taking all this into account, mRNA levels alone are not sufficient to predict protein levels (25). Thus, we decided to focus on the protein levels to draw conclusions, as they are a better indicator of the final effect.

In mature adipocytes, none of the extracts at the tested doses (from 10 to 150 µg/mL) reduced triglyceride accumulation after 24 hours of treatment, which is a period of time considered chronic in these cells (30,31). These results suggest that the potential anti-obesity effect of these extracts may not be evidenced in hypertrophic obesity.

5. Conclusions

In conclusion, the present study shows for the first time that although under our experimental conditions, the extracts of *Chlorella vulgaris, Nannochloropsis gaditana* and *Gracilaria vermiculophylla,* rich in peptides/proteins, are not able to reduce triglyceride accumulation in mature adipocytes, they show an anti-adipogenic effect in cultured adipocytes. Although in all cases, it seems that the extracts are not capable of acting throughout the whole adipogenic process, they are effective in suppressing pre-adipocyte differentiation, to a greater or lesser extent, and thus, in reducing triglyceride accumulation. This effect is mainly mediated by the transcription factor C/EBPα. Nevertheless, taking into account that an important limitation of *in vitro* studies is the impossibility to integrate the effects on different organs and to analyse their crosstalk, a conclusion concerning the beneficial effect of reducing adipogenesis on obesity and its co-morbidities cannot be drawn.

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Effect of *Gracilaria vermiculophylla* Macroalga on Non-Alcoholic Fatty Liver Disease in Obese Rats

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Abstract

Marine algae are valuable sources of bioactive compounds that have the potential to be used in the management of various pathologies. Despite the increasing prevalence of NAFLD, the absence of an approved effective pharmacological treatment with demonstrable effectiveness persists. In this context, the aim of the present study is to assess the effect of *Gracilaria vermiculophylla* red seaweed dietary supplementation on hepatic lipid accumulation, as well as on oxidative stress, inflammation and fibrosis- related markers on obese *fa/fa* Zucker rats fed with a standard diet, supplemented or not with 2.5% or 5% dehydrated *Gracilaria vermiculophylla*. After a six-week supplementation with the macroalga, no significant reduction in hepatic total lipid content or hepatic triglyceride content was observed. However, both doses were able to diminish hepatic NEFA concentration by reducing de novo lipogenesis and increasing mitochondrial biogenesis. Moreover, supplementation with the dose of 2.5% improved some oxidative stress and inflammation-related markers. Supplementation with the dose of 5% did not exert these clear beneficial effects. Thus, this study demonstrates that while *Gracilaria vermiculophylla* may not mitigate hepatic steatosis, it could exert protective effects on the liver by reducing NEFA content and enhancing oxidative stress and inflammation parameters.

Keywords: alga; *Gracilaria vermiculophylla*; non-alcoholic fatty liver disease; oxidative stress; Zucker rat

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) currently stands as a predominant contributor to chronic liver diseases worldwide, with an estimated prevalence of approximately 30% (1). Recently, it was renamed a metabolic (dysfunction)-associated fatty liver disease (MAFLD) in order to emphasise the underlying metabolic dysfunction (2,3). However, owing to the lack of consensus among all expert committees, the term NAFLD is used throughout the present manuscript.

NAFLD encompasses a variety of hepatic dysfunctions, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which may present with or without fibrosis and can progress to cirrhosis and hepatocellular carcinoma. Simple steatosis is characterized by an increased accumulation of lipids within the hepatocytes, exceeding 5% of liver weight. In NASH, this lipid accumulation is accompanied by inflammation and hepatocyte injury (4). NAFLD is strongly associated with insulin resistance, which causes an imbalance in lipid metabolism in favour of hepatic lipid accrual. The excessive accumulation of lipid content causes hepatocyte damage. Specifically, elevated levels of non-esterified fatty acids (NEFAs) can give rise to toxic lipid species, such as diacylglycerols, ceramides or lysophosphatidylcholine. This results in lipotoxicity, which triggers oxidative stress and the production of reactive oxygen species (ROS) (5,6). Increased oxidative stress plays a crucial role in the progression of NAFLD to NASH (7) because it damages cellular structures and function, causing hepatocyte injury and death (8). Presently, there exists no approved effective pharmacological treatment. Consequently, the scientific community is in pursuit of novel sources of molecules with potential utility in the prevention and treatment of NAFLD.

Marine macroalgae or seaweeds are considered a viable and sustainable source of both micro- and macronutrients that increase the nutritional value of the diet (9). Furthermore, they have gained a lot of interest due to their numerous and unique bioactive compounds, not present in terrestrial food sources. These include characteristic pigments, phenolic compounds, polyunsaturated fatty acids, polysaccharides or peptides (10). It is scientifically proven that these compounds exhibit the potential to improve human health through their documented antioxidant, anti-inflammatory, anticancer, antidiabetic, antihypertensive, antihyperlipidemic and antiobesity effects (9,11).

The red macroalga *Gracilaria* sp. is predominantly utilised for agar production and as a source of sulphated polysaccharides in the pharmaceutical and biotechnology sectors (12). Moreover, studies have described the potential of *Gracilaria* sp. to modulate glucose, cholesterol and lipid metabolism (13–16). In fact, in a preceding study conducted by our group, it was observed that an extract derived from *Gracilaria vermiculophylla*, particularly rich in proteins and peptides, demonstrated the ability to reduce triglyceride accumulation in cultured hepatocytes by increasing fatty acid oxidation (17). Nevertheless, there are no studies investigating the impact of dietary supplementation with *Gracilaria vermiculophylla* on in vivo models.

In this context, the aim of this study is to assess the influence of long-term dietary supplementation with *Gracilaria vermiculophylla*, a red macroalga, on hepatic lipid accumulation in Zucker obese (*fa/fa*) rats, a genetic model of steatosis. Additionally, the study aims to elucidate the potential underlying mechanisms involved. Furthermore, other parameters related with the early progression to NASH, such us oxidative stress and inflammation, are also assessed.

2. Materials and Methods

2.1. Macroalga Obtention and Characterisation

Gracilaria vermiculophylla specimens were harvested from the northwest Iberian coast and subsequently dehydrated by Porto Muiños S.L. (Cambre, Spain). *Gracilaria vermiculophylla* is a cartilaginous red macroalga (phyllum *Rhodophyta*) that arises from a discoid holdfast with a densely and irregularly branched thallus. Branches are cylindrical and slightly constricted at the base. The colour varies from dark red to reddish brown, occasionally appearing greenish or black, a characteristic that differentiates it from other species of the same genus such as *Gracilaria gracilis*, which has an intense red colour. It is mainly found in estuaries and intertidal habitats, often in shallow waters in sheltered areas influenced by freshwater. *Gracilaria vermiculophylla* tolerates broad ranges of temperature, light, salinity and nutrient levels. It usually grows unattached in loose-lying mats on mud and sand, and it is generally found in vegetative state with few reproductive structures (18,19).

Composition analysis was carried out by Corporación Laber S.L. (Santiago de Compostela, Spain). Briefly, total lipids were determined by the Weibull method, a variation of the Soxhlet method where the sample is subjected to hydrochloric acid digestion prior to lipid extraction. Saturated fatty acids were assessed by gas chromatography (GC) of fatty acid methyl esters (FAMEs). Protein content was determined by measuring the total nitrogen content using the Kjeldahl method. Simple carbohydrates were quantified by high-performance liquid chromatography with refractive index detector (HPLC-RI). Fibre content was assessed by an enzymatic–gravimetric method, which involved treating the sample with amylase, protease and amyloglucosidase. Ashes were examined by burning off the organic matter by heating the sample at 550 °C overnight to in a muffle furnace. Moisture of the samples was analysed through a drying process at 105 °C to a constant weight. Lastly, the carbohydrate content was calculated by the difference in 100 g of dehydrated macroalga. In the case of total polyphenols, these were quantified following the Folin-Ciocalteu method.

2.2. Animals, Diets and Experimental Design

The study was conducted on thirty-six male, homozygous, obese (*fa/fa*) Zucker rats and nine male lean (*Fa/?*) Zucker rats, aged eight weeks, purchased from Charles River Laboratories (Lyon, France). Animals were housed in polycarbonate cages (two rats per cage), placed in an air-conditioned room (22 ± 2 °C) with a 12 h light/dark cycle, and fed with a standard diet (2014 Global Diet, Envigo,

Italy) containing 4% lipids, 14.5% proteins and 48% carbohydrates and providing 2.9 kcal/g. After a 6-day adaptation period, the obese and lean rats were assigned to different experimental groups (n = 9/group). One group of obese rats was fed solely the standard diet (OC group), while two additional groups received the standard diet enriched with either 2.5% or 5% dehydrated *Gracilaria vermiculophylla* (LGV and HGV groups, respectively). The dehydrated macroalga was ground to powder using a blender, and then this powder was mixed daily with the standard diet until the mixture was homogeneous. Experimental groups that did not receive that macroalga were also provided with the same standard diet. Lean rats were assigned to the lean control group (LC) to function as the healthy control cohort in the study. Due to the fact that when compared to OC group, the HGV group significantly decreased its food intake shortly after the initiation of the experiment, a pair-fed (PF) group was introduced. The PF group received an amount of the standard diet equivalent to that consumed by rats in the HGV group on the preceding day. Water and food were provided ad libitum throughout the 6-week experimental period (long-term treatment), with the exception of the pair-fed group. Daily records of body weight and food intake were maintained. Additionally, the Energy Efficiency Ratio (EER) (body weight gain expressed in grams/energy intake expressed in kcal) was calculated.

Upon completion of the experimental period and subsequent to a 12 h fasting interval, blood samples were collected from the tail for glucose and insulin determinations. The animals were then euthanised through cardiac exsanguination under anaesthesia (chloral hydrate). The liver was weighed and promptly frozen. A section of the larger liver lobule from each animal was reserved for Oil Red O staining. Serum was extracted from blood samples following centrifugation (1000× *g* for 10 min, 4 °C). All samples were stored at −80 °C until analysis. The experiment adhered to the institution's guidelines for the care and use of laboratory animals (M20_2021_214).

2.3. Serum Parameters

dtaTriglyceride content was calculated taking into account the amount of free glycerol.

Insulin sensitivity was evaluated using two indices: the homeostatic model assessment for insulin resistance (HOMA-IR) and the revised quantitative insulin sensitivity check index (R-QUICKI). The HOMA-IR was calculated from basal insulin and glucose values using Matthews' formula (20):

$$\text{HOMA-IR} = (\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)})/22.5$$

R-QUICKI was calculated using fasting glucose, insulin and NEFA concentrations (21):

$$\text{R-QUICKI} = 1/(\log \text{fasting glucose (mg/dL)} + \text{fasting insulin } (\mu\text{U/mL}) + \text{fasting NEFAs (mmol/L)})$$

2.4. Hepatic Lipid Content

The lipid content in liver samples was assessed by analysing tissue sections embedded in the OCT embedding Matrix for Frozen Sections (Pioneer, PRC/OCT). These sections, cut into 5 μm thick samples using a Leica CM1850 Cryostat, were subsequently fixed in a 10% Formalin solution (neutral buffer, Sigma-Aldrich, HT501128) for 2 min. Following fixation, the slices were treated with 60% isopropanol and stained using freshly prepared oil red (Sigma-Aldrich, O0625-25g). After a 2 min wash with 60% isopropanol, the samples were counterstained with Mayer's Hematoxylin (Sigma MHS32) and then mounted with an aqueous mounting medium (22). No alterations were made to the histological microscopy images.

Moreover, liver samples weighing 100-200 mg were homogenised in 2 mL of buffer containing 10 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 250 mM sucrose (pH = 7.4). Commercially available spectrophotometric kits were used to measure hepatic triglyceride (1001313, Spinreact, Girona, Spain) and NEFA (434-91795, Fujifilm, Neuss, Germany) contents. Free glycerol (F6428, Sigma, Saint Louis, USA) was also quantified in order to perform free glycerol blanking for triglyceride measurements.

2.5. Enzyme Activities in Liver

Regarding the analysis of lipogenic enzymes, fatty acid synthase (FAS) activity was determined spectrophotometrically using the method outlined by Lynen (23) in the cytosolic fraction of liver homogenates. Briefly, liver samples (200-300 mg) were homogenised in 2 mL buffer (pH 7.4) containing 250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl, and then centrifugated (700 × *g*, 10 min, 4 °C). The supernatant was collected and subjected to a second centrifugation step (12,000 × *g*, 15 min, 4 °C). Following the second centrifugation, the supernatant was used to determine FAS activity from the rate of malonyl-CoA dependent nicotinamide adenine dinucleotide phosphate (NADPH) oxidation. The results were expressed as consumed NADPH nmol$\cdot$min$^{-1}\cdot$mg^{-1} protein.

With regard to fatty acid oxidation, the activities of carnitine palmitoyltransferase-1a (CPT-1a) and acyl-CoA oxidase (ACO) were measured in the mitochondrial/peroxisomal fraction of liver homogenates using the methods described by Bieber (24) and Lazarow (25), respectively. After the second centrifugation, the resulting pellet was re-suspended in resuspension buffer (pH 7.4) comprising sucrose (70 mM), mannitol (200 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (2 mM) and EDTA (1 mM). CPT-1a activity was expressed as released CoA nmol$\cdot$min^{-1} mg^{-1} protein, and ACO activity as NADH nmol formed$\cdot$min$^{-1}\cdot$mg^{-1} protein.

Concerning hepatic triglyceride release, microsomal triglyceride transfer protein (MTP) activity was assessed in liver samples obtained by homogenising 100 mg of liver in 1 mL of homogenisation buffer, which contained Tris-HCl (10 mM), NaCl (150 mM) and EDTA (1 mM) at pH 7.4. The homogenate was then centrifuged (7500 × *g*, 30 min, 4 °C). MTP activity was measured

fluorometrically in the supernatant by means of a commercial kit (MTP Activity Assay Kit, MAK 110, Sigma-Aldrich, St. Louis, USA) and expressed as a percentage of transference$\cdot h^{-1} \cdot mg^{-1}$ protein.

In all instances, total protein content from samples was quantified using the Bradford method (26), with bovine serum albumin (BSA) serving as the standard.

2.6. Western Blotting for Protein Expression Measurement

Diacylglycerol acyltransferase 2 (DGAT2), fatty acid transport protein 2 (FATP2), acetyl-CoA carboxylase (ACC), phosphorylated acetyl-CoA carboxylase (ACC), mitochondrial transcription factor A (TFAM), aquaglyceroporin 9 (AQP9), FAS, carbohydrate responsive element binding protein (CHREBP), nuclear respiratory factor 1 (NRF1), sequestosome-1 (P62), sirtuin 3 (SIRT3) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) were assessed by Western Blotting.

Liver samples (100 mg) were homogenised in 1 mL of cellular PBS (pH 7.4) containing protease inhibitors (100 mM phenylmethylsulphonyl fluoride and 100 mM iodoacetamide). The homogenates were then centrifuged (800 × *g*, 10 min, 4 °C) and the protein concentration of supernatants was measured using the Bradford method (26) with BSA as a standard.

For immunoblot analysis, 60 µg of protein was denaturalised at 95 °C for 3 min in Laemmli buffer and loaded into either 4-15% (DGAT2 and FATP2) or 4-20% (ACC, pACC, TFAM, AQP9, FAS, CHREBP, NRF1, P62, SIRT3, PGC1α) Mini-PROTEAN TGX Precast Gels (BioRad, Hercules, CA, USA). The proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, Cork, Ireland) by electroblotting, and later blocked with 5% casein and 0.5% BSA PBS-tween buffer for 2 h at room temperature. After washing, membranes were incubated overnight at 4 °C with specific antibodies targeting DGAT2 (1:1000, Abcam, Cambridge, UK, ab59493), FATP2 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-161311), ACC (1:1000, Cell Signaling Technology, Danvers, MA, USA, 4190), pACC (1:1000, Cell Signaling Technology, Danvers, MA, USA, 3661), TFAM (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-235588), AQP9 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA,sc-74409), FAS (1:1000, Abcam, Cambridge, UK,, ab128870), CHREBP (1:1000, NovusBio, Centennial, CO, USA, NB400-135), NRF1 (1:1000, Abcam, Cambridge, UK, ab175932), P62 (1:1000, Abcam, Cambridge, UK, ab56416), SIRT3 (1:500, Santa Cruz Biotechnology Dallas, TX, USA, sc-365175), PGC1α (1:1000, Abcam, Cambridge, UK, ab54481) and α-tubulin (1:1000, Cell Signaling Technology, Danvers, MA, USA, 2125).

Following the washing step, proteins were detected through a 2 h incubation with secondary antibodies, including anti-rabbit (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-2357), anti-goat (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-2354) and anti-mouse (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-516102) ones. The immunoreactive proteins were detected

using the Forte Western HRP substrate (WBLUF0100, Millipore, MA, USA), and the blots were imaged by scanning with the ChemiDoc™MP Imaging System (Bio-Rad, Hercules, CA, USA). Specific bands were identified with a standard loading buffer (Precision Plus Protein standards dual colour, 161-0374, Bio-Rad, Hercules, CA, USA). α-Tubulin was used for normalisation reference.

2.7. Gene Expression by Real-Time PCR

Total RNA was extracted from 100 mg of liver using TRIzol® (15596026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Genomic DNA was removed (DNase, Ambion, Foster City, CA, USA) and RNA concentration and quality (260/280 ratio) were determined using an RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with Recombinant DNase I (RNase-free) (2270A Takara Bio, Kusatsu, Japan). Total RNA (1.5 µg) was transcribed into complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Gene expression levels of actin alpha 2 smooth muscle (*Acta2*), macrophage mannose receptor 1 (*CD206*), collagen type I alpha 1 chain (*Col1a1*), C-reactive protein (*Crp*), adhesion G protein-coupled receptor E1 (*F4/80*), interleukin 1 beta (*Il1b*), matrix metallopeptidase 9 (*Mmp9*), TIMP metallopeptidase inhibitor 1 (*Timp1*), and transforming growth factor beta 1 (*Tgfb1*) were determined by Real-Time PCR in the presence of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using specific primer sequences (300 nM). *β-Actin* served as reference gene. Primer sequences are described in **Table 1**.

Table 1. Primer sequences for quantitative Real-Time PCR amplification.

Gene	Accession Number	Sense Primer 5'-3'	Antisense Primer 5'-3'
Acta2	NM_031004.2	GCC GAG ATC TCA CCG ACT AC	GTC CAG AGC GAC ATA GCA CA
Actb	NM_031144.3	CCC GCG AGT ACA ACC TTC T	CGT CAT CCA TGG CGA ACT
CD206	NM_001106123.2	ACT GCG TGG TGA TGA AAG G	TAA CCC AGT GGT TGC TCA CA
Col1a1	NM_053304.1	TCC TGG CAA GAA CGG AGA T	CAG GAG GTC CAC GCT CAC
Crp	NM_017096.4	TGT CTC TAT GCC CAC GCT GAT G	GGC CCA CCT ACT GCA ATA CTA AAC
F4/80	NM_001007557.2	CTC TTC CTG ATG GTG AGA AAC C	CCC ATG GAT GTA CAG TAG CAG A
Il1b	NM_031512.2	TGT GAT GAA AGA CGG CAC AC	CTT CTT CTT TGG GTA TTG TTT GG
Mmp9	NM_031055.2	AGC CGA CGT CAC TGT AAC TG	CCA GGA AGA CGA AGG GGA AG
Timp1	NM_053819.1	CAG CAA AAG GCC TTC GTA AA	TGG CTG AAC AGG GAA ACA CT
Tgfb1	NM_021578.2	CCT GGA AAG GGC TCA ACA C	TGC CGT ACA CAG CAG TTC TT

Acta2, Actin alpha 2 smooth muscle; *Actb*, beta actin; *CD206*, macrophage mannose receptor 1; *Col1a1*, collagen type I alpha 1 chain; *Crp*, C-reactive protein; *F4/80*, adhesion G protein-coupled receptor E1; *Il-1b*, interleukin 1 beta; *Mmp9*, matrix metallopeptidase 9; *Timp1*, TIMP metallopeptidase inhibitor 1; *Tgf b1*, transforming growth factor beta 1.

Monocyte chemoattractant protein 1 (*Mcp1*) and tumour necrosis factor α (*Tnfa*) were amplified using TaqMan probes (Mcp1: Accession Number NM_031530.1, Assay ID Rn00580555; Tnfα: Accession Number NM_012675.3, Assay ID Rn01525859) and the TaqMan Gene Expression Assay Mix (Applied Biosystems, Foster City, CA, USA). β-Actin (Accession Number NM_031144.3, Assay ID, Rn00667869) was used to normalise the expression levels.

All cDNA samples were amplified in an iCycler-MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Results were expressed as fold changes calculated using the $2^{-\Delta\Delta Ct}$ method (27).

2.8. Parameters Related to Oxidative Stress in Liver

Lipid peroxidation was determined spectrophotometrically by measuring the formation of thiobarbituric acid reactive species (TBARS) using a commercial kit (TBARS Assay Kit 10009055, Cayman Chemical Company, Ann Arbor, MI, USA). The TBARS concentration in the samples was calculated by means of a standard curve obtained with malondialdehyde (MDA) and results were expressed as nM MDA·mg^{-1} tissue.

The total antioxidant capacity was assessed employing the commercial kit OxiSelect™ Oxygen Radical Antioxidant Capacity (ORAC) activity assay (STA-345, Cell Biolabs, San Diego, CA, USA). Trolox solution was used to construct the calibration curve. The resulting ORAC values were expressed as µM Trolox equivalents·mg^{-1} protein.

The concentration of reduced glutathione (rGSH) in rat liver homogenates was colorimetrically assessed using a commercial kit (Glutathione Colorimetric Assay Kit, BioVision Incorporated K261, Milpitas, CA, USA), which relies on the glutathione recycling system in the presence of GSH and the DTNB fluorophore. The amount of GSH was calculated using a standard curve, and the results were expressed as µg rGHS·mg^{-1} protein.

The activity of glutathione peroxidase (GPx) was also assessed by measuring its H_2O_2 scavenging capacity using a colorimetric commercial kit (Glutathione Peroxidase Activity Colorimetric Assay Kit K762, BioVision Incorporated, Milpitas, CA, USA). The GPx levels in the samples were determined using a standard curve obtained with NADPH and results were expressed as mU GPx mg^{-1} protein.

Superoxide dismutase (SOD) was assessed spectrophotometrically using a commercial kit (Superoxide Dismutase Activity Assay Kit CS009, Sigma-Aldrich, St. Louis, MO, USA). This measurement involved quantifying the reduction in WST-1 formazan formation. SOD functions by quenching superoxide anions, leading to the conversion of WST-1 into WST-1 formazan. SOD activity was expressed as % inhibition rate.

Catalase activity was determined spectrophotometrically following the procedure described by Aebi (28), wherein the disappearance of H_2O_2 at 240 nm was monitored. Catalase activity was expressed as nmol·min^{-1}·μg^{-1} protein.

In all instances, an Infinite 200Pro plate reader (Tecan, Männedorf, Zurich, Switzerland) was used.

2.9. Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 24.0 (SPSS, Chicago, IL, USA). The normal distribution of data was confirmed by the Shapiro-Wilk test. LC, OC

and LGV groups on the one hand, and LC, OC, PF and HGV groups on the other hand were compared using a one-way ANOVA test, followed by the Newman-Keuls post hoc test. In the context of high-dose macroalga treatment, the inclusion of the pair-fed group facilitated the discernment of whether the alterations observed in the HGV group were attributable to the direct effect of the macroalga or were influenced by concomitant changes in food intake. Significance was assessed at the $p < 0.05$ level.

3. Results

3.1. Macroalga Composition

Dehydrated alga composition is summarised in **Table 2**. The analysis revealed that the major components of dried *Gracilaria vermiculophylla* are proteins (22.1%) and fibre (27.9%). Additionally, total polyphenol content accounted for 1180 mg/kg.

Table 2. Composition of *Gracilaria vermiculophylla*.

	Gracilaria vermiculophylla
Energy (kcal/100 g)	182
Total lipids (g/100 g)	1.40
Saturated fatty acids (g/100 g)	0.98
Total carbohydrates (g/100 g)	6.40
Simple carbohydrates (g/100 g)	<0.50
Proteins (g/100 g)	22.10
Fibre (g/100 g)	27.90
Ashes (g/100 g)	28.8
Moisture (g/100 g)	13.4

3.2. Body Weight, Energy Intake and Energy Efficiency Ratio

Figure 1A illustrates the trajectory of body weight across all groups throughout the entire experimental period. As expected, all obese Zucker rats showed higher body weight in comparison to their lean littermates during the intervention, as well as a greater body weight increase following the 6-week experimental period. The PF and HGV groups showed smaller body weight gain when compared to the OC group (**Figure 1B**).

The mean energy intake was higher in the OC group in comparison to the LC group. There were no differences between the OC and the LGV groups; however, in HGV, food intake was decreased, reaching values similar to those found in the LC counterpart (**Figure 1C**). In light of this circumstance, a pair-fed cohort (PF) was used elucidate whether changes induced by the elevated dosage of the alga were solely attributable to a reduction in food intake, or if additional direct metabolic effects were involved in the observed outcomes. When calculating the EER, a significant

difference was found between lean and obese rats. All obese rats showed a greater EER than lean rats, but no significant differences were observed among groups of obese rats (**Figure 1D**).

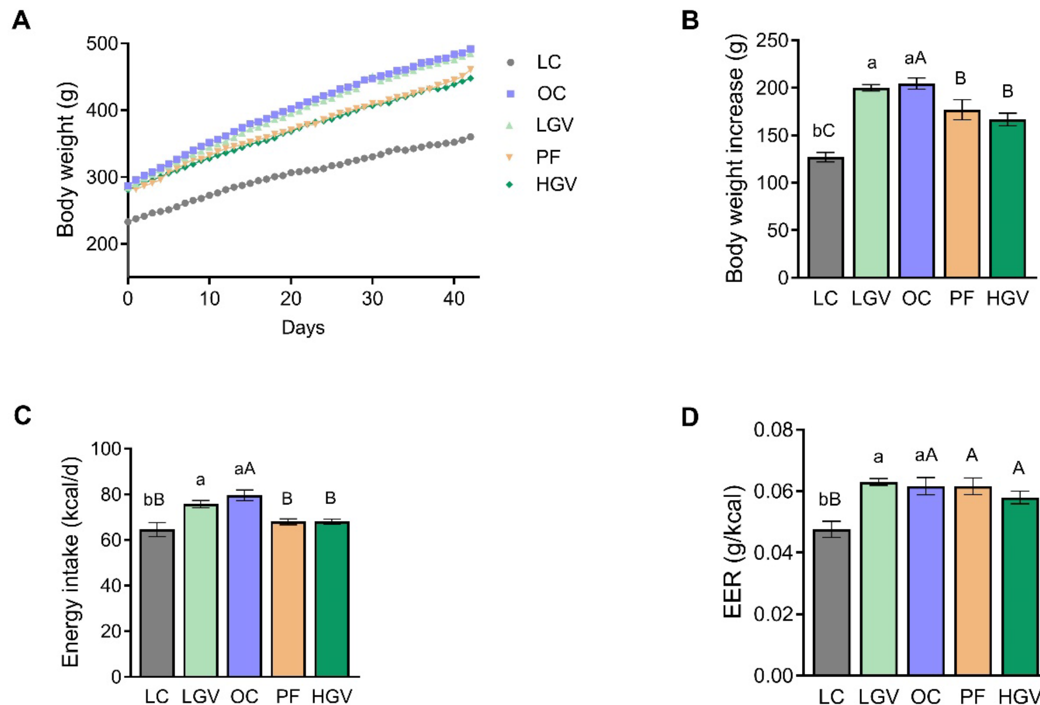

Figure 1. Body weight evolution curve (**A**), body weight increase (**B**), daily energy intake (**C**) and EER (**D**) of lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are presented as mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among the LC, OC, PF and HGV groups. Values not sharing a common letter are significantly different ($p < 0.05$). EER: Energy Efficiency Ratio.

3.3. Serum Biochemical Parameters

Serum glucose, insulin and NEFA levels were significantly higher in the OC group than in the LC cohort, suggesting insulin resistance (**Table 3**). This glycemic control alteration was confirmed by the values of both HOMA-IR and R-QUIKI indexes. LGV, HGV and PF groups reached physiological values of serum glucose. Regarding serum insulin levels, whereas the low dose of the alga did not modify this parameter, the high dose induced a significant reduction, which was similar to that observed in the PF group. When HOMA-IR was calculated, the treatment with the low dose of the alga did not modify this index, whereas in the high-dose and pair-fed groups, values were significantly decreased when compared with those of OC. Similar results were obtained in the case of R-QUICKY. Lastly, serum triglycerides were significantly higher in the OC rats than in their lean littermates. Among the experimental treatments, only the low dose of the alga induced a significant reduction (−26%), when compared to the OC **(Table 3**).

With regard to serum transaminases, the OC group exhibited higher ALT/GPT levels than the LC group. No changes were observed after treatment with the macroalga. In relation to AST/GOT, although the levels in the OC group did not differ from those in the LC counterpart, both groups supplemented with the macroalga demonstrated significantly higher values than the OC group. In the computation of the AST/ALT index, an indicative measure of hepatic disease, values were diminished in the OC group when compared with the LC cohort. Both groups receiving the macroalga along with PF exhibited higher values than the OC group. The ALP level, another parameter related to liver function, was increased in OC rats when compared to lean rats. Both groups receiving the macroalga showed increased levels in comparison to the OC group (**Table 3**).

Uric acid level augmented in OC rats when compared to the LC group. No significant differences were observed among the other experimental groups.

Table 3. Serum biochemical variables of rats from different experimental groups.

	LC	LGV	OC	PF	HGV
Glucose (mg/dL)	75 ± 7 ^{bB}	76 ± 12 ^{ab}	100 ± 8 ^{aA}	74 ± 4 ^{B}	78 ± 9 ^{AB}
Insulin (ng/mL)	0.8 ± 0.1 ^{bC}	25.3 ± 4.1 ^{a}	26.6 ± 1.5 ^{aA}	17.5 ± 3.2 ^{B}	15.8 ± 2.4 ^{B}
HOMA-IR	3.5 ± 0.7 ^{bC}	119.5 ± 28.2 ^{a}	174.2 ± 13.3 ^{aA}	70.8 ± 13.6 ^{B}	77.1 ± 15.4 ^{B}
TG (mg/dL)	26 ± 3 ^{bB}	151 ± 27 ^{a}	206 ± 50 ^{aA}	199 ± 33 ^{A}	219 ± 50 ^{A}
NEFA (mg/dL)	8.7 ± 0.9 ^{bB}	35.4 ± 4.4 ^{a}	29.3 ± 2.0 ^{aA}	32.0 ± 3.6 ^{A}	37.0 ± 6.5 ^{A}
R-QUICKI	0.38 ± 0.02 ^{aB}	0.29 ± 0.01 ^{b}	0.28 ± 0.01 ^{bC}	0.31 ± 0.01 ^{A}	0.31 ± 0.01 ^{A}
ALT/GPT (U/L)	56 ± 2 ^{bB}	119 ± 11 ^{a}	110 ± 12 ^{aA}	98 ± 7 ^{A}	119 ± 13 ^{A}
AST/GOT (U/L)	142 ± 8 ^{bB}	190 ± 9 ^{a}	144 ± 11 ^{bB}	166 ± 11 ^{AB}	199 ± 16 ^{A}
AST/ALT	2.6 ± 0.1 ^{aA}	1.8 ± 0.1 ^{b}	1.4 ± 0.1 ^{cC}	1.7 ± 0.1 ^{B}	1.6 ± 0.1 ^{B}
ALP (U/L)	278 ± 6 ^{cC}	515 ± 24 ^{a}	388 ± 15 ^{bB}	400 ± 15.1 ^{B}	542 ± 36 ^{A}
Uric acid (mg/mL)	1.6 ± 0.1 ^{bB}	5.0 ± 0.8 ^{a}	4.3 ± 0.4 ^{aA}	3.6 ± 0.5 ^{A}	6.4 ± 1.3 ^{A}

Values are presented as mean ± SEM. Lower cases represent ANOVA between LC, OC and LGV groups, and upper cases represent ANOVA between LC, OC, PF and HGV groups. Values not sharing a common letter are significantly different ($p < 0.05$). Experimental groups: HGV, *Gracilaria vermiculophylla* 5%; LC, lean control; LGV, *Gracilaria vermiculophylla* 2.5%; OC, obese control; PF, pair-fed. ALP: alkaline phosphatase, ALT/GPT: alanine aminotransferase, AST/GOT: aspartate aminotransferase, HOMA-IR: homeostatic model assessment for insulin resistance, NEFA: non-esterified fatty acid, R-QUICKI: revised quantitative insulin sensitivity check index, TG: triglyceride.

3.4. Liver Weight and Hepatic Index

Liver weight in rats from the OC group was significantly higher than in lean rats. The supplementation with the high dose of the macroalga, but not with the low dose, resulted in a significant reduction in this parameter (**Figure 2A**). However, this did not translate into significant changes in the hepatic index (**Figure 2B**).

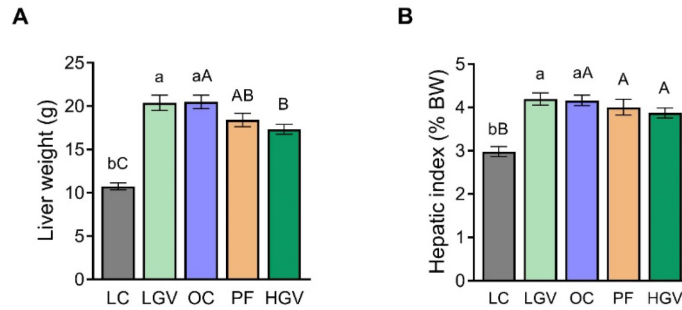

Figure 2. Liver weight (**A**) and hepatic index (expressed as percentage of body weight) (**B**) of lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). BW, body weight.

3.5. Hepatic Lipid Content

Genetically obese Zucker rats displayed a significant increase in hepatic total lipid content, triglycerides and NEFAs compared to the lean rats. *Gracilaria vermiculophylla* did not induce any changes concerning total lipid and triglyceride contents (**Figure 3A–C**); however, NEFA levels were significantly lower in the LGV and HGV groups than in the OC cohort (**Figure 3D**).

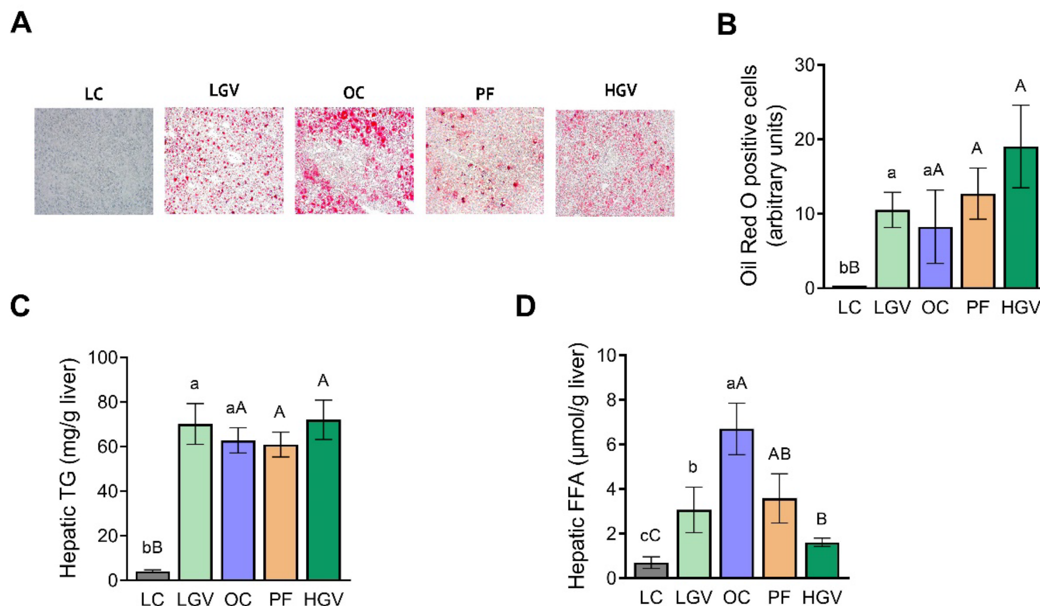

Figure 3. Representative Oil Red O staining (magnification x20) (**A**), quantification of neutral lipids by Oil Red O (**B**), triglyceride (**C**) and NEFA (**D**) content in livers of lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). NEFA: non-esterified fatty acid, TG: triglyceride.

3.6. Activities of Enzymes and Expression of Proteins Involved in Fatty Acid and Triglyceride Synthesis

Regarding de novo lipogenesis, FAS activity was higher in OC rats than in LC. This effect was reversed by both doses of the alga. In the case of the high dose, the reduction was significantly greater than that induced by food restriction in the PF group (**Figure 4A**). By contrast, FAS protein expression showed a tendency ($p = 0.06$) towards lower levels in the OC group when compared with LC. Although the low dose of the alga did not modify this parameter, the reduction observed in the OC group was completely reversed in the HGV and PF groups (**Figure 4B**). Concerning the pACC/totalACC ratio, recognised as an index of ACC activity (where a lower ratio indicates greater activation), no significant differences were observed among the experimental groups (**Figure 4C**). CHREBP expression, a transcription factor which regulates hepatic de novo lipogenesis, also remained unchanged among the experimental groups (**Figure 4D**).

Protein expression of AQP9 (**Figure 5A**) involved in glycerol uptake was increased in the OC group compared to the LC group, and dietary treatments did not induce significant changes. With regard to FATP2 responsible for fatty acid uptake, protein expression levels remained unaltered among the experimental groups (**Figure 5B**).

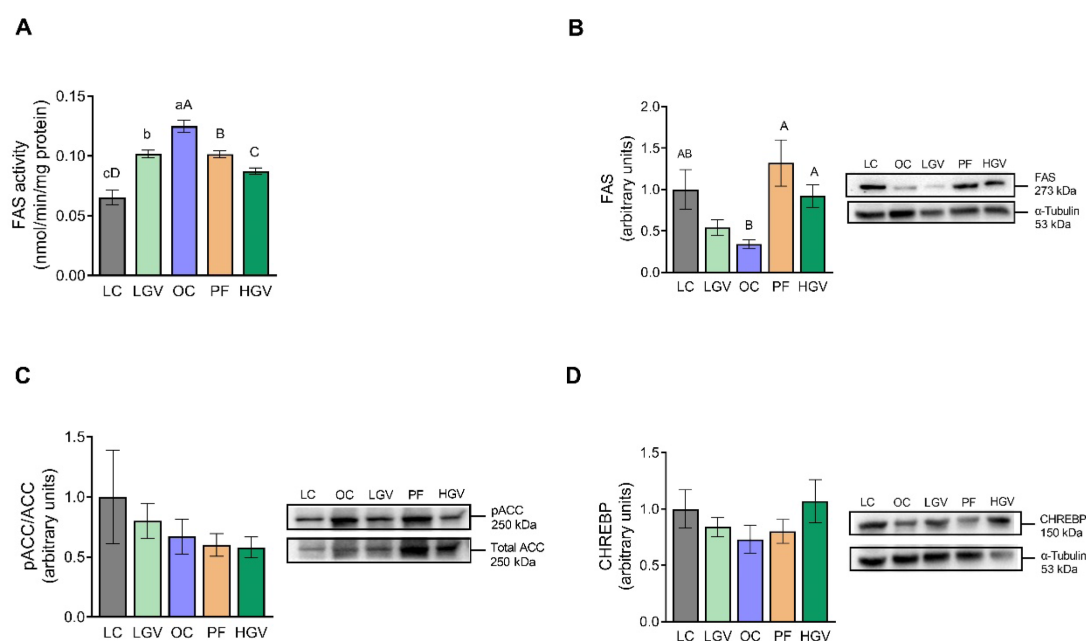

Figure 4. Activity of FAS (**A**), protein expression of FAS (**B**), phosphorylation ratio of ACC (**C**) and protein expression of CHREBP (**D**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). ACC: acetyl-CoA carboxylase, CHREBP: carbohydrate-responsive element-binding protein, FAS: fatty acid synthase.

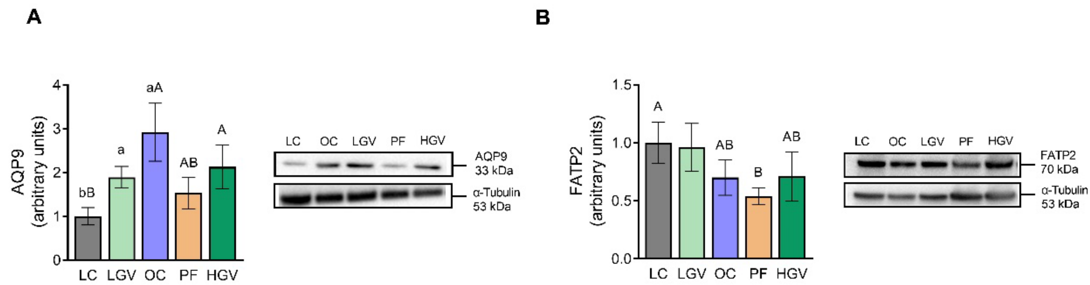

Figure 5. Protein expression of AQP9 (**A**) and FATP2 (**B**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are presented as mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. AQP9: aquaglyceroporin 9, FATP2: fatty acid transport protein 2.

Furthermore, the expression of DGAT2, an enzyme responsible for triglyceride assembly that displayed no differences between obese and lean rats, was not modified by the dietary treatments (**Figure 6**).

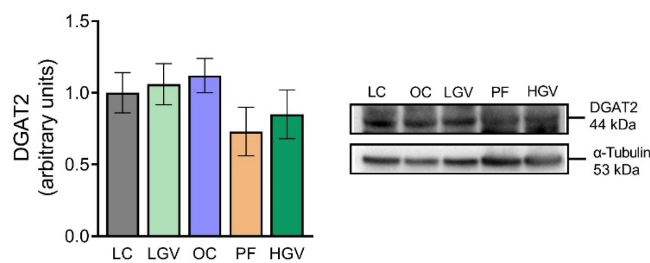

Figure 6. Protein expression of DAGT2 in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. DGAT2: diacylglycerol acyltransferase 2.

3.7. Activities of Enzymes and Expression of Proteins Involved in Fatty Acid Oxidation and Mitochondriogenesis

The activities of CPT-1a and ACO, key enzymes in mitochondrial and peroxisomal fatty acid oxidation, respectively, were measured. Both enzymes showed higher activity in the OC group in comparison to LC. Although supplementation with the low dose of the macroalga did not elicit any changes, both enzymes were decreased following supplementation with the high dose, reaching similar levels to those in lean rats, akin to the effects seen in the PF group (**Figure 7A,B**). Lastly, no changes in SIRT3 protein levels were observed among the experimental groups (**Figure 7C**).

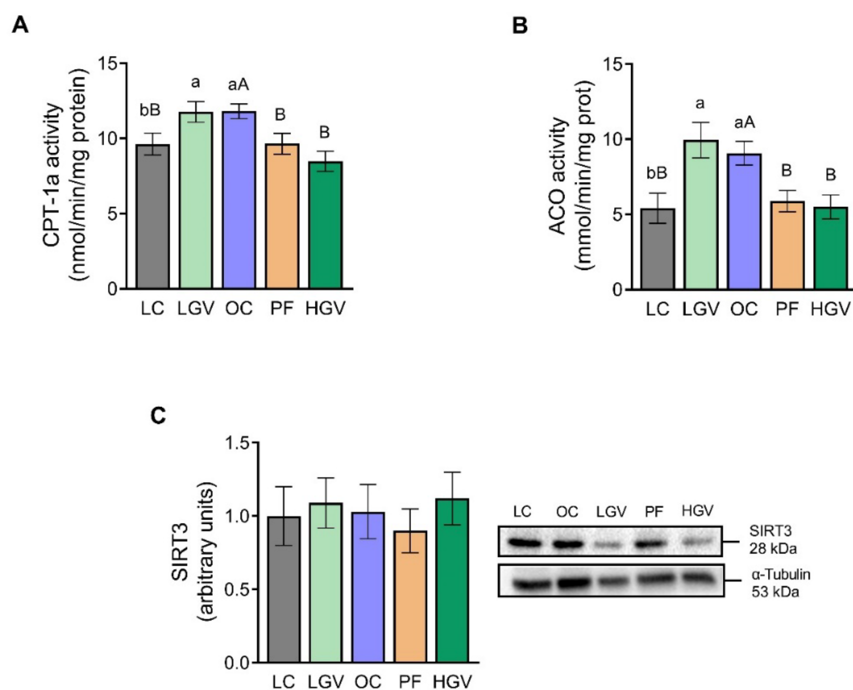

Figure 7. CPT-1a (**A**) and ACO (**B**) and protein expression of SIRT3 (**C**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). ACO: acyl-coenzyme A, CPT-1a: carnitine palmitoyltransferase-1a, SIRT3: sirtuin 3.

Concerning mitochondrial biogenesis, protein expressions of PGC1α, NRF1 and TFAM were measured. Acetylation levels of PGC1α remained unmodified among the experimental groups (**Figure 8A**). With regard to NRF1, rats in the OC group displayed a lower expression level than those in the LC group, and both doses of the macroalga were able to completely reverse this effect, reaching similar expression levels to those found in the LC group. In the PF cohort, NRF1 protein expression also increased compared to that in the OC group (**Figure 8B**). As for TFAM, reduced protein expression was observed in the OC group in comparison to LC. Rats receiving the low dose of the alga demonstrated increased protein expression, reaching the values yielded from the LC rats. However, the high dose induced no changes (**Figure 8C**).

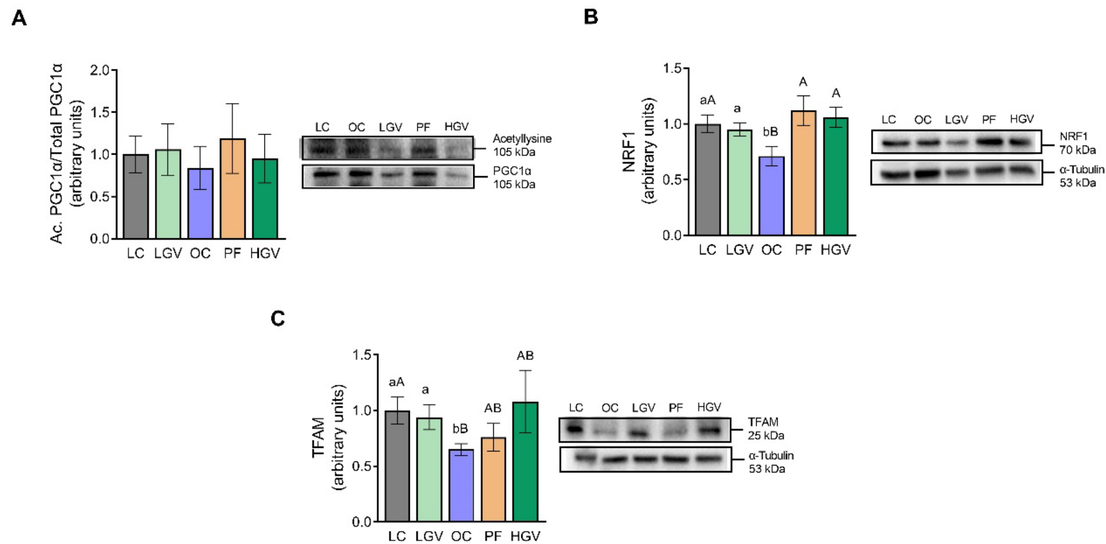

Figure 8. Acetylation ratio of PGC1α (**A**) and protein expression of NRF1 (**B**) and TFAM (**C**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha, NRF1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A.

3.8. Parameters Related to Triglyceride Secretion and Autophagy

No differences in the activity of MTP, an enzyme involved in triglyceride secretion from hepatocytes, were observed among the experimental groups (**Figure 9**). As for P62 protein levels considered to be a marker of the autophagic flux, a similar pattern without notable changes was observed among the experimental groups (**Figure 10**).

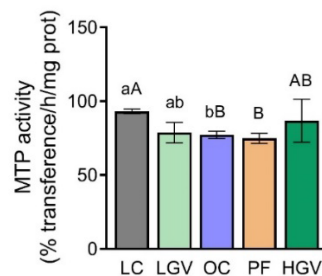

Figure 9. MTP activity in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). MTP: microsomal triglyceride transfer protein.

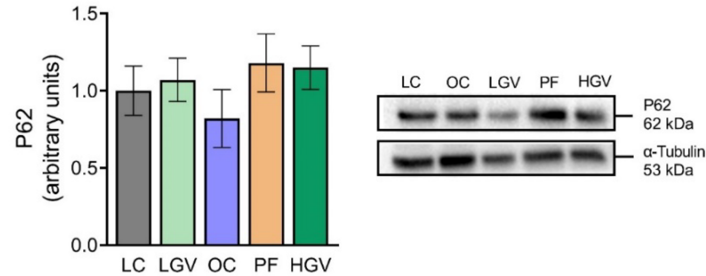

Figure 10. Protein expression of P62 in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. P62: sequestome-1.

3.9. Hepatic Oxidative Stress Markers

Regarding the activity of antioxidant enzymes, no differences were observed in SOD activity among the experimental groups (**Figure 11A**). By contrast, GPx activity was significantly lower in the OC group than in LC. The LGV group yielded no changes, while in the HGV group, GPx activity significantly decreased compared to the OC cohort. The PF rats similarly displayed reduced GPx activity in comparison with the OC group (**Figure 11B**). Concerning catalase, rats in the OC group exhibited a more reduced activity than those in the LC group, but no differences were observed among the other groups (**Figure 11C**). Regarding non-enzymatic antioxidant protection, no differences in rGSH levels were observed between the OC and LC groups. However, although not statistically significant ($p = 0.1$), values were increased by 30% in the LGV group when compared to OC (**Figure 11D**). Furthermore, MDA content, a marker of lipid peroxidation, was reduced in OC rats compared to their lean littermates. This reduction was reversed only in the HGV and PF groups (**Figure 11E**). Lastly, the total antioxidant capacity (Trolox equivalent) remained unaltered in the OC group compared to the LC counterpart. The administration of the low dose of the alga significantly increased this parameter in comparison to the OC group, but the high dose did not produce a similar effect. The PF group showed higher values than the OC cohort (**Figure 11F**).

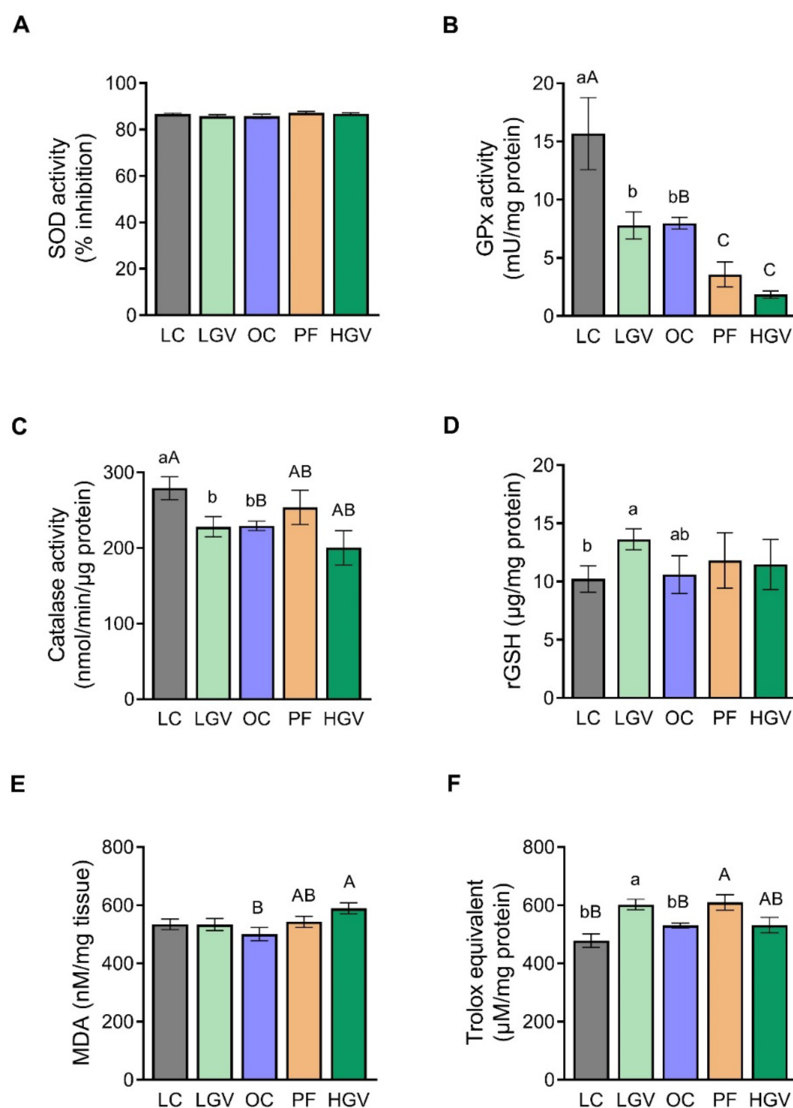

Figure 11. Activity of SOD (**A**), GPx (**B**) and catalase (**C**), and levels of rGSH (**D**), MDA (**E**) and Trolox equivalent (**F**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). GPx: glutathione peroxidase, rGSH: reduced glutathione, MDA: malondialdehyde, SOD: superoxide dismutase.

3.10. Gene Expression of Inflammation-Related Markers in Liver

Figure 12A shows the gene expression of *Il1b*, *Tnfa* and *Crp* inflammatory cytokines in liver. In all instances, the gene expression of these cytokines remained unaltered in the OC group compared to the LC cohort. Additionally, none of the treatments induced alterations in these cytokines.

Regarding macrophage markers (**Figure 12B**), the mRNA level of *Mcp1*, a chemokine that promotes hepatic infiltration of macrophages, did not differ among the experimental groups. By contrast, the

gene expression of *F4/80*, a marker of pro-inflammatory macrophages, was significantly up-regulated in the OC group in comparison to the LC group, while the supplementation with both doses of the alga decreased its expression to levels similar to those observed in the LC counterpart. The PF group showed intermediate values between the OC and HGV groups. Lastly, mRNA levels of *CD206*, a marker of anti-inflammatory macrophages, were not modified in the OC group in comparison to lean rats. Although no significant changes were observed among obese rats, gene expression was increased by 40%, 74% and 115% in LGV, HGV and PF, respectively, when compared to rats in the OC group.

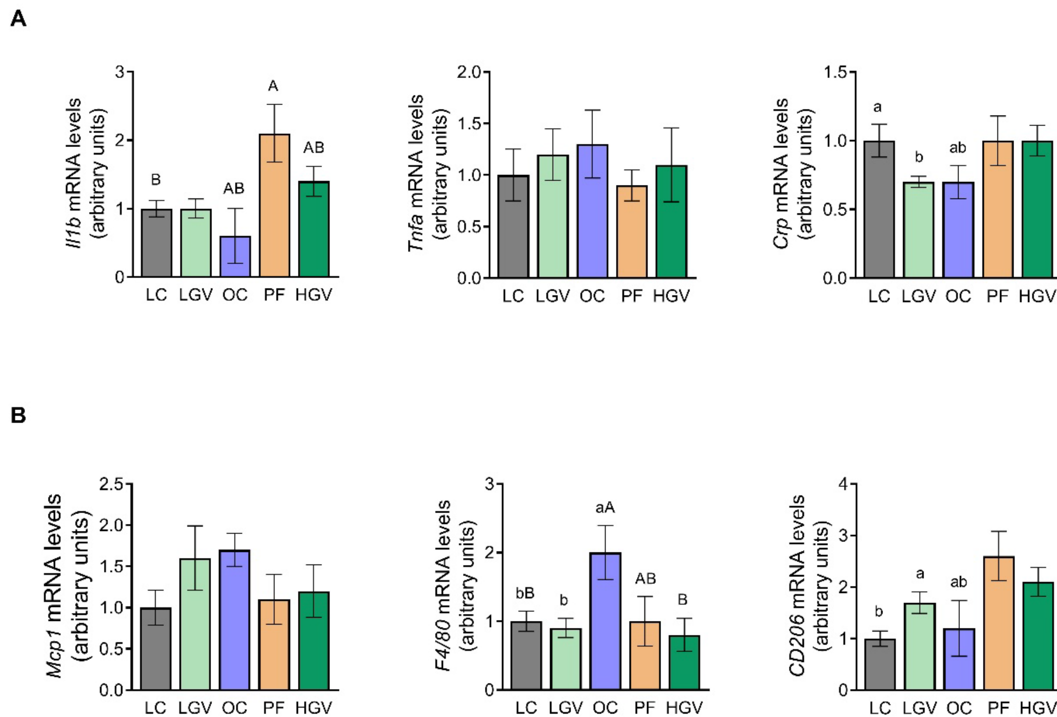

Figure 12. mRNA levels of inflammatory cytokines *Il1b*, *Tnfa* and *Crp* (**A**), and macrophage markers *Mcp1*, *F4/80* and *CD206* (**B**) in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different ($p < 0.05$). *CD206*: mannose receptor C, *Crp*: C-reactive protein, *F4/80*: adhesion G protein-coupled receptor E1, *Il1b*: interleukin 1b, *Mcp1*: monocyte chemoattractant protein 1, *Tnfa*: tumor necrosis factor α.

3.11. Gene Expression of Fibrogenic Markers in Liver

Acta2 mRNA levels were significantly higher in the OC group compared to the LC cohort. After the alga supplementation, while the low dose did not induce any change, rats in HGV showed increased *Acta2* gene expression when compared with OC. The PF group yielded intermediate values between the OC and HGV groups (**Figure 13A**). Regarding *Col1a1*, the OC group did not display any significant change in comparison to the LC counterpart. However, *Col1a1* expression decreased by 43% in LGV in comparison to OC, although statistical significance was not reached (**Figure 13B**).

Regarding *Timp1*, only a slight tendency towards higher gene expression level was observed in the OC group (p = 0.07) compared with the LC cohort. After the alga supplementation, no changes were induced (**Figure 13C**). *Tgfb1* mRNA levels remained unmodified among the experimental groups (**Figure 13D**). Regarding matrix metallopeptidases, enzymes involved in extracellular matrix degradation, *Mmp9* gene expression was measured. No differences were observed in the OC group in comparison to LC, and after supplementation with the low dose, no significant differences were observed in comparison to OC rats, although an increase of 68% was noted. HGV rats exhibited significantly increased *Mmp9* mRNA levels compared to the OC group. The PF cohort showed intermediate values between OC and HGV groups (**Figure 13E**).

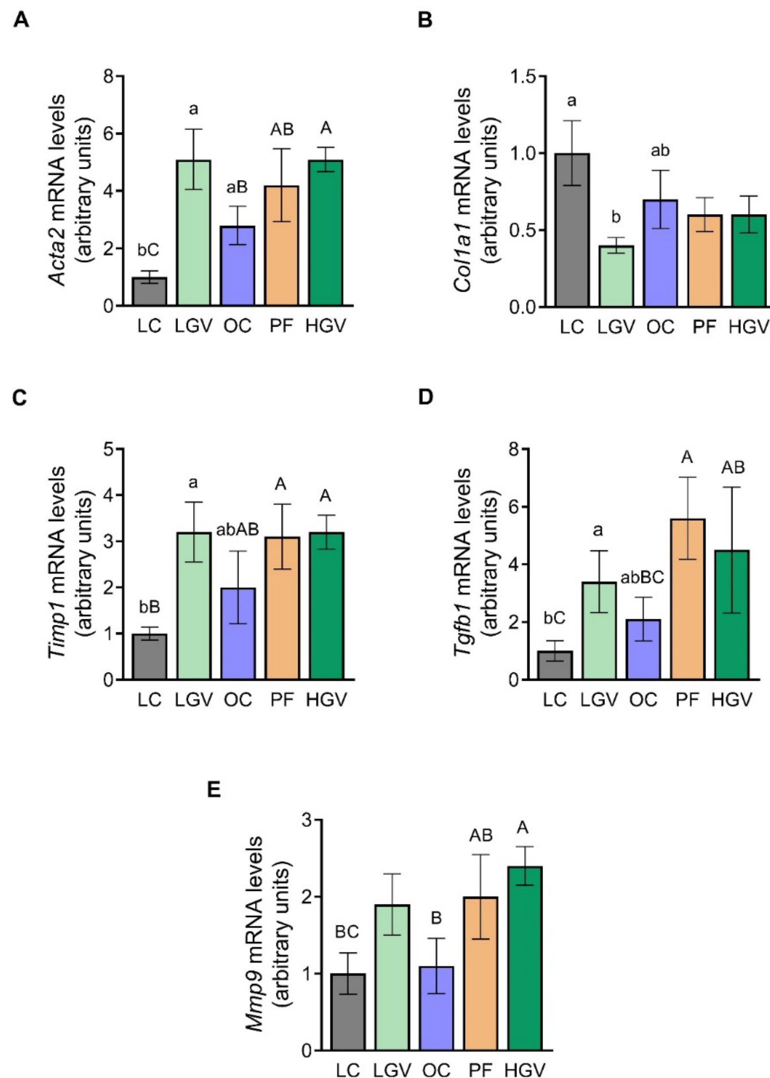

Figure 13. *Acta2* (**A**), *Col1a1* (**B**), *Timp1* (**C**), *Tgfb1* (**D**) and *Mmp9* (**E**) mRNA levels in liver from lean (LC) or obese Zucker rats fed with a standard diet (OC), a standard diet supplemented with 2.5% (LGV) or 5% (HGV) of *Gracilaria vermiculophylla* or a restricted amount of standard diet (PF). Values are mean ± SEM. Lower cases represent differences among LC, OC and LGV groups, and upper cases represent differences among LC, OC, PF and HGV groups. Bars not sharing common letters are significantly different (p < 0.05). *Acta2*: α-smooth muscle actin, *Col1a1*: collagen 1, *Mmp9*: matrix metallopeptidase 9, *Timp*: tissue inhibitor of matrix metalloproteases, *Tgfβ1*: transforming growth factor beta1.

Discussion

Marine macroalgae or seaweeds are garnering considerable attention due to their elevated nutritional value, particularly their rich content of protein, polyunsaturated fatty acids, fibres, minerals and vitamins (29). Additionally, they represent a valuable yet underutilized source of novel compounds with antioxidant, anti-inflammatory, antiviral or anticancer activities, among other potential applications (11). In fact, certain studies carried out in humans demonstrate that the consumption of the macroalgae can exert health benefits. For instance, it has been shown to improve the lipid profile (30), enhance insulin sensitivity (31) and glycemic control, and elevate levels of antioxidant enzymes (32).

With regard to hepatic steatosis, macroalgae consumption has shown to exert beneficial effects by decreasing hepatic fat deposition and other parameters related to fatty liver in experimental rat models (33–35) and mice (36–38). Moreover, a recent study conducted in humans revealed a negative association between seaweed intake and NAFLD, particularly among non-obese subjects (39).

In the present study, we explored the impact of administering the red seaweed *Gracilaria vermiculophylla* for six weeks on hepatic steatosis in obese (*fa/fa*) Zucker rats. The obese Zucker rat serves as a widely used model to study liver diseases associated with obesity (40,41). This model develops severe adiposity and impaired insulin sensitivity, fostering an elevated lipolytic flux originating from white adipose tissue, thus increasing plasmatic NEFA concentrations that reach the liver. Additionally, these rats exhibit heightened hepatic lipid synthesis and diminished oxidation, culminating in the development of liver steatosis (42,43).

Turning attention to glycemic control, glucose levels in the OC group were measured at 100 mg/dL, indicating a pre-diabetic stage. In contrast, glucose levels in the other experimental groups ranged from 74 to 78 mg/dL, signifying physiological values in these rats. These results suggest an improvement in insulin function in both alga-treated rats. The assessment of insulin levels revealed that in animals treated with the high dose of the macroalga, the improvement in insulin function could potentially be greater than that observed in animals treated with the low dose, as the serum concentration of this hormone was significantly reduced. The HOMA-IR index corroborated this suggestion. Taking into account that the obese rats exhibited increased serum NEFA levels, a metabolic condition often associated with impaired insulin sensitivity, the R-QUICKI index was also evaluated. The results were in accordance with those obtained from the assessment of HOMA-IR, indicating an improvement of insulin sensitivity following supplementation with the high dose of the alga. It is important to highlight that the positive effects induced by the high dose of the macroalga were attributed to the reduction in food intake resulting from this treatment, since the same effect was observed in the PF group.

Regarding serum triglycerides, it is noteworthy to mention that although no statistical differences were observed among obese rats, both OC and HGV groups exhibited triglyceride levels surpassing the threshold for hypertriglyceridemia (200 mg/dL). In contrast, the LGV and PF groups displayed values higher than physiological levels but below this threshold (150–199 mg/dL). For the LGV group, which showed a reduction of 26% in this parameter compared to the OC cohort, the triglyceride value reached almost physiological levels (151 mg/dL). This reduction could be explained, in part, by the effect of the macroalga on hepatic lipid metabolism, a topic discussed further.

Hepatic steatosis is characterised by increased accumulation of lipids in the liver, especially in the form of triglycerides. In a physiological status, only a relatively low quantity of triglycerides (less than 5%) is stored in cytoplasmic lipid droplets (44). However, in obese Zucker rats, hepatic triglyceride content is highly elevated (41). In the present experiment, as anticipated, obese rats exhibited higher total lipid content than lean Zucker rats, featuring increased amounts of triglycerides. These elevated concentrations contribute, at least in part, to the greater liver weight and hepatic index observed in obese rats. The heightened levels of triglyceride and NEFA in serum, could, in part, explain the increased lipid content observed in the livers of obese rats. Following supplementation with *Gracilaria vermiculophylla*, no changes in liver weight, hepatic lipid or triglyceride content were observed, suggesting that the macroalga supplementation was not effective in reducing hepatic steatosis.

To the best of our knowledge, this is the first study dedicated to investigating the effect of administering whole *Gracilaria vermiculophylla* on NAFLD. However, there is a study conducted in rats using 5% and 10% *Gracilaria changii* alga powder consumed with a high cholesterol/fat diet for eight weeks, where the treated groups exhibited ameliorated hepatic steatosis (45). One of the doses used by Chan et al. aligns with that in our study; however, the discrepancies between both studies could primarily stem from the use of different *Gracilaria* species, which may lead to variations in the composition of bioactive compounds (11,46). In addition, other differences in the experimental design may contribute to these discrepancies. Chan et al. employed a dietetic model of steatosis with an experimental period of eight weeks, while in the present study, a genetic model of steatosis and a shorter experimental period (six weeks) were used. Considering that approximately two weeks of a rat's live equate to one human year (47), a two-week disparity between the two protocols could indeed yield different outcomes. Based on these findings, it could be hypothesised that the alga may require longer treatment periods to modify hepatic lipid content, and different experimental models may respond divergently to the treatment.

Further studies on *Gracilaria* sp. have been documented, opting for the use of alga extracts, specific components or fractions rather than the entire alga. In this line, a study conducted in mice fed with a high fat diet observed that the administration of a sulphated polysaccharide from *Gracilaria lemaneiformis* was effective in reducing hepatic lipid deposition (14,15). In another study, the administration of *Gracilaria chorda* subcritical water extract in obese C57BL/6J mice ameliorated

hepatic lipid accumulation (13). The discrepancies between the present study and those reported in the literature may arise from the higher concentration of the mentioned bioactive compounds in the fractions and extracts compared with the whole alga. In addition, mice generally exhibit greater responsiveness to treatments than rats (48).

In hepatic steatosis, besides triglycerides, other important lipid species include NEFAs, which contribute even more significantly to hepatocyte injury, primarily through the formation of various toxic lipid species such as ceramides, diacylglycerols and lysophosphatidylcholine. These metabolites favour mitochondrial dysfunction, resulting in an elevated production of reactive oxygen species (ROS) (49). In the present study, supplementation with *Gracilaria vermiculophylla* led to a reduced hepatic NEFA content. Notably, in the case of the highest dose of the alga, the observed reduction was partially due to the decrease in food intake. This is evident as the PF group displayed intermediate values between the OC and HGV groups, with no significant differences between the PF and HGV cohorts.

In order to explain the reduction observed in NEFAs, the main enzymes involved in de novo lipogenesis were studied. The phosphorylation ratio of ACC was diminished by 33% in the OC group compared to their lean counterparts, indicating major ACC activation levels compared to those in lean rats. This effect, together with the enhanced FAS activity observed in obese rats, suggests an increase in de novo lipogenesis that may contribute to the hepatic pool of NEFAs. Supplementation with *Gracilaria vermiculophylla* reduced the increased FAS activity. Taking into account that the low dose of the macroalga did not modify FAS protein expression and, despite the increase after supplementation with the high dose, FAS activity decreased in both cases, it appears that this change in FAS activity occurred at the post-translational level (50). Thus, it can be proposed that *Gracilaria vermiculophylla* supplementation lowered hepatic NEFA content, in part, due to the decrease in de novo lipogenesis.

Additionally, we investigated whether changes in fatty acid oxidation were also involved either in the reduction of hepatic NEFAs or in serum triglycerides. This process occurs via β-oxidation and takes place in mitochondria and, to a lesser extent, in peroxisomes. The rate-limiting step for mitochondrial fatty acid oxidation is catalysed by CPT-1, and in peroxisomes, ACO catalyses the first reaction of β-oxidation. We analysed the activity of these two enzymes in the mitochondrial/peroxisomal fraction of hepatocytes, and subsequent to macroalga supplementation, only the high dose demonstrated the capacity to diminish the values. Nevertheless, this change was due to the reduction in food intake, as similar changes were observed in the PF group. Moreover, we noted an increase in the mitochondriogenesis with the low dose of the alga, as indicated by the increase in NRF1 and TFAM gene expression. This effect was less pronounced in the case of the low dose of the alga, as its supplementation only resulted in a significant increase in NRF1. On the other hand, the boost observed in the pair-fed rats may suggest that a lower food intake (a slight energy restriction) could

potentially restore NRF1 levels in non-treated obese rats. Additionally, the supplementation with the highest dose does not seem to independently affect mitochondrial biogenesis. These results may suggest that, in the case of the low dose, the alga can enhance fatty acid oxidation by increasing the number of mitochondria. Nonetheless, in the case of the high dose, it does not exert this direct effect. Taken together, these findings, along with the decrease in de novo lipogenesis, could explain the reduction observed in hepatic NEFA content as well as serum triglycerides (−26%).

Oxidative stress plays a central role in the progression of NAFLD towards NASH, since it triggers inflammatory and fibrogenic pathways (7). Zucker rats fed with a standard diet exhibit increased oxidative stress biomarkers at 14 weeks (51). This timeframe aligns with the age of the rats in the present study upon completion of the experimental period. Furthermore, certain algae are known to contain antioxidant molecules (52) and it has been demonstrated that specific compounds, such as N-acetylcysteine, possess the potential to improve oxidative stress independently of their ability to reduce lipid accumulation in the liver (53). On the one hand, the decrease in hepatic NEFA may imply a reduction in lipid peroxidation (8), and on the other hand, the increase in fatty acid oxidation can lead to elevated ROS production (54). Given this scenario, an investigation into oxidative stress was conducted.

Based on our findings, the activity of the antioxidant enzymes catalase and GPx was markedly reduced in obese rats. However, supplementation with *Gracilaria vermiculophylla* failed to improve this alteration; instead, the high dose of the alga induced an additional reduction. This effect was not a direct consequence of the alga itself, but rather associated with the reduction in food intake induced by this treatment. This is supported by the observation that rats from the PF group exhibited the same effect. Regarding the non-enzymatic antioxidant defense, rGSH was slightly increased ($p = 0.1$) by the low dose of the alga. In relation to the high dose, surprisingly, it led to an increase in lipid peroxidation, as evidenced by the enhancement of MDA, a phenomenon that appears to be unrelated to the reduction in food intake. Lastly, the total antioxidant capacity (Trolox) was increased by the low dose of the alga. Collectively, these results suggest that, under our experimental conditions, the low dose of the macroalga can exert some beneficial effects regarding oxidative stress, whereas the highest one seems to promote lipid peroxidation.

These effects are not in line with other studies in the literature. Chan et al. reported that rats fed with a high cholesterol/fat diet enriched with 5% and 10% *Gracilaria changii* whole algae powder for 8 weeks displayed improved lipid peroxidation (MDA levels) as well as antioxidant enzymes (catalase and GPx). However, SOD activity was only increased after supplementing with 10%, and no changes were observed with 5% (55). In another study where oligosaccharides from *Gracilaria lemaneiformis* were used, the authors observed antioxidant activity in a mice model of alcohol-induced liver damage. This was evident through a reduction in MDA levels, a boost in GSH levels and enhanced SOD activity (56). In an additional study, *Gracilaria birdiae* extract was found to increase hepatic catalase

activity and total antioxidant capacity, with no discernible impact on SOD and GSH (57). It is important to note that the observed discrepancies may be attributed to the significant differences in experimental designs between those studies and the present research.

It is well-established that Zucker rats do not spontaneously develop NASH unless exposed to an external stimulus such as a high-fat diet (58). Therefore, in the present study, rats were fed a standard diet, and consequently, the onset of NASH was not anticipated. Nevertheless, the analysis of potential changes in early biomarkers of inflammation and fibrosis could be of interest. Regarding inflammation, although mRNA levels of pro-inflammatory cytokines did not differ among the experimental groups, the reduction in *F4/80*, a marker of pro-inflammatory (M1) Kupffer cells observed in rats receiving *Gracilaria vermiculophylla*, suggests an anti-inflammatory effect of this macroalga.

As far as fibrosis is concerned, in general terms, the alga treatment did not have any significant effects on the studied markers (*Col1a1*, *Timp1*, *Tfgb1*). Nevertheless, our results showed an increase in hepatic *Mmp9* mRNA levels after supplementing rats with the macroalga. It is known that the activation of MMP-9 degrades collagen in liver, and for that reason, it is sometimes augmented in hepatic fibrosis (59), probably as a compensatory mechanism. However, in our study, since livers did not exhibit fibrosis or an increase in fibrotic markers, *Mmp9* is probably acting as a protective factor to prevent the potential development of fibrosis.

Regarding the limitations of this study, it is important to note that the precise composition and bioactive compounds found in *Gracilaria vermiculophylla* are not determined. This lack of knowledge hinders the identification of the specific component or components responsible for the observed biological effects. Nevertheless, it should be emphasized that, even in the case of having this information, the bioactive compounds should be commercially available in order to check their individual activities. Otherwise, they should be synthesized or extracted by a group of researchers with good expertise in this field. On the other hand, bioactive compound bioavailability should be determined, but the experimental conditions of the present study are not appropriate to investigate this issue. Thus, the fasting period used in the present design is not suitable. Moreover, since blood samples should be collected several times within a specific interval, the stress induced by this sample obtaining would likely alter some of the parameters evaluated in the present study. Consequently, an independent study carried out with a different experimental design would be necessary. Another limitation is that the effects of the alga on inflammation and fibrosis cannot be assessed in this study, since the obese Zucker rat does not spontaneously develop steatohepatitis. For this purpose, feeding these rats a high-fat diet would be required. Furthermore, a dose response study was not carried out in this work; instead, only two doses were used. This restricts our understanding of the effectiveness of this alga within a specific dosage range and, consequently, the fact that doses within that range could potentially be more effective than the low dose (2.5% in the diet) cannot be ruled out.

Moreover, it is worth noting that this study solely focused on male rats. Therefore, it remains uncertain whether the observed effects would be the same, lesser or even greater in females. This could be possible since there is evidence pointing to the fact that the development of steatosis occurs differently in males and females (60). Another important aspect is that it remains unknown why the consumption of a high dose of the alga resulted in a decrease in food intake. It could be due to a decrease in appetite, for instance, after modification of neuropeptides or simply because animals dislike the taste of seaweed; this requires further research. Additionally, the potential involvement of gut microbiota in the observed effects was not addressed. Finally, since this is a preclinical study, it is important to be aware that the translation of the observed effects to humans is not guaranteed. Consequently, further research is needed to evaluate the efficacy of the alga in clinical trials.

5. Conclusions

In summary, the findings reported in the present study indicate, for the first time, that while *Gracilaria vermiculophylla* may not reduce lipid and triglyceride accumulation in the liver, it does confer some beneficial effects, such as reductions in hepatic NEFAs, oxidative stress and inflammation makers. These effects are dose-dependent, and in the range used in the present study, only the low dose demonstrated effectiveness. Such effects probably make this alga a promising functional food that may have significant implications in the prevention of the appearance of complications associated with fatty liver.

Regarding future research, efforts should be made to identify the bioactive compounds responsible for the alga effects, as well as to determine their bioavailability. Further studies are also needed to determine the best choice for supplementation: some individual compounds, a combination of them or the whole alga, as in the present study.

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