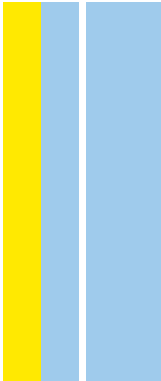


MESTRADO
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Discovery of novel compounds
from cyanobacteria
for the treatment of obesity
Susana Cação Lemos da Costa

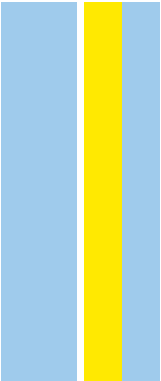
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Susana Cação Lemos da Costa. Discovery of novel compounds
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Discovery of novel compounds from cyanobacteria for the treatment of obesity

Dissertation for the master's degree in Environmental Toxicology and Contamination submitted to the Abel Salazar Institute of Biomedical Sciences from the University of Porto.

Supervisor: Doctor Ralph Urbatzka

Category: Postdoctoral researcher

Affiliation: Interdisciplinary Centre of Marine and Environmental Research (CIIMAR)

Co-supervisor: Doctor Mariana Reis

Category: Postdoctoral researcher

Affiliation: Interdisciplinary Centre of Marine and Environmental Research (CIIMAR)

Co-supervisor: Professor Victor Vasconcelos

Category: Full Professor

Affiliation: Sciences Faculty of Porto University

Category: President of the Board

Affiliation: Interdisciplinary Centre of Marine and Environmental Research (CIIMAR)

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Abstract

Obesity is one of the most life-threatening diseases, causing over 4 million deaths worldwide per year. This is an important matter since the number of obese individuals have nearly tripled in the past forty years. Furthermore, obesity is strongly associated to other morbidities, since majority of obese people are prone to develop diseases as diabetes, hypertension, high cholesterol, respiratory and cardiovascular complications and even some types of cancer. Alternatives for the current pharmaceuticals on the market, approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), are urgently needed and should, in particular, cause lower side effects. Aquatic organisms, such as cyanobacteria, have shown a high production of secondary metabolites with pharmacological activities that may be beneficial in the treatment of obesity.

In the present work, cyanobacteria were used to study their beneficial effects on obesity, by analysing their potential on repressing intestinal lipid absorption. Whole small animal assays based on zebrafish larvae were employed to screen for intestinal lipase and protease activity. Fractions with interesting activity were followed-up in secondary assays testing the uptake of short and long-chain fatty acids and cholesterol. Furthermore, alterations of the exposure to fractions were tested on main lipid classes and on the incorporation of fatty acids into main lipid classes, in order to understand if fractions would be able to target different modes of action of intestinal lipid absorption. Inhibition of lipase activity was observed in 11 fractions from the tested 263 fractions. Two fractions were identified that reduced the uptake of long-chain fatty acids (LEGE 07230 and LEGE 06001), while other two fractions reduced the cholesterol uptake (both from LEGE 07211). The analysis of main lipid classes and incorporation of fatty acids revealed different effects on the triglyceride hydrolysis and lipid processing, indeed highlighting that some of the bioactive fractions targeted different aspects of intestinal lipid absorption.

In the future, the responsible compounds for the bioactivities should be isolated from these promising strains and evaluated for their suitability as anti-obesity dietary supplements.

Resumo

A obesidade é uma das principais ameaças à saúde humana, causando mais de 4 milhões de mortes por ano em todo o mundo. Consequentemente esta patologia é um assunto importante, sendo que o número de indivíduos obesos triplicou nos últimos quarenta anos. A obesidade está fortemente associada a outras morbidades, sendo que a maioria da população obesa está mais predisposta a desenvolver doenças como diabetes, hipertensão, colesterol alto, complicações respiratórias e cardiovasculares e alguns tipos de cancro. Alternativas aos medicamentos disponíveis nos mercados, aprovados pela Food and Drug Administration (FDA) e pela European Medicines Agency (EMA), são urgentemente necessárias e devem, em particular, causar efeitos secundários menos severos. Organismos aquáticos, tal como cianobactérias, têm demonstrado uma alta produção de metabolitos secundários que poderão ser benéficos para o tratamento da obesidade.

No presente trabalho, cianobactérias foram usadas para estudar o seu efeito benéfico na obesidade, através da análise do seu potencial em reprimir a absorção intestinal lipídica. Ensaio com animais de tamanho pequeno, baseados em larvas de peixe-zebra foram realizados para fazer um rastreio da atividade intestinal da lipase e da protease. Frações com atividade interessante foram seguidas com ensaios secundários para testar o *uptake* dos ácidos gordos de cadeia curta e longa e do colesterol. Complementarmente, alterações da exposição às frações foram testadas nas principais classes lipídicas e na incorporação dos ácidos gordos nas principais classes lipídicas, de forma a poder distinguir se as frações seriam capazes de afectar diferentes modos de ação da absorção intestinal lipídica. Inibição da atividade da lipase foi observada em 11 frações das 263 frações testadas. Duas frações foram identificadas com atividade na redução do *uptake* de ácidos gordos de cadeia longa (LEGE 07230 e LEGE 06001), enquanto outras duas frações reduziram o *uptake* do colesterol (ambas da estirpe LEGE 07211). A análise das principais classes lipídicas e da incorporação dos ácidos gordos revelou diferentes efeitos na hidrólise dos triglicéridos e no processamento de lípidos, destacando, de facto, que várias frações influenciam diferentes aspetos da absorção intestinal lipídica.

No futuro, os compostos responsáveis pelas bioatividades devem ser isolados das estirpes promissoras e avaliados quanto à sua adequação como suplemento alimentar contra a obesidade.

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Abbreviations list

BBE	Blue Biotechnology and Ecotoxicology
BMI	Body mass index
BODIPY™ FL C5	4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoic Acid
BODIPY™ FL C16	4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid
BOGA	Biotarium for Aquatic Organisms
BTH	Butylated hydroxytoluene
C	Cholesterol
CD	Cluster of differentiation
CE	Cholesterol ester
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
CLS	Crown-like structure
CrtI	Solvent control
DMSO	Dimethyl sulfoxide
DPF	Days post fertilization
ECM	Extracellular matrix
EMA	European Medicines Agency
EtOH	Ethanol
FA	Fatty acids
FDA	Food and Drug Administration
FFA	Free fatty acids
GDP	Gross domestic product
KCl	Potassium chloride
LCFA	Long-chain fatty acids
LEGE-CC	Blue Biotechnology and Ecotoxicology Culture Collection
LPT	Lipoprotein transporter
MFI	Mean Fluorescence Intensity
MNP	Marine natural product
N₂	Nitrogen gas
NBD-Cholesterol	22-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3β-Ol
NP	Natural product

NPC1L1	Neiman-Pic C1-Like 1
ORO	Oil Red O
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PED6	N-((6-(2,4-Dinitrophenyl)Amino)Hexanoyl)-2-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoyl)-1-Hexadecanoyl-sn-Glycero-3-Phosphoethanolamine
PI	Phosphatidylglyceride
PLA₂	Phospholipase A ₂
PS	Phosphatidylserine
PTU	Phenylthiourea
SCFA	Short-chain fatty acids
SD	Standard deviation
T2DM	Type 2 Diabetes <i>Mellitus</i>
TG	Triglycerides
TLC	Thin Layer Chromatography
tRNA	Transfer Ribonucleic Acid
VLC	Vacuum Liquid Chromatography
WFCC	World Federation for Culture Collections
WHO	World Health Organization

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1. Introduction

1.1. Obesity

Obesity is considered an epidemic that has spread around the world, not only targeting adults but younger people as well. It is defined by the World Health Organization (WHO) as a health risk originated by an abnormal or excessive fat accumulation in the body (World Health Organization, 2000). A person is considered overweight with a body mass index (BMI) equal or more than 25 kg/m² and obese with a BMI equal or more than 30 kg/m² (Mitchell *et al.*, 2011). In the European Union alone, over half of the adult population is considered overweight, which is a major public health concern (World Health Organization, accessed on 05-07-2019). On the cellular level, obesity is an increased amount of fat cells, called adipocytes. This increment can be due to adipocyte hypertrophy, when there is an enlargement of the cells size, or by adipocyte hyperplasia, when new adipocytes are formed (Gupta, 2014). These cells were previously considered just as fat storing cells. Nowadays, they are recognized as important regulators of the metabolism and homeostasis in the body, producing hormones that regulate the metabolism of glucose and lipids, such as adiponectin, and hormones that regulate food intake and energy uptake, such as leptin (Castro *et al.*, 2016).

On mammals, there are two main types of fats that compose the adipose tissue: white adipocytes, responsible for the energy storage, and brown adipocytes, responsible for the thermoregulation, allowing the maintenance of body temperature. Obesity is a chronic inflammation disease and an abnormal accumulation of adipocytes is a serious health risk since it has many other essential roles in the body, such as immune responses, endocrine regulation, lactation and energy provision (Castro *et al.*, 2016). Therefore, several chronic diseases are consequences of obesity such as hypertension, respiratory and cardiovascular problems, diabetes (Type 2 Diabetes *Mellitus*, T2DM), sleep apnea, dyslipidaemia, musculoskeletal disorders and, ultimately, some forms of cancer which are nonetheless important and need to be solved for a healthier life of the patient (Mitchell *et al.*, 2011; Kang and Park, 2012; Rodgers *et al.*, 2012; Hruby and Hu, 2015; Castro *et al.*, 2016; Daneschvar *et al.*, 2016). Hence, obesity is an extremely complex metabolic disorder that occurs due to a positive energy balance between high caloric intake and low energy expenditure (González-Muniesa *et al.*, 2017). However, obesity does not solely occur due to an eating disorder or sedentary lifestyle that originate in the imbalance of the energy intake and expenditure, but it can, in fact, be influenced by several other aspects such as biological, environmental, developmental, socioeconomic and genetic factors (Blüher, 2019; González-Muniesa *et al.*, 2017; Hruby and Hu, 2015; Srivastava and Apovian, 2018). This disease has gained more importance in the last few years as the numbers are growing

and the solution seems to be far from being achieved. No other attempts made either by dieting, change in eating and lifestyle habits, surgery or even drugs, are getting results in solving this health threat in a permanent and healthy mode, despite efforts made by the medical community and private and governmental organizations (OECD/EU, 2017; Urbatzka *et al.*, 2018).

Obesity and its comorbidities have caused over 4 million deaths worldwide in 2015, representing more than 7% of all deaths that single year. Consequently, this disease is responsible for reducing life expectancy anywhere between 3 to 10 years (Afshin *et al.*, 2017; Blüher, 2019; Srivastava and Apovian, 2018). The etiology of obesity is, for these reasons, gaining more attention. However, it is a complex metabolic disorder due to its several possible complications and for the fact that, in the majority of cases, this disease has no single cause. Therefore, no easy, rapid and single cure has been found. Obesity affects over 28% of Portugal's population. Additionally, almost 40% of the population is considered overweight (Gaio *et al.*, 2018; Ministério da Saúde, 2018). These values reveal the pressing need to convert this disturbing situation and prevent the growth of this health hazard. Although a change in lifestyle and eating habits is being promoted more intensely nowadays, and in more urgent cases bariatric surgery is performed, these have not been enough to treat obesity and its associated comorbidities.

Additionally, obesity and its comorbidities carry a major economic burden, either to health care systems and to society, due to hospitalization, prescription drugs, days absent from work for short or long-term leaves, as well as the associated lower productivity at work and permanent disability. In Portugal, obesity and its comorbidities have been estimated to cost over EUR 199 million in one single year and the estimation for the global economic impact of obesity was US \$2.0 trillion. This represents almost 3% of the global gross domestic product (GDP) (Pereira and Mateus, 2003; Tremmel *et al.*, 2017). Comparing to other European countries, the estimated prevalence of this life-threatening disease in Portugal is one of the highest, along with the prevalence reported for most of the Great Britain countries (Gaio *et al.*, 2018). With the continued growth of obesity, half of the population will be considered obese or overweight by 2030, resulting in the rise of the associated health costs as well (Ng *et al.*, 2014; OECD/EU, 2017; Tremmel *et al.*, 2017).

1.1.1. Adipose tissue remodelling and expansion

Adipose tissue has the capacity to expand in a non-transformed state. This means that it can undergo continuous changes in a rapid manner to respond to alterations in nutrient deprivation and excess. This ongoing remodelling process of the adipocytes is accelerated in obese situations, which can cause the adipose tissue to reach the diffusional

limit of oxygen during growth to an almost unlimited capacity of tissue expansion, by either hyperplasia or hypertrophy. Hyperplasia is considered a healthier adipose tissue expansion, as the new adipocytes, originated from preadipocytes (precursor cells), are smaller, there is more space for lipid storage and a lower risk for cardiometabolic diseases, while hypertrophy is a pathological adipose tissue expansion where the adipocytes expand in size, as illustrated on Figure 1 (Gupta, 2014). This critical expansion can lead to various effects of adipose tissue dysfunction, including hypoxia, adipocyte cell apoptosis, extracellular matrix (ECM) overproduction, heightened state immune cell infiltration and subsequent pro-inflammatory responses, as well as a dysregulation of fatty acid fluxes (Lee *et al.*, 2010; Sun *et al.*, 2011). For these reasons, obesity is considered a complex multifactorial disease, as it can develop several other metabolic dysfunctions, commonly designated as obesity related comorbidities (Andolfi and Fisichella, 2018; Gustafson *et al.*, 2013; Hruby and Hu, 2015; Ren *et al.*, 2008).

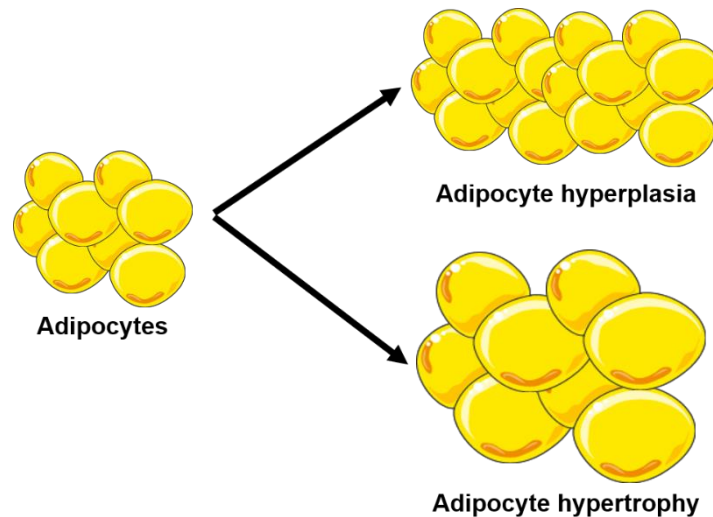


Figure 1 – Representative image of adipose tissue expansion by hyperplasia and hypertrophy.

Adipose tissue is primarily comprised of adipocytes and preadipocytes, pericytes, fibroblasts, endothelial cells, mast cells and immune cells (macrophages and T cells) (Lee *et al.*, 2010; Panina *et al.*, 2018). While new adipocytes populate the tissue, macrophages act like mediators of adipocyte remodelling by surrounding the dead tissue, creating crown-like structures (CLS). There is a thin balance between the populations of macrophages that are necessary to maintain proper adipocyte function and the ones that promote inflammation. In the lean state, resident immune cells (denominated M2 anti-inflammatory macrophages, regulatory T cells and eosinophils) promote tissue repair by clearing apoptotic and necrotic cells, remodelling ECM and angiogenesis in the expansion of adipose tissue, while in the obese state, mast cells, CD8 and CD4 T cells are the resident cells, which along with the heterogeneity of macrophages, undergo a transformation designated “phenotypic switching”, switching from an anti-inflammatory M2 state to a pro-

inflammatory M1 polarization state, losing their protective capacity (Lumeng *et al.*, 2007; Lee *et al.*, 2010; Schipper *et al.*, 2012). The disruption of the delicate balance between adipocytes and adipose tissue resident immune cells, that occurs in obesity, switching cells to a pro-inflammatory state, provides metabolic signals that mimic bacterial infection. In the case that the exposure to the promoted inflammation is prolonged, it can enhance adipose tissue inflammation and systemic metabolic abnormalities, such as insulin resistance and energy mobilization (Schipper *et al.*, 2012; Sun *et al.*, 2011).

While in a healthy expansion, preadipocytes are transformed into differentiated small adipocyte cells (hyperplasia), with subsequent vascularization, minimal induction of ECM and inflammation, in an unhealthy pathological expansion there is a rapid growth through enlargement of the already existing adipocytes (hypertrophy), causing limited vessel development and high transformation and infiltration of macrophages, inducing massive fibrosis and hypoxia. This critical expansion of fat cells promotes the development of adiposity-based chronic diseases comorbidities, which can range from chronic systemic inflammation, obesity-related insulin resistance, T2DM, non-alcoholic fatty liver and other metabolic syndromes (Mohamed *et al.*, 2014; Thomas and Apovian, 2017).

1.1.2. Anti-obesity medications and mechanisms of action

Obesity is a highly complex disorder and it has no single cause, making it extremely difficult to find a cure, and so, complementary strategies are required to overcome this disease. Although metabolic surgeries, such as bariatric surgery, are being performed and pharmacological treatments are also being used, these tend to have associated problems. In the case of surgery, although it shows results in weight loss, there are several surgical complications and reoperation may be required and, in the case of pharmacotherapy, weight loss is rarely sustained long-term and side effects may be associated (Kang and Park, 2012; Urbatzka *et al.*, 2018). For these reasons, reducing obesity requires a change in lifestyle and eating habits. A restriction of energy intake and a higher energy expenditure is needed to achieve a sustained weight loss, which is regarded as the best treatment for obesity and its comorbidities (Hill *et al.*, 2012; Dombrowski *et al.*, 2014). However, pharmacotherapy can aid in the process of a non-invasive weight loss journey.

Anti-obesity drugs can be divided into four groups, depending on their mechanism of action: drugs suppressing appetite, drugs increasing insulin sensitivity, drugs targeting sodium/glucose co-transporters and drugs decreasing lipid absorption (Castro *et al.*, 2016). As previously stated, many drugs have side effects associated with them, which can range from liver failure, increased heart rate, headache, hypoglycaemia, suicidal thoughts, nausea, carcinogenic and teratogenic potential, cognitive impairment and several other

reactions. For these reasons, many anti-obesity medications are prohibited or even withdrawn from the market by regulatory authority agencies, such as the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), when it is considered that the side effects do not outweigh the benefits (Kang and Park, 2012; Castro *et al.*, 2016; Daneschvar *et al.*, 2016). While the FDA approved five anti-obesity drugs for long-term use, there are only three medications approved by both the FDA and EMA, which are orlistat, naltrexone/bupropion combination and liraglutide (Yumuk *et al.*, 2015; Daneschvar *et al.*, 2016; Haslam, 2016). For a prospective drug to be approved by the regulatory authorities it is required that the difference in mean weight loss in clinical trials, after a 12-month period, to be statistically significant and of at least 5% compared to the placebo groups or at least 10% compared to the baseline weight. Additionally, at least 35% of patients must have reached that 5% weight reduction against the placebo-treated group and show metabolic improvements. Lastly, the drug must also be safe for long-term use and an assessment of the balance between the benefits and the risks of its use must be done (Castro *et al.*, 2016; European Medicines Agency, 2007; Kim *et al.*, 2013).

When certain drugs have been or were close to being approved by regulatory authorities but were not able to achieve the efficacy criteria of the 10% reduction of total body weight, a combination drug can be developed. The combination of naltrexone with bupropion culminated various advantages and resulted in the acceptance by both the FDA and EMA (Kang and Park, 2012).

Regarding drugs that decrease lipid absorption, by means of inhibition of the lipases, the gastrointestinal absorption of lipids will be lowered since the hydrolysis of dietary triglycerides will be prevented. Hence, fats will be excreted unchanged instead of being altered into fatty acids and monoglycerides, which are later absorbed by the enterocytes. For this reason, lipase inhibitors have already been used as an anti-obesity agent, such as orlistat (Castro *et al.*, 2016). Preventing the absorption of fat from a patients' diet will result in a lowered caloric intake that restrains the build-up of energy in the body, forcing it to use already stored energy, causing weight loss. Orlistat is a synthetic drug derived from lipstatin, which is a natural product isolated from the bacterium *Streptomyces toxytricini*. This approved anti-obesity drug is a potent and selective inhibitor of gastric and pancreatic lipases. It prevents the breakdown of dietary lipids resulting in an excretion of more than 30% of ingested fats in clinical trials. However, even though orlistat has been approved by the EMA in 2006, it has shown occasional acute kidney injuries, which may occur due to disturbances in fat absorption (Castro *et al.*, 2016; Kang and Park, 2012; Yumuk *et al.*, 2015).

Naltrexone and bupropion are synthetic drugs and have both already separately been approved for other clinical treatments. In combination, the opioid inhibitor and the

antidepressant result in an appetite suppressant drug, aiding in weight loss. However, although the mode of action of this drug is still not entirely understood and has shown side effects such as nausea, headache, dizziness, and may even have possible effects on the heart and blood vessels and increase the risk of seizures, this drug was approved by the EMA in 2015 (Kang and Park, 2012; Srivastava and Apovian, 2018; Yumuk *et al.*, 2015).

Lastly, liraglutide increases insulin and suppresses glucagon to control the blood sugar level, which is normally used to manage diabetes. This compound has shown to result in weight loss and was approved by the FDA in 2015. Some of its side effects are nausea and pancreatitis (Kang and Park, 2012; Srivastava and Apovian, 2018; Yumuk *et al.*, 2015).

Despite some more threatening side effects associated with the therapeutic use of these weight loss drugs, these have been approved due to their acceptable risk to benefit ratio. However, it is crucial and urgent to develop safer anti-obesity medications as part of a comprehensive strategy for disease management (Yumuk *et al.*, 2015).

Due to these harmful side effects from many compounds, the potential of natural products to be used as anti-obesity agents has, more recently, been under investigation as to encounter novel drug development strategies. There are several natural products that can be developed in an improved, effective and safe, pharmacological and pharmacokinetic way (Mohamed *et al.*, 2014). Nevertheless, it is important to not overlook the complexity of this metabolic disease as to create more effective and safer nutraceuticals that can battle obesity and its related comorbidities, which are urgently needed.

1.2. Natural products (NP) with anti-obesity bioactivity

A variety of natural products, ranging from crude extracts and isolated pure and natural compounds, such as aquatic and terrestrial plants, aquatic algae and bacteria as well as, fungi and animals, have bioactivity properties that can reduce and prevent obesity (Castro *et al.*, 2016; Fu *et al.*, 2016; Mohamed *et al.*, 2014). These natural products can aid in weight loss by targeting different parts of the body, depending on their molecular mechanism. One of the possible modes of action is the inhibition of lipases, which prevents the intestinal absorption of dietary fat. A wide variety of products, especially plant products, such as saponins, flavonoids, polyphenols and caffeine possess lipase inhibitory effects. Some varieties of tea, such as *Thea sinensis* (oolong tea) carry strong lipase inhibitors constituents. Marine algae such as *Caulerpa taxifolia* also present active lipase inhibitory constituents (Carten and Farber, 2009; Mohamed *et al.*, 2014). Natural products can also serve as appetite suppressants, regulating hunger through several hormonal and neurotransmitters such as histamine, serotonin and dopamine. The plant *Hoodia gordonii* is a natural appetite suppressant as it can significantly reduce the caloric intake, boosting

weight loss (Mohamed *et al.*, 2014). There are also natural products that are energy expenditure stimulants, which aid in the dissipation of excess energy as heat, such as *Garcinia cambogia*, *Silybum marianum* and *Panax ginseng* (Balaji *et al.*, 2016; Mohamed *et al.*, 2014). Another possible mechanism for natural products is the inhibition of adipocyte differentiation, several natural products such as chili peppers, *Glycine max*, *Curcuma longa*, fish and palm oil present interesting active constituents. Lastly, natural products can also regulate lipid metabolism either by decreased lipogenesis, which is achieved due to key enzymes as acetyl-CoA carboxylase, fatty acid synthase, carboxylesterase and malonyl-CoA, promoted by natural products such as *Lysimachia foenum-graecum*, or by increased lipolysis through stimulation of triglyceride hydrolysis, in order to diminish fat storage. Natural products such as *Zea mays L.* and *Glycyrrhiza glabra L.* contain active compounds that promote lipid metabolism (Balaji *et al.*, 2016; Mohamed *et al.*, 2014; Seo *et al.*, 2011). Various natural products have combined anti-obesity effects and are frequently present in our diets, such as ginger, pomegranate, celery, green tea, caffeine and peanuts (Afshin *et al.*, 2017; Yun, 2010).

1.3. Marine natural products (MNP) with anti-obesity bioactivity

The consumption of marine organisms and marine bioactive compounds were demonstrated to have the ability to reduce the incidence of chronic diseases, including obesity (Hu *et al.*, 2016; Suleria *et al.*, 2016). Fish, tunicates, sponges, crustaceans, fungi, cyanobacteria and algae produce secondary metabolites and contain compounds that possess anti-obesity activity. These compounds can contribute to fat loss through different mechanisms, either by lowering total cholesterol and triglyceride levels, inhibiting adipose tissue differentiation and lipogenesis, inhibiting pancreatic lipases activity or by inhibiting nervous system receptors that are involved in the mediation of food intake and body weight, for example. Thus, marine natural products can reduce inflammation and insulin resistance, aiding in weight loss (Castro *et al.*, 2016; Hu *et al.*, 2016).

Various compounds have strong weight loss potential, such as fucoxanthin, isolated from marine algae *Undaria pinnatifida*, which can reduce oxidative stress and has anti-diabetic and anti-obesogenic effects. Fucoxanthin was shown to stimulate energy expenditure causing a decrease in body weight gain and abdominal fat in C57BL/6J high-fat diet fed mice (Castro *et al.*, 2016; Yang *et al.*, 2019). A methanolic extract of *Eisenia bicyclis*, a marine brown alga, mainly composed by phlorotannins, inhibited pancreatic lipase and adipocyte differentiation, reducing lipid accumulation and glucose consumption (Castro *et al.*, 2016; Wan-Loy and Siew-Moi, 2016). Another compound with weight loss potential is fucoidan, a group of sulphated polysaccharides from marine brown algae that

can stimulate lipolysis used to mobilize stored energy during fasting or exercise (Castro *et al.*, 2016). Extracted from the brown alga *Sargassum henslowianum*, fucoidan can lower blood cholesterol and triglycerides levels in BALB/c high-fat diet fed mice (Wan-Loy and Siew-Moi, 2016). Lastly, alginates are a gelling polysaccharide extracted from marine brown algae that can modulate human appetite by producing postprandial satiety feeling, leading to a lowered food intake, having strong weight loss potential (Castro *et al.*, 2016; Jensen *et al.*, 2012).

1.3.1. Cyanobacteria as a potential source for the treatment of obesity

Cyanobacteria are often referred to as blue-green algae, however, they are not from the algae domain. These gram-negative photosynthetic prokaryotes are a phylum of bacteria and obtain their energy through photosynthesis, being able to produce and release oxygen, as a by-product. These microorganisms are morphologically and physiologically diverse and distributed in a broad spectrum of terrestrial and aquatic environments and conditions (Brito *et al.*, 2012; Mazard *et al.*, 2016). Cyanobacteria can be classified into five main subsections, where subsection I (Chroococcales) and II (Pleurocapsales) represent the unicellular bacteria and subsection III (Oscillatoriales), IV (Nostocales) and V (Stigonematales) comprise the filamentous strains (Brito *et al.*, 2012).

Based on unique and complex genetic pathways, cyanobacteria have developed unique interactions with other organisms, leading to the production of secondary metabolites that are regularly involved in causing or treating diseases. Although some cyanobacteria can trigger gastrointestinal illnesses and kidney and liver damages, they have various beneficial properties such as anti-cancer, anti-microbial and anti-inflammatory, for example. For these reasons, cyanobacteria have several biotechnological, pharmaceutical, cosmetic and industrial uses (Mazard *et al.*, 2016; Singh *et al.*, 2017).

Moreover, cyanobacteria are now considered a promising potential source of bioactive and beneficial components for the treatment of obesity as they contain interesting components such as carotenoids, phycocyanin, fibres, γ -linoleic acid and plants sterols to aid in weight loss. *Arthrospira* (formerly known as *Spirulina*), belonging to subsection III, has been consumed worldwide due to its beneficial effects on human health, it has anti-tumoral, anti-inflammatory, anti-hyperglycerolemia, anti-hypercholesterolemia and anti-viral activities (Castro *et al.*, 2016). The three major species that have therapeutic effects are *Arthrospira platensis*, *Arthrospira maxima* and *Arthrospira fusiformis*. The use of this cyanobacterium, as a dietary supplement, has shown lowered hepatic cholesterol and triglyceride levels in human clinical trials, thus aiding in the treatment of obesity (Castro *et*

al., 2016; Cheong *et al.*, 2010; Jarouliya *et al.*, 2015; Park and Lee, 2016; Szulinska *et al.*, 2017; Yang *et al.*, 2011). This cyanobacterium is commercially available to the public and does not require medical prescription.

Yoshinone A was isolated from *Leptolyngbya* sp. and acts as a suppressant of the adipogenic differentiation in 3T3-L1 mice cells. Although the mechanism of action is not well understood, this cyanobacterial compound is composed of a linear side chain and a γ -pyrone ring, in which its position is important to exhibit the inhibitory effect (Koyama *et al.*, 2016). Other cyanobacteria such as *Nostoc commune* var. *sphaeroides* Kützinger (*N. commune*) can aid in weight management and human health can benefit from its use. *N. commune* has the potential to reduce intestinal cholesterol absorption and can stimulate sterol excretion, through its hypocholesterolemic effect (Castro *et al.*, 2016; Rasmussen *et al.*, 2009; Yang *et al.*, 2011).

1.4. *In vivo* bioassays and small whole animal models for the study of obesity

Obesity is a type of lipid disorder and its prevalence has led to the necessity of knowing the lipid metabolism as to develop effective clinical treatments, without side effects. The development of effective therapeutics for lipid disorders, such as obesity, requires the understanding of the molecular mechanisms underlying lipid transport and lipid processing. Bioassays are, therefore, important for the screening of bioactivities of samples derived from natural compounds (Carten and Farber, 2009). While various studies are performed *in vitro*, using stable cell lines, this approach has limitations. These cells are less differentiated and possess altered levels of enzymes, there is no available cell line that can adequately capture all the cellular features of intestinal absorptive cells. Cultured cell models are predominantly comprised of a single cell type, which prevents them from being able to replicate the environment of a multicellular organ. Furthermore, *in vitro* cellular modes do not present several components that can influence lipid processing in live organisms, such as the interplay between different tissues and organs, that is particularly important for this complex disease. However, since *in vitro* studies cannot recreate the complex interplay of neural, chemical and hormonal cues, that regulate the metabolic processes in living animals, an advanced biological model of intestinal lipid absorption is needed to identify key molecular targets for therapeutic development and potential ligands, which can be achieved through *in vivo* bioassays, using small whole animal models (Carten and Farber, 2009; Giacomotto and Ségalat, 2010). Additionally, active compounds in *in vitro* studies often do not translate into effective drugs *in vivo* and may even show toxicity if tested in live organisms (Giacomotto and Ségalat, 2010).

Although there are several possible physiological models that can be used in *in vivo* studies, *Danio rerio* (zebrafish), a small freshwater teleost, has proven to be a powerful and reliable model to use in the study of vertebrate physiology and human disease, being an important model in drug development, presenting considerable logistical advantages against classical vertebrate models such as rats or mice, which are more expensive and time consuming for the initial screenings, in addition to the ethical conflicts when using rodent models (Carten and Farber, 2009; Giacomotto and Ségalat, 2010; Tabassum *et al.*, 2015). In comparison to mammals, zebrafish possess high physiological and genetic similarities, have well-developed endocrine and neural pathways, are highly sensitive to environmental alterations, have a relatively low maintenance cost, are of easy manipulation, models are available in adult and larval stages, have high fecundity and the larvae culture conditions are compatible with high-throughput screening (Giacomotto and Ségalat, 2010; Höltta-Vouri *et al.*, 2010). This model, even in the larval stage, has a rapid response to the efficacy and toxicity of compounds and develops rapidly in the first 24 to 48 hours after fertilization. The same gastrointestinal organs and similar cellular composition to the human body are observed, allowing for a wide range of biological processes and/or disease mechanisms studies on early developmental stages. Moreover, its small and transparent body allows for non-invasive imaging. For all these reasons, the use of zebrafish larvae in the study of the human lipidic metabolism is very advantageous, making them a physiological relevant whole animal model in initial drug discovery (Carten and Farber, 2009; Höltta-Vouri *et al.*, 2010; Tabassum *et al.*, 2015).

Although there are many advantages in using zebrafish as models in drug discovery, and these advantages keep increasing as the technology for their manipulation develops, there are some disadvantages to their use. It is important to remember that, even though their metabolism resembles one of mammals (endotherms), their type of organism is not the same, as they do not possess brown adipose tissue and therefore, their ectothermic organism and genome duplication can affect their metabolic and neuroendocrine regulation and, consequently, drugs may be metabolised in a different manner or rate, altering their function (Giacomotto and Ségalat, 2010; Höltta-Vouri *et al.*, 2010; Nguyen *et al.*, 2013). Inherent variability is associated with any type of *in vivo* studies, which can result in signal variation, hence, caution should be taken to minimize its biological variability. Therefore, larvae should be chosen with similar developmental stages and without any signs of sickness (Carten and Farber, 2009).

Many reports have been published using *Danio rerio* as models, including studies of obesity, and significant similarities have been observed in the lipid metabolism using fluorescent screening assays (Anderson *et al.*, 2011; Carten and Farber, 2009). Screening tools such as Nile Red, Oil Red O (ORO) and Sudan Black B have been the first lipophilic

dyes (lysochromes) to be used in this type of fluorescent obesity screening bioassays. These lysochromes can label a variety of lipids and lipid-containing structures, such as fatty acids, lipoproteins and triglycerides. Nile Red staining has been extensively used to identify lipid deposits and lipid-rich tissues in zebrafish larvae, allowing the identification of lipid reducing compounds that can be later validated on rodents (Carrasco Del Amor *et al.*, 2019; Carten and Farber, 2009; Costa *et al.*, 2019; Freitas *et al.*, 2019; Jones *et al.*, 2008; Urbatzka *et al.*, 2018). Further optical tools have been developed and studied in order to allow for a better discrimination between different lipids and lipid processing *in vivo* (Carten and Farber, 2009).

1.4.1. Zebrafish *in vivo* bioassay for intestinal lipid absorption

PED6 lipase reporter can be used to monitor lipid absorption in whole small animal models, such as zebrafish larvae, in real time. After its cleavage by phospholipase A₂ (PLA₂), PED6, that is built from a lysophospholipid covalently linked to a BODIPY fatty acid and a fluorescence quencher on the head group, releases a green fluorescence detectable at 488 nm in the gall bladder, intestine and liver, acting as a biosensor for lipase activity in live and healthy zebrafish larvae. Larvae that present defects in intestinal lipase activity or a hepatobiliary dysfunction will display an attenuated PED6 signal (Carten and Farber, 2009; Clifton *et al.*, 2010; Otis and Farber, 2013).

EnzChek, which is a casein labelled phosphoprotein with multiple red BODIPY fluorophores, detectable at 589 nm, reports the enzymatic function of protease. Proteases are also enzymes that help to break down food by transforming proteins into amino acids through the breaking of peptide bonds, which then the body utilizes as energy (López-Otín and Bond, 2009). The EnzChek protease screening assay is mostly used in conjunction with PED6, for the fact that with single fluorescent labelling techniques, interindividual variation may be observed. This can be minimized with the use of both reporters in combination. The combination creates a more physiologically relevant readout of the complexity of digestive processes *in vivo*. Moreover, EnzChek is used as a reporter of gastrointestinal toxicity since the ratio of lipase to protease activity has been used to screen for individuals with defects in exocrine pancreas function and impaired protein digestion (Carten and Farber, 2009; Otis and Farber, 2013).

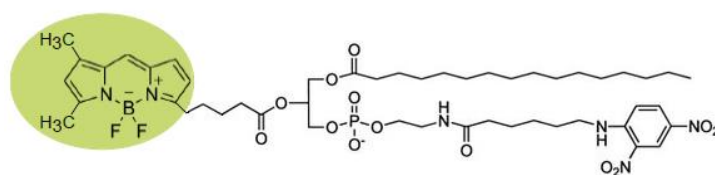


Figure 2 – Chemical structure of PED6 with the BODIPY-labelled acyl chain circled in green. Adapted from Anderson *et al.*, 2016.

The model compound used as a positive control was ezetimibe. This compound is a selective cholesterol absorption inhibitor in humans, which is metabolized by the intestine and liver (Clifton *et al.*, 2010). Since it prevents the transport of cholesterol from the intestinal lumen into the enterocytes of the small intestine, the intestinal absorption of cholesterol is inhibited, reducing the plasma cholesterol. It is also important to mention that ezetimibe's inhibition potency increases after being metabolized by the liver, which is another reason for the use of *in vivo* whole animal bioassays (Carten and Farber, 2009). In zebrafish larvae, ezetimibe has demonstrated absorption inhibition of triglycerides and related phytosterols as well as cholesterol reduction (Clifton *et al.*, 2010; Vavlukis and Vavlukis, 2018).

Following a primary assay with PED6 and EnzChek, the uptake of fatty acids and cholesterol can be identified using fluorescent lipid analogues such as BODIPYTM FL C16 (BODIPY-C16) for long-chain fatty acids, BODIPYTM FL C5 (BODIPY-C5) for short-chain fatty acids and NBD-Cholesterol.

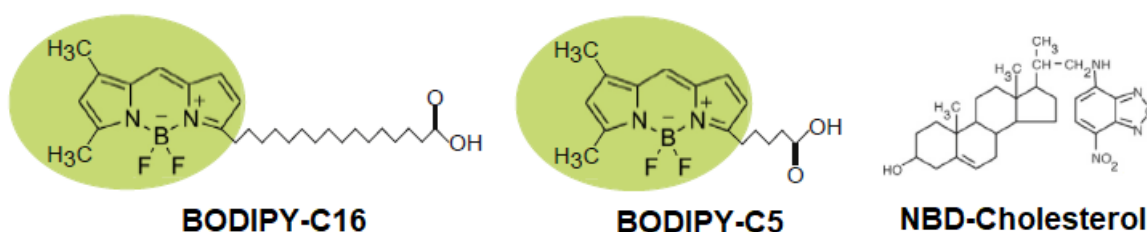


Figure 3 – Chemical structures of fatty acids (BODIPY-labelled acyl chain circled in green) and cholesterol analogues used in the bioassays. Adapted from Anderson *et al.*, 2016.

BODIPY-C16 and BODIPY-C5 are green fluorescent analogues for long-chain and short-chain fatty acids, respectively. They allow for visualisation of fatty acids uptake and metabolism (Carten *et al.*, 2011). Fatty acids are made of a carboxylic acid with a long aliphatic chain, either being saturated or unsaturated. These are important dietary sources of fuel for the body as they are important structural components of cells. The length of the aliphatic chain characterizes the fatty acid, which are mainly categorized between short and long-chain fatty acids. Short-chain fatty acids (SCFA) have aliphatic tails of a maximum of five carbons, while long-chain fatty acids (LCFA) have a tail of between thirteen to twenty-

one carbons. SCFA are primarily absorbed through the hepatic portal vein during lipid digestion, affecting the production of lipids, vitamins and energy. LCFA however, enter the blood first at the subclavian vein and help to sustain the structural integrity of cellular membranes, functioning as signalling molecules (Australian National Health and Medical Research Council, 2006; Nagy and Tiuca, 2017).

Cholesterol is a substance that is needed and produced by the body, as it helps to develop hormones, such as oestrogen and testosterone, vitamin D and other constituents that aid in the digestive process. Another function of cholesterol is to be integrated into membranes, aiding in membrane support, altering membrane fluidity and acting as a receptor. However, cholesterol is also found in meat, egg yolks, shellfish and other animal sourced foods. For this reason, cholesterol may accumulate in the body, specifically in the blood, which can ultimately cause plaque in the arteries when it combines with other substances, known as atherosclerosis. This is a threatening disease, associated with obesity, which can cause strokes or kidney problems, for example (American Heart Association, 2017; Medline Plus, 2016). NBD-Cholesterol is a continuously fluorescent lipid and allows visualisation of cholesterol absorption and investigation of lipid transport processing and lipid-protein interactions (Anderson *et al.*, 2011).

1.5. Main objectives

This work was integrated in the “CYANOBESITY” project. The development of this project will contribute to the discovery of novel cyanobacterial compounds that can tackle the threatening disease that obesity is. The aim is to develop innovative nutraceuticals, safe for human consumption, with beneficial effects on obesity and related comorbidities.

First and foremost, the main objective of the present work was to screen cyanobacteria strains for the reduction of intestinal lipid absorption in a whole small animal model, using a library of cyanobacterial fractions from CIIMAR’s Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). Specifically, the aim was to investigate lipase activity and determine protease activity as a proxy of intestinal toxicity. Bioactive fractions were followed-up in secondary assays to characterize their potential effects on the uptake of long and short-chain fatty acids and of cholesterol. Lastly, the analysis of the composition of lipids of exposed larvae was assessed by evaluating possible alterations of the main lipid classes, as well as the incorporation of long-chain fatty acids into the main lipid classes.

Overall, the evaluation of intestinal lipid absorption activity in an *in vivo* model should enable the identification of the most interesting cyanobacterial strains, which in the future could be analysed for the responsible compounds and developed into products for the treatment of obesity or other lipid disorders.

2. Materials and Methods

2.1. General procedures and material

Weighing was performed with an analytical Sartorius balance (0.01 mg deviation error). Ezetimibe (99.8% purity) used was from TargetMol, PED6, EnzChek, BODIPY-C5, BODIPY-C16 and NBD-Cholesterol from Invitrogen. Methanol, hexane, diethyl ether and acetic acid used in the Thin Layer Chromatography (TLC) analysis were 100% pure and chloroform was 99%. TLC analysis was performed using 10x10 cm glass-backed nano silica gel plates from Sigma-Aldrich. Cholesteryl palmitate, glyceryl trioleate (tripalmitin), palmitic acid, cholesterol, L- α -phosphatidylcholine, butylated hydroxytoluene, potassium chloride, primuline and tricaine (MS-222) used from Sigma-Aldrich.

2.2. Cyanobacteria culture

The BBE team (Blue Biotechnology and Ecotoxicology) holds its own Cyanobacterial Culture Collection (LEGE-CC) that is a member of World Federation for Culture Collections (WFCC). This collection, harbours over 1000 cyanobacterial strains from estuarine, freshwater and marine environments. Its database holds important information about each strain, including origin, culture conditions, morphological description and taxonomic-related data (Ramos *et al.*, 2018). From this collection, 263 cyanobacterial fractions from 45 strains belonging to freshwater and marine environments, of a variety of cyanobacterium orders, were selected for the bioassays. Cyanobacterial strains were cultured with Z8 culture medium, which integrates both macro and micronutrients (for marine strains, 25% of Tropical Marine salt and vitamin B12 was supplemented) at 25°C, under a light intensity of 10-30 $\mu\text{mol (m}^{-2}\text{s}^{-1})$ (Kotai, 1972). However, cyanobacteria grow slowly, therefore, the cultured strains and the ones selected for the present work were not the same.

The following cyanobacterial strains were selected for large-scale culture:

- 1) *Cyanobium* sp. LEGE 06137
- 2) *Nodosilinea nodulosa* LEGE 07084

The cyanobacterial strains selected for the bioassay are presented in Table A.II 1 in the Appendixes and were provided from the “CYANOBESITY” project.

The standard procedure for cyanobacterial growth was initiated with the culture in a 50 ml culture flask under sterile conditions using a Telstar Bio II Advance laminar flow chamber. Subsequently, part of the culture was transferred to an autoclaved flat-bottom conical glass flask with 500 ml of deionized water and the adjusted amount of Z8 medium as well as Tropical Marine salt and vitamins, if applied. The 50 ml culture flask was always kept as a back-up, in case there was a future contamination in the succeeding scale-ups. The scale-up process was then followed by a 4 l round flat-bottom glass flask, a 20 l Nalgene plastic

flask and, if necessary, for large-scale production, a 100 l plastic bag system. These were supplemented with air flux, for a continued medium circulation. For all the scale-ups, autoclaved deionized water and the adjusted amount of Z8 medium, with or without Tropical Marine salt and vitamin B12 were used and performed in sterile conditions.

Cyanobacterial biomass was harvested by centrifugation with 4700 rpm at 4°C for 20 minutes (Thermo Scientific Sorvall Bios 16). In marine strains, a final washing centrifugation cycle with deionized water was performed as to remove the salt from the harvested biomass. The collected biomass pellet was kept at -80°C and later, using a Telstar LyoQuest, the biomass was freeze-dried. The dried lyophilized biomass powder was stored at room temperature until subsequent extraction.

2.3. Extraction and fractionation

Two types of extractions were performed: Increased polarity extraction (ABC) and a polarity fractionation by Vacuum Liquid Chromatography (VLC). Increased polarity extraction is a simple extraction protocol usually done on new strains for screening purposes. This is achieved through sequential extraction with solvents of increasing polarity, typically done with hexane (non-polar) (A fractions), ethyl acetate (B fractions) and methanol (polar) (C fractions). Fractionation of biomass using VLC chromatography was performed to obtain a higher separation. The lyophilized biomass was soaked in dichloromethane with occasional stirring. The filtered extract was collected in a round-bottom flask and retained biomass was extracted again with a dichloromethane/methanol (2:1) mixture, until a colourless extract was obtained. After the crude extract was concentrated using a rotary evaporator (rotavapor), these were transferred to pre-weighted glass vials, the solvent was removed, and the crude extract was calculated.

VLC chromatography was then used to separate the components of the crude extract. The extract was solubilized with the dichloromethane/methanol mixture and absorbed to silica particles by solvent evaporation. The silica was then packed into a column and a stepwise mobile phase gradient from 100% hexane to 100% ethyl acetate and then to 100% methanol was used to elute the extract components, yielding different fractions. The collected fractions were transferred into pre-weighted glass vials after dried in a rotavapor. Solutions for bioactivity testing were prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 mg.ml⁻¹.

2.4. Zebrafish bioactivity screening

Zebrafish embryos were obtained from the Bioterium for Aquatic Organisms (BOGA), which develops and maintains aquatic laboratory animals under environmental,

nutritional and sanitary conditions. The embryos were collected on the day of fertilization (day 0), where they were cleaned manually, to discard any dead eggs and impurities, and incubated at 28°C in an aquarium with dechlorinated water, for their acclimatization. At 1 day post fertilization (DPF) zebrafish embryos were manually separated into petri dishes containing 20 ml of a salt medium with phenylthiourea (PTU), with a maximum density of 40 embryos per petri dish. The salt medium, which was previously prepared, consists of a marine sea salt water with a final concentration of 60 $\mu\text{g}.\text{ml}^{-1}$ and 200 μM of PTU, to suppress melanisation. Two days later, dead embryos and eggshells were removed, and the egg water was renewed. On 5 DPF, larvae were sorted by size and any larvae presenting malformations was discarded, this was done using a binocular microscope (Olympus SZX10).

In this work, optimization of assay conditions was performed for the fluorescent dyes of intestinal lipid absorption before using the cyanobacterial fractions. From the procedures presented in Farber *et al.*, 2001; Hama *et al.*, 2009; Clifton *et al.*, 2010; Anderson *et al.*, 2011; Carten *et al.*, 2011; Semova *et al.*, 2012; Falcinelli *et al.*, 2016, a few different conditions were tested and modified until consistent and reliable results were obtained for larval exposure to cyanobacterial extracts, resulting in an optimized protocol. All presented concentrations are final concentrations in the microwell plates, and all the substances used during the assays were stored at -20°C.

2.4.1. Lipase and protease activity

On 5 DPF six to eight replicates of zebrafish embryos were transferred from the petri dishes into a 48-microwell plate and the medium was removed with a syringe, leaving a leftover volume of 125 μl . In each assay, four replicate wells with DMSO (0.1%) (solvent control) and two replicate wells for each concentration (50 μM , 40 μM and 25 μM) of the positive control ezetimibe were included, while the remaining wells were used for the exposures to cyanobacterial fractions (10 $\mu\text{g}.\text{ml}^{-1}$), for 24h. The total volume of 750 μl was completed with new salt medium. The cyanobacterial strains and corresponding fractions can be found in Table A.II 1 in the Appendixes.

On 6 DPF, any existing eggshells and mortalities were removed and a combination of PED6 (0.5 $\mu\text{g}.\text{ml}^{-1}$) and EnzChek (5 $\mu\text{g}.\text{ml}^{-1}$) fluorescent dyes was added to the wells, homogenised and protected from light during 6h. PED6 and EnzChek were prepared on the day, as advised by the manufacturer. Throughout the assays, larvae were kept incubated at 28°C. After the 6h staining, the wells were cleaned and refilled with 750 μl of fresh salt medium as to eliminate fluorescence in the background when imaging. Fractions that

presented activity were re-analysed, in an independent assay through the same process as described above, as to confirm activity and reliability of the results.

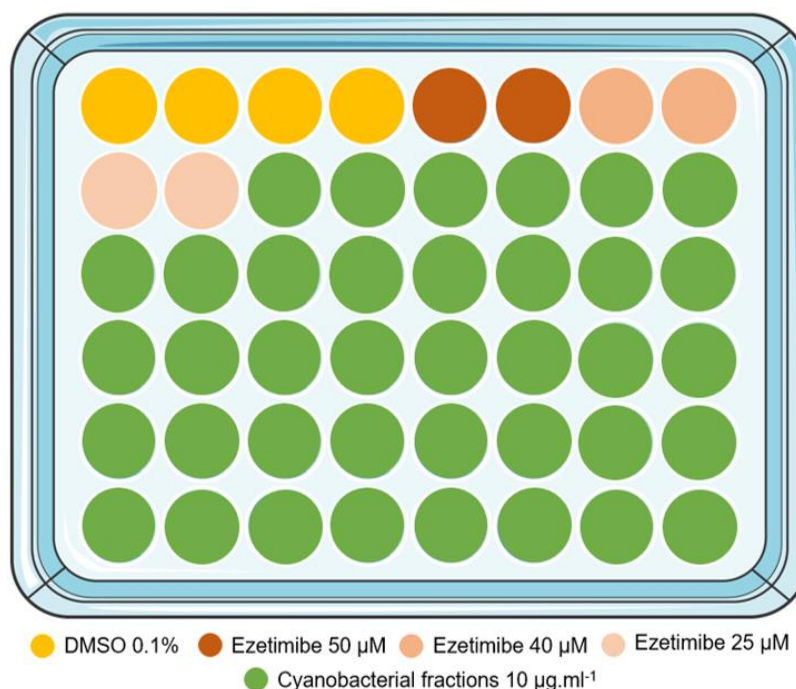


Figure 4 – Representative image of a 48-microwell plate layout for the bioassays.

2.4.2. Fatty acids and cholesterol uptake

On 5 DPF six to eight replicates of zebrafish embryos were transferred from the petri dishes into a 48-microwell plate and the medium was removed with a syringe, leaving a leftover volume of 125 µl. In each assay plate, two replicate wells with DMSO (solvent control) and one replicate well for each concentration of the positive control ezetimibe were included and the remaining wells were used for the 24h exposure to the cyanobacterial fractions. Total volume of 750 µl was completed with new salt medium and concentrations used were the same as for the lipase and protease assay. Only bioactive fractions that presented moderate/good ($\geq 30\%$ inhibition) and reliable results in both independent lipase activity (PED6) assays were selected for this examination. On 6 DPF, any existing eggshells and mortalities were removed. Each analogue was used in a different plate, BODIPY-C16 (diluted in 99% ethanol (EtOH)), BODIPY-C5 and NBD-Cholesterol (both diluted in 100% DMSO) with a final concentration of 0.8 µM. The plates were protected from light, homogenised and kept for 6h at 28°C. Afterwards, the wells were cleaned and refilled with 750 µl of fresh salt medium, as to eliminate background fluorescence.

2.4.3. Image acquisition and analysis

Zebrafish were anesthetized with 15 μ l of tricaine (MS-222, 0.03%) and visualized under a fluorescence microscope (Leica DM6000 FS) in the FITC (PED6, BODIPY-C16, BODIPY-C5 and NBD-Cholesterol) and in the TRITC (EnzChek) fluorescence channels. Fluorescence intensity was adjusted for the DMSO group at the beginning of every experiment and maintained for all other groups of the same experiment. Quantification of the intestinal fluorescence intensity was performed using ImageJ software program with an appropriate plug-in for zebrafish analysis. Mean fluorescence intensity values obtained from ImageJ analysis were used to calculate percentages of lipase and protease activity, as well as the uptake of fatty acids and cholesterol against DMSO-treated larvae.

2.4.4. Lipid extraction, identification of main lipid classes and fatty acid processing

Thin Layer Chromatography (TLC) has also been performed, on larval lipid extracts, to determine whether BODIPY analogues were metabolized by the zebrafish and to detect and identify the functional groups of the lipids, separated by this technique.

Two plates were prepared for the lipid extraction experiment. One of the plates was incubated with BODIPY-C16 for 6h, as to assess fatty acid incorporation into lipids, while the other plate remained without the fluorescent dye for analysis of lipid classes. The selected cyanobacterial fractions were the ones that presented moderate/good ($\geq 30\%$ inhibition of PED6) and reliable results in both lipase activity assays. Exposure conditions were described before. After exposure, a fixed number of zebrafish (6 larvae) from each well was transferred to a 2 ml Eppendorf tube. To each Eppendorf tube 15 μ l of tricaine anaesthesia (MS-222, 0.03%) was added, following a centrifugation (VWR MicroStar 17R) for two minutes (8000 rpm at 20°C). These were kept on an ice rack and the supernatant was discarded, as to leave the larvae dry. The Eppendorf tubes were kept on -80°C and protected from light before lipid extraction. The lipid extraction procedure was an adaptation of Folch *et al.*, 1957 and Flynn *et al.*, 2009. The zebrafish samples kept at -80°C were homogenised, using an Ultra-Turrax (Ystral X1020), with 500 μ l of 2:1 chloroform/methanol solution, which dissolves all the lipids and has a rapid evaporation, containing 0.01% of butylated hydroxytoluene (BTH) that acts as an antioxidant, preventing lipid oxidation. After addition of 100 μ l of 0.9% potassium chloride (KCl) to each Eppendorf, these were kept on ice for 5 minutes, vortexed and centrifuged at 2,000 g for 30 minutes (20°C). With a glass Pasteur pipette the lower layer was transferred to pre-weighted and identified 2 ml glass vials. Each tube was dried to completion with nitrogen gas (N₂) and weighted again to obtain the weight of the lipids.

For the TLC, two solvent mixtures were prepared. Solvent 1 was a mixture of 60:30:5 chloroform, methanol and deionized water, while solvent 2 a mixture of 80:20:1.5 hexane, diethyl ether and acetic acid. A mixture of lipid standards was prepared with cholesteryl palmitate for the lipid class of cholesterol ester (CE), tripalmitin for the lipid class of triglycerides (TG), palmitic acid for the lipid class of free fatty acids (FFA), cholesterol (C) and L- α -phosphatidylcholine (PC), each at 10 mg.mL⁻¹. To the dried lipid samples, 20 μ L of the solution of 2:1 chloroform/methanol with BHT was added to dilute and prepare the samples for the TLC plate set-up. The whole experiment was divided into six TLC plates (three with and three without BODIPY-C16 analogue). In each plate, 10 μ L of the lipid mixture and 10 μ L of samples were added.

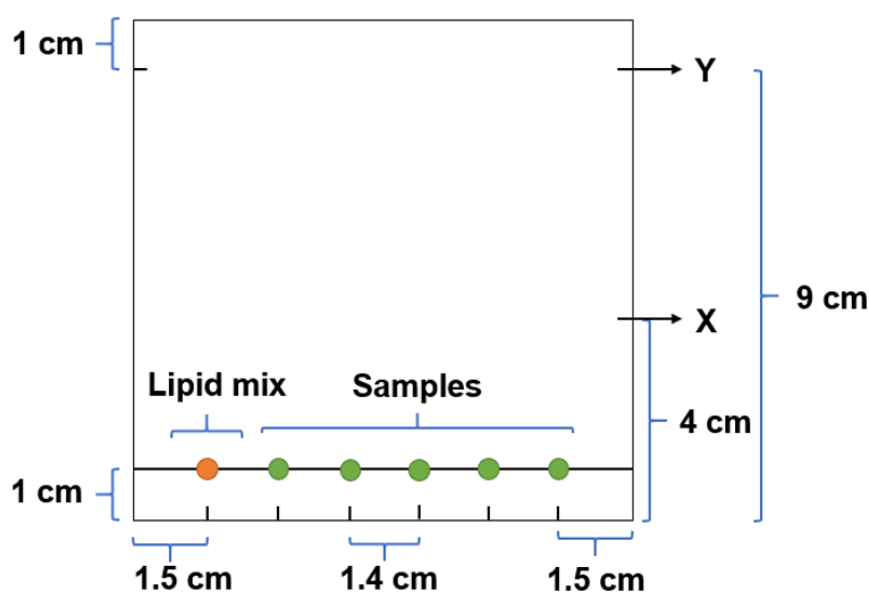


Figure 5 – TLC plate schematic representation of assay preparation and layout.

The process for the TLC consisted of two consecutive runs of solvent mixture 1 (10 ml) to the X mark, which separates the more polar phospholipids, and one run with solvent mixture 2 (10 ml) until the Y mark, which separates the neutral lipids. Between each run, the TLC plate was air-dried completely. For visualization of lipids on the TLC plates of samples without BODIPY-C16, primuline solution (0.01% concentration) was sprayed onto the plates. Primuline solution was made with 60% acetone/40% deionized water, protected from light and kept refrigerated. TLC plates with BODIPY-C16 were visualized directly by their incorporated fluorescent lipid analogue.

2.4.4.1. TLC image acquisition and analysis

For image acquisition, completely dried TLC plates were visualised using the molecular imager BIO-RAD Gel Doc™ XR+. The plates sprayed with primuline were

visualised using SYBR Green settings, filter 1 and an exposure time of 0.735 seconds, while plates with the analogue BODIPY-C16 were visualised using Alexa 488 settings, filter 2 and 12.929 seconds of exposure time. For both, the gel type selected was Bio-Rad PROTEAN II xi Gel with UV Transillumination, optimization of faint bands and highlighted saturated pixels was selected. Analysis was performed using ImageJ software by plotting profiles of each sample run and each band, selecting the peaks to obtain integrated density (considering intensity and the peak area) and their relative percentages.

2.5. Statistical analysis

Statistical differences analysis was performed for zebrafish exposed to the eleven promising strains, from the PED6 screening, SCFA, LCFA and cholesterol uptake using Prism 8 program (GraphPad). The assumption for ANOVA was tested by a normality and lognormality test (homogeneity of variance) by Anderson-Darling, D'Agostinho & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality test (p value < 0.05). If the assumption was met, the difference between the solvent control group (DMSO) and all other fractions was analysed by One-Way ANOVA with Dunnett's posthoc test (p value < 0.05). If the data had non-parametric distribution, the analysis was made by Kruskal-Wallis with Dunn's posthoc test (p value < 0.05). Data were represented as box-whisker plots with values in 5-95 percentiles and fractions with significance differences were indicated by the symbol *.

3. Results and Discussion

3.1. Cyanobacteria growth

Cyanobium sp. LEGE 06137 and *Nodosilinea nodulosa* LEGE 07084 were selected for large-scale culture. This process is ongoing and continued growth is required, in order to obtain a significant yield of biomass for further experiments.



Figure 6 – *Cyanobacteria* growth culture. 50 ml back-up culture flasks of both strains (on the left), 20 l Nalgene flasks of *Cyanobium* sp. (on the middle) and 100 l plastic bag system of *Nodosilinea nodulosa* (on the right).

3.2. Zebrafish bioactivity screening

3.2.1. Lipase and protease activity

The lipase activity reporter PED6, used in the present work, is designed to present an increased fluorescence when cleavage by phospholipase A₂ enzyme (PLA₂) occurs. This fluorescence is observed in the gall bladder and intestines of zebrafish larvae (Carten and Farber, 2009; Sato *et al.*, 2016), as illustrated in Figure 7. Fluorescent lipid reporters enable the direct observation of the metabolism of lipids in live zebrafish larvae, which generates a visible readout of lipid processing.

If the selected cyanobacterial fractions have the potential to lower the gastrointestinal absorption of phospholipids or to inhibit the intestinal lipase activity, the fluorescence intensity of PED6 will be lowered. DMSO-treated groups (solvent control) will show the intensity of the normal functioning of these digestive enzymes, that act upon the lipids. A positive control with a model compound, ezetimibe, was included, known to reduce PED6 activity in zebrafish as well as intestinal cholesterol absorption (Clifton *et al.*, 2010; Park, 2013).

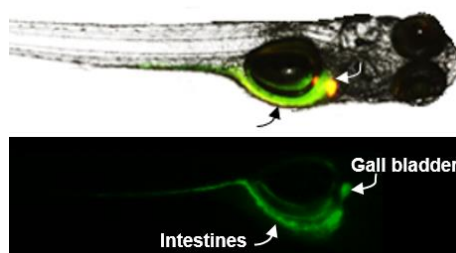


Figure 7 – Representation of the PED6 fluorescence (in green) in the gall bladder (top image in white arrow) and intestines (top image in black arrow) in live zebrafish (top image lipase and protease activity, bottom image solely lipase activity).

From the total of 263 cyanobacterial fractions that were tested in this work, 3 fractions (1.1%) presented strong inhibition of lipase activity ($\geq 50\%$ inhibition) and 8 fractions (3%) presented moderate inhibition ($\geq 30\%$ inhibition), some of them illustrated in Figure 8 and the data representation of all fractions on Figure 9. The results of the first screening were based on a defined cut-off for bioactivity of $\geq 30\%$.

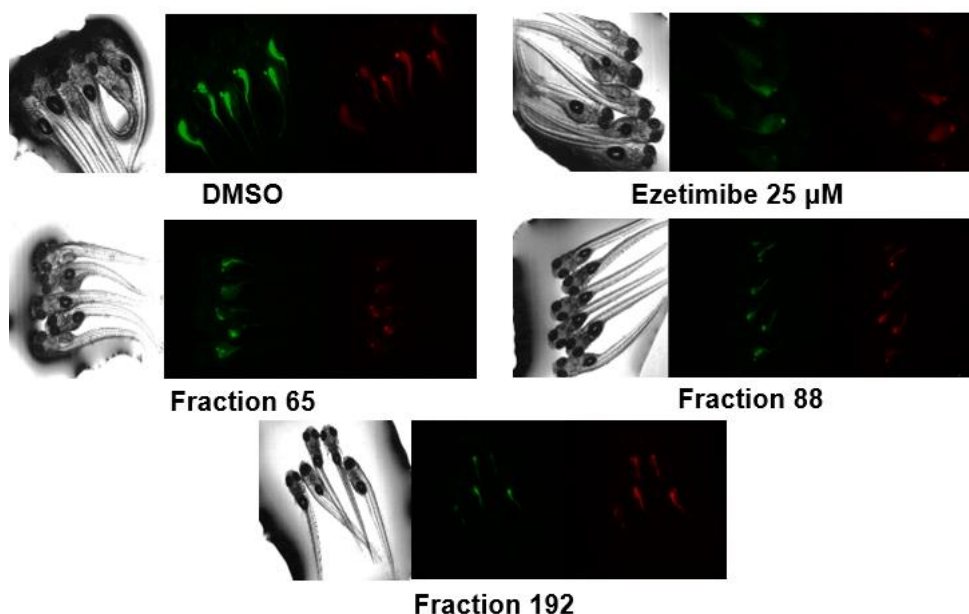


Figure 8 – Representative images of zebrafish PED6 and EnzChek bioassay. Fraction 65 (*Synechocystis* sp. LEGE 07211) with moderate inhibition, fraction 88 (*Tychonema* sp. LEGE 06363) and fraction 192 (*Leptolyngbya mycoidea* LEGE 06118) with strong inhibition. Brightfield images on the left, PED6 images on the middle (green fluorescence) and EnzChek images on the right (red fluorescence).

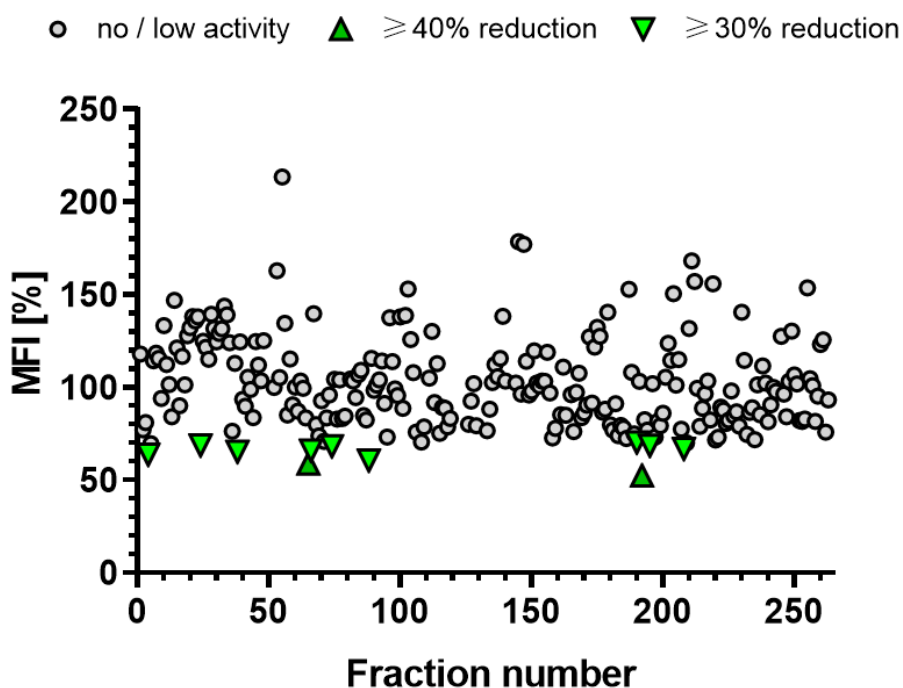


Figure 9 – Data of all 263 fractions used in the PED6 assay are represented as mean fluorescence intensity (MFI) relative to the solvent control (DMSO).

The 11 fractions (4.2%) that showed the best results in lipase inhibition were selected for further examination. These were fraction 4 (*Planktothrix mougeotii* LEGE 07230), fraction 24 (*Nodosilinea* sp. LEGE 06001), fraction 38 (*Microcystis aeruginosa* LEGE 91094), fraction 65, 66 and 74 (*Synechocystis* sp. LEGE 07211), fraction 88 (*Tychonema* sp. LEGE 06363), fraction 190, 192 and 195 (*Leptolyngbya mycoidea*, LEGE 06108, LEGE 06118 and LEGE 06009, respectively) and fraction 208 (*Pseudanabaena aff. persicina* LEGE 07163). From these fractions, 4 belonged to freshwater strains (*Planktothrix mougeotii*, *Microcystis aeruginosa*, *Synechocystis* sp. and *Tychonema* sp.) and 3 to marine strains (*Nodosilinea* sp., *Leptolyngbya mycoidea* and *Pseudanabaena aff. persicina*). Bioactivity of those selected strains was confirmed in an independent assay, using again 6 to 8 replicate individuals per group. Statistical analysis confirmed that five fractions showed $\geq 30\%$ inhibition and two fractions showed $\geq 40\%$ inhibition (fraction 65 and 192), which were statistically different against DMSO. All fractions presented moderate activity in the inhibition of intestinal lipases, in regard to our initial classification (Figure 10 and Table 1).

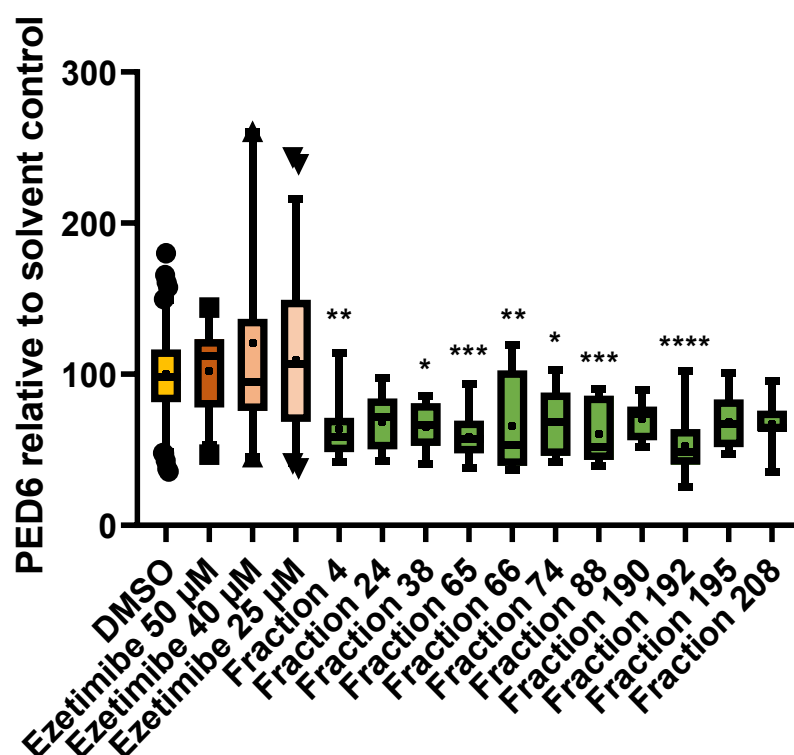


Figure 10 – Lipase activity in zebrafish PED6 assay exposed to cyanobacterial fractions. Data are shown from 12 to 16 individuals derived from two independent assays ($n=12-16$). Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO (0.1%) treated group. Data are represented as box-whisker plots, statistical differences were analysed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 1 – Inhibition of lipase activity in percentage (mean $\pm$ standard deviation, SD).

Fraction	Strain	Species	Inhibition $\pm$ SD (%)
4	LEGE 07230	<i>Planktothrix mougeotii</i>	36.34 $\pm$ 18.12
38	LEGE 91094	<i>Microcystis aeruginosa</i>	34.83 $\pm$ 14.46
65	LEGE 07211	<i>Synechocystis</i> sp.	41.23 $\pm$ 13.32
66	LEGE 07211	<i>Synechocystis</i> sp.	34.17 $\pm$ 18.93
74	LEGE 07211	<i>Synechocystis</i> sp.	31.80 $\pm$ 13.10
88	LEGE 06363	<i>Tychonema</i> sp.	39.47 $\pm$ 11.67
192	LEGE 06118	<i>Leptolyngbya mycoidea</i>	47.66 $\pm$ 18.91

In previous studies with the PED6 assay in zebrafish larvae, 50-70% of lipase inhibition was described for pure compounds, such as 1-methyl-3-[2-(methylamino)phenyl]quinoxalin-2-one and clofazimine (Clifton *et al.*, 2010). For this reason, 30-40% inhibition of lipase activity in our work can be considered as a good result, since fractions present a mixture of many compounds, and single compounds contained therein are diluted by inactive compounds.

From the 263 fractions, 3.04% of the fractions caused 100% mortality. These were the fractions 49, 50, 51, 79, 80, 128, 152 and 256 (no image acquisition was performed in these cases), while fraction 14 and 89 caused 50% mortality. However, it should be noted that some fatalities may be due to handling and, it is important to understand that, for the fractions with a high rate of mortality at a final concentration of 10 $\mu\text{g}.\text{mL}^{-1}$, a lower concentration may function as a positive anti-obesity activity (not tested in this work). An extremely strong reduction of lipid absorption may cause malformations and lead to death, as a balanced lipid content is crucial for life maintenance. Especially, since the first few days post fertilization, the embryo development is dependent on the continuous supply of nutrients by the yolk sac, since there was no external feeding (Carten and Farber, 2009).

There are several mechanisms of action that can be proposed for the reduction of PED6 fluorescence. Any present compound in the fractions that can affect the cholesterol levels in the larvae, may cause inhibition of a proper emulsification, digestion and uptake of the lipase activity reporter due to a bile acid deficiency (Rubinstein *et al.*, 2005). Decrease of fluorescence can also occur due to compounds that directly prevent uptake of lipids across the brush border of the intestine (terminal carbohydrate digestions site). Additionally, damage can occur to the intestinal system, due to certain compounds, that prevent lipid absorption and proper processing. Larva swallowing ability can also be impaired by some compounds, resulting in the lack of fluorescence in the intestine (Rubinstein *et al.*, 2005). Moreover, possible lack of fluorescence in the gall bladder may be due to a mutation that

has the ability to alter lipid absorption or due to a mutation that affects tRNA synthesis. For these reasons, viability and/or swallowing function is required to be tested in repetition assays (Carten and Farber, 2009).

Although the principal target of the chosen model compound, ezetimibe, is the inhibition of the cholesterol transport protein Neiman-Pick C1-Like 1 (NPC1L1) (Phan *et al.*, 2012; Park, 2013), it was demonstrated to inhibit lipase activity at 50 μM in the zebrafish model (Clifton *et al.*, 2010). However, these results were not confirmed in our work and ezetimibe did not reduce PED6 activity at the chosen concentrations (25 μM , 40 μM and 50 μM). In fact, ezetimibe-treated larvae showed high variability between plates and zebrafish replicas, as well as mortalities, even at lower concentrations (Figure 10).

PED6 has mainly been used to assay intestinal lipid processing in zebrafish mutants (such as fat-free and *cloche*) as they fail to properly absorb this reporter, however, a significant variability of fluorescence was observed for wild-type siblings in the larval digestive tract (Rubinstein *et al.*, 2005; Sadler *et al.*, 2005; Schlegel and Stainier, 2006; Hama *et al.*, 2009). The variability in the intensity of the lipase reporter may be due to interindividual differences in the ingestion of the reporters, intestinal microenvironment and differences in the developmental stages of the larvae, although measures were taken to diminish these variations. The use of PED6 in conjunction with EnzChek, has been demonstrated to create a more physiologically relevant readout of the digestive process *in vivo* (Carten and Farber, 2009; Hama *et al.*, 2009). Although larvae were chosen according to their size, their internal development may present some differences and zebrafish selection can be done according to their yolk sac size. Additionally, larvae swallowing ability also impacts on the fluorescence signals, which can be addressed by using microspheres.

During PED6 bioassay, two parameters for toxicity of the fractions were used, that allowed continuous tracking of possible toxicity. One of the parameters was by the mortality of the larvae, after 24h or 48h of exposure. The protease reporter, EnzChek, was also used as a proxy for toxicity, since it allows confirmation whether general digestive processes might be affected (data from the 263 fractions tested in our work are represented on Figure 11). EnzChek has been utilized as a screen for larvae with impaired exocrine pancreas, since it is known to secrete pancreatic juice containing diverse digestive enzymes, including lipases and proteases, that are critical for intestinal absorption of nutrients (Carten and Farber, 2009; Hama *et al.*, 2009; Otis and Farber, 2013). For this reason, fractions that had good results in inhibiting lipase activity, but presented low activity of protease enzymes, were considered potentially toxic and not used in further tests. However, for all the promising fractions, EnzChek analysis showed moderate to high activity of proteases not significantly different from the solvent control, confirming no defects in exocrine pancreas function, and no impaired digestion of proteins (Figure 12).

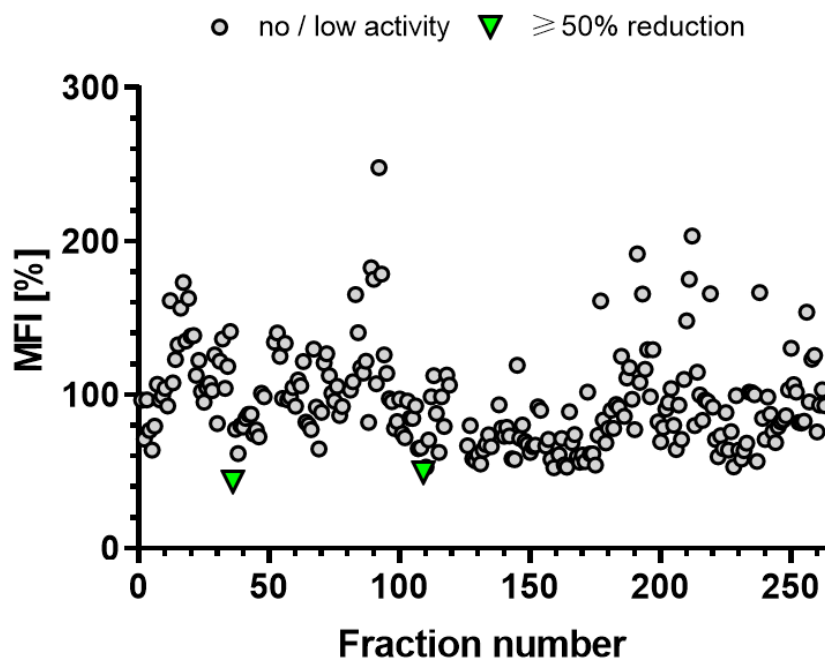


Figure 11 – Data of all 263 fractions used in the EnzChek assay are represented as mean fluorescence intensity (MFI) relative to the solvent control (DMSO).

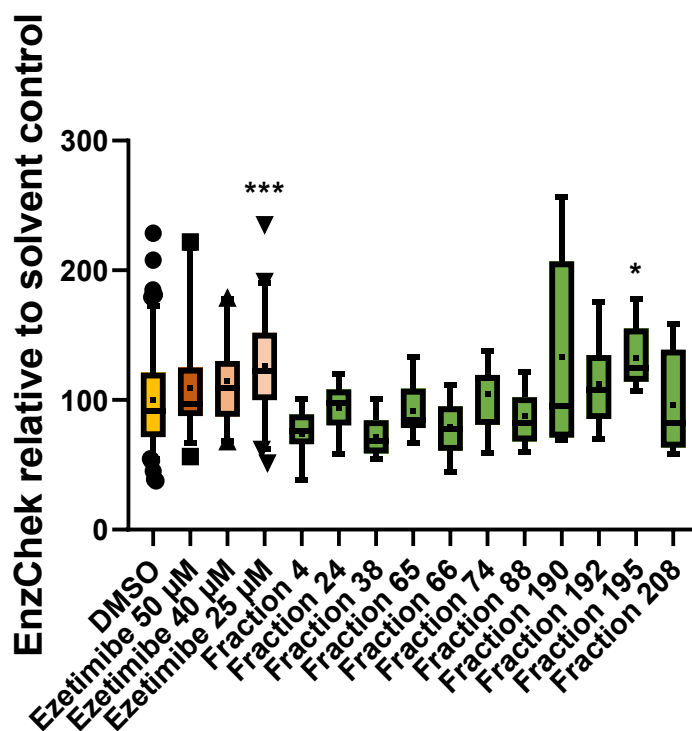


Figure 12 – Protease activity in zebrafish EnzChek assay exposed to cyanobacterial fractions. Data are shown from 12 to 16 individuals derived from two independent assays ($n=12-16$). Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO (0.1%) treated group. Data are represented as box-whisker plots, and statistical differences were analysed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.2.2. Fatty acids and cholesterol uptake

The 11 fractions that showed moderate inhibition of lipases, with no intestinal toxicity, were tested with three different fluorescent lipid analogues in separate bioassays. The use of BODIPY-C16 and BODIPY-C5, allowed for visualisation of the uptake and metabolism of long and short-chain fatty acids, respectively. These secondary assays were performed to evaluate if dietary fatty acids are differentially absorbed and processed by the intestine absorptive cells, which would allow to further characterize the mode of action of bioactive fractions (Clifton *et al.*, 2010). Figure 13 shows the statistical analysis of BODIPY-C5 relative to DMSO solvent control (Ctrl). The uptake of short-chain fatty acids (BODIPY-C5) was not reduced by any of the promising cyanobacterial fractions, in concordance with previous studies, which have shown that BODIPY-C5 usually showed a less pronounced reduction of the uptake and metabolism of the SCFA, comparing to the LCFA and cholesterol analogues (Clifton *et al.*, 2010). Studies in Caco-2 and HepG2 cell lines, also confirmed these findings (Ahowesso *et al.*, 2016). Shorter chains of fatty acids, which are transported by passive diffusion, showed no effect, while there was a clear inhibition of LCFA uptake and metabolism (Ahowesso *et al.*, 2016).

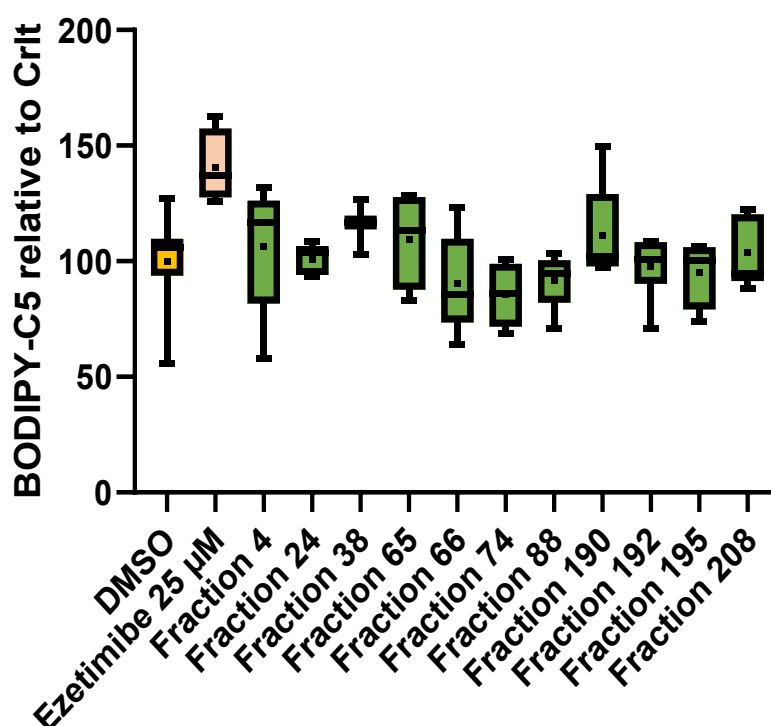


Figure 13 – Short-chain fatty acids uptake in zebrafish BODIPY-C5 assay. Values from 6 to 8 replicate individuals from a single assay are expressed as mean fluorescence intensity (MFI) relative to the DMSO (0.1%) treated solvent control group. Data are represented as box-whisker plots, and statistical differences were analysed by Kruskal-Wallis with Dunn's posthoc test.

In the case of long-chain fatty acids, statistical analysis revealed that two fractions reduced their uptake by 34.96% (LEGE 07230 *Planktothrix mougeotii*) and 42.87% (LEGE 06001 *Nodosilinea* sp.), as illustrated on Figure 14 and 15, confirming the findings of previous studies described above (Ahowesso *et al.*, 2016; Clifton *et al.*, 2010). BODIPY-C16 accumulates into lipid droplets and lipoproteins, which allowed simultaneous *in vivo* monitorization of multiple fat storing organs and tissues (Carten *et al.*, 2011).

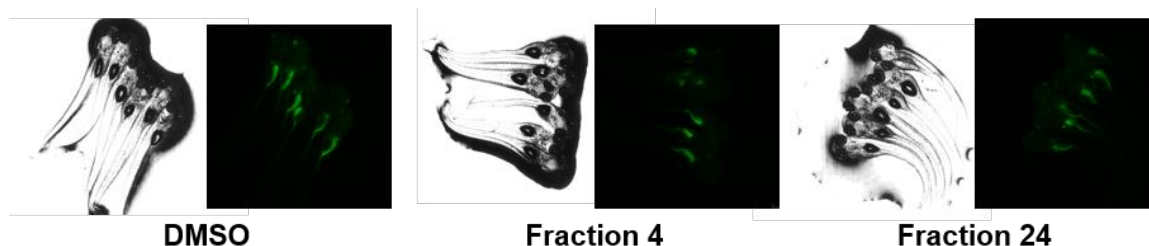


Figure 14 – Representative images of zebrafish long-chain fatty acids uptake. Fraction 4 (*Planktothrix mougeotii* LEGE 07230) and fraction 24 (*Nodosilinea* sp. LEGE 06001) showed inhibition of LCFA uptake. Brightfield images on the left and BODIPY-C16 images on the right (green fluorescence).

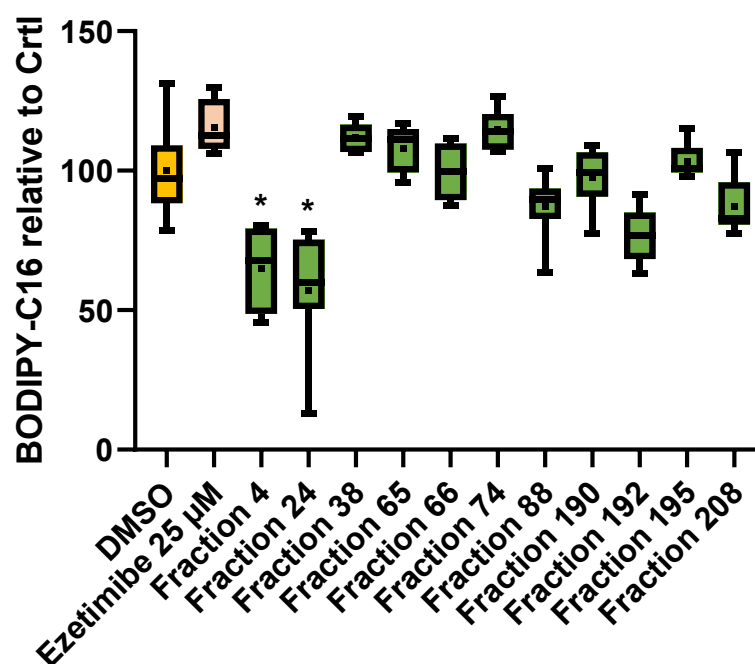


Figure 15 – Long-chain fatty acids uptake in zebrafish BODIPY-C16 assay. Values of 6 to 8 replicates of individuals from a single assay are expressed as mean fluorescence intensity (MFI) relative to the DMSO (0.1%) treated solvent control group. Data are represented as box-whisker plots, and statistical differences were analysed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The clear difference between the inhibition of the metabolism within the long and short-chain fatty acids, also observed in other studies, may be due to the differences between their uptake. SCFA enter the blood directly and bind to serum proteins for transportation, while LCFA require their incorporation into lipoprotein particles to be transported from the enterocytes to the liver (Ahowesso *et al.*, 2016; Clifton *et al.*, 2010).

Although ezetimibe's principal target is the inhibition of the cholesterol transport protein NPC1L1 (Phan *et al.*, 2012; Park, 2013), it was shown to reduce the uptake of long-chain fatty acids at 50 μ M (Clifton *et al.*, 2010). However, we could not confirm this effect in the present work.

Analysis of cholesterol absorption was performed with NBD-Cholesterol using the selected bioactive fractions. Again, this secondary assays should help to characterize the mode of action of the bioactive fractions (Clifton *et al.*, 2010). The mechanism underlying the uptake of cholesterol differs from that of the fatty acids, since cholesterol requires the sterol transporter to move from the intestinal lumen into the enterocytes, while fatty acids require proteins and lipoproteins to be transported.

In previous studies, compounds such as N-(2,5-Dimethoxyphenyl)-4-thien-2-yl-1,3-thiazol-2-amine and 2-[3-(Furan-2-ylmethyl)-4-oxospiro[6H-benzo[h]quinazoline-5,1'-cyclopentane]-2-yl]sulfanylacetonitrile inhibited cholesterol absorption of almost 70% (Clifton *et al.*, 2010). However, in the present work, statistical analysis showed that cholesterol absorption was solely reduced by fractions 65 (26.39%) and 74 (36.25%) of the species *Synechocystis* sp., however, these reductions in cholesterol uptake did not reach the significance level of $p < 0.05$ (Figure 16 and 17).

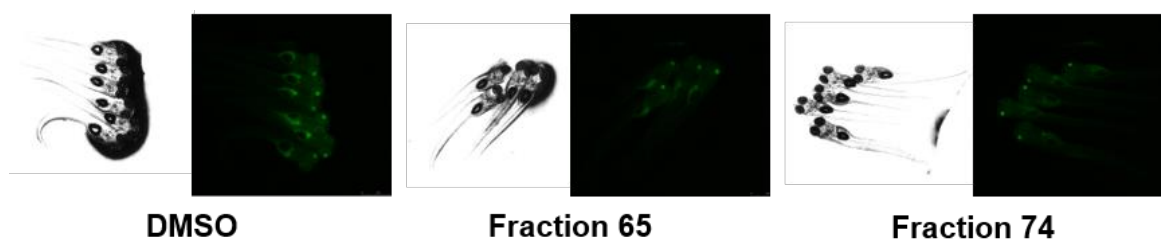


Figure 16 – Representative images of zebrafish cholesterol uptake. Fraction 65 and fraction 74 (*Synechocystis* sp. LEGE 07211) showed moderate inhibition activity. Brightfield images on the left and NBD-Cholesterol images on the right (green fluorescence).

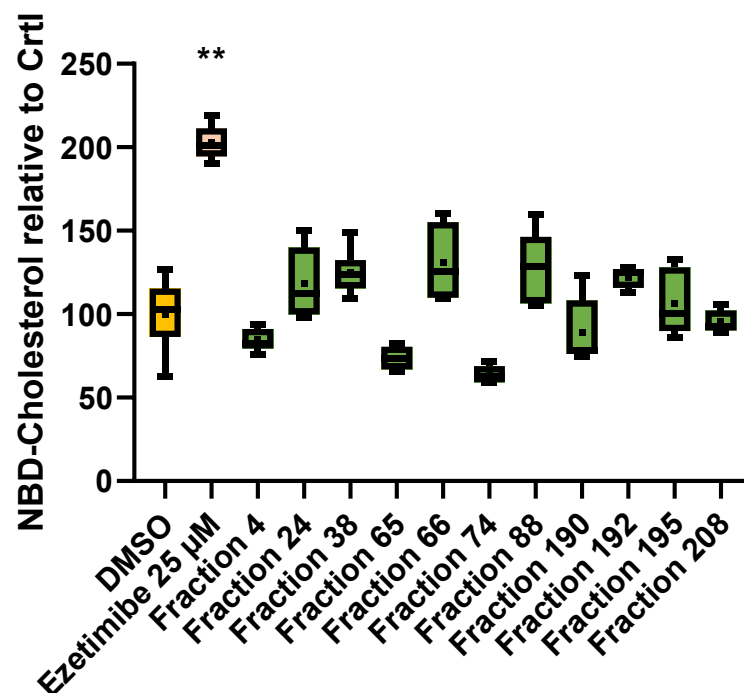


Figure 17 – Cholesterol uptake in zebrafish NBD-Cholesterol assay. Values from 6 to 8 replicates from a single assay are expressed as mean fluorescence intensity (MFI) relative to the DMSO (0.1%) treated solvent control group. Data are represented as box-whisker plots, and statistical differences were analysed by Kruskal-Wallis with Dunn's posthoc test and are indicated to the solvent control with the symbol * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

It is important to note that these results are preliminary, and a confirmation should be done by an additional independent assay. A higher number of replicates will allow to obtain better and more reliable results. Although the final concentration of NBD-Cholesterol chosen for this bioassay was low (0.8 μM), some fluorescence in the eyes and body of the larvae (background) was still noticeable, so conditions for upcoming assays still require some adjustments.

The model compound ezetimibe was shown to treat hypercholesterolemia in humans (Mauro and Tuckerman, 2003; Patel *et al.*, 2003). In zebrafish, a 50-70% inhibition of cholesterol absorption was shown for ezetimibe using NBD-Cholesterol analogue. NPC1L1, the target of ezetimibe, is encoded in the genome of zebrafish and the uptake of cholesterol from the intestinal lumen into the intestinal cells is facilitated by this transport protein (Clifton *et al.*, 2010; Phan *et al.*, 2012; Park, 2013; Quinlivan *et al.*, 2017). It is important to note that regulation of the activity of the cholesterol transport protein NPC1L1, is still largely uncharacterized (Quinlivan *et al.*, 2017). However, these results were not confirmed in our work, but in contrast, an increase in cholesterol uptake was observed, supporting the unreliability of ezetimibe as a positive control.

3.2.3. Quantification of main lipid classes and fatty acids processing

TLC analysis was performed to visualize the metabolism and incorporation of different lipid classes from larval lipid extracts, and to characterize further the alterations on lipid metabolism by exposure of zebrafish larvae to bioactive fractions.

Two different characterization of lipid alterations were performed. First, primuline staining was used to observe the quantity and alteration of the main lipid classes by the cyanobacterial fractions, as illustrated on Figure 18.

Second, the exposure of larvae to BODIPY-C16 analogue visualizes the incorporation of the fluorescent lipid analogue into different lipid classes, as illustrated on Figure 20.

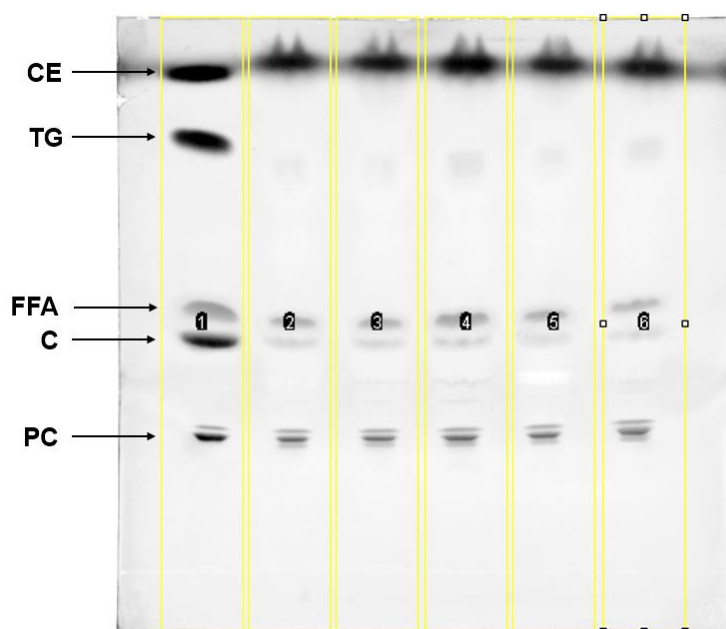


Figure 18 – TLC separation of main lipid classes of zebrafish exposed to cyanobacterial fractions and representation of the band selection for plotting the profiles. Lipid classes were identified by a mix of known lipid standards (first lane). Visualization of lipids were done by spraying with primuline. Second lane: Fraction 24; Third lane: Fraction 38; Fourth lane: Fraction 65; Fifth lane: Fraction 66; Sixth lane: Fraction 74. Cholesteryl palmitate (CE); tripalmitin (TG); palmitic acid (FFA); cholesterol (C) and L- α -phosphatidylcholine (PC).

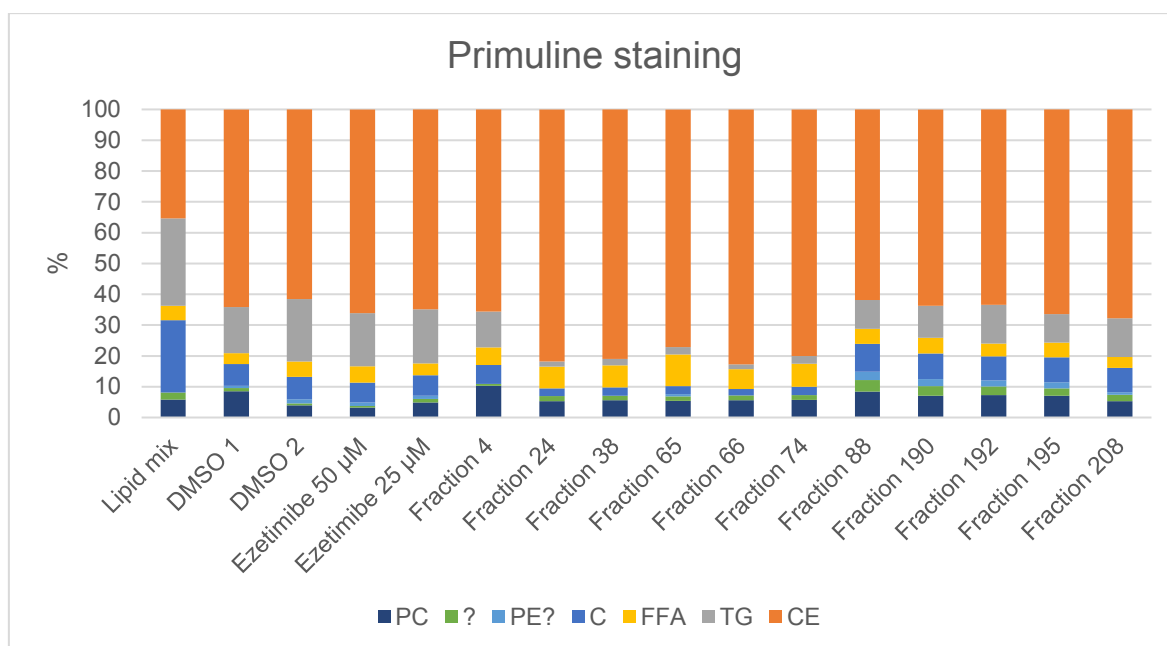


Figure 19 – TLC analysis for the staining of lipid classes using primuline. Results are presented in percentages.

The primuline TLC methodology revealed a strong reduction of triglycerides for fractions 24 (LEGE 06001), 38 (LEGE 91094), 65, 66 and 74 (LEGE 07211) (all tested on the same TLC plate), and a moderate reduction on fractions 4 (LEGE 07230), 88 (LEGE 06363) and 195 (LEGE 06009). However, a plate effect cannot be excluded for the fractions from 24 to 74, which generates uncertainty and a confirmation by another assay is needed. The reduction is concordant with the PED6 assay. If there is a reduction on the activity of the enzymatic lipases, there is a lowered metabolism of triglycerides and their conversion into monoglycerides and fatty acids is prevented (Castro *et al.*, 2016). However, other effects can also not be ruled out. Since fractions are a mixture of many compounds, various single compounds contained therein could target different proteins/enzymes or biological signalling pathways. Fractions could act on the reduction of neutral lipids as it was shown for many bioactive fractions on the zebrafish Nile red fat metabolism assay (Costa *et al.*, 2019). In general, the analysis of major lipid classes was in accordance with Fraher *et al.*, 2016, which showed that cholesterol and cholesteryl esters form the major lipid classes, while triglycerides compose just a small percentage of lipids at this stage. TLC analysis of main lipid classes using primuline staining can be observed on Figure 19.

Zebrafish larvae are a powerful tool to use in the study of triglycerides digestion and the absorption, metabolism and distribution of free fatty acids, derived from dietary lipids. Although fluorescent analogues of fatty acids have been well developed and enable the visualisation of the metabolism of fatty acids, there is a need for specific methods to assay digestion of triglycerides (Sæle *et al.*, 2018). The TLC assay performed in this work has allowed for the visualisation of the metabolism of TG lipid class.

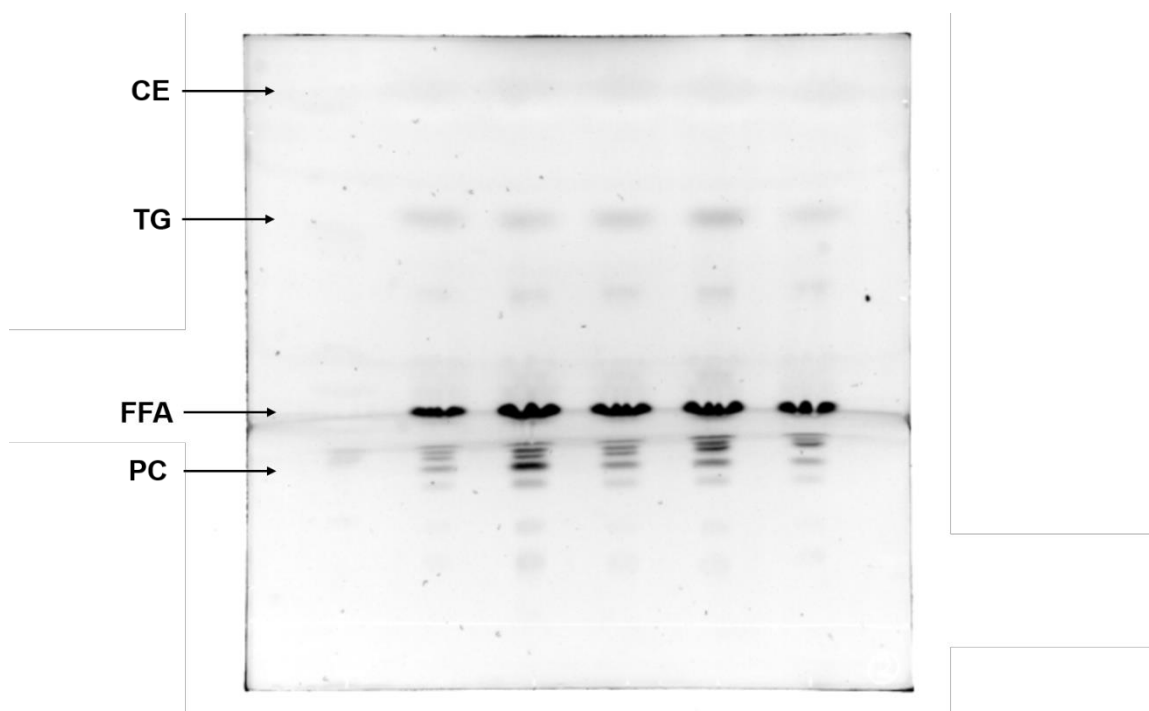


Figure 20 – TLC separation of main lipid classes of zebrafish exposed to cyanobacterial fractions. BODIPY-C16 was incorporated into different lipid classes and visualized by green fluorescent TLC. First lane: Lipid mix; Second lane: Fraction 24; Third lane: Fraction 38; Fourth lane: Fraction 65; Fifth lane: Fraction 66; Sixth lane: Fraction 74. Cholesteryl palmitate (CE); tripalmitin (TG); palmitic acid (FFA); cholesterol (C) and L- α -phosphatidylcholine (PC).

In the following assay, we exposed zebrafish to BODIPY-C16 and analysed the incorporation of this long-chain fatty acid into different lipid classes, presented on Figure 21. The following strains showed a strong reduction of the incorporation of LCFA into triglycerides: 38 (LEGE 91094), 74 (LEGE 07211), 88 (LEGE 06363), 192 (LEGE 06118), 195 (LEGE 06009) and 208 (LEGE 07163). These results clearly demonstrate that those fractions altered the processing of lipids in the zebrafish larvae.

If results are compared to the alterations of the main lipid classes (primuline staining), different groups can be distinguished, which highlight different changes of the lipid metabolism, and hence the possibility of different targets or involved mechanisms of action. The first is characterized by a reduction of TG without alterations of LCFA incorporation (fractions 24 (LEGE 06001), 65 and 66 (LEGE 07211)). Here, we propose that the reduction of the TG level is affected by the reduced lipase activity as seen in the PED6 assay. A reduced capacity of TG hydrolysis derived from the yolk sac would directly translate into a reduced FA uptake via the enterocytes and finally, in a lower TG level on the larvae. The second group is characterized by a reduction of TG with reduced LCFA incorporation into TG (fractions 38 and 74). In this group, we observe the reduction of both analysed possibilities to reduce whole TG level of the zebrafish larvae, either via reduced TG hydrolysis or via reduced FA incorporation and hence TG synthesis. The third group is characterized by no alterations of TG levels, but a reduced incorporation of LCFA into TG

(fractions 88, 192, 195 and 208). Here, we observe a clear inhibition of lipid processing, however, this is not translated into an overall reduced TG level.

Ezetimibe at a final concentration of 25 μM has also shown a reduced incorporation of triglycerides, which confirms ezetimibe's ability to inhibit other lipids other than the ones that have been formerly described in literature (Park, 2013).

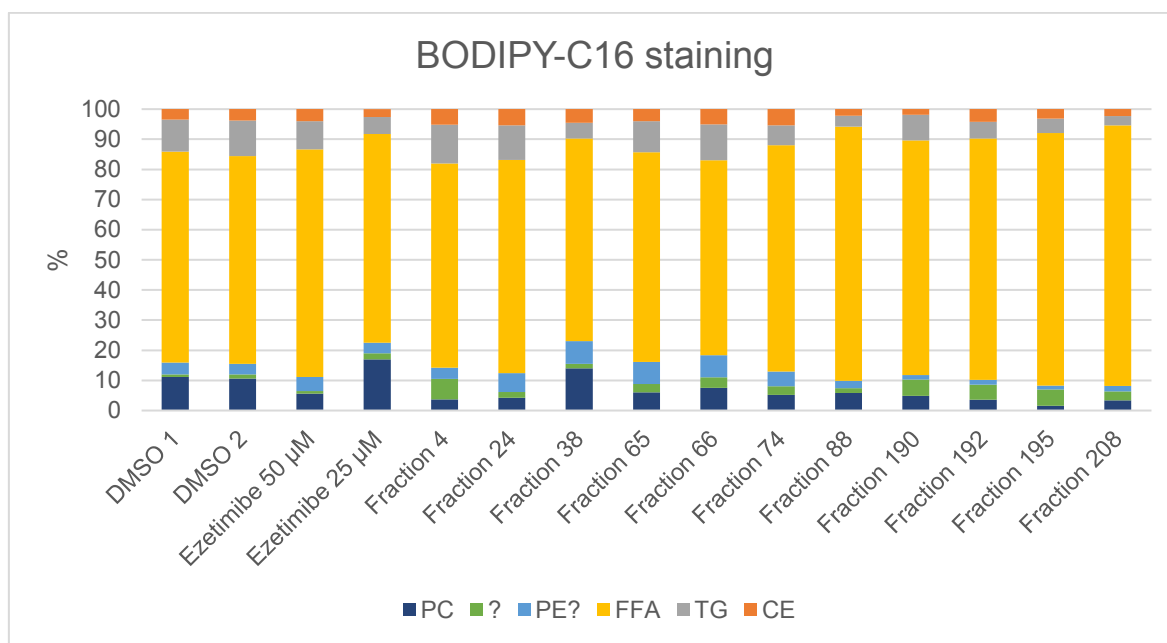


Figure 21 – TLC analysis for the metabolization and incorporation of different lipid classes using BODIPY-C16 analogue. Results presented in percentages.

Analysis of the TLC results, both with primuline or BODIPY-C16 analogue staining, showed additional bands derived from the used PC standard, labelled “PE?” and “?”, illustrated on Figure 19 and 21. These impurities are other lipids contained in the PC (L- α -phosphatidylcholine) lipid standard, which has only a purity of 65%. From a comparison to other TLC literature, we speculate that one of these bands is PE, phosphatidylethanolamine, which is the second most abundant glycerophospholipid in eukaryotic cells. For the lipid class identified with “?”, it is possibly a small lipid molecule named phosphatidylinositol (PI) from the class of phosphatidylglycerides or phosphatidylserine (PS) from the glycerophospholipid class (Christie, 2011).

In conclusion, TLC results identified alterations of the specific metabolic processing of fatty acids, both into phospholipid and neutral lipid synthesis. Both primuline and BODIPY-C16 TLC analysis are regarded as preliminary assays and a confirmation is necessary to have at least 3 replicates. Further studies with other chain length BODIPYs may reveal disturbances of other metabolic pathways of the analogues, based on their chain length. BODIPY analogues, such as BODIPY-C16 studied in the present work, serve as

tools to assess the physiological effects of drug treatments, which aid in the development of therapeutics for metabolic diseases, such as obesity (Carten *et al.*, 2011).

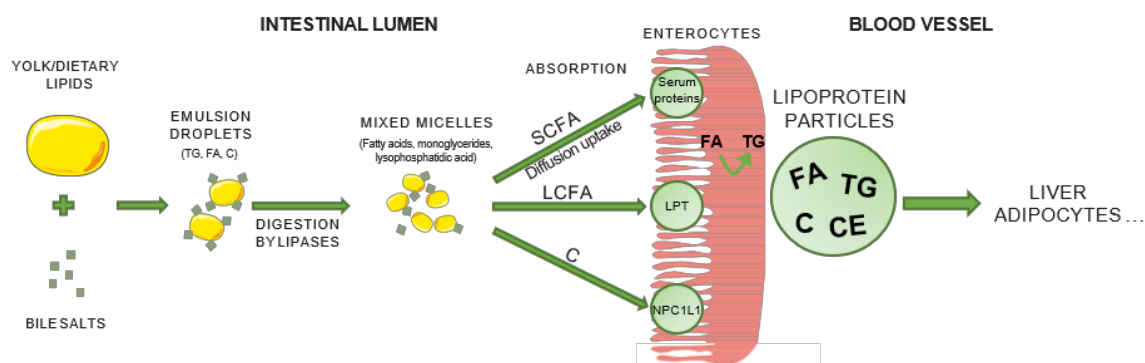


Figure 22 – Representation of the intestinal metabolism of dietary lipids. In the intestine, triglycerides are broken down by enzymatic lipases and bile salts, originating in fatty acids and monoglycerides. These are then subsequently moved to the absorptive enterocytes, converting the fatty acids and monoglycerides back into triglycerides. These are packaged together with lipoproteins, excreted from the cells and transported into the blood flow. TG: Triglycerides; FA: Fatty acids; C: Cholesterol; SCFA: Short-chain fatty acids; LCFA: Long-chain fatty acids; LPT: Lipoprotein transporter; NPC1L1: Neiman-Pick C1-Like 1 (transport protein); CE: Cholesterol ester.

3.3. Future perspectives

Ezetimibe effects were not consistent in our work, having shown great variability between plates and zebrafish replicas, as well as mortalities, even at lower concentrations. On our assays, ezetimibe at a final concentration of 50 μM and 40 μM , caused a mortality of about 70%. Although at a final concentration of 25 μM ezetimibe did not cause any mortality, its activity on inhibiting lipases and the uptake of the fatty acids and cholesterol was not as expected from previous studies (Clifton *et al.*, 2010; Phan *et al.*, 2012; Park, 2013). For this reason, future work will include other possible model compounds in order to obtain effective and reliable results for this type of zebrafish bioassays, namely controlling the quality of each assay.

Upcoming studies will focus on the repetition assays of fatty acids and cholesterol uptake as well as for the lipid extraction and quantification of main lipid classes and lipid incorporation of the promising cyanobacterial fractions. The identification of fractions with different modes of actions, helps to select a few fractions, which will be forwarded to additional works. The metabolite profiling of fractions will give an insight to whether the strains produce new metabolites or already known metabolites. From the most interesting fractions, the responsible compounds will be isolated in order to elucidate their chemical structures.

Finally, the protein targets of isolated compounds should be revealed, for example by application of bioactive thermal protein profiling (Carrasco Del Amor *et al.*, 2019).

4. Conclusions

The main aim of the present work was to screen cyanobacterial strains for the reduction of intestinal lipid absorption in whole small animal models, namely zebrafish larvae. The work focussed on investigating lipase activity and determining whether protease activity can be a proxy for intestinal toxicity. Eleven fractions, of a total of 263, were identified with a reduced lipase activity and no disruption of the protease activity.

Secondary assays were applied for those promising fractions. The effects on lipid uptake of long and short-chain fatty acids and cholesterol uptake were analysed. Two fractions decreased the uptake of long-chain fatty acids (*Planktothrix mougeotii* LEGE 07230 and *Nodosilinea* sp. LEGE 06001), while other two fractions showed a moderate, although not significant, reduction of cholesterol uptake (both from *Synechocystis* sp. LEGE 07211).

Lastly, the composition of main lipid classes and the incorporation of long-chain fatty acids into main lipid classes was assessed in exposed zebrafish larvae. Three main groups were identified, where we could relate the reduced lipase activity to different defects in lipid processing or triglyceride hydrolysis. These analyses helped to understand that fractions targeted different aspects of intestinal lipid absorption, which highlighted the diversity of cyanobacterial fractions for future applications. The use of the advanced whole small organism assays based on zebrafish larvae were very useful for this work, since such a complexity cannot be modelled in more simplistic models, such as cellular assays.

5. References

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6. Appendixes

Appendix I – Macro and micronutrients composition of Z8 medium

Table A.I 1 – Composition and concentration of stock solutions for the preparation of the Z8 medium.

Solution A	
Reagent	Concentration (g.l ⁻¹ UPW*)
Sodium Nitrate (NaNO ₃)	46.7
Calcium Nitrate Tetrahydrate (Ca(NO ₃) ₂ .4H ₂ O)	5.9
Magnesium Sulphate Heptahydrate (MgSO ₄ .7H ₂ O)	2.5

Solution B	
Reagent	Concentration (g.l ⁻¹ UPW*)
Potassium Phosphate Dibasic (K ₂ HPO ₄)	3.1
Sodium Carbonate (Na ₂ CO ₃)	2.1

FE-eDTA Solution	
Reagent Solution	Concentration (ml.l ⁻¹ UPW*)
Iron(III) Chloride Colution (FeCl ₃)	10
Sodium EDTA Solution (EDTA-Na)	9.5

Iron(III) chloride Solution	
Reagent	Concentration (g.l ⁻¹ HCl)
Iron(III) Chloride Hexahydrate (FeCl ₃ .6H ₂ O)	28

Sodium EDTA Solution	
Reagent	Concentration (g.l ⁻¹ NaOH)
EDTA	39

Micronutrients Solution	
Reagent Solution	Concentration (ml.l ⁻¹ UPW*)
1 to 12	10
13 and 14	100

Solutions 1-14 (Micronutrients)	
Reagent	Concentration (g.l⁻¹ UPW*)
1 – Sodium Tungstate Dihydrate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$)	0.33
2 – Ammonium Paramolybdate Dihydrate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 2\text{H}_2\text{O}$)	0.88
3 – Potassium Bromide (KBr)	1.2
4 – Potassium Iodide (KI)	0.83
5 – Zinc Sulfate Heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	2.87
6 – Cadmium Nitrate Tetrahydrate ($\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$)	1.55
7 – Cobalt(II) Nitrate Hexahydrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$)	1.46
8 – Copper(II) Sulfate Pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	1.25
9 – Nickel(II) Ammonium Bi-Sulfate Hexahydrate ($\text{NiSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$)	1.98
10 – Chromium(III) Nitrate Nonahydrate ($\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$)	0.41
11 – Vanadium pentoxide (V_2O_5)	0.089
12 – Aluminium Potassium Bi-Sulfate Dodecahydrate ($\text{Al}_2(\text{SO}_4)_3\text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$)	4.74
13 – Boric Acid (H_3BO_3)	3.1
14 – Manganese(II) Sulfate Tetrahydrate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$)	2.23

***UPW – Ultrapure Water**

Appendix II – Cyanobacterial strains selected for the bioassays

Table A.II 1 – Cyanobacterial fractions selected for the lipase and protease bioactivity assay.

Strain	Species	Fraction
LEGE 07230	<i>Planktothrix mougeotii</i>	1 to 9
LEGE 07196	<i>Tychonema</i> sp.	10 to 18
LEGE 06001	<i>Nodosilinea</i> sp.	19 to 27
LEGE 06079	<i>Synechocystis</i> sp.	28 to 35
LEGE 91094	<i>Microcystis aeruginosa</i>	36 to 44
LEGE 07084	<i>Nodosilinea nodulosa</i>	45 to 53
LEGE 06104	<i>Nodosilinea nodulosa</i>	54 to 62
LEGE 07211	<i>Synechocystis</i> sp.	63 to 74
LEGE 07212	Unidentified <i>Oscillatoriales</i>	75 to 84
LEGE 06363	<i>Tychonema</i> sp.	85 to 95
LEGE 00237	<i>Limnothrix</i> sp.	96 to 105
LEGE 03283	<i>Nodosilinea</i> sp.	106 to 115
LEGE 07175	<i>Cyanobium</i> sp.	126 to 137
LEGE 07075	Unidentified <i>Synechococcales</i>	138 to 146
LEGE 11428	<i>Synechococcus</i> sp.	147 to 155
LEGE 00060	Unidentified <i>Oscillatoriales</i>	156 to 164
LEGE 00247	<i>Cuspidothrix issatschenkoi</i>	165 to 173
LEGE 00246	<i>Dolichospermum</i> sp.	174 to 182
LEGE 06152	<i>Nodosilinea nodulosa</i>	183 to 185
LEGE 06102	<i>Leptolyngbya</i> cf. <i>halophilla</i>	186 to 188
LEGE 06108	<i>Leptolyngbya mycoidea</i>	189 to 191
LEGE 06118	<i>Leptolyngbya mycoidea</i>	192 to 194

Strain	Species	Fraction
LEGE 06009	<i>Leptolyngbya mycoidea</i>	195 to 197
LEGE 07167	<i>Leptolyngbya fragilis</i>	198 to 200
LEGE 07160	<i>Pseudanabaena</i> aff. <i>curta</i>	201 to 203
LEGE 07169	<i>Pseudanabaena</i> aff.	204 to 206
LEGE 07163	<i>Pseudanabaena</i> aff. <i>persicina</i>	207 to 209
LEGE 06144	<i>Pseudanabaena</i> sp.	210 to 212
LEGE 06194	<i>Pseudanabaena</i> sp.	213 to 225
LEGE 06098	<i>Cyanobium</i> sp.	226 to 218
LEGE 06134	<i>Cyanobium</i> sp.	219 to 221
LEGE 07186	<i>Cyanobium</i> sp.	222 to 224
LEGE 06113	<i>Cyanobium</i> sp.	225 to 227
LEGE 06137	<i>Cyanobium</i> sp.	228 to 230
LEGE 06097	<i>Cyanobium</i> sp.	231 to 233
LEGE 06139	<i>Cyanobium</i> sp.	234 to 236
LEGE 07171	<i>Synechococcus nidulans</i>	237 to 239
LEGE 07172	<i>Synechococcus</i> sp.	240 to 242
LEGE 06005	<i>Synechococcus</i> sp.	243 to 245
LEGE 06026	<i>Synechococcus</i> sp.	246 to 248
LEGE 06099	<i>Synechocystis salina</i>	249 to 251
LEGE 06155	<i>Synechocystis salina</i>	252 to 254
LEGE 07173	<i>Synechocystis salina</i>	255 to 257
LEGE 06013	<i>Romeria</i> sp.	258 to 260
LEGE 07310	<i>Romeria</i> aff. <i>gracilis</i>	261 to 263

Appendix III – Work dissemination: Poster communication

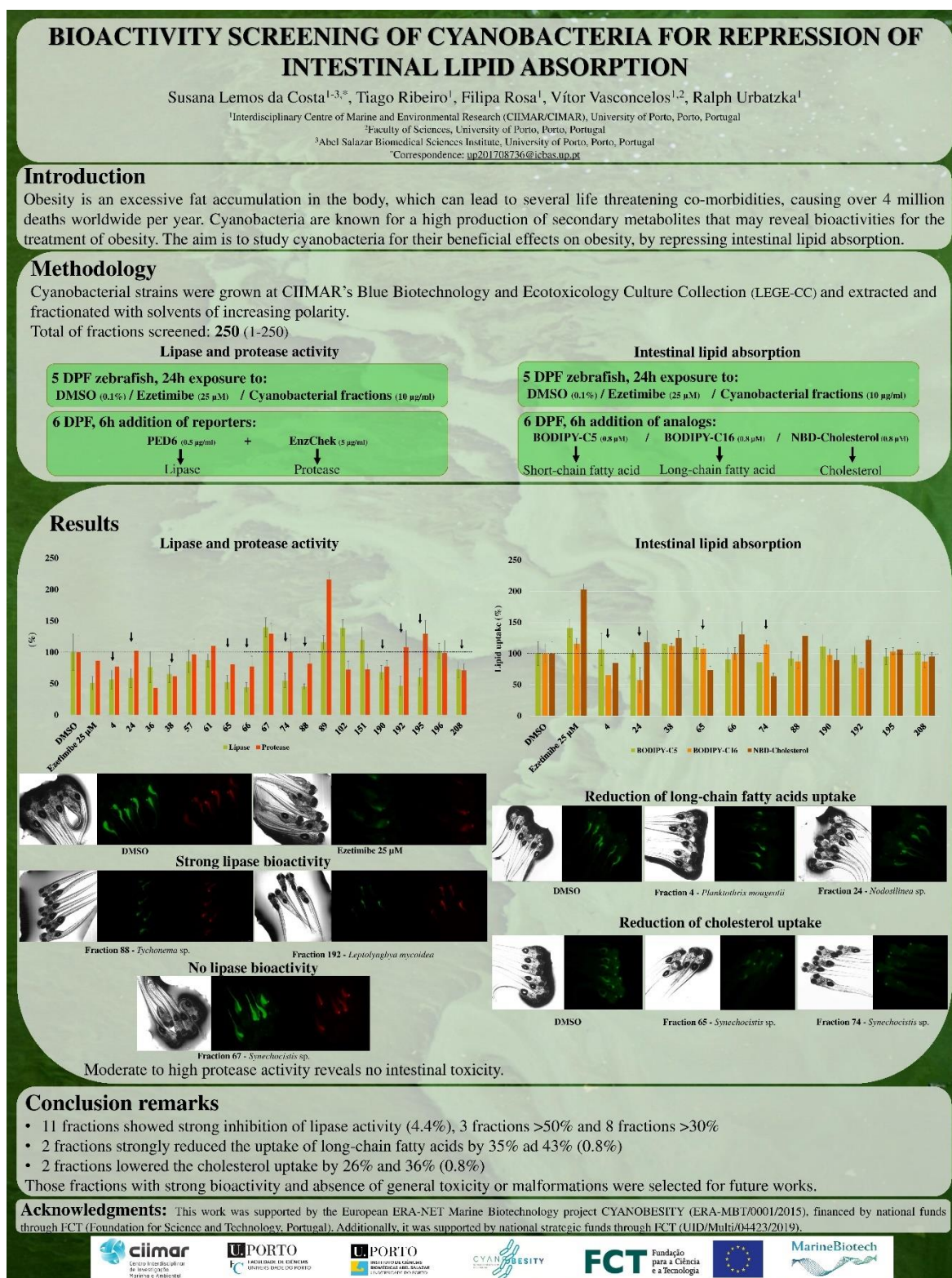


Figure A.III 1 – Poster communication for XVI International Symposium on Marine Natural Products | XI European Conference on Marine Natural Products 2019.

BIOACTIVITY SCREENING OF CYANOBACTERIA FOR OBESITY AND OBESITY-RELATED CO-MORBIDITIES WITHIN THE FRAMEWORK OF THE CYANOBEESITY PROJECT



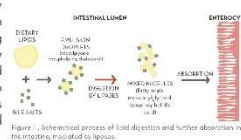
Marta Belver¹, Susana Lemos², Vilas Vasconcelos², Ralph Urbatzki¹
 1 - Université de La Rochelle
 2 - CIIMAR, Interdisciplinary Centre of Marine and Environmental Research
 *E-mail address: marta.belver.cacao@etu.univ-lr.fr

INTRODUCTION

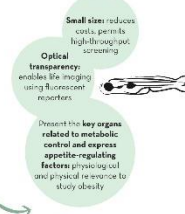
Obesity is a multifactorial disorder characterized by a sustained positive energy balance leading to excessive fat accumulation in different body regions. This condition raises the risk of developing several comorbid diseases such as type II diabetes, cardiovascular diseases and some forms of cancer. Moreover, obesity has become an increasingly global health problem that urgently needs to be mitigated by novel effective, specific and non-invasive treatments.

Cyanobacteria, also known as blue-green bacteria, are a group of gram-negative photoautotrophic prokaryotes regarded to produce a wide variety of secondary metabolites, some of which have shown lipid-reducing and anti-inflammatory bioactivities. Thus, these could be an interesting resource to develop drugs or nutraceuticals to fight obesity and its related comorbidities.

Digestive lipases are related to the absorption of lipids in the intestine, so their inhibition has been approached to reduce excessive lipid uptake in obese individuals.



Zebrafish larvae as a relevant animal model

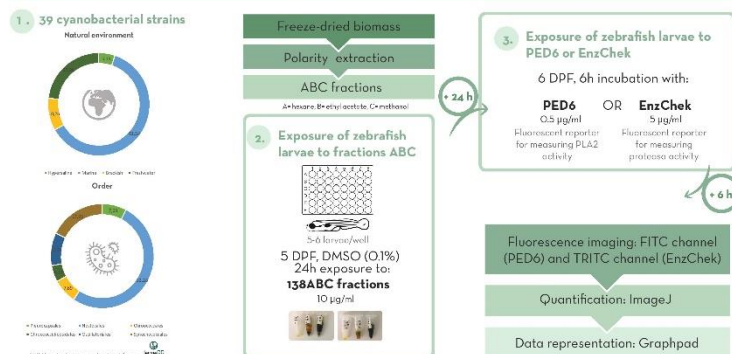


The main aims of this study were:

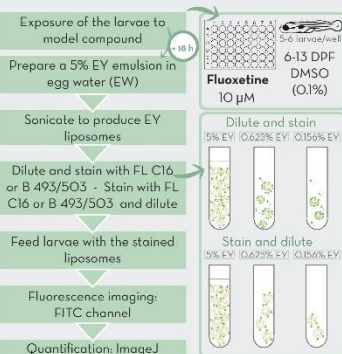
- To screen cyanobacterial extracts that could interfere with intestinal lipid uptake by repressing the digestive phospholipase A2 (PLA2) without interfering the intestinal activity of proteases.
- To optimize a novel assay using fluorescent-stained egg yolk (EY) liposomes, to further develop screenings of cyanobacterial extracts for their potential of appetite-reduction

METHODS

Screening of cyanobacterial fractions with potential lipase inhibition activities (PED6 and EnzChek)



Optimization of an appetite regulation assay



RESULTS

It was aimed to find cyanobacterial fractions parallelly inhibiting PLA2 (reducing PED6 signal), without influencing protease-dependent digestive processes (maintaining EnzChek signal).

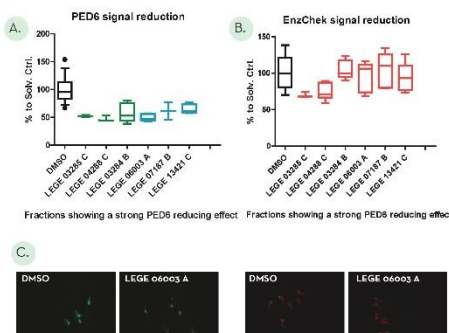
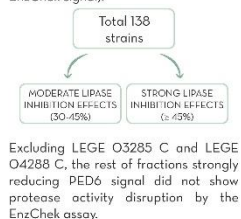
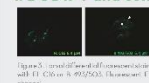


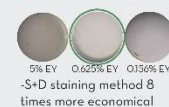
Figure 3. A) Box plot showing PED6 signal reduction in zebrafish larvae. B) Box plot showing EnzChek signal reduction in zebrafish larvae. C) Fluorescence images of zebrafish larvae stained with PED6 and EnzChek.

1. BODIPY and concentration:



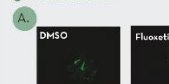
FL C16 at 6.4 µM:
 -Less background signal
 -Less unspecific staining (eyes, gallbladder)

2. EY concentration and staining approach:



EY 0.625% stained by S-D:
 -Staining more detectable than with 0.156% EY
 -Zebrafish may not ingest lipids as passively as with 5% EY

3. Fluoxetine:



Fluoxetine 10 µM:
 -Staining more detectable than with 0.156% EY
 -Zebrafish may not ingest lipids as passively as with 5% EY

CONCLUSIONS

The most promising fractions characterized by strong inhibition of lipid uptake, absence of protease activity and absence of toxicity were LEGE 03284 B, LEGE 06003 A and LEGE 13421 C. The best conditions for the zebrafish feeding assay were the use of 0.625% EY liposomes, stained with the method S + D at 6.4-M with FL C16.

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Figure A.III 2 – Poster communication for the presentations of the Applied Blue Biotechnology International Master course at La Rochelle University 2019.