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The impact of bacteria-microalgae co-cultivation on the growth and productivity of *Tisochrysis lutea*

UNIVERSIDADE DO ALGARVE

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Prof. Dr. João Varela – UAlg, CCMar, GreenCoLab

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Nowadays, the lack of essential resources, such as food and water, has been the cause of worldwide concern. Therefore, new alternatives are being increasingly researched to tackle these global demands. Microalgae are a possible alternative to obtain compounds (e.g., proteins and fatty acids) that can be applied to several economic sectors, such as the food, feed or pharmaceutical industries. In nature, these eukaryotes share their habitat with various microorganisms. With them, microalgae can establish a wide variety of interactions, ranging from parasitism to mutualistic relations, which often involve the exchange of compounds, such as organic matter and vitamins, required for the development and survival of the involved organisms. However, despite microalgae potential for industrial purposes, the impact of their naturally occurring bacteriome in the cultures is still overlooked or viewed negatively since bacteria are often considered as undesired contaminants. In this thesis, 145 bacteria were identified in the industrial cultures of the microalga *Tisochrysis lutea*, a species produced primarily for the aquaculture industry, due to the high content of polyunsaturated fatty acids (PUFA) and pigments. Of those, 7 strains showed potential to improve the productivity or biochemical composition of *T. lutea* upon co-culture with this microalga. Simple "tailor-made" bacterial mixes were then designed to optimize the production of this haptophyte. Among the 21 combinations assayed, 6 were associated with a significant productivity increase, ranging from 30 to 58%. In addition, 5 combinations were correlated with elevated concentrations of methylcobalamin and 3 with enhanced concentrations of some PUFA (16:2*n*-6, 18:3*n*-3α, and 22:5*n*-3). Through this work, we enhanced the biomass production and productivity of bioactive compounds in *T. lutea* at the laboratory scale by adding custom bacterial blends to the already xenic cultures of this microalga. This represents a significant step to improve the industrial production of this haptophyte with important biotechnological applications.

Keywords: Haptophyte; Bacteria blends; Mutualism; Co-culture; Methylcobalamin; Growth-promoting.

Resumo

Atualmente, recursos essenciais, como água, alimento e recursos energéticos, estão a ser consumidos a ritmos alarmantes, sendo, por isso, causa de preocupação em todo o mundo. Como consequência, está a aumentar a pesquisa de alternativas sustentáveis. Nesse âmbito, as microalgas estão a ser estudadas como uma opção alternativa para suprir algumas destas necessidades, visto que na sua biomassa conseguem acumular uma grande diversidade de compostos, como pigmentos, proteínas, ácidos gordos e vitaminas, que podem ser utilizados em várias indústrias, tais como, a farmacêutica, cosmética, bioenergia, suplementação alimentar e ração animal. No entanto, a produção à escala industrial destes microrganismos ainda acarreta elevados custos, sendo necessário otimizar a produção industrial de microalgas, de forma a recolher maiores quantidades de biomassa, contendo os compostos desejados, com o menor custo possível. Na natureza, estes eucariontes unicelulares estão presentes em praticamente todo o tipo de habitats aquáticos (marinhos e água doce) e também em alguns terrestres, onde coexistem com outros microrganismos, como, por exemplo, fungos ou bactérias. Microalgas e bactérias podem estabelecer entre si interações, que podem ser antagonistas, no caso de competição ou parasitismo, ou benéficas, como, por exemplo, o mutualismo. Relações mutualísticas estabelecem-se essencialmente através de trocas de compostos, como matéria orgânica, fatores de crescimento, vitaminas e compostos inorgânicos (CO_2), na maioria das vezes essenciais para o normal desenvolvimento e sobrevivência de ambos os organismos. Por exemplo, as bactérias são capazes de produzir vitaminas, como a vitamina B_{12} (ou cobalamina), que são essenciais para várias espécies de microalgas, e em troca recebem matéria orgânica, produzida pelas algas. No entanto, apesar de estas interações serem reportadas em ambientes naturais, o efeito de bactérias em culturas de produção industrial de microalgas ainda é pouco estudado, especialmente considerando que as bactérias, na sua maioria, são classificadas como organismos invasores que contaminam culturas. Apesar disto, alguns estudos recentes apontam a co-cultura de microalgas com outros microrganismos (fungos ou bactérias) como uma alternativa para otimizar a produção industrial de microalgas, de modo a obter não só maiores quantidades de biomassa, mas também biomassa de melhor qualidade, enriquecida em compostos de interesse. A microalga *Tisochrysis lutea* é uma espécie de interesse industrial atualmente, essencialmente devido ao seu perfil nutricional. A sua biomassa contém elevadas quantidades de ácidos gordos polinsaturados, como o ácido

docosahexenoico, que é essencial na dieta de peixes, crustáceos, e outros organismos aquáticos, especialmente nas fases iniciais do crescimento, sendo por isso importante para a indústria de aquacultura. Esta microalga também têm um alto conteúdo de pigmentos (essencialmente fucoxantina) com atividade antioxidante e anti-inflamatória. No decorrer desta tese, cerca de 145 bactérias foram isoladas e identificadas a partir de amostras recolhidas em reatores industriais utilizados na produção de *T. lutea*. A maioria das bactérias identificadas estavam incluídas nas classes *Gammaproteobacteria* e *Actinomycetes*, enquanto *Bacilli*, *Cytophagia* e *Saprospiria* foram as classes menos representadas. De acordo com informação bibliográfica recolhida para as espécies identificadas, 40 foram selecionadas para ensaios de co-cultura, sendo que inicialmente apenas um isolado bacteriano era inserido na cultura de *T. lutea*. No decorrer do primeiro ensaio, no dia 5, as culturas inoculadas com 8 dos isolados colapsaram, enquanto, no final do ensaio, 16 outras bactérias estiveram associadas a efeitos negativos no crescimento da microalga, visto que a concentração celular nestas culturas era significativamente menor do que nas culturas de controlo. Contrariamente a isto, as co-culturas com o isolado 80 (*Roseovarius* sp.) registaram um aumento significativo nas contagens celulares da microalga. As 13 bactérias associadas a culturas com os melhores resultados de produtividade de *T. lutea* e 2 bactérias reportadas na literatura como produtoras de vitamina B_{12} foram selecionadas para um novo ensaio em co-cultura com a microalga. Neste ensaio, a bactéria que, quando inoculada com *T. lutea*, levou ao maior crescimento da microalga foi o isolado 84, identificado como *Haliea salexigens*. O crescimento bacteriano também foi quantificado, por CFU, no início e fim da experiência. Enquanto em 5 das bactérias testadas (*Halomonas alkaliphila*, *Cyclobacterium* sp., *Roseovarius* sp., *Priestia flexa* e *Haliea salexigens*), a concentração bacteriana por mL de cultura aumentou, em outras 4 estirpes (*Arenibacter* sp., *Microbacterium* sp., *Brevibacterium* sp. e *Pseudidiomarina maritima*), aconteceu o oposto, registando-se uma diminuição da carga bacteriana nas culturas, ao longo do ensaio. No fim desta experiência, a biomassa recolhida foi coletada para análises bioquímicas. Apenas a cultura contendo o isolado 78 (*Microbacterium* sp.) obteve um aumento significativo no conteúdo de proteínas. Porém, nos perfis de ácidos gordos e de pigmentos, nenhuma das culturas inoculadas com os isolados selecionados demonstraram aumentos significativos. Em relação às medições de concentração de metilcobalamina, uma forma comum de vitamina B_{12} em organismos vivos, as culturas contendo isolados reportados na literatura como produtores de vitamina B_{12}, nomeadamente *Halomonas alkaliphila*, *Roseovarius* sp. and *Alcaligenes faecalis,* atingiram as

maiores concentrações deste nutriente. Considerando o conjunto de resultados obtidos ao longo da fase I do projeto, 7 dos isolados testados foram selecionados para a fase seguinte, onde foram combinados em pares e novamente co-inoculados com a microalga. Das 21 combinações de bactérias testadas, 6 (7/132, 8/78, 8/116, 78/84, 80/132 and 84/132) corresponderam a um crescimento mais elevado de *T. lutea*, em cerca de 30 a 58%, quando comparado com o controlo. Em culturas contendo algumas destas misturas bacterianas (78/84, 80/132 and 84/132), esta melhoria no crescimento, também correspondeu a elevadas concentrações de metilcobalamina. Também foram detetados aumentos nas concentrações de alguns ácidos gordos polinsaturados, nomeadamente hexadecadienóico (16:2*n*-6), α-linolénico (ALA, 18:3*n*-3α) e/ou docosapentenóico (DPA, 20:5*n*-3), nas culturas onde foram adicionadas as combinações de *Microbacterium* sp. com *Alcaligenes faecalis*, *Marinobacter* sp. e *Haliea salexigens*, e também nos replicados inoculados com a combinação de *Marinobacter* sp. com *Alcaligenes faecalis*. Contudo, no conteúdo proteico da microalga e no perfil de pigmentos não se registaram melhorias em comparação com o controlo. Para concluir, ainda são necessários mais testes para melhor entender as dinâmicas da comunidade após a adição das combinações bacterianas, e como podemos aproveitar as interações entre estes microrganismos para melhorar produtividade e produção de compostos biológicos de interesse industrial. Para além disto, uma validação destes resultados em culturas de escala industrial é necessária, visto que todos os resultados foram obtidos em culturas de escala laboratorial.

Palavras-chave: Microalgas; Bactéria; Mutualismo; Co-cultura; *Tisochrysis lutea*; Promotor de crescimento.

Index

Figure Index

Table index

Abbreviations list

AA - Arachidonic acid

ALA - α-Linolenic acid

ANOVA - Analysis of variance

BHT - Butylhydroxytoluene

BLASTn - Nucleotide Basic Local Alignment Search Tool

CC - Cellular concentration

CCMAR - Center of Marine Sciences

CFU - Colony-forming units

Chl - Chlorophyll

CO_2 - Carbon dioxide

CTRL - Control

DOC - Dissolved organic carbon

DOM - Dissolved organic matter

DHA - Docosahexaenoic acid

DW - Dry weight

EPA - Eicosapentaenoic acid

FA - Fatty acids

FAME - Fatty Acid Methyl Ester

GC-MS - Gas Chromatography-Mass Spectrometry

HAB - Harmful Algal Blooms

HPLC - High Performance Liquid Chromatography

IAA - Indole-3-Acetic Acid

MA - Marine Agar

MeCbl - Methylcobalamin

MUFA - Monounsaturated fatty acids

N - Nitrogen

NB^+ - NUTRIBLOOM PLUS®

NCBI - National Center for Biotechnology Information

NGS - Next-Generation Sequencing

nr/nt - non-redundant nucleotide

O_2 - Molecular oxygen

OD - Optical density

P - Phosphorus

PCA - Plate Count Agar

PCB - Plate Count Broth

PCR - Polymerase Chain Reaction

PES - Polyethersulfone

PTFE - Polytetrafluoroethylene

PUFA - Polyunsaturated fatty acids

QQ - Quantile-Quantile

QS - Quorum-sensing

SA - Stearidonic acid

SD - Standard deviation

SFA - Saturated Fatty Acids

SW - Seawater

TPBR - Tubular photobioreactors

UV - Ultraviolet

1.Introduction

With the increase in world population and environmental-related concerns (e.g., climate change), the world is considering and implementing a circular economy approach to tackle the rising global demands for food, water, and energy resources (Khan et al., 2018; Tait et al., 2019; Araújo et al., 2021; Y. Fu et al., 2021). These needs have led to the search for sustainable alternatives, and in this context, microalgae have emerged as a noteworthy option (Benedetti et al., 2018; Khan et al., 2018; Y. Fu et al., 2021).

Microalgae are a widely diverse group of single-cell eukaryotic microorganisms commonly found in either freshwater or marine habitats and some terrestrial environments (Ahamed et al., 2015; Fulbright et al., 2018; Cunha et al., 2020). These microscopic organisms, mostly photosynthetic, are essential for aquatic and global ecosystems. As primary producers, they utilize sunlight and inorganic substrates, such as atmospheric carbon dioxide (CO_2) and nitrogen (N), to produce organic matter and molecular oxygen (O_2), thus playing a vital role in the regulation of many nutrient cycles (e.g., carbon) (Natrah et al., 2014; Ahamed et al., 2015; Cooper & Smith, 2015; Cunha et al., 2020; Hamidi et al., 2020).

Unlike traditional crops, microalgae do not require arable land or large amounts of freshwater for cultivation since they can grow in salt or wastewater, needing only inorganic CO_2, light, and access to essential nutrients (e.g., vitamins and minerals) (Khan et al., 2018; Giraldo et al., 2019; Di Caprio, 2020; Tong et al., 2023). Besides, they can grow faster and at higher photosynthetic rates than terrestrial plants, thereby minimizing environmental impacts of production while still ensuring the production of high-value biomass since, during the photosynthetic process, they can convert atmospheric CO_2 into valuable bioactive compounds, such as lipids, proteins, pigments, carbohydrates, and other metabolites (Chua & Schenk, 2017; Tandon et al., 2017; Edelmann et al., 2019; Cao et al., 2019; Onyeaka et al., 2021). This enriched biomass can be used in various applications, involving the production of biofuels, biofertilizers, cosmetics, pharmaceuticals, food, and feed (**Figure 1.1**) (Ahamed et al., 2015; Bai et al., 2015; Cunha et al., 2020).

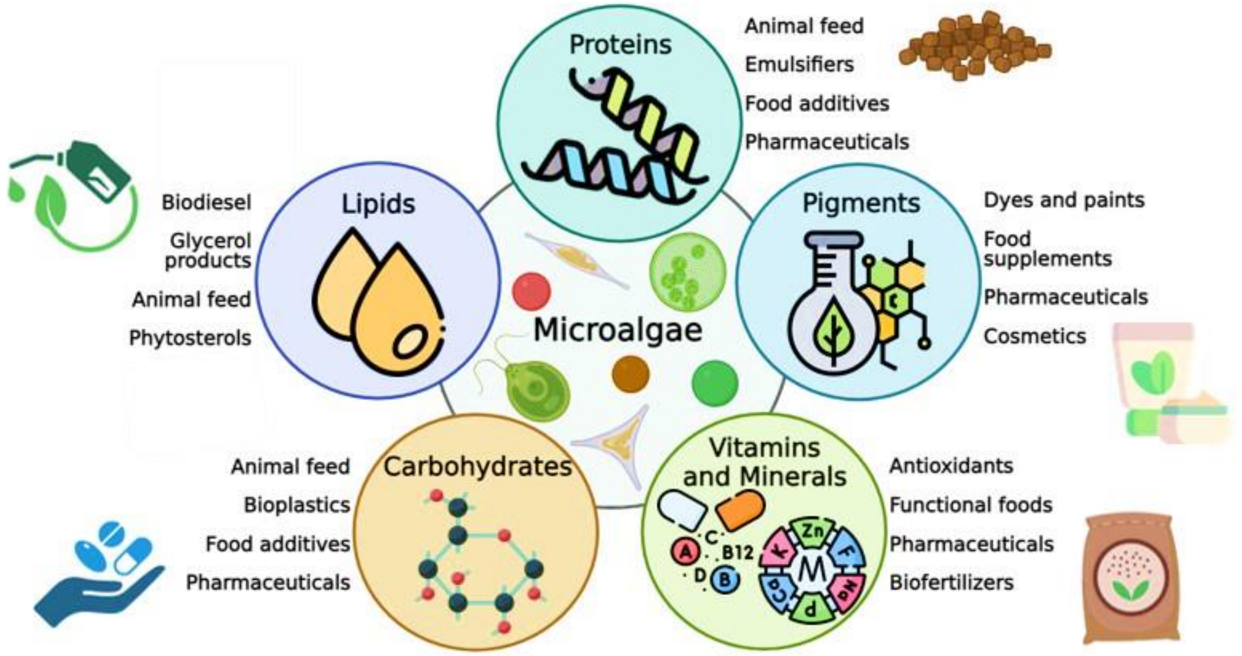

Figure 1.1-Industrial applications of high-value compounds found in microalgae biomass. Adapted from Machado et al. (2022). This figure was designed using resources from Flaticon.com and BioRender.com.

For instance, the marine haptophyte *Tisochrysis lutea* is currently being produced industrially as aquaculture feed for larval stages of several organisms, such as fish and bivalves, due to its high content of polyunsaturated fatty acids (PUFA) (da Costa et al., 2017; Gangadhar et al., 2020).

Despite all these advantages, developments in the area and optimized cultivation techniques with lower production costs are still necessary to turn microalgae into a commercially attractive alternative for industrial purposes (Chua & Schenk, 2017; Benedetti et al., 2018; Khan et al., 2018; Cunha et al., 2020; Duran et al., 2021).

For this purpose, optimization of microalgae industrial production has been the focus of recent research to obtain higher-quality biomass and better yields.

Microalgae are cultivated in closed or open cultivation systems (Duran et al., 2021).

In closed cultivation systems (e.g., tubular photobioreactors), growth conditions are more controlled, and exposure to the outside environment is low. CO_2 fixation in these systems is enhanced, primarily due to improved mixing, light capture, and nutrient uptake, thus resulting in higher growth rates (Deore et al., 2020; Duran et al., 2021; Kukwa & Chetty, 2021). However, the capital and operational costs of the necessary infrastructures and production cycles in this kind of system are high, especially compared to the costs of an open system approach (Deore et al., 2020; Duran et al., 2021).

For this reason, open systems are the most widely used cultivation systems for microalgal industrial production. These systems are simpler, cheaper, and easier to operate than closed systems (Benedetti et al., 2018; Deore et al., 2020; Y. Fu et al., 2021). However, in open systems (e.g., raceway ponds), the culture is exposed to the surrounding environment. When under outdoor conditions, temperature and irradiation can fluctuate considerably, making it difficult to control the abiotic factors of the overall cultivation process (Deore et al., 2020; Duran et al., 2021). For this reason, the cultures are often not under optimal conditions leading to lower productivity (Duran et al., 2021; Lian et al., 2022).

Nevertheless, in both closed and open systems, when growth is carried out photoautotrophically, it is nearly impossible to maintain axenic conditions, therefore, during cultivation, microalgae will most likely coexist and interact with a wide variety of organisms, such as fungi, bacteria, protozoa, and even other microalgal strains (Le Chevanton et al., 2016; Biondi et al., 2017; Krug et al., 2019; Deore et al., 2020; Duran et al., 2021).

In nature, microalgae also share their habitat with several other organisms and can interact with them. For instance, the organic compounds produced by microalgae, during their photosynthetic process, can be taken up by other coexisting microorganisms and they can directly serve as food for fish and zooplankton, thus influencing aquatic food webs (Natrah et al., 2014; Ahamed et al., 2015; Hom et al., 2015; Kouzuma & Watanabe, 2015; Samo et al., 2018).

Microalgae can also impact other species through the formation of harmful algal blooms (HAB) that can lead to toxin accumulation in food webs and loss of life in aquatic ecosystems. Other repercussions include public health-related issues, like poisoning through consumption of seafood products, and substantial economic problems, often related to massive fish loss in fisheries (Hom et al., 2015; Kim et al., 2015; Lam et al., 2018).

Nevertheless, just like microalgae influence every other aquatic organism, they are also affected by the presence of an ample variety of microorganisms coexisting in their natural habitats, such as bacteria (Helliwell et al., 2018; Berthold et al., 2019).

Bacteria are a group of diversified small-sized prokaryotic organisms with various shapes. They are virtually ubiquitous, due to their ability to withstand almost every environmental condition, including extremes in salinity and temperature (Hamidi et al., 2020). These microorganisms are vital to the global biosphere since they influence biogeochemical cycles by participating in nutrient exchanges and remineralization and decomposition of organic matter and, consequently, influencing energy and matter transfers between other organisms (Lauro et al., 2009; Samo et al., 2018; Hamidi et al., 2020; Pushpakumara et al., 2023).

These microorganisms and microalgae are the most abundant groups in aquatic habitats (Ramanan et al., 2016; Berthold et al., 2019; Pushpakumara et al., 2023).Therefore, by sharing their environment, bacteria and microalgae interact with one another, and, in some cases, they can rely on each other to grow, survive and fulfill their roles in aquatic habitats (Seymour et al., 2017; Samo et al., 2018; Berthold et al., 2019; Yao et al., 2019; Pushpakumara et al., 2023).These microbe-microbe associations are crucial to global ecosystems since they can impact nutrient cycles, aquatic food webs, and even climate (Ramanan et al., 2015, 2016; Fuentes et al., 2016; Seymour et al., 2017).

These interactions range from antagonistic connections to beneficial relations (Grossart et al., 2005; Seymour et al., 2017).

Competition and parasitism are the most common negative interactions (**Figure 1.2**).

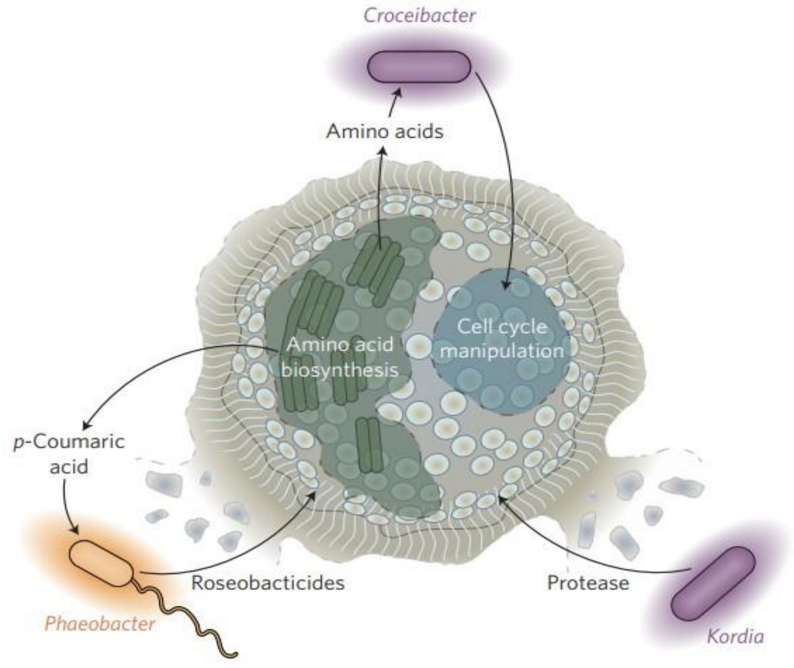

Figure 1.2-Examples of reported antagonistic interactions between microalgae and bacteria. From Seymour et al. (2017).

Regarding competition, since they share the same habitat, bacteria and microalgae may influence each other negatively by competing for the same inorganic nutrients, namely iron and phosphorus (P) (Cooper & Smith, 2015; Seymour et al., 2017; Fulbright et al., 2018; Tait et al., 2019).

In the case of parasitism, one or both organisms involved act negatively toward the other, often secreting harmful compounds into their surroundings, which may lead to the death of the affected organism (Lutzu & Dunford, 2018).

Bacteria can be antagonists for microalgae, through the production of apoptosis-inducing and algicidal compounds, such as serine proteases, that decrease algal growth and may cause algal death (Le Chevanton et al., 2013; Kim et al., 2015; Fuentes et al., 2016; Fulbright et al., 2018; Lutzu & Dunford, 2018). For instance, to obtain organic matter, *Kordia algicida*, a flavobacterium, secrete proteases that can lyse marine diatoms cells like *Skeletonema costatum, Thalassiosira weissflogi and Phaeodactylum tricornutum* (**Figure 1.2**) (Paul & Pohnert, 2011; Amin et al., 2012; Seymour et al., 2017). Also, some bacteria belonging to the genus *Vibrio* display algicidal activity against several microalgae (e.g., dinoflagellates) through the production of β-cyano-L-alanine, a metabolite linked to cell lysis (M. Wang et al., 2020).

Bacteria can also bind themselves to microalgae cells, causing cell lysis through direct contact (Amin et al., 2012; Qu et al., 2014; M. Wang et al., 2020). For example, *Saprospira* sp. is reported to be able to attach itself to diatom cells, releasing microtubule-like products that seem to be connected to microalgal cell lysis (Amin et al., 2012).

On the other hand, microalgae can also attack surrounding bacteria for their benefit by secreting antibacterial products, such as fatty acids (FA) and glycosides, that can suppress bacterial growth (Amin et al., 2012; Natrah et al., 2014; Qu et al., 2014; Liu et al., 2020). One known example of this antibacterial activity is related to the microalga *Chlorella vulgaris*, which produces chlorellin, an extracellular metabolite that can suppress *Staphylococcus aureus* growth (Natrah et al., 2014). Furthermore, *Phaeodactylum tricornutum* is often associated with the production of FA with antibacterial activity (e.g., eicosapentaenoic and palmitoleic acids), particularly against Gram-positive bacteria (Amin et al., 2012; Natrah et al., 2014).

Additionally, several microalgae species can meddle with Quorum-sensing (QS), a form of communication between bacteria cells that involves the secretion of signaling molecules that when recognized by receptors, will regulate the expression of several genes related to specific ecological functions of bacteria (e.g., secretion of virulence factors and formation of biofilms) (Rajamani et al., 2008; Chi et al., 2017; Muras et al., 2018). By releasing compounds that either affect signal receptors or mimic signal molecules and bind to receptors, microalgae can interfere with QS communication and, consequently, with the regulation of several processes in bacterial populations (Natrah et al., 2014; Hom et al., 2015; Chi et al., 2017). For instance, the microalga *Chlamydomonas reinhardtii* produces a molecule named lumichrome, which shares structural similarities with bacterial signals. As a result, it can be recognized by signal receptors and activate them, hence stimulating gene transcription in *Pseudomonas aeruginosa* (Rajamani et al., 2008; Natrah et al., 2014; Zhang et al., 2020).On the other hand, diatoms are reported as producers of enzymes, like haloperoxidases, that can induce alterations in signal molecules, preventing the binding to their targets and, consequently, interfering with QS communication (Amin et al., 2012; Natrah et al., 2014).

Conversely, microalgae and bacteria can also favor each other through mutualistic interactions, in which both organisms benefit from the other's actions (**Figure 1.3**).

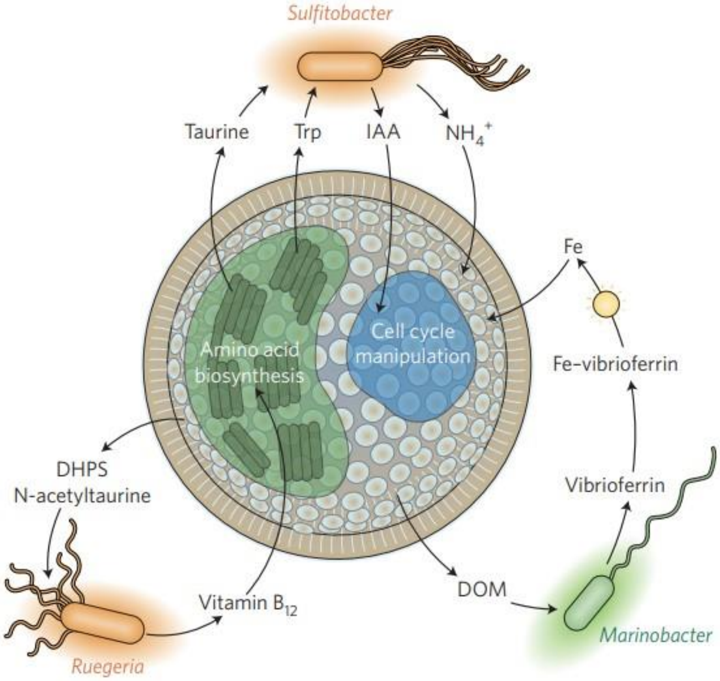

Figure 1.3-Examples of mutualistic interactions between microalgae and bacteria. From Seymour et al. (2017) .

These favorable microalgae-bacteria relationships, usually involve the exchange of essential nutrients between both groups (de-Bashan et al., 2016; Helliwell et al., 2018; González-González & De-Bashan, 2021). For instance, microalgae can supply organic carbon macromolecules to bacteria, while, in exchange, bacteria provide micronutrients and growth-promoters, necessary for algal development (**Figure 1.4**) (Fulbright et al., 2018; Helliwell et al., 2018; González-González & De-Bashan, 2021).

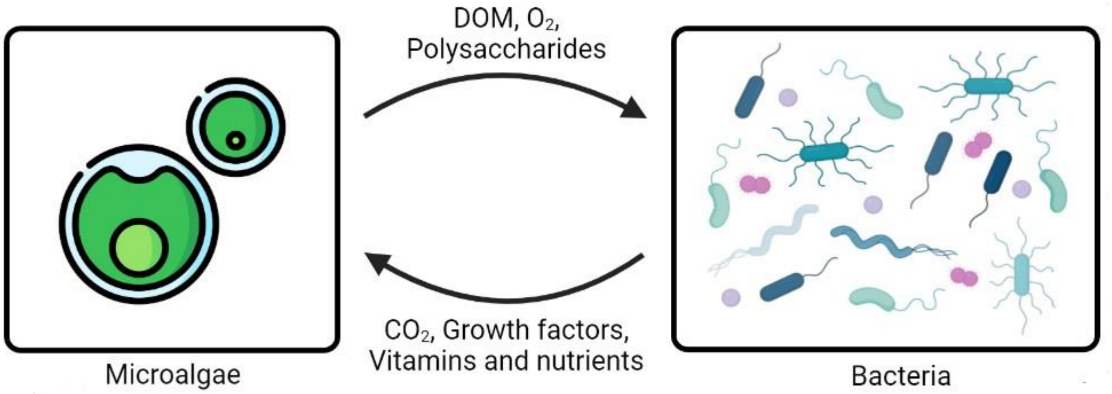

Figure 1.4-Examples of compounds, such as dissolved organic matter (DOM) or vitamins (e.g., vitamin B_{12}), that are often exchanged between microalgae and bacteria in mutualistic associations. This figure was designed using resources from Flaticon.com and BioRender.com.

This is believed to occur in natural settings regarding B vitamins, namely cobalamin (also known as vitamin B_{12}). Currently, at least half of algal species, including *Tisochrysis lutea,* are considered auxotrophic for cobalamin, as they cannot grow without this micronutrient (Croft et al., 2005; Grant et al., 2014; Nef et al., 2019). This vitamin is vital for the algae's metabolism, since it is a required cofactor for B_{12}-dependent methionine synthetase, an enzyme involved in the methionine synthesis. This amino acid is essential to protein synthesis, as it is encoded in the start codon of all open reading frames of protein-coding genes (Croft et al., 2005; Grant et al., 2014; Helliwell et al., 2018; Prabaningtyas et al., 2021). However, the synthesis of this vitamin cofactor is complex, since the transformation of uroporphyrinogen III, a precursor molecule, into cobalamin requires several enzymatic steps (Croft et al., 2005; Doxey et al., 2015; Nef et al., 2019). Unlike some bacteria (e.g., *Rhodobacteraceae*) and archaea (e.g., *Thaumarchaeota*), which through aerobic or anaerobic pathways can synthesize cobalamin, eukaryotes, including microalgae, do not have the necessary enzymes to complete this process (Croft et al., 2005; Doxey et al., 2015; Nef et al., 2019). Furthermore, levels of this vitamin in water environments seem insufficient to support algal growth, suggesting that an exogenous source is required (Helliwell et al., 2018). Thus, since some prokaryotes appear to be the only known producers of this complex nutrient, this can indicate they are the most likely source of cobalamin for microalgae through a mutually beneficial association (Croft et al., 2005; Kazamia et al., 2012).

The vitamin B_{12}-carbon trade has also been observed in a lab study, where *Halomonas* sp. bacterium, found in co-culture with the dinoflagellate *Amphidinium operculatum*, was able to sustain the growth of this microalga in axenic conditions, without the need to add vitamin B_{12} to the culture media, while receiving, in exchange, organic carbon (Croft et al., 2005).

Some algae species can also get this vitamin through a commensalism relationship. Commensalism is defined as an interaction between two organisms, in which only one is benefited by their partner, while the other is neither harmed nor favored by the other's actions (Lutzu & Dunford, 2018). A reported case of this type of interaction, regarding vitamin B_{12}, is the interaction between the microalga *C. reinhardtii* and the bacterium *Mesorhizobium loti*. In this situation, *C. reinhardtii* can obtain vitamin B_{12}, produced by the bacteria, despite *M. loti* not receiving any algal organic carbon, from *C. reinhardtii* (Lutzu & Dunford, 2018).

In addition, bacteria can provide microalgae with the means to obtain inorganic micronutrients that otherwise are not available to be taken up by them. Iron, for instance, is an indispensable nutrient for microalgae since it is necessary for photosynthesis and other cell processes (e.g., redox reactions) (Amin et al., 2009, 2012). However, due to its poor solubility, this inorganic element is available in very low amounts in aquatic environments (Amin et al., 2009). Therefore, for microalgae to obtain this vital nutrient, several bacterial strains, such as *Marinobacter* sp., produce siderophores, such as vibrioferrin, that, by binding to iron, improve the solubility of this micronutrient, thus facilitating assess to it by microalgae (**Figure 1.3**) (Amin et al., 2009, 2012; Fuentes et al., 2016; Seymour et al., 2017). Microalgae use this soluble form of iron in photosynthesis, releasing organic matter taken up by bacteria (Fuentes et al., 2016; Seymour et al., 2017).

However, mutualistic interactions between these microorganisms are not always connected to a nutrient exchange. For example, some bacteria have been implicated in producing metabolites with antimicrobial activity, thereby protecting microalgae from pathogenic species (Lian et al., 2018; Liu et al., 2020).

Nevertheless, the separation between a cooperation or antagonism association is very narrow. All these interactions can be highly variable, changing from species to species and according to abiotic factors, such as light intensity or nutrient concentration, and biotic factors, such as the physiology of the organisms involved (Grossart et al., 2005; Ahamed et al., 2015; Seymour et al., 2017; Lutzu & Dunford, 2018; Tait et al., 2019; Zhang et al., 2020).

An example of these shifts between positive and negative interactions is observed between the coccolithophore microalga *Emiliania huxleyi* and some bacterial species belonging to the *Roseobacter* clade, namely *Phaeobacter gallaeciensis*. Upon active growth, this bacterium provides nutrients to *E. huxleyi*. These nutrients can be vitamins and growth-promoting phytohormones, and also tropodithietic acid, which can be used by the microalga for protection against harmful bacteria. In return, *E. huxleyi* supplies the bacterium with dissolved organic carbon (DOC). However, upon reaching the stationary phase of algal growth, *p*-coumaric acid is released by the microalgae, consequently stimulating the secretion of troponoids and other roseobacticides by *P. gallaesciensis*, leading to algal death and, consequently, the release of organic matter for the bacteria (Ramanan et al., 2016; Seymour et al., 2017).

Despite this, mutualism associations appear to be more common in natural environments than parasitic interactions when considering interactions between eukaryotic microalgae and prokaryotic bacteria (Seymour et al., 2017).

Nevertheless, the effect of these invading bacteria in microalgae industrial cultures is still overlooked, especially considering that, until recently, these organisms were viewed as culture contaminants, prejudicial for algal growth (Biondi et al., 2017; Lutzu & Dunford, 2018; Lian et al., 2021, 2022).

Like in nature, some bacteria can act as culture contaminants by behaving as parasites, decreasing growth, causing algal death, or as competitors by removing nutrients that are essential for algae growth (Lam et al., 2018; Lian et al., 2021). Therefore, the action of these microorganisms can reduce productivity and, in the worst cases, even lead to the collapse of entire cultures, causing substantial economic losses for the industrial producers (Frenken et al., 2017; Lam et al., 2018). For instance, some strains linked to *Pseudoalteromonas* sp. and *Microbacterium* sp. have already been reported as prejudicial organisms for microalgae cultures (Lian et al., 2022).

The invading organisms may also pose a health safety issue if they are present in cultures meant to be used in food and feed industries, especially if these microorganisms are toxin-producers (Lam et al., 2018; Di Caprio, 2020).

Despite this, recent studies also confirmed that these microbes can positively influence algal growth and productivity (Le Chevanton et al., 2013; Berthold et al., 2019; Lian et al., 2021).

Furthermore, after adding mutualistic bacteria, microalgae-bacteria interactions in natural habitats appear equivalent to those in industrial cultures (González-González & De-Bashan, 2021).

For this reason, researchers have even started to consider if axenic cultures are a good option for cultures since bacteria and microalgae seem to thrive in the presence of one another in both industrial cultures and natural settings (Padmaperuma et al., 2018). Moreover, adding specific bacterial strains to the microalgae in culture has been suggested to enhance productivity and biomass quality (Le Chevanton et al., 2013).

For example, a study involving the microalga *Chlorella sorokiniana* and the bacteria *Azospirillum brasilense* and *Bacillus pumilus* showed that co-culturing this microalga with each of these prokaryotes individually resulted in higher growth of *C. sorokiniana* and enhanced amounts of lipids, carbohydrates, and chlorophyll (Chl) *a* in the algal biomass (Amavizca et al., 2017).

Considering this, recent studies propose that bacteria with reported benefits for microalgae can be joined to form small artificial microalgae-bacteria consortia to improve productivity and biomass quality (Berthold et al., 2019; González-González & De-Bashan, 2021).

While taking advantage of the mutualistic associations between microalgae and bacteria, the designed microalgae-bacteria consortia can be applied to large-scale industrial cultures to protect cultures from unwanted organisms, overcome nutrient deficiency, improve growth and production of high-value products, or even to facilitate acclimation to culture conditions, such as temperature or salinity, thus reducing operational costs and cultivation limitations (Le Chevanton et al., 2013; Lian et al., 2018; Berthold et al., 2019; Liu et al., 2020).

For this reason, several studies have been made concerning this theme in recent years. For instance, an artificially designed microalgae-bacteria consortium was created using four bacterial strains (*Flavobacterium* sp., *Hyphomonas* sp., *Rhizobium* sp. and *Sphingomonas* sp.), reported as growth enhancers, and inoculated with the microalga *C. vulgaris*. The co-culture had higher growth rates and lipid content when compared to the control (CTRL) axenic culture of *C. vulgaris* (Cho et al., 2015).

In addition, bacteria have also been considered for biomass harvesting since some induce cell aggregation, flocculation, and cell disruption processes necessary for the recovery of compounds of interest, thereby reducing the costs and biomass losses related to these procedures (Fuentes et al., 2016; González-González & De-Bashan, 2021). For instance, adding a *Citrobacter* sp. strain to *Chlorella pyrenoidosa* simplified the harvesting process of this microalga through flocculation.

Before inoculation with the bacterium, the harvesting process would require metal ions and pH alterations that were no longer needed after co-inoculation with this bacterium (He et al., 2022).

These artificially designed microalgae-bacteria consortia have also been studied regarding bioremediation industries for removing toxic compounds (e.g., heavy metals and pesticides) from water sources. For instance, in a recent study, bacterial species with genes known to encode for enzymes involved in the degradation of glyphosate were selected to create a mixture that could be added to *C. protothecoides*. This microalga is known for its bioremediation capacity, thus creating a consortium that could potentially remove the herbicide glyphosate from water. Results showed that the co-culture of this microalga with *Sinomonas atrocyanea*, *Pseudomonas stutzeri* and *Comamonas odontotermitis* reduced glyphosate amounts in wastewater (Borella et al., 2023).

However, despite the recent advances made regarding the possible roles of bacteria in industrial cultures of microalgae, the effects of the naturally present bacteriome on microalgae industrial cultures still need to be characterized.

Therefore, considering that a whole bacteriome of interacting organisms surrounds microalgae in industrial cultivation systems, the question remains whether a tailor-made microalgae-bacteria consortium, designed with the best-performing bacterial strains can be a valid approach to further optimize the industrial production of highly valuable microalgal species.

One of the species currently being produced in industrial scale cultures is *T. lutea* (**Figure 1.5**), a small golden-brown marine haptophyte initially isolated from seawater (SW) habitats in Tahiti (Bendif et al., 2013; Gangadhar et al., 2020; Gonçalves de Oliveira-Júnior et al., 2020; Bigagli et al., 2021).

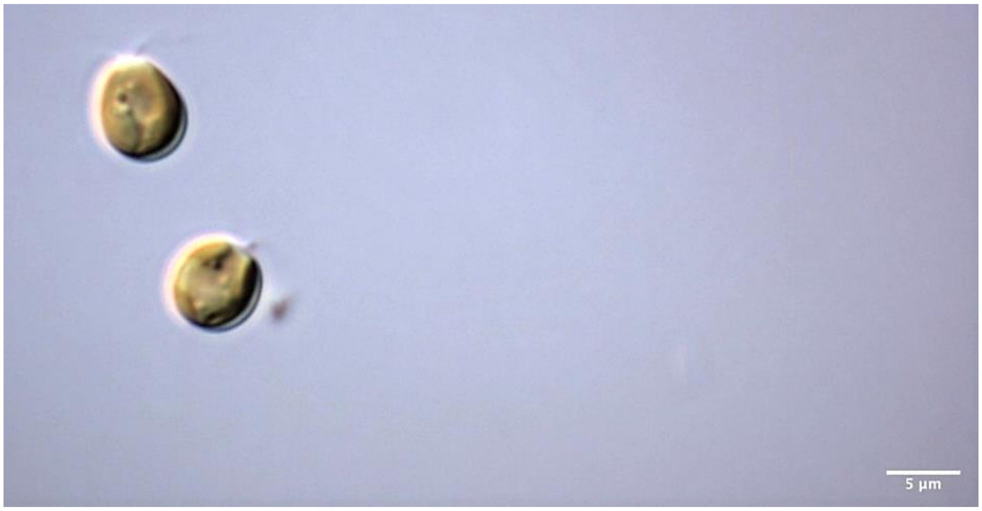

Figure 1.5-*Tisochrysis lutea* microscopic photography using DIC at a 400x magnification. Scale bar = 5 µm. (Photo by Gabriel Bombo).

This microalga, previously known as *Isochrysis galbana* (T-ISO), is cultivated during warmer seasons (spring and summer), and is produced industrially mainly for aquaculture since biomass harvested from *T. lutea* is rich in PUFA, namely docosahexaenoic acid (DHA), important especially in the early growth phases of aquatic species (da Costa et al., 2017; Gangadhar et al., 2020; Gonçalves de Oliveira-Júnior et al., 2020; Bigagli et al., 2021; H. Pereira et al., 2021). Moreover, this marine species has a relatively small size and fragile cell wall, thus facilitating the ingestion and digestion process by young larvae (Nalder et al., 2015; da Costa et al., 2017). Currently, the aquaculture industry utilizes this biomass as feed to fish and mollusk larvae, crustaceans, and rotifers, which are then fed to fish (Almutairi et al., 2020; Gangadhar et al., 2020; Andriopoulos et al., 2022).

T. lutea is also rich in other high-valuable products, such as vitamins, phenolic compounds, and pigments, such as fucoxanthin (Gonçalves de Oliveira-Júnior et al., 2020; Bigagli et al., 2021; Mohamadnia et al., 2021). For this reason, extracts from this microalga are increasingly being used in pharmaceutical and health-related studies since, for instance, it has been reported that fucoxanthin has antioxidant, anti-cancer and anti-inflammatory bioactivities (Gangadhar et al., 2020; Gonçalves de Oliveira-Júnior et al., 2020; Bigagli et al., 2021; H. Pereira et al., 2021).

Given the importance of this microalga in a wide variety of industries nowadays and its potential to be used in many more, studies have been conducted to optimize its production and cultivation. Some of these studies have addressed the possibility of specific changes in culture conditions toward growth and biomass quality enhancement (da Costa et al., 2017).

For example, the induction of thermal stress in several cultures of different strains of *T. lutea* has led to changes in fatty acid (FA) composition in two strains (Gachelin et al., 2021). Another study tested the effect of several N concentrations on *T. lutea* production. The results showed that N depletion maximized lipid content and improved the composition of FA and the amount of total carbohydrates and carotenoids (Almutairi, 2020). However, total protein and Chl content decreased when N was lacking in the culture medium, and growth rates were also lower (Almutairi, 2020).

In its natural aquatic habitat, *T. lutea*, like most haptophytes, is believed to depend on cobalamin (vitamin B_{12}) to grow (Nef et al., 2022). Since only some bacteria and archaea produce this vitamin, they are the most probable source of vitamin B_{12} for *T. lutea* (Nef et al., 2022). This may indicate that, throughout time, mutualistic interactions were developed between this marine microalga and its surrounding bacteria for the former to acquire B_{12}. Recent evidence suggested that the growth and metabolism of *T. lutea* are affected by axenic conditions (Nef et al., 2019, 2022).

For this reason, a recent study has been made by adding specific bacterial strains, known producers of vitamin B_{12}, and extracts of these strains to *T. lutea* cultures and effects were evaluated (Nef et al., 2022). Results showed that axenic and xenic cultures had similar growth when vitamin B_{12} was present in the culture media. However, in media lacking B_{12}, xenic cultures had higher growth rates than axenic cultures, suggesting that this mutualistic association is possibly facultative, only occurring under nutrient depletion (Nef et al., 2022). Furthermore, in cultures containing bacterial extracts, *T. lutea* retrieved vitamin B_{12} from the growth medium (Nef et al., 2022).

So, if *T. lutea* can inherently interact with bacteria in its natural environment, and its growth can be improved in xenic cultures, in cases of nutrient depletion, can the naturally present bacteriome in *T. lutea* industrial cultures be the way to optimize production and enhance the nutritional value of this microalga?

2.Objectives

The main goal of this work is to develop bacterial mixes that can be added to *Tisochrysis lutea* industrial cultures to improve productivity and stimulate the production of high-value compounds (e.g., FA, proteins, pigments, and vitamin B_{12}).

Specific objectives were designed to achieve the main goal:

I. Identification of bacteria associated with *T. lutea* industrial cultures.
II. In co-inoculation trials, bacterial isolates were added to *T. lutea* cultures, to discover which strains correlated with higher growth and higher concentrations of products of interest in the microalgal biomass.
III. Design of tailor-made bacterial consortia.
IV. Evaluation of microalgal productivity and biochemical value of the biomass harvested from *T. lutea* cultures after co-inoculation with the tailored bacterial mixes.

3.Materials and methods

3.1. Experimental design

This work was performed between September 2022 and April 2024 in the Marine Biotechnology Research Laboratory (Marbiotech) in the Center of Marine Sciences (CCMAR) of the University of Algarve, under the scope of Tamára Santos' PhD project entitled "Microbiome mediated improvement of industrially produced microalgae".

The project was divided into three main parts, described in **Figure 3.6**:

I. Identification of bacterial species naturally associated with *T. lutea* industrial cultures.

II. Co-inoculation trials of *T. lutea*: Bacteria
   a. In the first part of the work, a single bacterial isolate was inoculated into *T. lutea* cultures, and its effects on microalga growth and biomass quality were assessed in *T. lutea* growth and biomass quality.
   b. For the second phase, the best performing bacterial strains from stage I were combined to form simplified bacterial mixtures to be added to *T. lutea* cultures, and their effect on the microalga biochemical profile and productivity was assessed.

III. Validation of the best-performing "tailor-made" bacterial consortium.

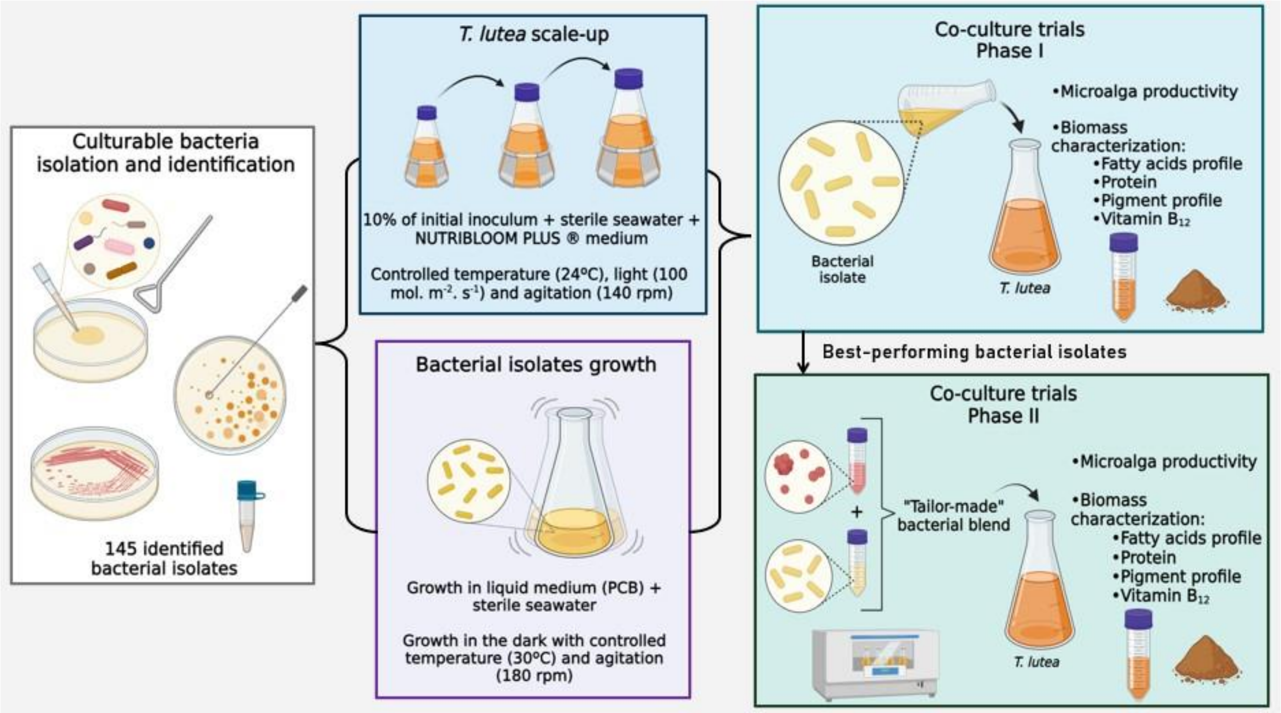

Figure 3.6-Schematic representation of the experimental design of the project. Created with BioRender.com.

3.2. Microalga production and sampling

T. lutea, a marine microalga, was sampled in prior works. Microalga samples were collected from industrial cultures in Necton facilities between June and October 2019, and 2020 since *T. lutea* production usually occurs during late spring, summer, and early autumn.

Six complete microalga production cycles, from inoculation until biomass harvesting, were sampled in 6 independent tubular photobioreactors (TPBR; **Figure 3.7**), with a working volume from 15 to 19 m^3. Sampling was performed weekly.

Figure 3.7-*Tisochrysis lutea* production in TPBR at Necton facilities (Photo by Tamára Santos)

3.3. *Tisochrysis lutea* scale-up

The working species *T. lutea* was provided by Necton (Olhão, Portugal). A scale-up process was conducted, where a starting inoculum of the microalga was added to sterile Erlenmeyer Flasks containing seawater (SW) supplemented with NUTRIBLOOM PLUS® medium (NB^+), a commercial product from Necton, at a final concentration of 4 mL. L^{-1}. The initial culture volume was 50 mL, but with each passage, the total volume of the culture increased, until a maximum of 200 mL was reached. Cultures in the exponential growth phase were used as a starting inoculum for the new cultures in a volume corresponding to 10% of the total new culture volume (**Figure 3.8**).

To avoid loss of culture, a 200 mL culture of *T. lutea* was maintained with successive passages.

Before the beginning of each trial, an inoculum of the microalga was transferred to a previously sterilized 1 L bottle containing the same culture media and left to grow under light and aeration conditions until the exponential growth phase was achieved (**Figure 3.9**).

In each culture system, the initial volume, after inoculation, was marked, and, when necessary, volume loss through water evaporation was compensated with previously sterilized distilled water.

To maintain sterility, all materials and waters used were autoclaved at 120ºC, and the NB^+ culture medium was filtered using a 0.20 μM polyethersulfone (PES) filter (Filtropur S, SARSTEDT, Germany). Also, prior to sterilization, SW was filtered in a filtration system connected to a vacuum pump to remove debris and solids. In addition, the salinity of SW was estimated using a seawater refractometer (HI96822 Seawater Refractometer, Hanna Instruments, USA) and, if necessary, adjusted with distilled water to a final value of 33 ppt, which is optimal for most marine microalgae.

Growth was monitored through optical density readings (OD) at 680 nm and 750 nm (BioTek Synergy Neo2 multi-mode reader, BioTek Instruments Inc., USA), two wavelengths often used for estimation of biomass concentration in microalgae (Griffiths et al., 2011).

Figure 3.8-*Tisochrysis lutea* culture in exponential growth phase (back) and culture immediately after passage with an inoculum of 10% (front)

Figure 3.9-*Tisochrysis lutea* culture in 1 L bottle with light and aeration.

3.4. Assessment of growth parameters in *T. lutea*

A starting inoculum of *T. lutea* was utilized to inoculate three 2 L bottles, with a working volume of 1 L each, according to the procedure described in the previous section (**Figure 3.10**).

The cultures were grown under light and aeration conditions that were altered following microalgae growth.

To follow the growth of *T. lutea* in the three replicates, regular samples were taken every 2/3 days, for the dry weight (DW) estimation, OD at 680 nm and 750 nm, and cellular concentration (CC) determination.

Figure 3.10-*Tisochrysis lutea* culture (in triplicate) prepared for the assessment of growth parameters (Dry weight, optical density spectrophotometry and cellular concentration) in this microalga.

3.4.1. Dry Weight (DW)

The dry weight of cultivated *T. lutea* was estimated using a moisture analyzer (Moisture Analyzer AND MS-70, A&D Instruments LTD., United Kingdom).

Glass microfiber filters of 47 mm (1.2 µm, VWR) were washed using a filtration system and a vacuum pump with 10 mL of distilled water, followed by 10 mL of a previously prepared ammonium formate solution (31.5 g. L^{-1}, VWR), to remove any salts that could interfere with the analysis (Zhu & Lee, 1997; Khaw et al., 2021).

The filters were then transferred to an oven at 50ºC and left to dry for 48 hours. The filter weights were registered (initial weight) prior to utilization.

Afterward, samples containing between 5 to 20 mL of liquid culture were filtered, and then a washing step with 10 mL of the ammonium formate solution was performed.

Using clamps, the filters carrying the samples were placed in the moisture analyzer and left to dry at 130ºC before weighing (final weight) (**Figure 3.11**).

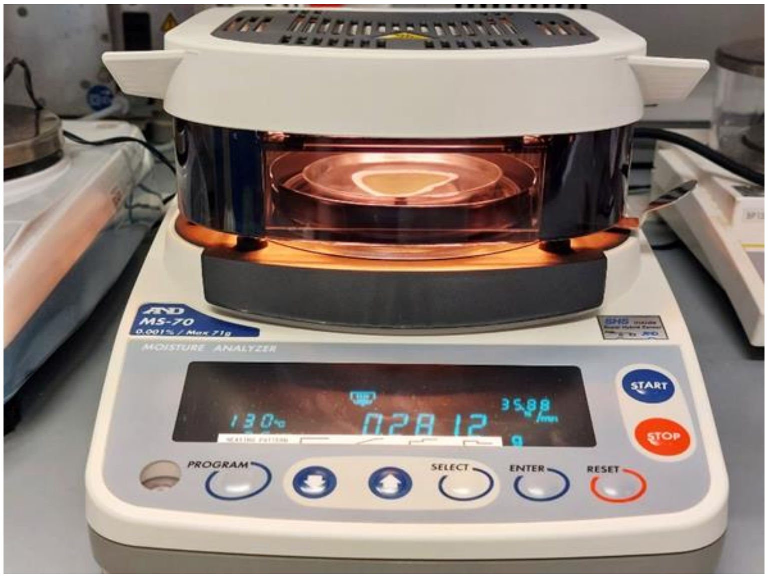

Figure 3.11-Sample harvested from the *T. lutea* culture drying at 130ºC in the moisture analyzer.

To ensure complete dryness of the samples, the drying procedure in the moisture analysis was repeated at least once for each sample.

The dry weight of cultures was estimated with **Equation 1**:

$$DW\ (g.L^{-1}) = \frac{(Final\ weight - Initial\ weight)}{Sample\ Volume} \qquad (\mathbf{1})$$

3.4.2. Optical density spectrophotometry (OD)

The OD of the cultures was read using a microplate reader (BioTek Synergy Neo2 multi-mode reader, BioTek Instruments Inc., USA). Bacterial growth was followed by measuring light absorption at a wavelength of 600 nm, whereas microalgae growth was monitored at 680 nm and 750 nm.

After sample collection, 200 μL aliquots were transferred onto a 96-well plate (in duplicate), and absorbance was read at both wavelengths. If the resulting absorbance values exceeded 1.0, samples were diluted with SW.

3.4.3. Cellular Concentration (CC)

CC was assessed by optical microscopy (400x amplification) using a Neubauer-improved counting chamber (Marienfeld Superior, Germany).

Briefly, 100 μL of each sample was fixed with 10 μL of Lugol solution (5%, Panreac AppliChem ITW Reagents).

After homogenization, 10 μL of the sample was transferred to two chambers.

The cell count was kept between 30 and 300. For higher concentrations, samples were diluted in new Eppendorf tubes containing SW.

CC was calculated according to **Equation 2**:

$$Cellular\ Concentration\ (cells.mL^{-1}) = Dilution\ \times Cell\ count\ \times\ 10^{4} \qquad \textbf{(2)}$$

3.5. Bacteria isolation and identification

Bacterial isolation from the naturally occurring bacteria in *T. lutea* industrial cultures was performed before this work.

Briefly, it was performed by inoculating the collected samples into non-selective heterotrophic media.

For the inoculation, 100 µL of the suspension was subjected to serial dilutions and inoculated onto solid medium plates of Plate Count Agar (PCA, 23.5 g. L^{-1}, Liofilchem), non-selective media rich in nutrients, and Marine Agar (MA, 52.35 g. L^{-1}, US Biological Life Sciences), an appropriate medium to grow most culturable marine bacteria. Before inoculation, the culture media was sterilized and autoclaved at 120ºC to prevent contamination. The plating and inoculation procedures occurred in a class II flow cabinet (Telstar Bio II Advance 4, TELSTAR Technologies

S. L., Spain) previously sterilized with germicidal ultraviolet (UV) radiation. Plates were incubated at 27ºC, in the dark, for 24 to 72 hours. The identification of morphologically distinct colonies occurred through visual observation. Selected colonies were then isolated using several cross-streaking steps onto new plates containing the same culture media.

Afterward, bacterial DNA extraction was carried out using the Quick-DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA), following the manufacturer's instructions. Accordingly, an isolated bacterial colony was placed in 500 µL of Genomic Lysis Buffer, provided in the kit, and homogenized mechanically. Afterward, the sample was centrifuged at 10,000 *g* for 5 minutes, and the resulting supernatant was transferred to a Zymo-Spin IICR Column, being centrifuged again, at the same speed, for 1 minute. The column was transferred to a new collection tube, and 200 µL of DNA Pre-Wash Buffer was added, followed by a new centrifugation step at 10,000 *g* for 1 minute. After that, 500 µL of g-DNA Wash Buffer was added, and the samples were centrifuged in the same conditions as the previous step. Finally, the spin column was transferred to a new tube, and 50 µL of DNA Elution Buffer was added, followed by an incubation at room temperature of 5 minutes and a spin at maximum speed to release the DNA from the column.

The quantity and quality of the DNA were assessed by spectrophotometry analysis conducted utilizing a NanoDrop OneC micro-UV-visible spectrophotometer (Thermo Scientific, USA). An electrophoresis procedure involving a 1% agarose (Nzytech) gel visualization was also performed. The extracted DNA was then stored at -20ºC until utilization.

The extracted bacterial DNA was amplified by a Polymerase Chain Reaction (PCR) using the primers F27:5' – AGA GTT TGA TCG TGG CTC AG-3' and R1492: 5' - TAC GGY TAC CTT GTT ACG ACT-3', as proposed elsewhere (Weisburg et al., 1991). A PCR reaction mix was prepared as described in **Table 3.1**.

Table 3.1 -Composition of the prepared master mix for the PCR amplification of the 16S rRNA gene in the bacteria isolates.

Mix Components	**Volume per reaction**
Water for Molecular Biology	5.95 µL
5x Colorless GoTaq® Flexi Buffer	4.00 µL
$MgCl_2$ solution (25 mM)	1.60 µL
dNTP Mix (10 mM)	1.25 µL

F27 Primer (10 μM)	1.00 μL
R1492 Primer (10 μM)	1.00 μL
GoTaq® G2 Flexi DNA Polymerase (5U/ μL)	0.20 μL

Afterward, 15 μL of the prepared mix was added to each well of the PCR plate, followed by 5 μL of the bacterial DNA. The PCR plate was placed in a 2720 Thermal Cycler (Applied Biosystems, USA), according to the conditions described in **Table 3.2**.

Table 3.2-Amplification program selected for the PCR amplification of the 16S region of DNA extracted from the bacterial isolates.

Cycles	Program steps	Temperature	Time
1	Denaturation	95ºC	5 minutes
35	Denaturation	95ºC	30 seconds
	Annealing	50ºC	30 seconds
	Elongation	72ºC	1.5 minutes
1	Extension	72ºC	10 minutes
-	Hold	4ºC	∞

Subsequently, the amplified DNA was sequenced at a CCMAR sequencing facility equipped with an Applied Biosystems 3130XL DNA sequencer (Life Technologies BV, Porto, Portugal). Afterward, the 16S sequences were processed using CLC Genomics Workbench 21 (QIAGEN, Aarhus, Denmark) software and analyzed using the nucleotide BLASTn tool of the National Center for Biotechnology Information (NCBI) against the non-redundant nucleotide (nr/nt) database to obtain the identification of each isolated bacteria. Descriptions of the higher taxonomical levels corresponding to the identified bacteria, were also obtained upon further research on the taxonomy database of NCBI. Typically, only results with identity percentages above 94.5% were considered identified down to the genus level, while a 97% similarity is required for an identification of a strain down to the species level (Valenzuela-González et al., 2015). However, during this work, several attempts of identification were made for each isolate, so lower identity percentages were also considered if the obtained genus/species identification remained the same upon the processing of different sequences and, if the growth and colony characteristics of the isolates matched those reported for bacteria identified with a given taxon.

3.6. Co-culture trials

3.6.1. Bacterial isolates growth

After the identification of bacterial isolates present in association with *T. lutea*, a bibliographic review was made, in which the bacterial species previously reported as growth promoters, vitamin B_{12}-producers or bioactive molecules inducers were selected for the growth trials, resulting in a total of 40 isolates. The bacteria isolates were maintained at 4ºC throughout the project on PCA and MA plates (**Figure 3.12**). Furthermore, to prevent the loss of any of the isolated species, colonies of each isolate were also placed into tubes containing glycerol as cryoprotectant and cryopreserved at -80ºC.

Figure 3.12-Isolated bacterial colonies in PCA plates (Photo by Tamára Santos).

Before each growth trial, an isolated colony of each selected bacterium was transferred to a sterile liquid medium containing Plate Count Broth, a previously prepared mixture of tryptone (5 g. L^{-1}, Biolife), glucose (1g. L^{-1}, Scharlau) and yeast extract (2.5g. L^{-1}, Biolife), dissolved in SW (**Figure 3.13**). Before inoculation, the liquid medium was supplemented with a glucose stock solution (250 g L^{-1}, Scharlau) to achieve a final concentration of 15 g. L^{-1}. Sterile conditions were ensured through the material and medium sterilization at 120ºC and irradiation with UV in a type II flow chamber (Telstar Bio II Advance 4, TELSTAR Technologies S. L., Spain).

Afterward, liquid cultures were placed in an incubator (Incubated Shaker IIS-4075R, from JEIO TECH), under controlled conditions, at 30ºC in the dark, with constant agitation (180 rpm) for 48h. A 10% inoculum was then transferred to a fresh PCB medium supplemented with glucose (15 g. L^{-1}, Scharlau) for another 24 hours. Growth was monitored by turbidity check through OD spectrometry at 600nm, a frequently used wavelength to monitor bacteria in liquid culture, since it provides reliable results without causing any harmful effects on the culture (Chapman et al., 2021).

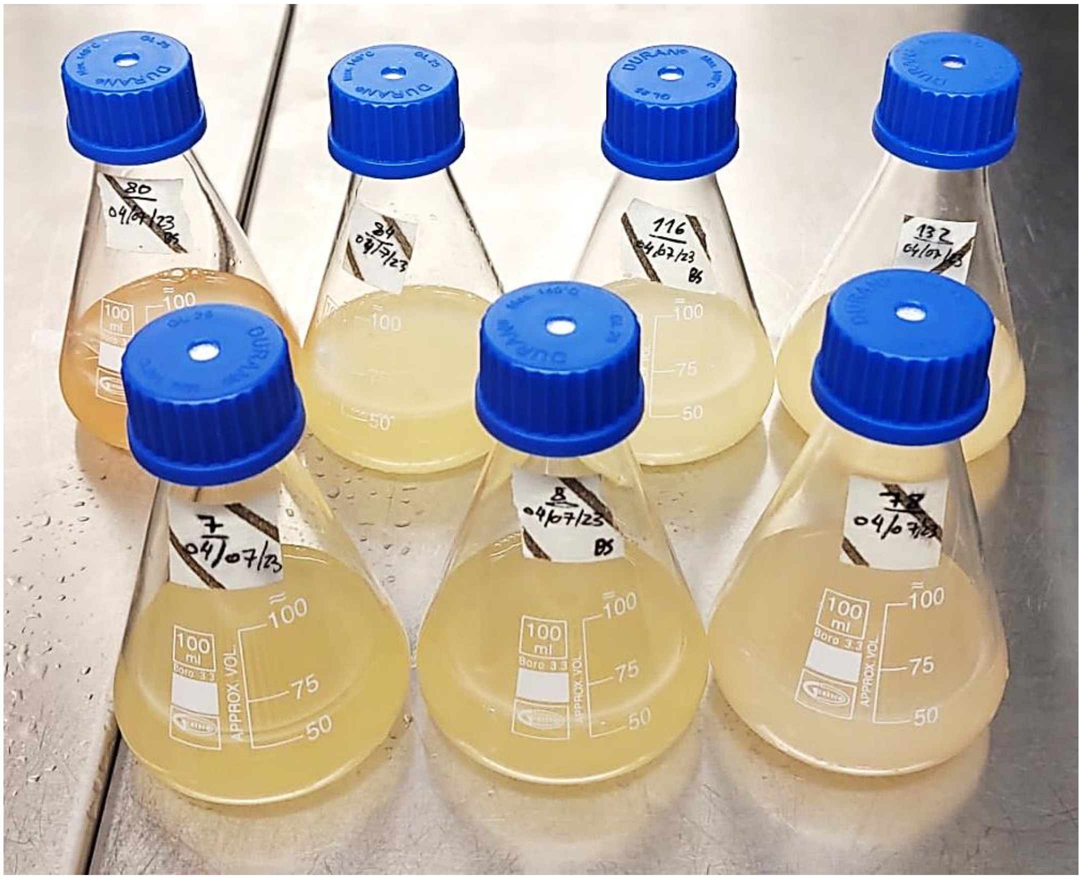

Figure 3.13-Bacterial isolates grown in liquid PCB media.

3.6.2. Microalga-bacteria co-culture (Phase I)

The selected bacteria were co-cultured with *T. lutea* in two trials to discover which bacterial strains improved the growth and productivity of this marine haptophyte.

All the tested treatments were performed in duplicate, with the only difference between them being the bacterial isolate(s) added to the culture. The bacterial strains, already in liquid PCB medium, were centrifuged at 3000 *g* for 2 minutes and resuspended in SW before inoculation into the culture, which already containing the *T. lutea* inoculum.

Furthermore, four CTRL cultures were prepared for each experiment without adding any of the selected bacterial isolates to the xenic *T. lutea* inoculum.

In all trials, the cultures were grown in an incubator (Incubated Shaker IIS-4075R, from JEIO TECH) at 24ºC with continuous light (100 mol. m^{-2}. s^{-1}) and agitation (140 rpm). Moreover, the same culture media, composed of sterile SW, supplemented with 4mL. L^{-1} of NB^{+} was used across all the experiments.

Aseptic conditions were ensured throughout the trials by sterilizing all the utilized material and SW at 120 ºC in an autoclave (Autoclave-steam sterilizer 2540 ML, Tuttnauer). In addition, before sterilization, SW underwent the same methodology as the stock cultures of *T. lutea*. Moreover, all the experimental procedures conducted before and during the co-culture trials were performed inside a flow chamber (Telstar Bio II Advance 4, TELSTAR Technologies S. L., Spain), previously irradiated with germicidal UV for 15 minutes.

During the trials, 1 mL samples were collected to monitor microalgae growth, through CC and OD spectrometry measurements at 680 nm and 750 nm and bacterial turbidity, with OD readings at 600 nm (**Figure 3.14**). The visual aspect of cultures and alterations in cell morphology were also assessed.

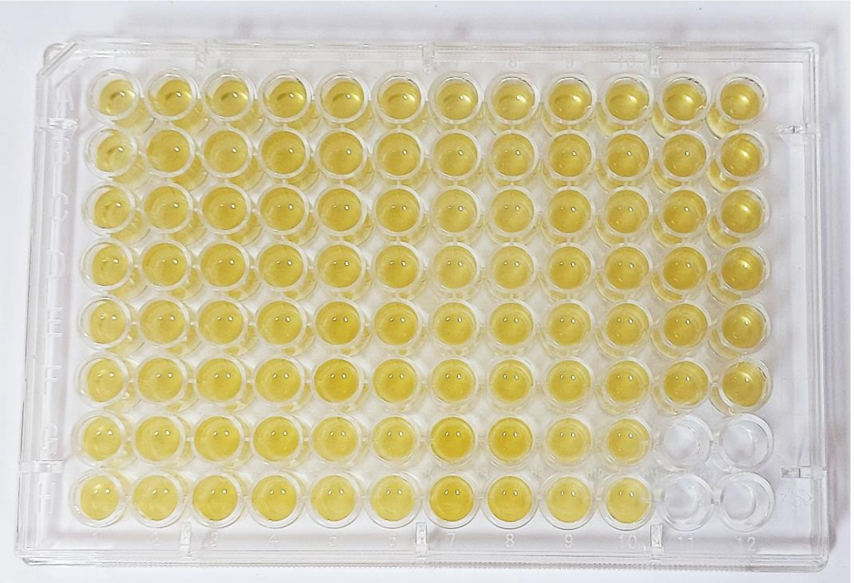

Figure 3.14-Plate prepared for optical density measurements at 600, 680 and 750 nm of samples collected from microalga-bacteria co-cultures.

3.6.2.1. Microalga-bacteria co-culture trial 1 (IT1)

The 40 initially selected isolated bacterial strains were inoculated with *T. lutea* in a growth trial that ended one week after inoculation or sooner in case of culture collapse.

Cultures were grown in sterile culture T-flasks, with ventilation caps, to a final volume of 40 mL per replicate. Since the assay was conducted in T-flasks, the agitation was set at 115 rpm for this trial instead of the previously planned 140 rpm to prevent spills.

Samples of each replicate were collected at the beginning and end of the trial.

At the end of the trial, the strains that improved microalgae growth were selected for a second isolate trial. The bacteria reported in the literature as vitamin B_{12} producers were also chosen for utilization in the second trial.

3.6.2.2. Microalga-bacteria co-culture trial 2 (IT2)

The bacterial isolates selected at the end of the first trial were co-inoculated with *T. lutea* in a new trial that lasted 8 days.

In this case, cultures were grown in sterile 100 mL Erlenmeyer flasks, with ventilation caps containing 50 mL of culture per replicate.

Samples were collected four times during the trial (at the beginning and end of the experiment and on days 3 and 6). Additionally, samples collected in this assay were used to estimate bacterial load in each replicate, using the colony-forming units (CFU) method.

At the end of the trial, microalgal biomass was harvested by centrifugation at 7,000 *g* for 5 minutes (Beckman Coulter Avanti J-25 High-Performance centrifuge). The collected biomass was then freeze-dried and stored for nutritional and biochemical analysis.

Bacterial isolates associated with improved growth and biochemical profile in *T. lutea* were selected for the final part of the project.

3.6.2.2.1. Colony-forming units (CFU)

CFU were counted to estimate bacterial abundance throughout the first isolate trial.

Briefly, 100 µL of each sample was serially diluted with sterile SW and spread onto solid medium plates containing either plate count agar (PCA) or marine agar (MA), depending on the bacterial isolate. Afterward, the plates were incubated for 24 hours in the dark at 30ºC.

The dilutions chosen for plating for the samples collected at the beginning of the growth trials were 10^{-3} to 10^{-4}, while for the samples collected at the end of the growth trials, the selected dilutions were 10^{-3} to 10^{-5}.

Final estimation of CFU per mL was calculated with **Equation 3**:

$$\frac{CFU}{mL} = Number\ of\ counted\ colonies\ \times Dilution\ \times Volume\ in\ the\ plate \qquad (3)$$

3.6.3. Microalga-bacteria co-culture (Phase II)

Seven bacterial isolates correlated with higher microalgal productivity and biomass quality were combined into pairs and inoculated with *T. lutea* for the project's second phase.

The cultures were inoculated into ventilated 100 mL Erlenmeyer flasks, to a final volume of 50 mL per replicate.

Liquid bacterial cultures were centrifuged at 3000 *g* for 2 minutes and resuspended in SW, before being added to the cultures.

Upon the inoculation of the microalga, bacterial isolates were added one by one to the microalgal growth medium.

All the treatments were in co-culture for 11 days. During that time, *T. lutea* reached the stationary growth phase, in the same culture conditions (temperature, agitation and irradiation) as the Phase I trials.

Sample collection occurred every two days, except for the beginning and end of the trial, in which samples were taken on days 0 and 1 and days 10 and 11, respectively. Each sample containing 1 mL of culture was divided for cell counts and OD readings (at 600 nm for bacteria and, 680 nm and 750 nm for the microalga).

The culture state was also assessed throughout the experiment by visual observations of the general aspect and color of the cultures.

To conclude the assay, biomass was harvested by centrifugation at 7,000*g* for 5 minutes (Beckman Coulter Avanti J-25 High-Performance centrifuge). Afterward, it was freeze-dried and stored at -4ºC to analyze the biochemical and nutritional profile.

3.7. Biochemical analysis

3.7.1. Fatty acids profile

Determination of the fatty acid methyl esters (FAME) profile was obtained following a modified Lepage and Roy procedure, as described in H. Pereira et al. (2012).

Glass beads were added to 2 mL Eppendorf tubes containing approximately 10 mg of dry biomass. Then, 1.5 mL of a methanol/acetyl chloride mixture (20:1, v/v) was added to the samples and homogenized by a mixer mill (Retsch MM400) at 30 Hz for 3 cycles of 1 minute.

The resulting mixture was transferred to derivatization vessels, followed by a 1 mL addition of hexane.

The vessels were placed in a water bath at 70ºC for one hour, followed by a cooldown in ice for 15 minutes.

Afterward, 1 mL of distilled water and 4 mL of hexane were added to the samples. The tubes were then placed in a vortex in two cycles of 30s and centrifuged for 5 minutes at 2,000 *g*.

Subsequently, the top fraction containing the mix of FA dissolved in hexane was transferred to a new glass vial. The centrifugation and extract collection steps were repeated following a new addition of 4 mL of hexane.

Anhydrous sodium sulfate (ThermoScientific) was added to the collected fractions in excess, followed by a filtration step with a 0.2 µm filter (Whatman Puradisc, PTFE). The filtered fractions were evaporated with an N flow and resuspended in 500 µL of HPLC-grade hexane.

Samples fatty acid profile was analyzed in a Gas Chromatography-Mass Spectrometry (GC-MS, Scion TQ 456-GC, Bruker), with a ZB5-MS Plus capillary column (25 m x 0.25 mm internal diameter, 0.25 µm film thickness, Phenomenex). Helium was used as a carrier gas, in splitless injection mode, with 1 µL injections at 300ºC and a working flow of 1 mL min^{-1}. The temperature profile of the GC program was the following: an initial 1 min hold at 60ºC, then a 30ºC increase/min until 120ºC, followed by 4ºC elevation/min to 250ºC and a final 20ºC increase/min to 300ºC, followed by a hold for 4 minutes at that temperature. For the MS procedure, the transfer line and source temperatures were set at 260ºC and 220ºC, respectively.

The FAME was quantified using calibration curves for each compound, designed using an external standard mix (Supelco® 37 Component FAME Mix, Sigma-Aldrich, Sintra, Portugal). If any of the encountered FAME didn´t have a correspondent calibration curve in the external standard mix, the calibration curve of the FAME with the closest structure was used (H. Pereira et al., 2012).

3.7.2. Protein

About 1 to 2 mg DW of the samples was used to estimate protein content through total N content determination by an Elementar Analyzer model Vario III, according to the manufacturer's instructions.

Following the information described in Lourenço et al. (2004), the used N-to-protein conversion factor was 4.59, a specific value reported for *Isochrysis galbana*, and total protein content was calculated using **Equation 4**:

$$Total\ protein\ content = \%\ Nitrogen\ \times 4.59 \qquad \textbf{(4)}$$

3.7.3. Pigment profile

Approximately 5 to 6 mg of dry biomass were used to obtain carotenoid and Chl profiles in the samples.

After adding 1 mL of a methanol solution containing 0,03% butylhydroxytoluene (BHT, Sigma-Aldrich) to the samples, these were disrupted by a mixer mill (Retsch MM400) at 20 Hz for 3 cycles of 1 min, with 30s breaks, in ice, between cycles.

The samples were then centrifuged at 12,000 g for 6 min, and the supernatant was collected in a new amber vial. This collection process was repeated until both the pellet and supernatant were colorless.

The combined collected fractions were evaporated using a gentle N flow overnight.

The resulting extract was resuspended in 1 mL of HPLC-grade methanol and filtered with a 0.22 µm nylon filter, before injection in HPLC.

The pigment profile was determined by HPLC (Chromaster, Hitachi, VWR) with Diode array detector (5430 DAD, Hitachi, VWR), with a Purospher® STAR RP-18 endcapped chromatographic column (250 × 2.1mm × 5 µm) (Merck), at 27ºC, with a flow rate of 1 mL.min^{-1} for 40 minutes, based on what was previously described by Couso et al. (2012) and Schüler et al. (2020).The injection volume was set to 50 µL. Compounds in the pigment profile were identified through comparison with standards at 450 nm and the concentration of each pigment was determined based on the respective standard calibration curve.

3.7.4. Vitamin B_{12}

The concentration of vitamin B_{12}, in the form of methylcobalamin (MeCbl), was determined using a Greencolab internal method, in which Amberlit® was used to purify the sample.

Analysis was performed in HPLC (Chromaster, Hitachi, VWR) with a diode array detector (5430 DAD, Hitachi, VWR) and a C18 column (150 x 4.6mm, 5 µm) and the injection volume was 50 µL. This compound was identified by comparison with the calibration curves of the standard.

3.8. Statistical analysis

Statistical analysis was performed using the statistics program GraphPad Prism version 8.0.2 (GraphPad Software, Boston, Massachusetts USA).

Parametric assumptions were made by analyzing the Quantile-Quantile (QQ) plot and descriptive statistics.

Statistical differences for the results obtained during Phase I were determined with One-way ANOVA test with Dunnett multiple comparisons test for growth and biochemical analysis, and an unpaired *t*-test with Welch's correction for bacterial concentration analysis, all to a significant level of $\rho<0.05$ (95% confidence).

For the results of Phase II (CC and biochemical analysis), differences were estimated with a One-way ANOVA, followed by Tukey post-hoc comparisons test, to a significant level of $\rho<0.05$ (95% confidence).

Results outside normal distribution were analyzed through a non-parametric Mann-Whitney Test with 95% confidence ($\rho<0.05$).

4.Results and Discussion

4.1. Assessment of growth parameters

Monitoring microalgal growth is essential to estimate productivity and assess the culture state throughout the cultivation process (Sarrafzadeh et al., 2015; Schagerl et al., 2022; Thiviyanathan et al., 2024). For this reason, it was necessary to select procedures that allowed us to accurately follow the growth and biomass production of *T. lutea* during the co-culture trials.

Considering this, the growth of *T. lutea* was evaluated with three widely used methods to follow the growth of the microalga in cultivation: DW, OD and CC (Sarrafzadeh et al., 2015; Thiviyanathan et al., 2024). For that purpose, three 1 L cultures of *T. lutea* were prepared as described in a previous section (**section 3.4**), and they were maintained until day 18 when *T. lutea* was confirmed to be in the stationary growth phase. Results obtained in this analysis were used to construct a growth curve for each method (**Figures 4.15-4.17**).

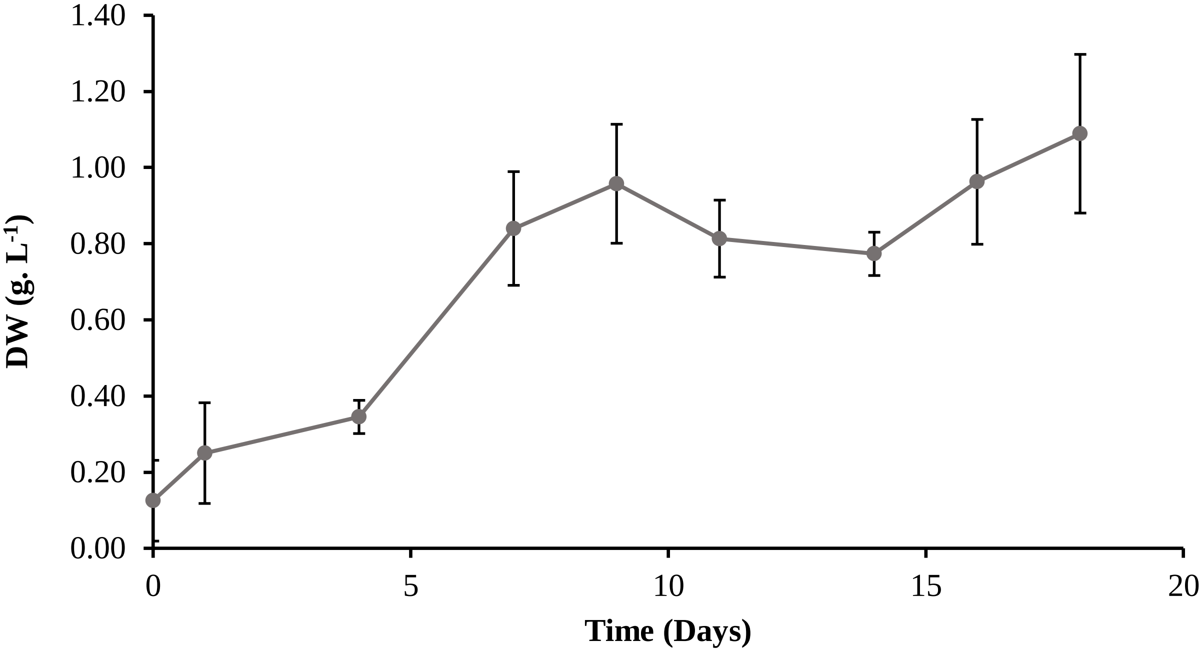

Figure 4.15- Dry weight of *T. lutea* grown for 18 days. Data is presented as mean values ± SD (*n*=3)

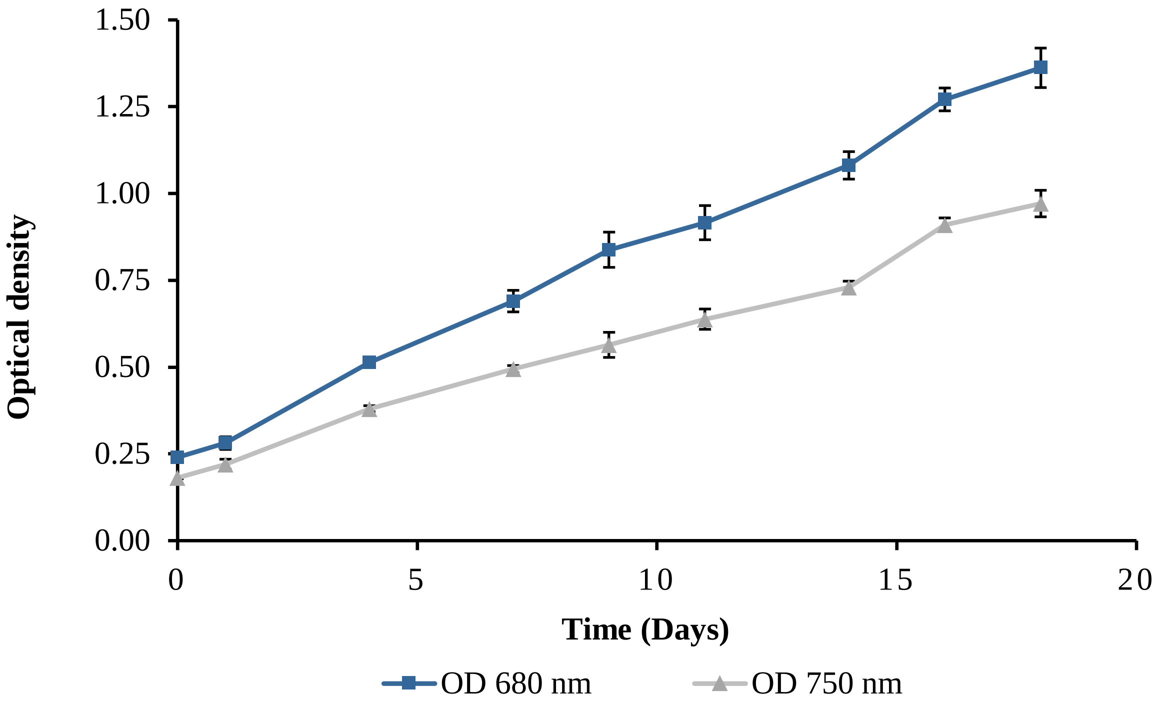

Figure 4.16-Optical density measured at 680 nm and 750 nm of *T. lutea* in culture for 18 days. Data is presented as mean values ± SD (*n*=3)

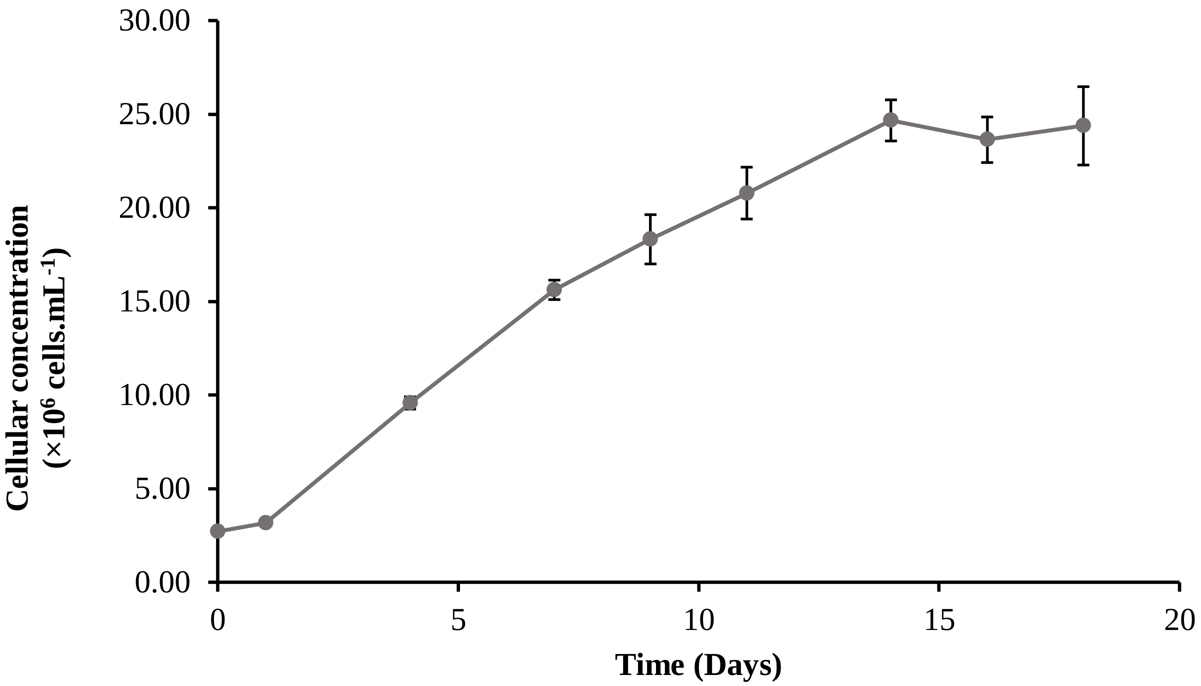

Figure 4.17-CC (x10^6 cells. mL^{-1}) of *T. lutea* maintained in culture for 18 days. Data is presented as mean values ± SD (*n*=3)

Generally, microalgae growth can be characterized by five main phases: an initial lag phase, for acclimatization to culture conditions, a log or exponential growth curve when growth is faster, a declining growth phase when the algae are still growing but slower than before, and finally a stationary growth phase, when the microalgae stabilize and is longer fast multiplying, followed by a death phase with a decrease in the number of viable cells (Blair et al., 2014; Sandgruber et al., 2023).

As shown in the growth curves, the curves plotted using the OD and cell concentration measurements (**Figures 4.16** and **4.17**) follow a typical microalgal growth curve. However, the curve drawn utilizing DW data does not correctly represent the expected growth curve for this microalga from day 9 onwards. This can be related to the loss of some biomass during the filtration process (Schagerl et al., 2022). In samples with higher cellular concentrations, the filtration process is longer and requires higher pressures to overcome filter clogging. These conditions could cause small leaks in the filters, resulting in loss of biomass prior to the drying and weighting process (Schagerl et al., 2022).

Conversely, *T. lutea* growth throughout time as estimated by CC enabled the identification of the corresponding growth phases from the lag phase until the stationary phase (**Figure 4.17**).

In addition, the curves obtained following OD readings at 680 nm and 750 nm (**Figure 4.16**) are also closer to a standard growth curve for this group of microorganisms. However, in this case, it was not possible to differentiate growth phases, or identify where these phases begin, toward the end of the curve.

Nevertheless, it must be taken into account that while absorbance can be used for estimation of biomass of a given microalga in a suspended culture, these values can be influenced by a wide variety of factors, such as pigment content or the presence of suspended particles and contaminants in the samples, and therefore are not an accurate assessment of biomass concentration in cultures (Chioccioli et al., 2014; Nielsen & Hansen, 2019; Thiviyanathan et al., 2024).

For this reason, to correctly estimate and monitor growth utilizing this technique, it was necessary to directly link values obtained through OD spectrometry with dry biomass weight and CC in cultures, through the construction of a standard linear correlation for each microalgae species and culture conditions (Griffiths et al., 2011; Sarrafzadeh et al., 2015; Nielsen & Hansen, 2019).

Considering this, correlations between OD vs. DW, and OD vs. CC were established, considering the data collected from both the 680 nm (**Figures 4.18** and **4.19**) and 750 nm (**Figures 4.20 and 4.21**) readings, since these were the two wavelengths used to monitor microalgae growth.

In the correlations involving the absorbance values obtained for the 680 nm wavelength, between DW and OD (**Figure 4.18**), the obtained linear equation was $DW = 0.7851 \times OD_{680} + 0.0566$ with a $r^2 = 0.8223$ ($\rho<0.001$), while between CC and OD (**Figure 4.19**), the equation was $CC = 20.906 \times OD_{680} - 0.8338$ with a $r^2 = 0.9314$ ($\rho<0.0001$).

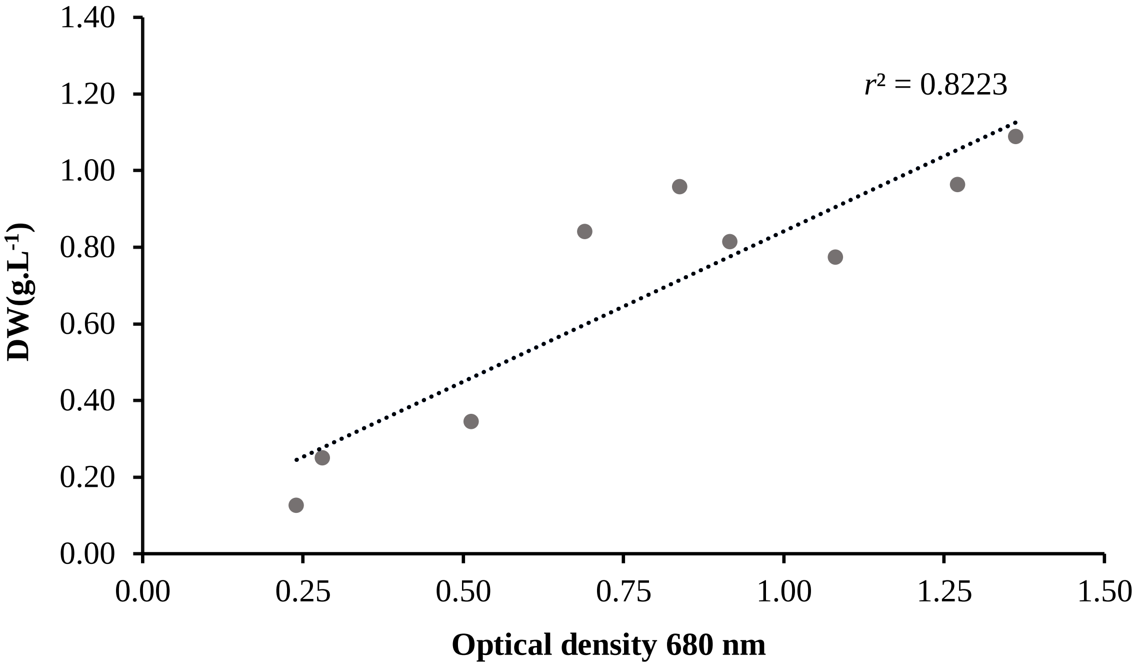

Figure 4.18 - Linear correlation between optical density measurements at 680 nm and dry weight (g. L^{-1}), with an r^2 coefficient of 0.8223 ($\rho<0.001$).

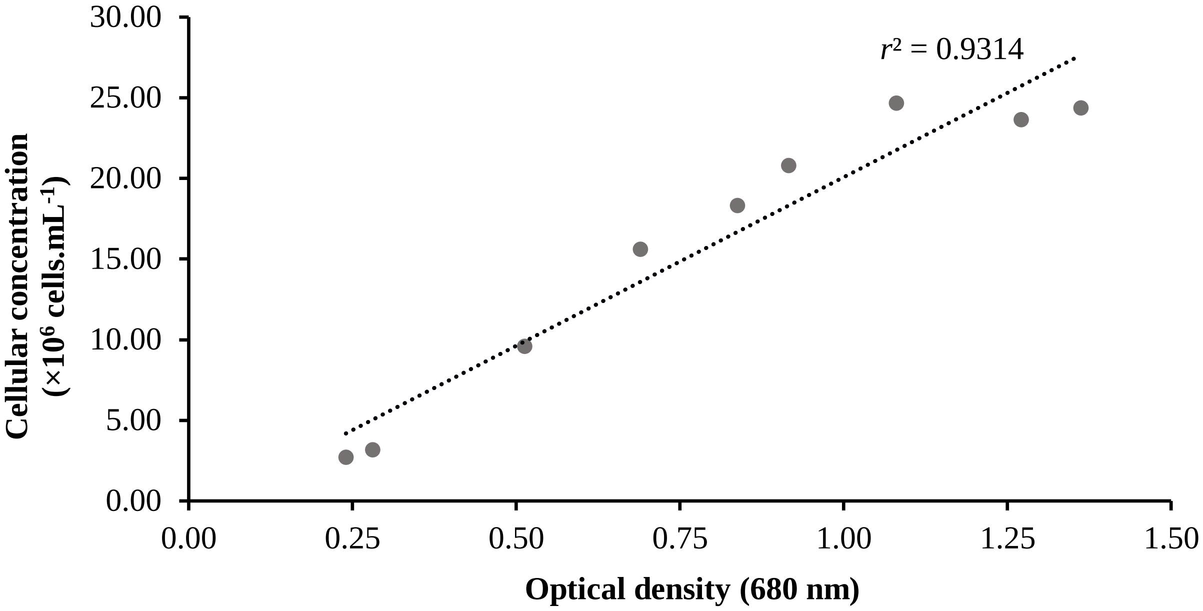

Figure 4.19-Linear correlation between optical density measurements at 680 nm and cellular concentration (x10^6 cells. mL^{-1}), with a r^2 coefficient of 0.9314 ($\rho<0.0001$).

Regarding the correlations where the OD measurements at 750 nm were utilized, the following equations were obtained: between DW and OD (**Figure 4.20**), DW = 1.1258 × OD750 + 0.0475, with a $r^2 = 0.8037$($\rho<0.01$), and between CC and OD (**Figure 4.21**), CC = 29.824 × OD750 – 0.9884, with a $r^2 = 0.9010$ ($\rho<0.0001$).

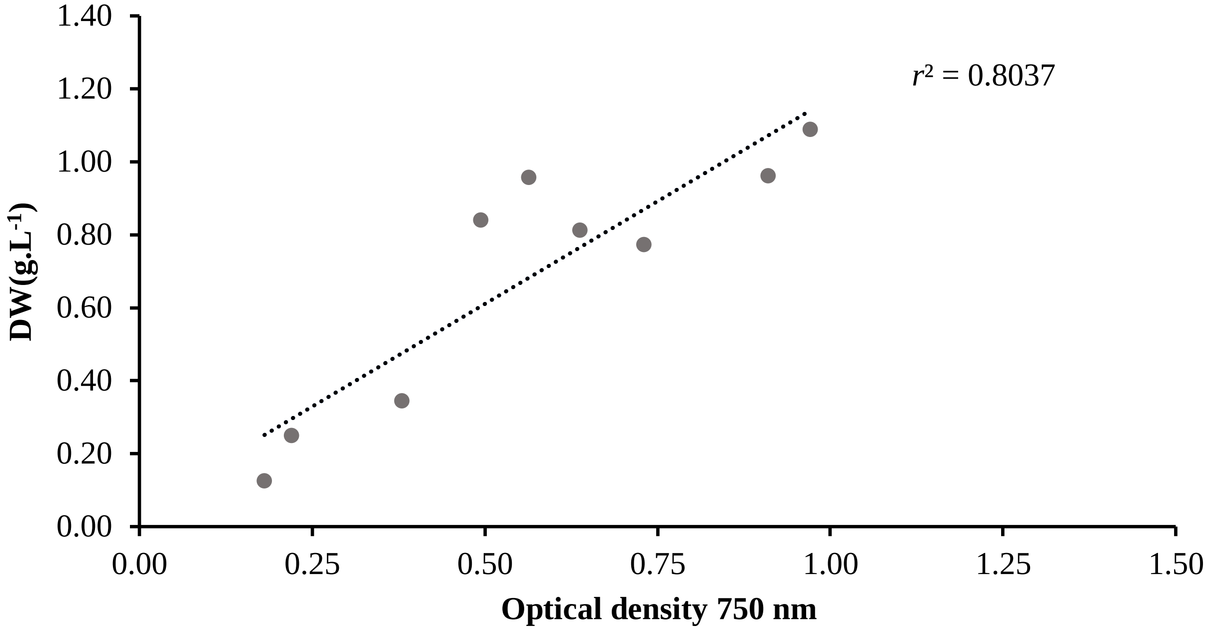

Figure 4.20-Linear correlation between optical density readings at 750 nm and dry weight (g. L^{-1}), with a r^2 coefficient of 0.8037 (ρ<0.01).

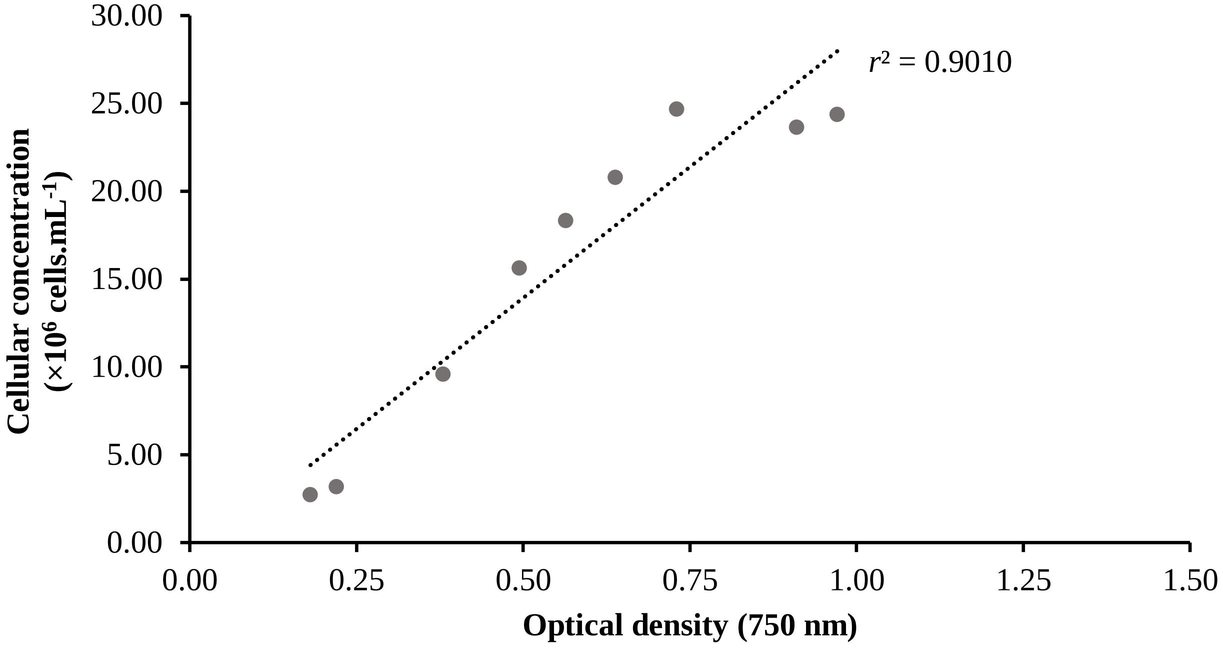

Figure 4.21-Linear correlation between optical density readings at 750 nm and cellular concentration (× 10^6 cells. mL^{-1}), with a r^2 coefficient of 0.9010 (ρ<0.0001).

As can be observed in the figures above (**Figures 4.18** to **4.21**), considering the results for both wavelengths, the relationship between DW and OD had lower correlation coefficients than the equations obtained with the CC and OD results, which can be related to the irregular growth curve obtained in the DW analysis (**Figure 4.15**). Even though the latter showed to be less representative of the evolution of *T. lutea* in culture, thus not corresponding to what occurred during the cultivation time, the correlations found were all significant.

However, the two linear equations obtained using the absorbance values at 680 nm had higher r^2 coefficients than those obtained under the 750 nm results, further demonstrating the higher reliability of these values.

While the goal of this procedure was to determine the best approach to follow the growth of *T. lutea* in culture before the co-culture trials, the fact that the microalga culture is not under axenic conditions, cannot be ignored, especially considering that it has been previously reported in literature that the presence of other microorganisms in microalgal cultures can interfere with both OD and DW measurements (Griffiths et al., 2011; Chioccioli et al., 2014; Nielsen & Hansen, 2019).

For instance, OD measurements at 750 nm are considered reliable for the estimation of microalgal growth in cultures since absorption at this wavelength is not heavily influenced by pigment content, unlike readings at 680 nm, a wavelength where chlorophyll (Chl) has high absorption (Griffiths et al., 2011; Nielsen & Hansen, 2019). However, the presence of contaminants, such as bacteria, in the culture medium, may cause interference in readings at 750 nm. In that case, readings at 680 nm may be more precise, since they measure absorbance values that are linked directly to Chl content and thus to photoautotrophic microorganisms like microalgae. Therefore, in mixed cultures, this is probably an expedited methodology to better distinguish between algae and contaminants present in the cultures (Griffiths et al., 2011; Nielsen & Hansen, 2019; Di Caprio, 2020).

Taken this together, the OD wavelength selected for CC estimation for the inoculation in the co-culture trials was 680 nm, since it had the highest correlation value (r^2=0.9314) among the assessed growth parameters. Moreover, OD readings and cell counts were selected to monitor *T. lutea*, during the following co-culture trials.

4.2. Identification and selection of bacterial isolates

To understand how specific bacteria can affect *T. lutea* during cultivation, it was essential first to identify the bacterial community associated with this marine microalga strain produced in tubular photobioreactors. Subsequently, bacteria were identified through 16S rRNA sequencing. According to the NCBI taxonomy database, 145 bacteria, split across 27 families and 37 genera, were identified in these cultures. These results are presented in **Figure 4.22** and **Table 4.3**.

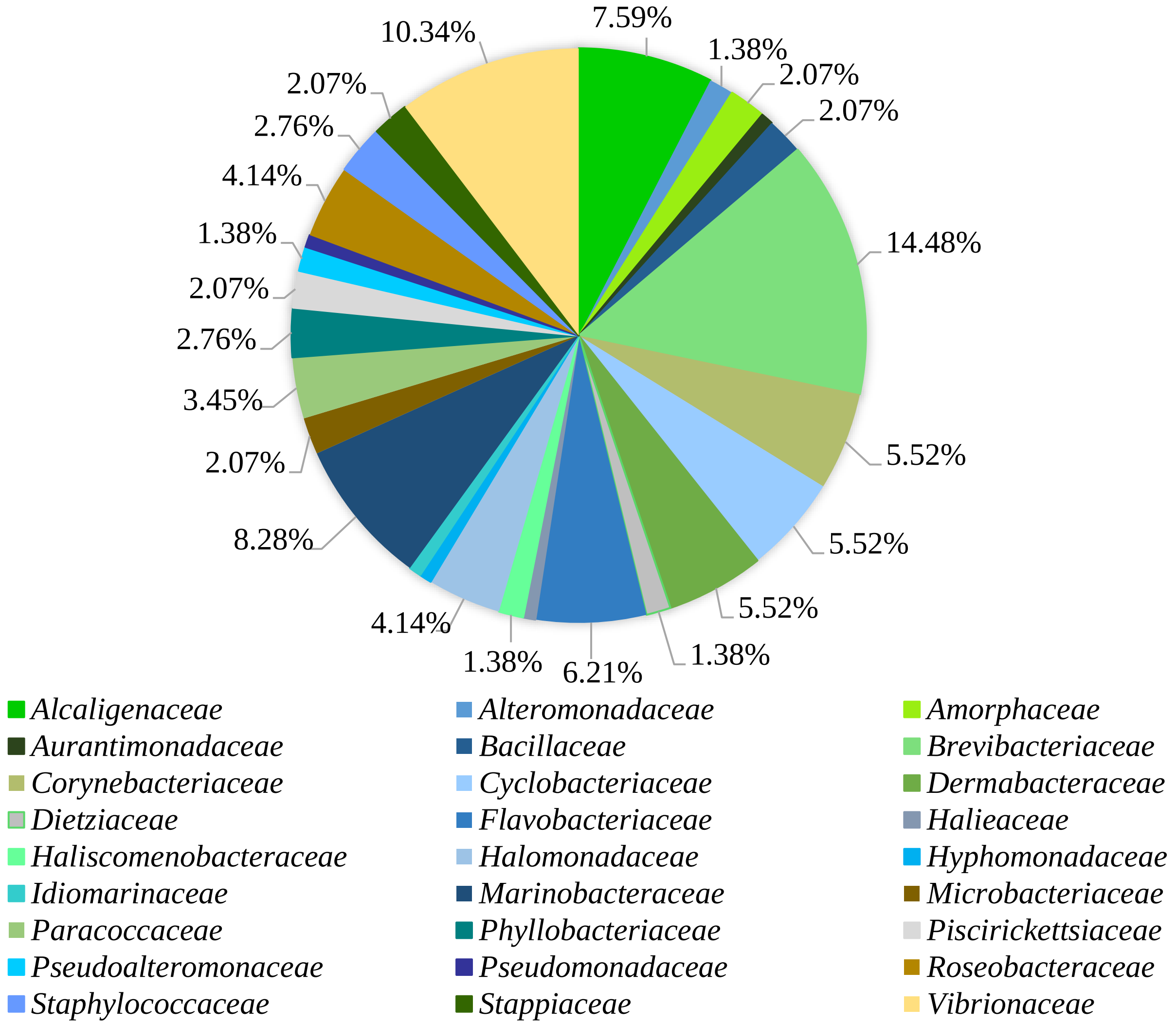

Figure 4.22-Relative abundance of the culturable bacterial families (%) isolated from cultures of *Tisochrysis lutea* produced in industrial tubular photobioreactors at Necton facilities between June and October (2019 and 2020).

Table 4.3-Description of families, genera and species identified in the 145 bacteria isolated from cultures of *Tisochrysis lutea* produced in industrial tubular photobioreactors at Necton facilities between June and October (2019 and 2020).

Identified families	**Identified genera**	**Identified species**	**Nº of isolates**
Alcaligenaceae	*Alcaligenes*	*Alcaligenes faecalis*	11
Alteromonadaceae	*Alteromonas*	*Alteromonas marina*	1
		Alteromonas sp.	1
Amorphaceae	*Acuticoccus*	*Acuticoccus yangtzensis*	2
	Amorphus	*Amorphus suaedae*	1
Aurantimonadaceae	*Martelella*	*Martelella mediterranea*	1
Bacillaceae	*Bacillus*	*Bacillus* sp.	1
	Oceanobacillus	*Oceanobacillus oncorhynchi*	1
	Priestia	*Priestia flexa*	1
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium epidermidis*	2
		Brevibacterium sp.	19
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium glyciniphilum*	8
Cyclobacteriaceae	*Cyclobacterium*	*Cyclobacterium marinum*	5
		Cyclobacterium sp.	3
Dermabacteraceae	*Brachybacterium*	*Brachybacterium paraconglomeratum*	8
Dietziaceae	*Dietzia*	*Dietzia aurantiaca*	2
Flavobacteriaceae	*Arenibacter*	*Arenibacter palladensis*	4
		Arenibacter sp.	1
	Feifantangia	*Feifantangia zhejiangensis*	1
	Flagellimonas	*Flagellimonas marinaquae*	2
	Xanthomarina	*Xanthomarina gelatinilytica*	1
Halieaceae	*Haliea*	*Haliea salexigens*	1
Haliscomenobacteraceae	*Phaeodactylibacter*	*Phaeodactylibacter luteus*	2
Halomonadaceae	*Halomonas*	*Halomonas alkaliphila*	3
		Halomonas sp.	1
		Halomonas titanicae	2
Hyphomonadaceae	*Hyphomonas*	*Hyphomonas* sp.	1
Idiomarinaceae	*Pseudidiomarina*	*Pseudidiomarina maritima*	1
Marinobacteraceae	*Marinobacter*	*Marinobacter adhaerens*	5
		Marinobacter flavimaris	1
		Marinobacter nauticus	3
		Marinobacter sp.	3
Microbacteriaceae	*Microbacterium*	*Microbacterium* sp.	3
Paracoccaceae	*Phycocomes*	*Phycocomes zhengii*	2
	Tritonibacter	*Tritonibacter mobilis*	3

Phyllobacteriaceae	*Nitratireductor*	*Nitratireductor* sp.	3
	Pseudohoeflea	*Pseudohoeflea suaedae*	1
Piscirickettsiaceae	*Methylophaga*	*Methylophaga muralis*	1
		Methylophaga sp.	2
Pseudoalteromonadaceae	*Pseudoalteromonas*	*Pseudoalteromonas* sp.	1
		Pseudoalteromonas tetraodonis	1
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas* sp.	1
Roseobacteraceae	*Ponticoccus*	*Ponticoccus alexandrii*	1
	Roseovarius	*Roseovarius aquimarinus*	1
		Roseovarius nanhaiticus	1
		Roseovarius sp.	1
	Sulfitobacter	*Sulfitobacter alexandrii*	1
		Sulfitobacter sp.	1
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus*	3
		Staphylococcus sp.	1
Stappiaceae	*Labrenzia*	*Labrenzia aggregata*	3
Vibrionaceae	*Vibrio*	*Vibrio alginolyticus*	10
		Vibrio brasiliensis	1
		Vibrio hepatarius	2
		Vibrio neocaledonicus	1
		Vibrio sp.	1

In general, *T. lutea* culture is diverse, containing a wide variety of bacterial families with only a few members each, compared with the total number of culturable isolates obtained.

Brevibacteriaceae, a family in the order *Micrococcales*, was the most abundant taxon, with 21 isolates corresponding to approximately 14.5% of the identified bacteria. Next in abundance were bacterial families associated with the class *Gammaproteobacteria*, such as *Vibrionaceae* and *Marinobacteraceae*, with relative abundances of about 10.3% and 8.3%, respectively, and *Betaproteobacteria*, like *Alcaligenaceae*, with roughly 7.5% of the total isolates (**Figure 4.22**).

Conversely, other bacterial genera also associated with the phylum *Pseudomonadota*, namely *Martelella* and *Hyphomonas*, included in the class *Alphaproteobacteria*, and *Haliea*, *Pseudidiomarina* and *Pseudomonas*, gammaproteobacterial isolates, registered the lowest abundance in cultures (0.69%), with only one identified bacterial isolate present in the culture per each of these genera **(Figure 4.22**).

Prior works have already identified many of these bacterial groups in coexistence with microalgae (Sandhya & Vijayan, 2022). For instance, a previous study showed that

Alphaproteobacteria and *Gammaproteobacteria* appeared as the predominant groups in xenic laboratory cultures of three strains of *Isochrysis galbana* following a 16S rDNA gene sequencing. These classes, included in the phylum *Pseudomonadota*, were also reported by the same study as prevalent in cultures of *Thalassiosira pseudonana* and *Nannochloropsis oceanica* (Ling et al., 2020). Considering the results of this study, the dominant bacterial groups in the established communities appear to remain the same across the tested strains, with differences between microalgal species cultures, occurring in lower taxonomical levels, such as genera (Ling et al., 2020).

In line with those results (Ling et al., 2020), the class *Gammaproteobacteria* was also dominant in *T. lutea* cultures, according to **Table 4.3** and **Figure 4.22**. Nevertheless, in these samples, *Alphaproteobacteria* had lower relative abundancies (15.86%) than the class *Actinomycetes* (28.97%).

Overall, in the samples of *T. lutea*, isolates belonging to the classes *Gammaproteobacteria* and *Actinomycetes* were prevalent, since more than 58% of identified bacteria in the cultures belonged to either one of these taxonomical groups, while the class *Saprospiria* was the most scarce among the identified classes, with a relative abundance surrounding 1.3%, corresponding to the two bacteria identified as *Phaeodactylibacter luteus*, a species classified as part of the family *Haliscomenobacteraceae*.

However, these variabilities in results regarding bacterial presence in microalgae cultures may be explained by shifts in the established communities, associated with differences in growth phases of each microalgae species and environmental or culture conditions to which they are exposed to (Grossart et al., 2005; Poretsky et al., 2014; Fulbright et al., 2018; Lee et al., 2019). Furthermore, the manipulation of cultures or exposure to outside factors can allow for external strains to enter the cultures, affecting interactions within the already existing community and influencing its usual composition and structure (Biondi et al., 2017; Fulbright et al., 2018).

Moreover, since we used specific culture media (MA and PCA) for isolation of bacteria, it is not possible to fully analyze the whole community, since we are limiting the number of isolated organisms to only those that are able to grow on the selected medium and under the chosen isolation and culture conditions (Carraro et al., 2011; Zampieri et al., 2021). Perhaps, the use of culture-independent methods, such as Next-Generation Sequencing (NGS), would allow for a broader view of the whole bacterial community, rather than only analyzing some isolated culturable bacteria, thus overcoming the limitations associated with the isolation and culture of bacteria from these complex samples (Cameron et al., 2021).

Despite *Brevibacteriaceae* having the highest abundance, other bacterial families, like *Flavobacteriaceae and Roseobacteraceae* showed higher diversity within them since we could identify various species belonging to different genera. However, we could not identify 19 of the 21 *Brevibacterium* isolates down to the species level (**Table 4.3**). This occurred in approximately 30% of the isolated bacteria, which meant that 44 of the 145 isolates could only be identified down to the genus level. For example, species identification was not possible in any of the bacteria included in the genera *Nitratireductor* or *Microbacterium* (**Table 4.3**).

As previously mentioned, identification of the bacterial isolates was achieved through the amplification and sequencing of the 16S rRNA gene, a gene often used as a marker to identify prokaryotic microorganisms, since it contains highly conserved regions common to all prokaryotes intertwined with highly variable regions (V1 to V9), which allow for the distinction of different taxonomic levels (Johnson et al., 2019). The primers used to identify the bacteria present in *T. lutea* cultures were the F27 and R1492, a pair of primers designed to amplify almost the entire length of the 16S gene (**Figure 4.23**), that, when the PCR reaction is successful, result in sequences with more than 1000bp (Heuer et al., 1997). By amplifying several of the variable regions of this gene, it is possible to reach higher taxonomical levels, such as genera, with better efficiency.

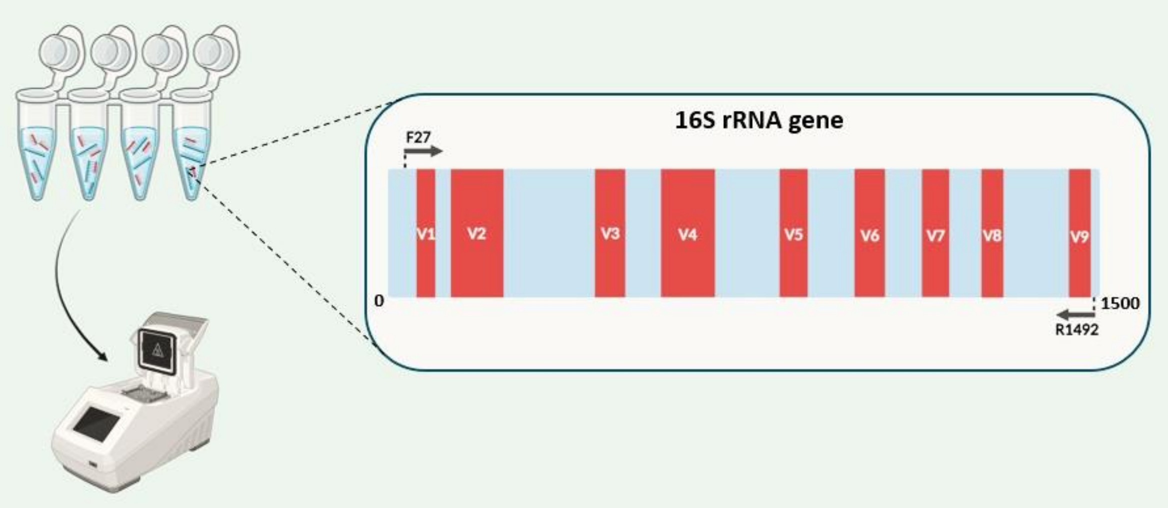

Figure 4.23-Amplyfication of 16S rRNA gene through a PCR reaction, using the primers F27 and R1492, designed to amplify not only conserved regions (blue areas), but also the 9 variable regions (V1 to V9) of this gene. Adapted from Yang et al. (2016). Created with BioRender.com

However, in several cases, it can still be challenging to distinguish between organisms at a species level, especially when they share the exact identification in the taxonomic levels above since only a few variations existed in the sequences of species included in the same genera (Poretsky et al., 2014). This limitation is further aggravated when the subject of the work involves the study of a community. Since the conserved regions of 16S are the target sequences of the utilized primers, it is possible to amplify bacterial or archaeal DNA that does not belong to the isolate of interest, if the extracted DNA is contaminated (Wintzingerode et al., 1997). Thus, if some analyzed bacteria were not completely isolated as we initially thought, that could result in some interferences and possible mismatches in the sequencing reads. Moreover, the presence of unculturable organisms that did not grow on the plates but could be present in the original samples of the cultures could also interfere with the sequencing procedure. Consequently, these issues may decrease the accuracy of the sequencing procedure, impacting the identification of the bacterial isolates and resulting in identifications only down to the genus level, as lower identity percentages are reached.

In their natural environment, microalgae interact with bacteria in several ways, ranging from negative to positive (Seymour et al., 2017). For this reason, the selection process for the bacteria with the potential to benefit *T. lutea* was necessary after obtaining the corresponding identification for the isolated bacteria. Considering this, bibliography research was conducted for each bacterial isolate individually before advancing to the co-culture trials.

First, species that, as far as we could tell, were not previously identified in either aquatic habitats or in association with microalgae and that could probably originate from a contamination in the manipulation of the samples were removed. This is the case of the isolate *Brevibacterium epidermidis*. This species is usually found in human skin, and while the *Brevibacterium* genus is not thought to be pathogenic, some strains, including *B. epidermidis*, have been associated with some opportunistic infections in compromised patients (Funke et al., 1997; Forquin-Gomez et al., 2014). Therefore, it is likely that its appearance in the isolated plates could result from contamination during the operation of the cultures, samples, or materials.

Another factor of exclusion was pathogenicity towards humans or towards microalgae, fish and other aquatic organisms since *T. lutea* is widely produced mainly for aquaculture (Gangadhar et al., 2020).For example, the genus *Staphylococcus* is known to have several pathogenic species (e.g., *S. aureus* and *S. saprophyticus*) that can affect not only humans but also other animals

(Rosenstein & Götz, 2013). Members of the *Vibrio* genus are also reported as pathogenic towards humans, larvae and fish. For instance, *V. alginolyticus*, a bacterium commonly found in marine environments, has been associated with several cases of infection in humans and animals, some of which have led to severe illness and even the death of the infected organism (K. Fu et al., 2016). This *Vibrio* bacterium is also viewed as a pathogen for fish, mollusks, and crustaceans, along with other *Vibrio* spp., such as *V. harveyi*, *V. anguillarum* and *V. parahyemolyticus* (Molina-Cárdenas & Sánchez-Saavedra, 2017; Le et al., 2020; Androutsopoulou & Makridis, 2023). For this reason, the presence of *Vibrio* spp. organisms in aquaculture cultures have been associated not only with loss of life but also heavy economic losses (Molina-Cárdenas & Sánchez-Saavedra, 2017). Moreover, interactions between *Vibrio* sp. and microalgae have been described as antagonistic, as not only *Vibrio* sp. can act as algicidal, but also some microalgae (e.g., *Isochrysis galbana* and *Tetraselmis suecica*) have demonstrated antimicrobial activity against *Vibrio* strains (Li et al., 2014; M. Wang et al., 2020; Molina-Cárdenas et al., 2022; Soto-Rodriguez et al., 2022).

Pseudomonas sp. is another genus that includes some harmful species, like *P. aeruginosa*, a bacterium, found in both terrestrial and aquatic environments, responsible for opportunistic infection cases, and *P. protegens*, a species associated with the production of compounds with adverse effects on several microorganisms, including the microalga *C. reinhardtii* (Kimata et al., 2004; Rose et al., 2021). However, despite this, some *Pseudomona*s strains (e.g., *P. composti*) showed beneficial effects on various microalgae in previous studies, promoting growth or lipid content (Fukami et al., 1997; Sureshkumar et al., 2014; Berthold et al., 2019).

Since it was not possible to achieve an accurate species identification of the single *Pseudomonas* sp. isolate (isolate 19) found in *T. lutea* cultures, it was not possible to assume that this bacterium isolate was a pathogen or algicidal species or a potentially advantageous organism for the microalga. Taking this into consideration, this isolate was selected for further trials. Other isolates, whose identification down to the species level was not possible, like *Methylophaga* sp., *Brevibacterium* sp., *Nitratireductor* sp. and *Microbacterium* sp., were also chosen for the co-culture trials, following the same criteria applied to the *Pseudomonas* sp. isolate.

On the other hand, species associated with beneficial effects on the microalgae cultures or known producers of important bioactive metabolites for the microalgae were selected for the co-culture trials. For instance, bacteria included in the order *Rhodobacterales* have often been associated with the production of nutrients necessary for microalgae. An example is *Sulfitobacter* sp., which promoted the growth of a diatom, through the secretion of the phytohormone indole-3-acetic acid (IAA), a known auxin (Amin et al., 2015).

Moreover, *Halomonas* spp. (e.g., *Halomonas alkaliphila*) were reported as cobalamin producers, and, in a previous study, a *Halomonas* strain was able to sustain the growth of two different microalgae species (Croft et al., 2005; Nef et al., 2022).

Furthermore, despite being associated with some opportunistic infections, *Alcaligenes faecalis* can produce cobalamin, and some *Alcaligenes* strains have been reported for their growth-promoting effects on microalgae, so, for this reason, it was also chosen for further trials (Fukami et al., 1997; Prabaningtyas et al., 2021).

In the end, the selected species were inoculated into liquid media, identical to the culture media chosen for the growth of bacteria during the co-culture trials (described in the previous **section 3.6**) (**Figure 4.24**). Several isolates, identified as *Roseovarius nanhaiticus*, *Sulfitobacter alexandrii*, *Alteromonas marina*, *Arenibacter palladensis* and others did not grow on the selected liquid medium (PCB), and for this reason, these isolates were also excluded from the following trials. However, since some of these bacteria are commonly found in marine environments, it is possible that the used liquid media did not have the necessary components for the development of these strains or that other conditions, such as the pH, were not adequate for the growth of the listed bacterial isolates. Hence, if another culture medium (e.g., Marine Broth) was used, perhaps it would be possible to mimic optimal growth conditions for these strains, thus allowing them to develop and grow.

Figure 4.24- Growth of bacterial isolates in liquid medium (Photo by Tamára Santos)

In addition, in the case of repeated isolation of bacteria belonging to a given species (e.g., *Corynebacterium glyciniphilum*, *Tritonibacter mobilis, Halomonas alkaliphila* and *Halomonas titanicae*), the isolates registering higher OD_{600nm} (**Supplementary Data 1**), were the selected for the co-culture experiments.

The exception to this criterion was the previously selected isolates based only on the genus identification since identification down to the species level was not achieved (e.g., *Brevibacterium* sp.). For these, if growth on the liquid media occurred, then those isolates were selected. Conversely, failure of growing in the selected media led to the exclusion of those bacteria for the next stage of the project.

Therefore, this selective procedure allowed us to choose 40 bacteria for the Phase I co-culture trials (**Table 4.4**).

Table 4.4-Identification of the 40 bacterial isolates selected for the co-culture trials with *T. lutea*, according to the BLASTn against the nr/nt NCBI database.

Extraction Code	Probable ID	Query Cover (%)	% Identity	GenBank Accession code
7	*Marinobacter nauticus*	100.0	100.0	MT325887.1
8	*Marinobacter* sp.	97.00	93.85	KR004809.1
14	*Roseovarius aquimarinus*	100.0	99.38	OR654359.1
17	*Corynebacterium glyciniphilum*	100.0	99.67	MK414897.1
19	*Pseudomonas* sp.	100.0	99.62	MT187898.1
22	*Halomonas alkaliphila*	100.0	99.74	CP024811.1
33	*Pseudoalteromonas* sp.	100.0	99.84	MN889178.1
45	*Sulfitobacter* sp.	92.00	95.53	MZ262822.1
47	*Arenibacter* sp.	100.0	99.28	MN784288.1
60	*Halomonas titanicae*	100.0	99.71	CP054580.1
69	*Hyphomonas* sp.	100.0	97.96	MK029430.1
73	*Marinobacter* sp.	100.0	99.06	CP045367.1
74	*Brevibacterium* sp.	100.0	94.37	LC133762.1
75	*Cyclobacterium* sp.	100.0	92.40	KY770661.1
78	*Microbacterium* sp.	100.0	98.84	MH712125.1
80	*Roseovarius* sp.	100.0	100.0	MH650971.1
81	*Priestia flexa*	100.0	99.72	MW110779.1
84	*Haliea salexigens*	99.00	100.0	OR654371.1
86	*Brevibacterium* sp.	100.0	100.0	ON810589.1
88	*Flagellimonas marinaquae*	100.0	99.29	AP027268.1

95	*Methylophaga muralis*	99.00	98.21	KX279370.1
96	*Marinobacter adhaerens*	99.00	97.74	MT507042.1
99	*Methylophaga* sp.	99.00	97.32	OQ055141.1
103	*Cyclobacterium marinum*	88.00	95.28	LT601135.2
105	*Brevibacterium* sp.	96.00	96.95	MF431766.1
106	*Nitratireductor* sp.	100.0	99.84	MT457434.1
107	*Nitratireductor* sp.	100.0	100.0	MT457434.1
108	*Microbacterium* sp.	100.0	90.91	MK967164.1
109	*Feifantangia zhejiangensis*	100.0	99.84	NR_148332.1
114	*Cyclobacterium* sp.	94.00	92.55	KY770661.1
116	*Alcaligenes faecalis*	100.0	93.16	MH029146.1
123	*Tritonibacter mobilis*	93.00	98.33	MK493584.1
131	*Brevibacterium* sp.	100.0	100.0	MT433875.1
132	*Pseudidiomarina maritima*	100.0	99.59	KT986182.1
133	*Microbacterium* sp.	100.0	99.83	KU560428.1
138	*Cyclobacterium marinum*	100.0	100.0	LT601135.2
147	*Cyclobacterium* sp.	100.0	93.55	LT630358.2
149	*Marinobacter adhaerens*	100.0	95.09	MT507043.1
156	*Marinobacter* sp.	100.0	99.83	MT457438.1
157	*Nitratireductor* sp.	100.0	100.0	MT457434.1

4.3. Microalga-bacteria co-culture (Phase I)

4.3.1. Isolate trial 1 (IT1)

For the first co-culture trial with *T. lutea*, bacterial isolates with reported beneficial effects on microalgae growth and associated with the production of bioactive compounds were selected. The assay lasted for 7 days for most of the selected isolates. However, for 8 of the selected strains, the trial ended on day 5 upon culture collapse. The effects of the bacteria on the microalga were assessed through cell counts immediately after inoculation and on the last day of the trial (either day 5 or 7) (**Figure 4.25**).

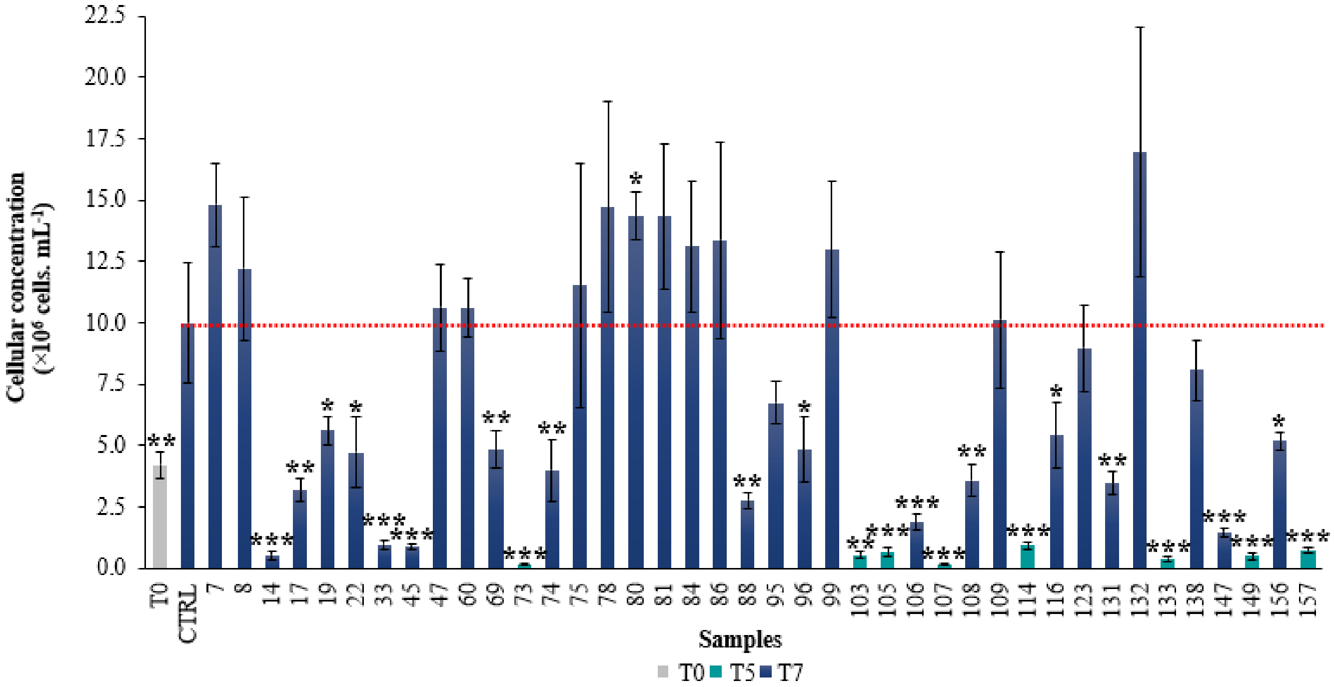

Figure 4.25-Cellular concentration of *T. lutea* (×10^6 cells. mL^{-1}), immediately after inoculation (T0) and at the end of the trial (T5 and T7), without (CTRL) and in co-culture with bacterial isolates (7 to 157). Bars correspond to the mean values ± SD. Bars marked with "*" represent significant difference levels (ρ<0.05, ρ<0.01, ρ<0.001, ρ<0.0001, respectively) when compared to the CTRL (ANOVA One-way with Dunnett multiple comparison test, ρ<0.05).

As shown in **Figure 4.25**, of the 40 tested strains, 25 showed significant differences compared to the CTRL cultures. Of those, 24, corresponding to 60% of the total tested bacteria, had a negative impact on the growth of the microalga, having lower cellular concentrations than the CTRL (9.98 ± 2.46 ×10^6 cells. mL^{-1}).

Furthermore, 18 of those 24 isolates did not contribute to *T. lutea* growth since the addition of these bacteria to the microalgal cultures probably resulted in algicidal activity with CC of *T. lutea* being lower at the end of the trial at the inoculation day (4.20×10^6 cells. mL^{-1}).

In addition, for 8 of these bacteria the trial ended prematurely (day 5) upon culture collapse (**Figure 4.26**).

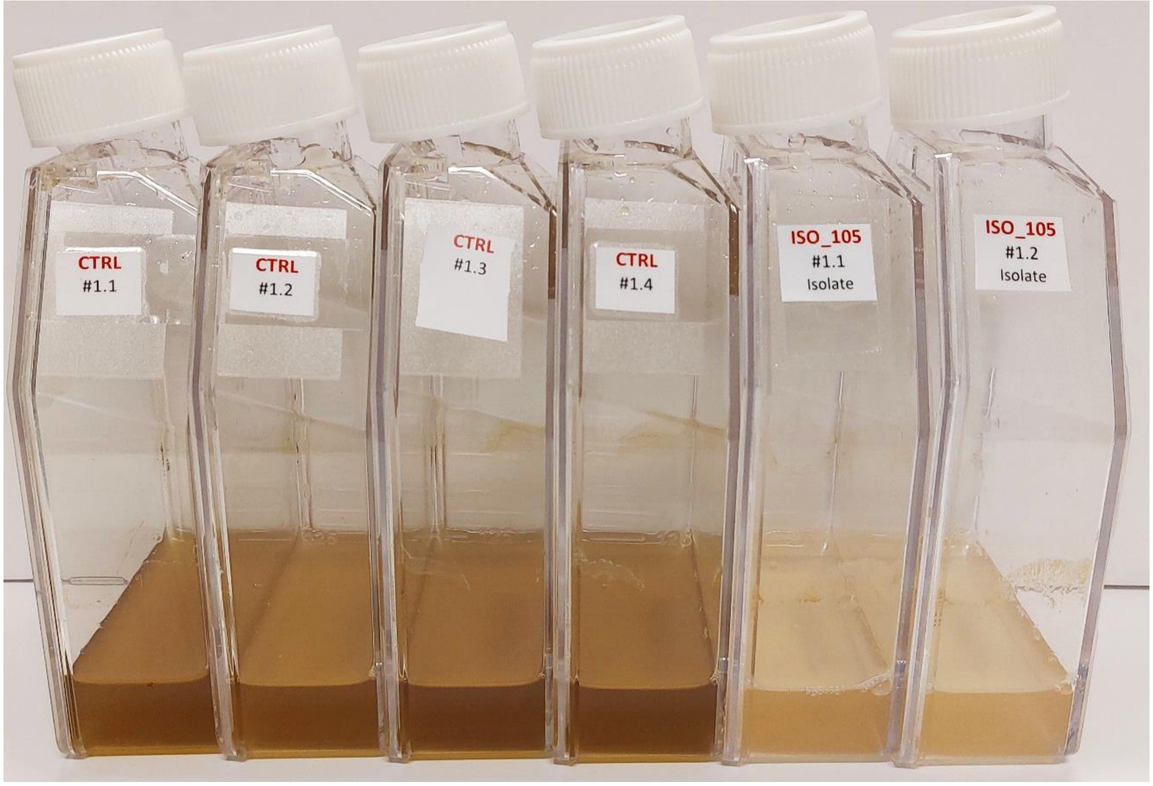

Figure 4.26-Visual aspect of cultures of *T. lutea* after 5 days of co-culture without the addition of the selected bacterial isolates (CTRL) and with the addition of isolate 105, identified as *Brevibacterium* sp. (ISO_105)

Among these strains were isolates identified as *Nitratireductor* sp. bacteria. In a previous study, co-culture of *Nitratireductor* sp. with *N. oceanica* increased biomass production for this microalga (Liu et al., 2020). However, the co-culture of *T. lutea* with any of the *Nitratireductor* sp. isolates tested in this assay (isolates 106, 107 and 157) resulted in algal cell death (**Figure 4.25**).

The same happened in several other tested isolates belonging to the genera *Roseovarius* and *Sulfitobacter*, belonging to the family *Roseobacteraceae* (isolates 14 and 45), and the genus *Marinobacter*, included in the family *Marinobacteraceae* (isolates 73 and 149). Bacteria in these families are frequently found in cultures and have previously been reported as beneficial for microalgae (Amin et al., 2009, 2015; Buchan et al., 2014; Cooper & Smith, 2015). However, adding these isolates to *T. lutea* was linked to a significant decrease in algal growth during co-cultivation. Furthermore, for the two *Marinobacter* strains, the experiment ended following culture collapse, at day 5, although isolate 80, identified as a *Roseobacteraceae* bacterium belonging to the genus *Roseovarius* sp., had a positive effect on the cultures since its inclusion in *T. lutea* cultures significantly enhanced their growth (**Figure 4.25**).

Moreover, while no other bacteria assessed registered significant improvements in the growth of *T. lutea*, we observed several isolates with the potential to enhance the productivity of this microalga, achieving higher CC values than that of the CTRL. Among these, isolates 7, 8, 60, 84, 99 and 132, belonged to class *Gammaproteobacteria*, 47 and 109 to the *Flavobacteriia*, 78 and 86 to *Actinomycetes*, whereas 75 and 81 were identified as part of *Cytophagia* and *Bacilli*, respectively.

Since the conditions of cultivation were the same across the experiment, these different outcomes may be related to the specific needs of each organism, consequently affecting the interactions between members of these communities. Thus, these results seem to be in concordance with literature reports that the effects of bacteria on microalgae and vice-versa may be species-specific or vary according to the available conditions, needs and physiological state of each of the involved organisms (Ahamed et al., 2015; Fuentes et al., 2016; Tait et al., 2019).

In addition, we need to consider that these bacterial isolates are being inserted into an already established xenic community, meaning that the effect of the addition of these bacteria to the cultures may be causing shifts in the surrounding community, that can influence interactions among microorganisms, thus indirectly affecting the microalga.

Considering these results, the 13 best performing strains were selected for further trials. In addition, given the link between vitamin B_{12} and *T. lutea* in natural settings, mentioned previously in the introduction (**section 1**), 2 bacterial strains reported in the literature as cobalamin producers were also selected for the subsequent trial, despite not showing any clear improvements in microalga productivity when in co-culture with *T. lutea* during this assay. The list of the 15 bacterial isolates selected for the second trial is given in **Table 4.5**.

Table 4.5-List of bacteria selected for the second trial

Isolate Code	ID
7	*Marinobacter nauticus*
8	*Marinobacter* sp.
22	*Halomonas alkaliphila*
47	*Arenibacter* sp.
60	*Halomonas titanicae*

75	*Cyclobacterium* sp.
78	*Microbacterium* sp.
80	*Roseovarius* sp.
81	*Priestia flexa*
84	*Haliea salexigens*
86	*Brevibacterium* sp.
99	*Methylophaga* sp.
109	*Feifantangia zhejiangensis*
116	*Alcaligenes faecalis*
132	*Pseudidiomarina maritima*

4.3.2. Isolate trial 2 (IT2)

The isolates selected according to the results of the previous trial were tested again in co-culture with *T. lutea.*

The assay lasted 8 days, and sampling occurred on days 0, 3, 6 and 8. The collected samples were utilized for cell counts and OD readings, allowing us to assess the state of the cultures throughout the experiment. These results are presented in **Figures 4.27** and **4.28**.

According to the data presented in **Figure 4.27**, the addition of the isolate identified as *Haliea salexigens* (isolate 84) was correlated with an apparent enhancement in the productivity of *T. lutea* following 8 days of co-culture compared with the CTRL cultures.

On the other hand, the insertion of isolates 22 and 60, identified as members of the genus *Halomonas*, and 81, identified as *Priestia flexa*, in the cultures had a negative effect on the cultures, resulting in lower CC of *T. lutea*, at the end of this assay (**Figure 4.27**).

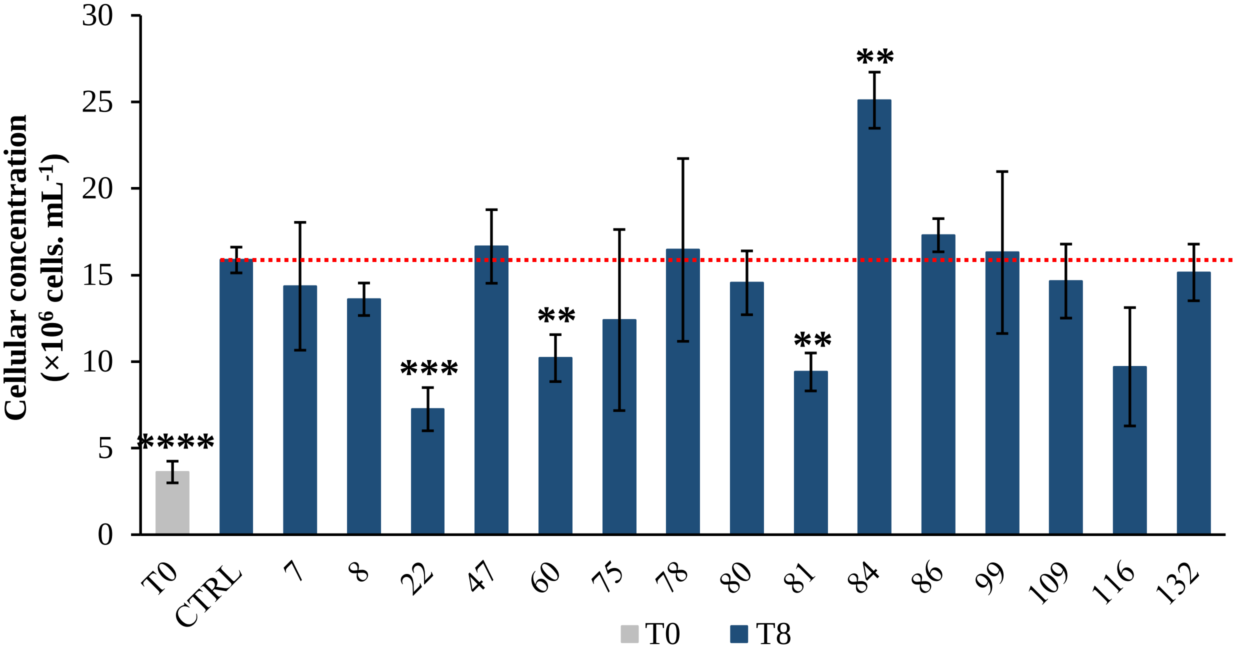

Figure 4.27-Cellular concentration (x10^6 cells. mL^{-1}) of *T. lutea* after inoculation (T0) and after 8 days of culture (T8) with bacterial isolates and without the selected bacteria (CTRL). Bars correspond to the mean values ± SD. Bars marked with "*" represent significant differences when compared to the CTRL (ANOVA One-way with Dunnett multiple comparison test, $\rho<0.05$).

When compared to the results of the previous experiment with these isolates (**section 4.3.1**), *T. lutea* cultures inoculated with isolates 7, 8, 60, 75, 78, 80 and 132 registered similar CC across both assays, while isolates 47, 84, 86, 99, 109 and 116 appeared to have an increase in CC in the second trial. In addition, the culture containing isolate 22 also had a growth increase in this assay despite still having significantly lower CC than the CTRL after 8 days of cultivation. On the contrary, the cultures with the addition of isolate 81 registered a decrease in the number of cells compared to the results obtained in the first trial, thus having lower CCs than the CTRL cultures used for this assay. However, it is noteworthy to point out that the CTRL cultures showed better productivity in the second trial, represented by the higher CC values achieved in this experiment ($15.87\pm0.74 \times10^6$ cells. mL^{-1}) than at the end of the first assay ($9.98\pm2.46 \times10^6$ cells. mL^{-1}).

Through visual observations of the cultures, we can hypothesize that these alterations in the results across both assays, particularly in the CTRL, may be associated with the optimization of the culture conditions by changing the type of culture flasks and, by consequence, the agitation. With lower agitation, it was possible to observe some flocs of the microalga, probably indicating that agitation was not entirely adequate.

Furthermore, in this assay, samples were collected at the beginning and end of the trial and in previously selected middle points (days 3 and 6). CC was also analyzed at those points to investigate the evolution of the cultures throughout the experiment. Those data were used in the construction of growth curves for each of the isolates. In **Figure 4.28**, we can observe the growth curves of isolates 22, 60, 81 and 84 since these isolates showed apparent differences from the CTRL cultures at the termination of the trial.

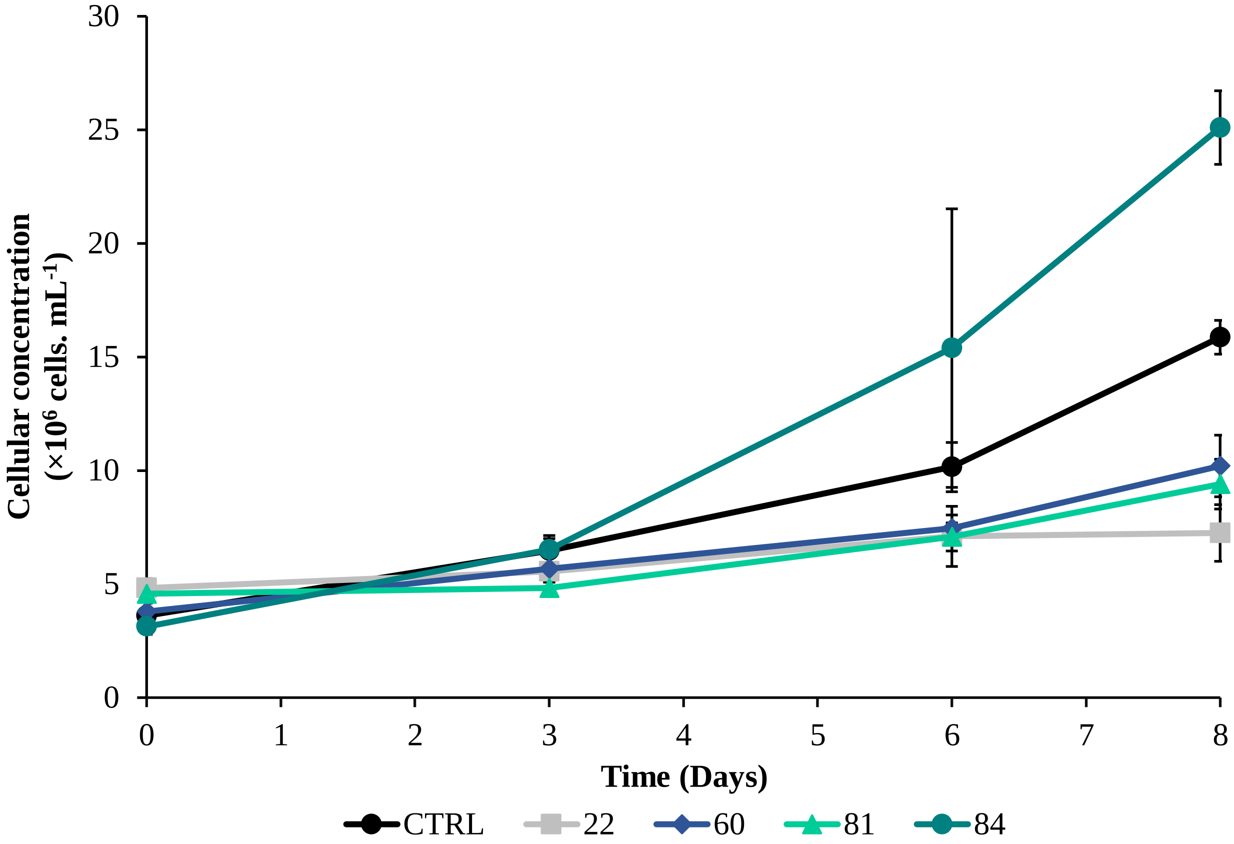

Figure 4.28-Cellular concentration of *T. lutea* (x10^6 cells. mL^{-1}) in the control cultures without the addition of bacterial isolates (CTRL) and in the cultures containing bacterial isolates 22, 60, 81 and 84, throughout the duration of the trial, with sampling points at days 0, 3, 6 and 8.

According to the growth curves drawn in **Figure 4.28**, the cultures containing the added bacterial isolates seemed similar to those where no additional bacterial isolate was added (CTRL) in the first 3 days of culture, most likely corresponding to the time needed to adapt to the culture conditions.

From day 3 onwards, the period most likely to correspond to the exponential growth phase of the microalga. The different bacterial treatments seem to have provoked alterations in the established community, influencing *T. lutea* growth, since, as it can be visualized in the graph, at day 6, the cultures inoculated with the bacterium 84 started to emerge above the CTRL. In contrast, the cultures where the isolates 22, 60 and 81 were added, appeared to register lower concentrations than the CTRL. These tendencies remained until the end of the experiment, seeing that, at day 8, the treatment cultures with isolate 84 achieved the highest CC among all. In contrast, the cultures containing the bacteria 22, 60 or 81 ended the trial with values below that of the CTRL cultures.

To understand how adding the selected bacterial isolates influenced bacterial concentration in the co-cultures, samples collected immediately after inoculation (T0) and at the end of the experiment (T8) were also used to estimate the bacteria present in the cultures, through the CFU method. The results are represented in **Figure 4.29** for the bacteria grown in MA and **Figure 4.30** for the ones grown in the PCA medium.

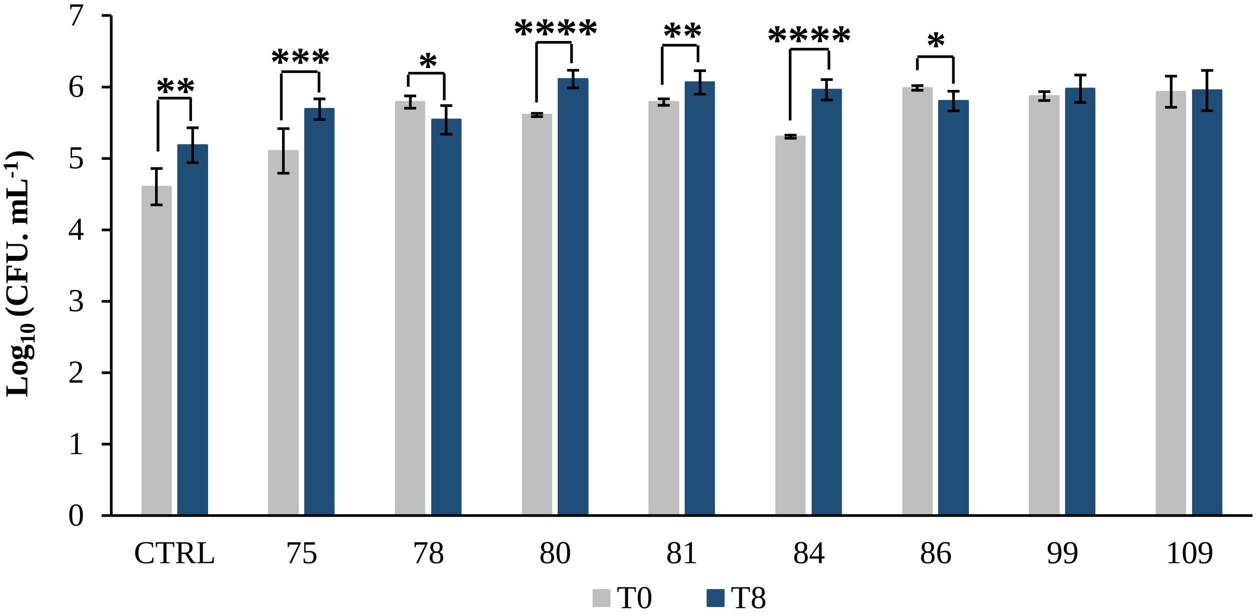

Figure 4.29-Evolution of the number of bacteria (Log$_{10}$ (CFU. mL^{-1})) present in *T. lutea* cultures during the trial, without (CTRL) and with the addition of bacterial isolates 75, 78, 80, 81, 84, 86, 99 and 109, measured through the count of colony-forming units in marine agar (MA) plates, immediately after inoculation (T0) and at the end of the assay (T8). Bars correspond to the mean values ± SD. Bars marked with "*" represent significant differences between T0 and T8 for each of the tested treatments (Unpaired T-test with Welch's correction, $\rho<0.05$).

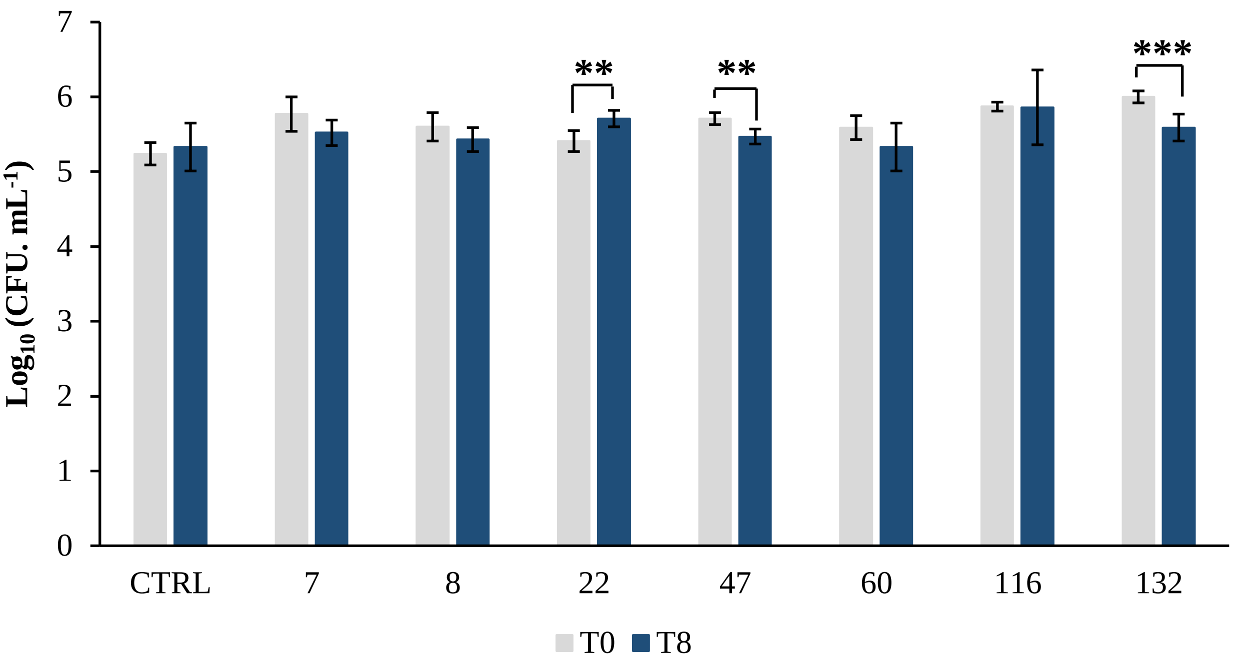

Figure 4.30-Evolution of the number of bacteria (Log$_{10}$ (CFU. mL^{-1})) present in *T. lutea* cultures during the trial, without (CTRL) and with the addition of bacterial isolates 7, 8, 22, 47, 60, 116 and 132, measured through the count of colony-forming units in plate count agar (PCA) plates, immediately after inoculation (T0) and at the end of the assay (T8). Bars correspond to the mean values ± SD. Bars marked with "*" represent significant differences between T0 and T8 for each of the tested treatments and controls (Unpaired T-test with Welch's correction, $\rho<0.05$)

According to **Figures 4.29** and **4.30**, the treatments with the bacteria 22, 75, 80, 81 and 84 showed increased total bacteria concentration from T0 to T8. Moreover, it was also possible to verify an increase in the counted bacteria in the CTRL cultures, containing only the original community present in the industrial samples of the *T. lutea* cultures, but only on those grown in the MA plates. These results are in accordance with literature reports, where an increase in the total bacterial concentration at the latest stages of microalgae growth is expected (Sureshkumar et al., 2014).

Furthermore, the results related to *T. lutea* growth (**Figure 4.27**) during the same period also need to be considered. When looking at the results obtained for both microalgae and bacterial growth regarding the cultures containing isolates 22, 75, 80, 81 and 84, while the bacterial concentration increases for all treatment groups, different responses can be observed in the CC of *T. lutea* (**Figures 4.27, 4.29-4.30**). For instance, the inoculation with bacterial strains 22 and 81 resulted in a decline in *T. lutea* growth compared to the CTRL cultures (**Figure 4.27**). Thus, these results indicate that adding these isolates to the cultures can be associated with antagonistic effects on *T. lutea*. Moreover, we can hypothesize that this response may be related to nutrient competition rather than an algicidal interaction since, despite the reduced growth, *T. lutea* still reached higher cell concentrations after 8 days of co-cultures with these bacteria than the values registered immediately after the inoculation (T0). Opposite effects were observed in the replicates containing the bacterium 84 inoculum, seeing that *T. lutea* was able to achieve higher CC in co-culture with this isolate than in the CTRL cultures, thus pointing to the existence of a mutually beneficial interaction between the established community and the microalga, following the addition of the *Haliea salexigens* strain to the culture (**Figures 4.27-4.28**).

Conversely, microalgal cultures to which isolates 47, 78, 86 and 132 were added registered a decreased total number of bacterial colonies (**Figures 4.29-4.30**). A possible justification for this outcome is that in response to the addition of these bacteria to the culture, the microalga or other microorganisms present in the cultures reacted negatively, thus inducing an antimicrobial response associated with the decline in the concentration of total bacteria following the 8 days of co-culture. However, this response did not seem to have influenced microalgal growth since cultivating *T. lutea* with these bacteria had similar results to that of the CTRL.

Nonetheless, according to the results represented in **Figures 4.29 and 4.30**, there seems to be a higher variation between T0 and T8 in the concentration of bacteria grown in MA plates, with the CTRL. In fact, 6 of the 8 treatments showed significant differences between the beginning and end of the assay, while for the bacteria inoculated in PCA plates, the CTRL counts remained similar throughout the trial, along with most of cultures containing the bacterial isolates. In the latter case, only 3 of those showed significant differences between days 0 and 8. However, MA is a culture media enriched in nutrients required by most heterotrophic marine bacteria that can mimic the natural conditions these organisms encounter in their natural marine habitats (Rodrigues & de Carvalho, 2022) . Thus, it is likely that MA is a better option to grow a broader range of the bacteria found in *T. lutea* cultures than PCA, a general non-selective medium for bacterial growth (McIlwaine et al., 2024). In addition, several of the bacteria isolated from *T. lutea* (**section 4.2**) could not grow on PCA but did grow on solid MA plates, further reinforcing this hypothesis.

Moreover, the NB^+ culture medium selected for all the growth trials is a medium that also contains nutrients, such as nitrogen, phosphorus, iron and vitamins, essential for marine microalgae, such as *T. lutea*. Since these nutrients are also crucial for the growth of other microorganisms (e.g., heterotrophic marine bacteria) it is possible that this liquid medium also promoted bacterial growth on the co-cultures (Rodrigues & de Carvalho, 2022).

In addition to all these results, it must be considered that plate counting methods, such as CFU, often underestimate the bacterial presence in microalgae culture. These methods do not consider bacteria that are not able to grow under the selected culture conditions, such as culture medium and temperature (Di Caprio, 2020). Thus, the results obtained with this technique may not accurately measure the total bacterial concentration in the *T. lutea* cultures.

4.3.3. Biochemical analysis

4.3.3.1. Fatty acids profile

The FAME profile was determined in the biomass at the beginning and end of the experiment both from the CTRL cultures and the co-cultures (containing the selected bacterial isolates from 7 to 132) (**Table 4.6**).

The total FAME concentration across samples ranged between 23.26 mg. g^{-1} (cultures with the bacterial isolate 80, *Roseovarius* sp.) and 138.13±34.57 mg. g^{-1} (CTRL) (**Table 4.6**). The value determined for the CTRL is higher to the previously reported reference values for this species. However, depending on the growth phase at the time of the biomass harvesting and extraction conditions the FA content can go up to 320 mg. g^{-1} (Gnouma et al., 2017; Andriopoulos et al., 2022; García-García et al., 2024). Furthermore, it is noteworthy that variations in each of the estimated FAME concentrations were noticed among the CTRL, resulting in higher variations among biological replicates, thus contributing to more considerable errors and probable deviations from actual concentration values.

Overall, the CTRL registered total concentrations of 37.06±9.83 mg. g^{-1}, 37.68±7.61 mg. g^{-1} and 63.39±17.13 mg. g^{-1} of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, respectively (**Table 4.6**). Thus, these samples had a higher content of PUFA, about 46% of total FAME content, and nearly equal portions of SFA and MUFA, equivalent to 27% each. These contents were similar to reference values previously mentioned in other works (Gachelin et al., 2021; Y. Y. Wang et al., 2022; Wu et al., 2023). Also, according to previously reported data, within the detected PUFA, the *n*-3 FA were the most abundant, resulting in lower *n*-6/*n*-3 ratios, with positive impact on fish and human health (Strobel et al., 2012; Almutairi, 2020; Nagappan et al., 2021).

Regarding the results obtained for the tested co-cultures containing the selected bacterial isolates, concentrations ranged from 7.26 to 29.54±1.39 mg. g^{-1}, for total SFA, 6.41 to 30.00±1.39 mg. g^{-1}, regarding the total MUFA, and 9.60 to 48.90±4.58 mg. g^{-1} for the total PUFA, thus corresponding to SFA values ranging from 27% to 37%, 25% to 30% for MUFA and between 32% to 45% of PUFA, with the lower values corresponding to the results obtained for the replicates with the isolate 80, identified as *Roseovarius* sp., and the highest with the values registered in the group 8, where the *Marinobacter* sp. isolate was added. Moreover, the cultures containing isolates 7 and 8, identified within the genus *Marinobacter* (*Marinobacter nauticus* and *Marinobacter* sp.), reached higher concentrations of total FAME, with 95.46±1.69 mg. g^{-1} and 108.44±7.36 mg. g^{-1}, respectively (**Table 4.6**). Previously, a strain also identified as *Marinobacter* sp. has been associated with changes in the fatty acid composition of *Isochrysis galbana*, when in co-culture with the microalga, enhancing the concentrations of hexadecadienoic acid (16:2*n*-6), stearidonic acid (SA, 18:4*n*-3) and DHA (DHA, 22:6*n*-3), while decreasing the amount of α-linolenic (ALA,18:3*n*-3), eicosadienoic (20:2*n*-6) and arachidonic (AA, 20:4*n*-3) acids, in comparison with axenic cultures of this eukaryote (Y. Y. Wang et al., 2022). While no improvements have been noticed in the concentrations of any of the FA detected in the analyzed co-cultures of *Tisochrysis lutea* with the *Marinobacter* isolates, taken together, these data suggest that the addition of these bacteria to the cultures can influence the fatty acid profile of this marine haptophyte although results may vary according to the species and bacterial concentration in the culture (Y. Y. Wang et al., 2022; Wu et al., 2023).

Unlike the previous strain, some of the assessed bacterial isolates (80 to 132) decreased the total FAME concentration of cultures relative to the values registered at the beginning of the experiment (T0, 52.39±4.63 mg. g^{-1}) (**Table 4.6**).

The predominant FAME found in the samples were the SFA myristic (C14:0) and palmitic (C16:0) acids, the palmitoleic (C16:1*n*-7) and oleic (18:1*n*-9c) acids, two MUFA, and the PUFAs SA (C18:4*n*-3) and DHA(C22:6*n*-3) (**Table 4.6**), in accordance to what was previously described regarding this marine microalga (Custódio et al., 2014; Gnouma et al., 2017; Almutairi, 2020).

Conversely, other FA, such as lauric (12:0) and eicosenoic (20:1) acids, were present in low amounts in some of the tested samples (**Table 4.6**). Furthermore, these compounds were not commonly detected in other reports on the fatty acid profile of either *I. galbana* or *T. lutea* (Custódio et al., 2014; Y. Y. Wang et al., 2022). Thus, we could hypothesize that these were most likely produced by either the bacterial isolates added to the cultures or other microorganisms in the already established community surrounding *T. lutea*. For instance, the fatty acid lauric acid found in the cultures with the bacterial isolates 7, 8, 22, 47, 60, 75, 78 and 116, has been reported to be produced by bacterial strains, including *Marinobacter* sp. (Y. Y. Wang et al., 2022). However, since this SFA was also detected in the samples collected at T0 and the CTRL, it is most likely that its production is associated with the surrounding microbial community rather than the introduction of one of the selected strains.

Aquaculture is nowadays the main industrial application of *T. lutea*, mainly to feed larvae of bivalves, mollusks, fish and crustaceans (da Costa et al., 2017; García-García et al., 2024). During these early growth stages, these animals require FA, namely eicosapentaenoic acid (EPA, 20:5*n*-3) and DHA (22:6*n*-3), essential for their survival and development (Nalder et al., 2015; Nagappan et al., 2021). Regarding these two PUFA, the CTRL cultures obtained the highest concentrations, containing 0.51±0.19 mg. g^{-1} of EPA and 21.38±6.22 mg. g^{-1} of DHA. The DHA concentration results are consistent with prior reports regarding this species, while the EPA content seem slightly below expected (García-García et al., 2024). Among the bacterial groups, the cultures inoculated with the *Marinobacter* spp. isolates achieved the highest concentrations, with the microalgal cultures containing isolate 7 replicates having the highest EPA concentration (0.37±0.01 mg. g^{-1}), while cultures with isolate 8 had the highest DHA concentration (15.92±1.45 mg. g^{-1}).

Table 4.6-FAME profile of biomass collected at the beginning (T0) and end of the second isolate trial for the control cultures without the further addition of bacteria (CTRL) and each of the tested bacterial isolates (7 to 132). Values are expressed in FAME concentration (mg. g^{-1}) ± SD (n=2 for the tested samples, except the bacterial isolate 80, where n=1). The values marked with one or more "*****" show different significance levels (ρ<0.05, ρ<0.01, ρ<0.001, ρ<0.0001, respectively) when in comparison with the CTRL (ANOVA One-way with Dunnett multiple comparison test, ρ<0.05). When results are marked with n.d., the corresponding FAME was not detected in the samples.

FAME (mg. g^{-1})	T0	CTRL	7	8	22	47	60	75	78	80	81	84	86	99	109	116	132
C12:0	0.05 ± 0.01**	0.26 ± 0.01	0.25 ± 0.04	0.23 ± 0.01	0.23 ± 0.02	0.22 ± 0.02	0.22 ± 0.01	0.24 ± 0.03	0.13 ± 0.001*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.13	n.d.
C14:0	5.93 ± 0.18	19.20 ± 4.73	13.00 ± 0.30	15.17 ± 1.09	11.54 ± 1.59	11.53 ± 4.26	10.88 ± 0.72	12.62 ± 0.10	9.87 ± 0.18	3.79	7.45 ± 1.45	7.43 ± 0.67	8.08 ± 0.38	6.18 ± 0.62	6.47 ± 0.20	5.08 ± 3.07	5.92 ± 0.23
C15:0	0.39 ± 0.01	0.66 ± 0.19	0.65 ± 0.01	0.72 ± 0.04	0.51 ± 0.04	0.52 ± 0.17	0.50 ± 0.06	0.45 ± 0.02	0.41 ± 0.01	0.18	0.31 ± 0.05	0.30 ± 0.002	0.33 ± 0.01	0.28 ± 0.003	0.30 ± 0.003	0.22 ± 0.11	0.27 ± 0.002
C16:0	4.71 ± 0.08	13.76 ± 4.24	10.18 ± 0.04	10.71 ± 0.28	8.54 ± 0.24	7.49 ± 2.57	7.97 ± 0.48	8.64 ± 0.01	5.98 ± 0.21	2.33	5.45 ± 1.15	4.90 ± 0.26	5.09 ± 0.03	4.23 ± 0.06	4.32 ± 0.04	4.49 ± 0.09	3.94 ± 0.11
C17:0	0.10 ± 0.005	0.12 ± 0.01	0.12 ± 0.002	0.11 ± 0.004	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.002	0.11 ± 0.004	0.07 ± 0.0003	0.07	0.10 ± 0.01	0.07 ± 0.003	0.08 ± 0.01	0.09 ± 0.0002	0.09 ± 0.002	0.09 ± 0.005	0.07 ± 0.01
C18:0	0.45 ± 0.05	2.35 ± 0.56	1.98 ± 0.01	2.05 ± 0.04	1.83 ± 0.03	1.47 ± 0.51	2.13 ± 0.26	1.68 ± 0.16	0.86 ± 0.12	0.90	1.63 ± 0.34	0.93 ± 0.06	0.88 ± 0.15	1.12 ± 0.20	1.03 ± 0.06	2.01 ± 2.01	0.88 ± 0.12
C20:0	0.15 ± 0.01	0.29 ± 0.04	0.26 ± 0.01	0.23 ± 0.02	0.21 ± 0.01	n.d.	0.24 ± 0.03	0.26 ± 0.04	0.16 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:0	0.22 ± 0.01	0.41 ± 0.06	0.33 ± 0.003	0.32 ± 0.03	0.26 ± 0.01	0.23 ± 0.02	0.26 ± 0.01	0.31 ± 0.02	0.20 ± 0.02	n.d.	0.22 ± 0.02	0.19 ± 0.02	0.32 ± 0.07	0.21 ± 0.01	0.25 ± 0.03	0.18 ± 0.01	0.17 ± 0.002
ΣSFA	11.98 ± 0.33	37.06 ± 9.83	26.78 ± 0.29	29.54 ± 1.39	23.24 ± 1.95	21.55 ± 7.56	22.30 ± 1.56	24.29 ± 0.02	17.67 ± 0.24	7.26	15.16 ± 3.03	13.82 ± 0.90	14.78 ± 0.18	12.11 ± 0.74	12.45 ± 0.14	12.36 ± 1.12	11.27 ± 0.01
C14:1 *n*-5	0.10 ± 0.003	0.23 ± 0.03	0.20 ± 0.02	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.17 ± 0.01	0.21 ± 0.003	0.12 ± 0.01	0.14	0.20 ± 0.02	0.15 ± 0.004	0.16 ± 0.02	0.19 ± 0.01	0.18 ± 0.004	0.17 ± 0.02	0.14 ± 0.02

C16:1 *n*-7	2.74 ± 0.01	7.24 ± 1.81	4.85 ± 0.24	5.60 ± 0.61	4.12 ± 0.56	3.80 ± 1.53	4.08 ± 0.31	3.99 ± 0.06	3.25 ± 0.16	1.51	3.01 ± 0.55	2.65 ± 0.31	2.77 ± 0.23	2.46 ± 0.37	2.12 ± 0.02	2.11 ± 1.15	1.92 ± 0.12
C17:1 *n*-7	0.33 ± 0.01	0.48 ± 0.26	0.36 ± 0.16	0.71 ± 0.07	0.21 ± 0.01	0.39 ± 0.28	0.38 ± 0.04	0.37 ± 0.01	0.36 ± 0.004	0.15	0.32 ± 0.08	0.35 ± 0.01	0.35 ± 0.03	0.37 ± 0.01	0.35 ± 0.005	0.25 ± 0.07	0.33 ± 0.01
C18:1 *n*-9c	2.03 ± 2.40	27.85 ± 6.30	19.03 ± 0.71	21.45 ± 0.68	14.78 ± 1.95	13.49 ± 5.06	13.84 ± 2.57	16.86 ± 0.70	12.94 ± 0.39	3.80	7.14 ± 1.57	8.43 ± 0.99	9.56 ± 0.42	6.72 ± 0.23	7.91 ± 0.76	6.60 ± 1.98	6.90 ± 0.02
C18:1 *n*-9t	1.71 ± 0.10	1.59 ± 0.30	1.65 ± 0.002	1.81 ± 0.03	1.87 ± 0.16	1.28 ± 0.45	1.98 ± 0.19	1.45 ± 0.15	1.03 ± 0.05	0.81	1.66 ± 0.22	0.90 ± 0.02	0.90 ± 0.05	0.81 ± 0.02	1.02 ± 0.01	1.41 ± 0.83	0.82 ± 0.05
C20:1 *n*-9	0.09 ± 0.01	0.10 ± 0.0002	0.10 ± 0.01	0.09 ± 0.003	0.08 ± 0.01	0.05 ± 0.07	0.09 ± 0.004	0.05 ± 0.06	n.d.	n.d.	n.d.	0.06 ± 0.0002 ****	n.d.	n.d.	n.d.	n.d.	n.d.
C22:1 *n*-9	0.21 ± 0.004	0.20 ± 0.02	0.17 ± 0.03	0.16 ± 0.01	0.16 ± 0.07	0.13 ± 0.05	0.12 ± 0.01	0.13 ± 0.002	0.08 ± 0.001	n.d.	0.11 ± 0.005	0.09 ± 0.01	0.10 ± 0.01	n.d.	n.d.	n.d.	0.08 ± 0.01
ΣMUFA	7.20 ± 2.53	37.68 ± 7.61	26.35 ± 0.35	30.00 ± 1.39	21.42 ± 2.77	19.31 ± 7.47	20.66 ± 3.14	23.05 ± 0.95	17.80 ± 0.59	6.41	12.44 ± 2.45	12.63 ± 1.28	13.84 ± 0.54	10.55 ± 0.58	11.57 ± 0.74	10.54 ± 2.40	10.20 ± 0.03
C16:2 *n*-6	0.14 ± 0.005	0.34 ± 0.09	0.26 ± 0.01	0.27 ± 0.01	0.22 ± 0.06	0.23 ± 0.05	0.22 ± 0.02	0.23 ± 0.002	0.23 ± 0.01	0.10	0.20 ± 0.005	0.17 ± 0.01	0.21 ± 0.03	0.24 ± 0.0004	0.18 ± 0.01	0.22 ± 0.001	0.14 ± 0.02
C18:2 *n*-6	2.67 ± 0.04	3.42 ± 1.48	1.98 ± 0.19	2.37 ± 0.28	1.79 ± 0.23	1.60 ± 0.65	1.61 ± 0.002	1.42 ± 0.87	1.40 ± 0.09	1.08	1.42 ± 0.30	1.11 ± 0.09	1.03 ± 0.001	1.21 ± 0.02	0.82 ± 0.02	1.00 ± 0.41	0.76 ± 0.04
C18:3 *n*-3α	2.89 ± 1.01	1.39 ± 0.42	1.01 ± 0.26	1.10 ± 0.17	0.86 ± 0.09	0.92 ± 0.37	0.81 ± 0.05	0.89 ± 0.05	0.66 ± 0.03	0.28	0.61 ± 0.11	0.63 ± 0.01	0.59 ± 0.11	0.51 ± 0.01	0.46 ± 0.06	0.40 ± 0.28	0.46 ± 0.07
C18:3 *n*-6γ	0.80 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C18:4 *n*-3	14.22 ± 0.25	33.87 ± 8.14	22.97 ± 1.09	26.88 ± 2.47	18.98 ± 2.96	18.17 ± 6.75	18.24 ± 1.42	18.58 ± 0.22	16.11 ± 1.19	6.17	13.99 ± 2.51	11.78 ± 0.59	12.85 ± 0.89	12.07 ± 1.45	10.32 ± 0.001	8.32 ± 8.38	9.52 ± 0.72
C20:3 *n*-6	0.14 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

C20:4 *n*-6	0.20 ± 0.01	0.29 ± 0.03	0.27 ± 0.01	0.26 ± 0.001	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.02	0.26 ± 0.01	0.16 ± 0.005	0.17	n.d.	0.19 ± 0.01	0.19 ± 0.03	n.d.	n.d.	n.d.	n.d.
C20:5 *n*-3	0.33 ± 0.01	0.51 ± 0.19	0.37 ± 0.01	0.35 ± 0.06	0.29 ± 0.01	0.27 ± 0.08	0.24 ± 0.03	0.28 ± 0.01	0.18 ± 0.01	0.14	0.22 ± 0.02	0.25 ± 0.05	0.20 ± 0.004	0.21 ± 0.02	0.19 ± 0.001	0.19 ± 0.02	0.16 ± 0.02
C22:5 *n*-3	1.48 ± 0.07	2.19 ± 0.57	1.56 ± 0.08	1.76 ± 0.13	1.20 ± 0.20	1.13 ± 0.57	1.14 ± 0.09	1.19 ± 0.004	0.91 ± 0.05	0.28	0.59 ± 0.13	0.61 ± 0.03	0.56 ± 0.02	0.46 ± 0.02	0.49 ± 0.02	0.37 ± 0.16	0.41 ± 0.004
C22:6 *n*-3	10.34 ± 0.36	21.38 ± 6.22	13.92 ± 0.73	15.92 ± 1.45	10.53 ± 1.65	10.01 ± 5.53	10.10 ± 0.74	11.21 ± 0.01	8.90 ± 0.57	1.39	4.37 ± 1.47	5.55 ± 0.50	5.15 ± 0.04	3.19 ± 0.27	4.04 ± 0.27	2.31 ± 2.07	3.30 ± 0.31
ΣPUFA	33.20 ± 1.77	63.39 ± 17.13	42.33 ± 2.34	48.90 ± 4.58	34.11 ± 5.21	32.56 ± 14.01	32.59 ± 2.27	34.07 ± 0.70	28.57 ± 1.97	9.60	21.40 ± 4.54	20.28 ± 1.30	20.78 ± 0.74	17.90 ± 1.74	16.50 ± 0.36	12.82 ± 11.33	14.75 ± 1.10
ΣFAME	52.39 ± 4.63	138.13 ± 34.57	95.46 ± 1.69	108.44 ± 7.36	78.77 ± 9.93	73.42 ± 29.03	75.55 ± 6.97	81.41 ± 1.67	64.04 ± 2.80	23.26	49.00 ± 10.01	46.73 ± 3.47	49.40 ± 1.46	40.56 ± 3.06	40.52 ± 1.24	35.72 ± 14.84	36.21 ± 1.12
Σ*n*-3	29.26 ± 1.69	59.33 ± 15.53	39.83 ± 2.17	46.00 ± 4.29	31.87 ± 4.91	30.50 ± 13.30	30.54 ± 2.33	32.16 ± 0.29	26.77 ± 1.86	8.25	19.78 ± 4.23	18.82 ± 1.19	19.34 ± 1.07	16.45 ± 1.76	15.50 ± 0.35	11.59 ± 10.92	13.84 ± 1.13
Σ*n*-6	3.94 ± 0.08	4.05 ± 1.60	2.50 ± 0.21	2.90 ± 0.29	2.24 ± 0.30	2.05 ± 0.70	2.05 ± 0.04	1.91 ± 0.88	1.80 ± 0.11	1.35	1.62 ± 0.31	1.47 ± 0.11	1.44 ± 0.06	1.46 ± 0.02	1.00 ± 0.03	1.22 ± 0.41	0.90 ± 0.06
Σ*n*-6/ Σ*n*-3	0.13	0.07	0.06	0.06	0.07	0.07	0.07	0.06	0.07	0.16	0.08	0.08	0.07	0.09	0.06	0.11	0.07
ΣMUFA/ ΣSFA	0.60	1.02	0.98	1.02	0.92	0.90	0.93	0.95	1.01	0.88	0.82	0.91	0.94	0.87	0.93	0.85	0.91
ΣPUFA/ ΣSFA	2.77	1.71	1.58	1.66	1.47	1.51	1.46	1.40	1.62	1.32	1.41	1.47	1.41	1.48	1.32	1.04	1.31

4.3.3.2. Protein

Protein content of the harvested biomass was estimated through an elemental analysis of the total N content determined in the harvested biomass. Values measured for N percentages were converted into protein using a previously reported N-to-protein conversion factor for *Isochrysis galbana*, as *T. lutea* was formerly known (Lourenço et al., 2004). The results are shown in **Figure 4.31**.

Overall, in all the tested samples, protein content in *T. lutea* biomass varied between 20.69% ± 0.51%, registered at T0, and 28.43% ± 5.54%, obtained for the cultures containing the isolate 116, with the CTRL cultures registering 23.90% ± 0.17% (**Figure 4.31**). These values are slightly higher than those previously reported. However, these alterations can be related to the bacterial presence in the harvested biomass (Skrede et al., 2011; Gnouma et al., 2017).

Moreover, in most treatments, the co-culture of the bacterial isolates with *T. lutea* did not correlate with any significant variation in protein content compared with that of the CTRL cultures (**Figure 4.31**).

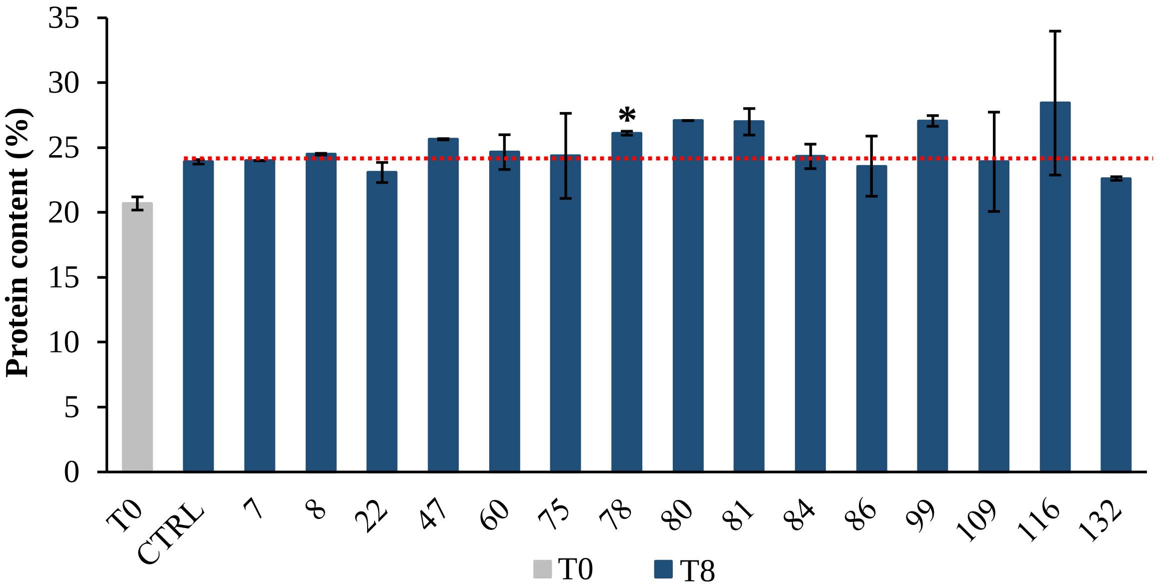

Figure 4.31-Protein content (%) in the biomass of *T. lutea*, harvested immediately after inoculation (T0) and after 8 days of culture (T8) with the selected bacterial isolates (7 to 132) or without the addition of bacteria (CTRL). Bars correspond to the mean values between biological replicates ± SD (*n*=2). Bars marked with "*" indicate significant differences from the CTRL (ANOVA One-Way with Dunnett multiple comparison test, $\rho<0.05$).

Nonetheless, the inoculation of the bacterium 78 within the *T. lutea* cultures showed an enhancement in the protein amount in the collected biomass (26.1089% ± 0.1520%). In addition, the cultures containing the isolates 47, 80, 81, 99 and 116, despite not having any significant effect, also showed potential to improve protein content in the biomass, with the estimated protein amounts ranging from 25.64%±0.05% to 28.43%±5.54% (**Figure 4.31**).

4.3.3.3. Pigment profile

The pigment profile of *T. lutea* was assessed before and after the co-cultivation with the bacterial isolates (**Table 4.7**). According to **Table 4.7**, the only pigments found in the biomass samples were the carotenoids fucoxanthin, lutein and *β*-carotene.

The fucoxanthin concentrations varied between 3.515±0.082 mg. g^{-1}, detected in the co-cultures containing the isolate 60, and 7.866±2.216 mg. g^{-1}, for the CTRL cultures. In the meantime, the lutein concentration ranged from 0.138±0.018 mg. g^{-1}, at T0, to 2.044±0.145 mg. g^{-1}, at the stationary phase of the CTRL samples, while the *β*-carotene content was between 1.610± 0.056 mg. g^{-1} (isolate 81) and 3.516±1.180 mg. g^{-1} (T0) (**Table 4.7**). Therefore, it seems as though the addition of the selected bacterial strains to the cultures do not correlate with any significant improvements in the pigment profile of *T. lutea*. Moreover, after 8 days of co-cultures with the isolates, some cultures registered significant decreases in the pigment content compared with the CTRL. For instance, the inoculation of the microalga with the bacterial strains 8 and 99 reduced the lutein concentration. At the same time, the addition of the bacterial isolates 22, 60, 81, 109 and 132 were associated with a reduction in the concentration of *β*-carotene (**Table 4.7**).

Nonetheless, *T. lutea* is a microalga known to have an enriched pigment composition, containing carotenoids (primarily fucoxanthin, but also other xanthophylls) and Chl *a* and *c* (Beuzenberg et al., 2017; Gonçalves de Oliveira-Júnior et al., 2020). However, only three pigments (fucoxanthin, lutein and *β*-carotene) were found in the tested samples across all CTRL and treatment replicates at both T0 and T8. Thus, an optimization of the method utilized for the detecting and quantifying of the pigment profile of *T. lutea* is required for further trials.

Table 4.7-Concentration of the carotenoids fucoxanthin, lutein and β-carotene (mg. g^{-1}) measured in *T. lutea* biomass collected at the beginning (T0) and end of the trial (T8), after culture without (CTRL) and with the selected bacterial isolates (7 to 132). Values indicate pigment concentration mean values ± SD. Different letters (a, b, c) correspond to different levels of significance ($\rho<0.05$, $\rho<0.01$, $\rho<0.001$, respectively) in comparison to the CTRL (ANOVA One-way with Dunnett multiple comparison test, $\rho<0.05$).

Samples	**Pigment concentration (mg. g^{-1})**		
	Fucoxanthin	**Lutein**	**β-Carotene**
T0	5.749 ± 1.143	0.138 ± 0.018***	3.516 ± 1.180
CTRL	7.866 ± 2.216	2.044 ± 0.145	3.168 ± 0.428
7	4.310 ± 0.648	1.541 ± 0.294	2.221 ± 0.426
8	4.568 ± 0.169	1.575 ± 0.059*	2.183 ± 0.071
22	3.969 ± 0.351	1.335 ± 0.088*	1.895 ± 0.135*
47	4.183 ± 0.243	1.431 ± 0.120	2.008 ± 0.270
60	3.515 ± 0.082	1.217 ± 0.085**	1.648 ± 0.091*
75	3.632 ± 0.429	1.270 ± 0.241	1.617 ± 0.274
78	3.826 ± 0.555	1.247 ± 0.167	1.744 ± 0.299
80	5.942	0.930	1.941
81	5.055 ± 0.062	0.924 ± 0.043**	1.610 ± 0.056*
84	4.429 ± 0.593	1.310 ± 0.221	1.872 ± 0.357
86	4.696 ± 0.028	1.448 ± 0.185	2.056 ± 0.283
99	5.730 ± 0.306	1.044 ± 0.071**	2.156 ± 0.059
109	3.896 ± 0.654	1.286 ± 0.098*	1.718 ± 0.098*
116	4.208 ± 3.434	1.254	1.242 ± 1.130
132	4.096 ± 0.073	1.320 ± 0.071**	1.957 ± 0.154*

4.3.3.4. Vitamin B_{12}

The content of vitamin B_{12} at the beginning of the assay (T0) and stationary phase (T8) of the co-cultures was determined through HPLC analysis (**Figure 4.32**). The concentration of this micronutrient was measured only in the form of MeCbl, a biologically active form of vitamin B_{12} that works as the cofactor of methionine synthase (Heal et al., 2014; J. Pereira et al., 2019).

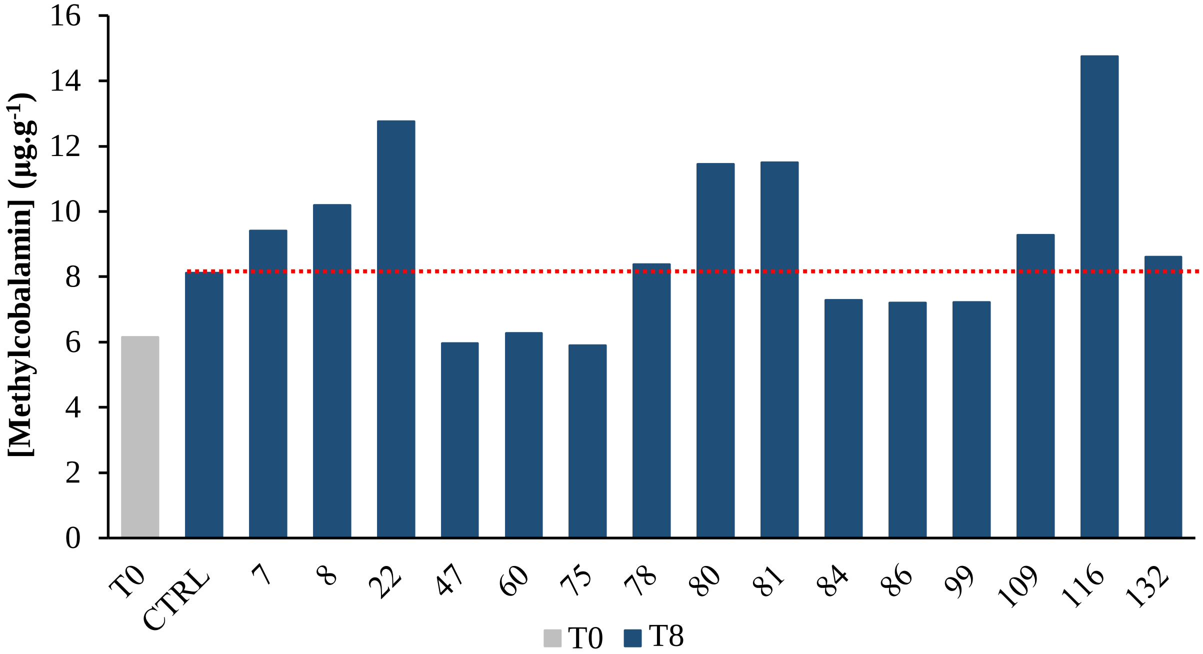

Figure 4.32-Concentration of vitamin B_{12} in the form of methylcobalamin (µg. g^{-1}) in the biomass collected from *T. lutea* cultures, after inoculation (T0) and after 8 days of culture (T8) without (CTRL) and with the added bacterial isolates (7 to 132) (*n*=1).

Following what is described in Figure 4.30, the CTRL cultures contained approximately 8 µg. g^{-1} of MeCbl at stationary phase (T8), while at the beginning of the trial (T0), the measured concentration was approximately 6 µg. g^{-1}. Within the treatments with the selected bacterial isolates, 9 of those, namely the microalgal cultures containing strains 7, 8, 22, 78, 80, 81, 109, 116, 132 appeared to have higher concentrations of MeCbl after 8 days of co-culture in comparison with the CTRL (**Figure 4.32**). In the meantime, cultures with the remaining bacteria presented lower concentrations of the tested compound, with 2 of the isolates (47 and 75) leading to a total concentration of MeCbl even lower than the value determined at T0 (5.96 µg. g^{-1} and 5.90 µg. g^{-1}, respectively) (**Figure 4.32**).

Overall, in all the tested biomass samples, the concentration of MeCbl varied between 5.90 and 14.75 µg. g^{-1}, with the lowest concentration corresponding to the culture sample to which isolate 75 was added. In contrast, the highest concentration was found in the sample containing the biomass collected after the co-culture with the bacterium 116 (**Figure 4.32**). As mentioned, this isolate was identified as *A. faecalis*, a bacterium reported as a vitamin B_{12} producer (Prabaningtyas et al., 2021). Other isolates, like 22 and 80, identified as *H. alkaliphila* and *Roseovarius* sp., also achieved higher concentrations of MeCbl when compared to the CTRL. Similarly to isolate 116, these 2 bacterial species are either identified as producers of vitamin B_{12} (*H. alkaliphila*) or are included in taxa (clade *Rhodobacteraceae* and genus *Halomonas*) often associated to the secretion of this micronutrient (Nef et al., 2022).

However, despite this, the increase in MeCbl concentration did not correlate with the observed growth improvements in *T. lutea* cultures when co-cultivated with added bacteria, since cell concentrations were below the CC determined for the CTRL. In a previous study, Nef et al. (2022) had reported that the co-culture of this marine haptophyte with vitamin B_{12} -producing bacterial strains, *Phaeobacter porticola* and a *Halomonas* sp. strain closely related to *H. alkaliphila*, in medium with and without further supplementation with vitamin B_{12}, was not associated with any significant differences regarding the growth performance of *T. lutea* when compared to CTRL cultures. Therefore, the results obtained during this assay, supported by prior reports, do suggest that the exchange of vitamin B_{12} from bacterial origin to cobalamin-dependent microalgae through mutualistic relations may be influenced by the interacting species, their ratio or the culture conditions.

4.3.4. Selection of bacteria for the bacterial mixes

When reviewing the results related to Phase I, none of the utilized bacterial isolates was able to enhance all the tested parameters in co-culture with *T. lutea*. However, some showed the potential to improve either productivity or production of some biomolecules of interest.

Firstly, the CC was selected as the parameter that could better extrapolate the effect of the co-inoculation with the bacterial isolates on *T. lutea* productivity. According to the CC results obtained at the first growth trial with the bacteria (**section 4.3.1-Figure 4.25**), the addition of isolate 80, identified as *Roseovarius* sp., to the *T. lutea* cultures resulted in a growth improvement of the microalga, in comparison to the CTRL. Furthermore, the bacterium 132 (*Pseudidiomarina maritima),* was not associated with an overall enhancement in the growth of this haptophyte, but one of its replicates registered the highest CC among all the samples. Therefore, this strain could have the potential to improve *T. lutea* growth. Regarding the second trial, the cultures inoculated with the bacterium 84, *H. salexigens*, overperformed not only the CTRL cultures but also every other bacterial treatment group, thus improving the growth of the microalga following the eight days of co-culture (**Figures 4.27-4.28**). Moreover, across both growth trials the response of *T. lutea* to the addition of either *Roseovarius* sp. or *Pseudidiomarina maritima* in the cultures appeared to be similar since the CC values remained equivalent in the co-cultures with these strains from the first to the second trial. In contrast, the co-inoculation of *T. lutea* with strain 84 improved the overall CC of the microalga from the first assay to the second.

On the other hand, the addition of other isolates, such as the *Marinobacter* strains 7 and 8, to the cultures did not correlate with any improvements to the proliferation of *T. lutea* when compared to CTRLs (**Figure 4.25 and 4.27**). However, in co-culture with these strains *T. lutea* obtained nearly equal CC results to the CTRL (**Figure 4.27**). On top of that, the replicates containing these isolates were associated with higher concentrations of lutein and *β*-carotene among all the tested treatment groups (**Table 4.7**). They were also the best-performing strains concerning the FAME profile (**Table 4.6**). Besides, species included in the genus *Marinobacter* have been continuously associated with beneficial interactions with microalgae, improving growth and biomass quality (Fuentes et al., 2016; Lian et al., 2018; Y. Y. Wang et al., 2022).

Alternatively, the bacterium identified as *Microbacterium* sp. (isolate 78) was associated with an enhancement in the protein content compared to that of the CTRL (**Figure 4.31**). In similar conditions, *T. lutea* culture with the addition of strain 116 (*Alcaligenes faecalis*) reached the highest concentration of MeCbl (**Figure 4.32**). Furthermore, this data agrees with prior reports that classified *A. faecalis* as a vitamin B_{12}-producing bacterium (Prabaningtyas et al., 2021).

Considering these results, 7 bacterial isolates were selected for the project's next phase, where every possible combination of two bacteria with the chosen isolates was tested in co-culture with *T. lutea* until the stationary phase (**Table 4.8**).

Table 4.8-List of bacterial isolates selected for Phase II

Isolate code	ID
7	*Marinobacter nauticus*
8	*Marinobacter* sp.
78	*Microbacterium* sp.
80	*Roseovarius* sp.
84	*Haliea salexigens*
116	*Alcaligenes faecalis*
132	*Pseudidiomarina maritima*

4.4. Microalga: bacteria co-culture (Phase II)

Bacterial isolates selected according to the results of the previous experiments were combined into pairs to form small bacterial mixes to be inoculated with *Tisochrysis lutea*, following the conditions formerly mentioned in **section 3.6.3**. The objective was to evaluate whether combinations of bacteria could outperform inoculations using only one isolate.

A total of 21 pairs of isolates, corresponding to every possible combination of two bacteria that could be designed with the seven chosen strains, were tested. The cultures were maintained until OD values at 680 nm indicated that *T. lutea* was in a stationary phase across all treatments, which happened at day 11 of the experiment. Samples were collected for CC determination during the experiment to assess the culture state (**Figure 4.33**).

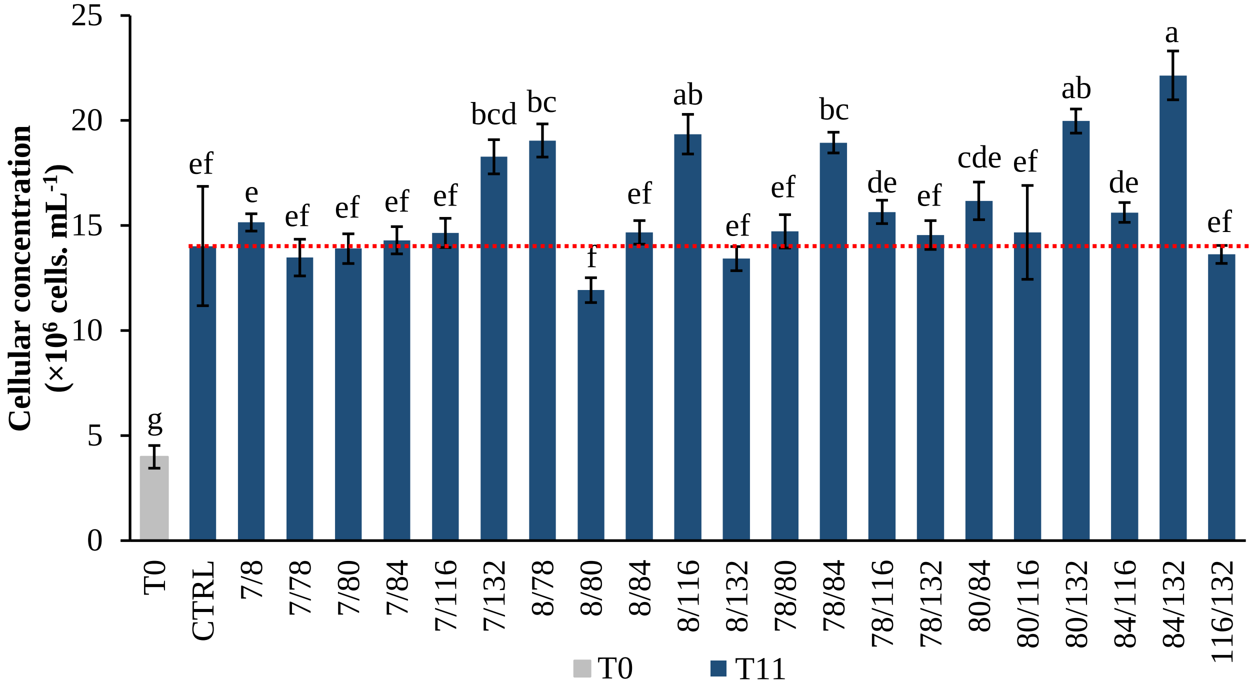

Figure 4.33-Cellular concentration of *T. lutea* (×10^6 cells. mL^{-1}) at the beginning (T0) and at stationary phase (T11), without the addition of bacterial isolates (CTRL) and in co-culture with the designed bacterial mixes (from 7/8 to 116/132). Bars represent mean values ± SD. Different letters (a to f) showcase significant differences among treatments (ANOVA One-Way with Tukey post hoc test, $\rho<0.05$)

Firstly, the CTRL cultures achieved similar CC results from the previous growth trial from Phase I and this Phase II assay, at T0 and T11, registering 3.99±0.54 ×10^6 cells. mL^{-1} and 14.03±2.84 ×10^6 cells. mL^{-1}, respectively, while in the previous trial, these groups obtained 3.61±0.70 ×10^6 cells. mL^{-1} and 15.87±0.74 ×10^6 cells. mL^{-1} (**Figures 4.27 and 4.33**).

In comparison, when added to the cultures some of the selected bacteria were associated with better growth results of *T. lutea*, combined in pairs with others rather than as single isolates. For instance, in co-culture with isolate 116 (*Alcaligenes faecalis*), *T. lutea* had CC results below the CTRL in both trials from Phase I, achieving 5.40±1.34 ×10^6 cells. mL^{-1} and 9.70±3.42 ×10^6 cells. mL^{-1}, for each of the assays respectively, but when this bacterium was inoculated into the microalga cultures in combination with the other selected isolates, the CC of *T. lutea* varied between 13.63±0.43 ×10^6 cells. mL^{-1} (mix 116/132) and 19.35±0.94 ×10^6 cells. mL^{-1} (mix 8/116) (**Figures 4.27 and 4.33**).

Overall, the insertion of any of the designed bacterial combinations in the cultures was not associated with any harmful effects on the growth of this microalga, since all the treatments presented higher microalgal cell concentration than at T0 (3.99±0.54 $\times10^6$ cells. mL^{-1}) (**Figure 4.33**).

Furthermore, according to Figure 4.31, 6 of the bacterial mixtures (7/132, 8/78, 8/116, 78/84, 80/132 and 84/132) did correlate with improvements in the growth of *T. lutea,* in comparison with CTRL cultures (14.03±2.84$\times10^6$ cells. mL^{-1}). This increase may be related to the duration of the assay since while the growth trials with the isolates lasted at most 8 days when *T. lutea* was co-inoculated with the tailor-made mixes, it only reached stationary phase at day 11 (**Figure 4.34**). While the CTRL cultures registered a decay after day 8 of the experiment, the cultures where these 6 bacterial mixes were added still registered a slight rise in CC concentration between days 8 and 11 (**Figure 4.34**). It is possible that the bacterial strains inserted in the surrounding community of *T. lutea* could produce or recycle nutrients in the culture media and making them available to the microalga, thus promoting growth and sustaining the microalga for longer periods, therefore delaying the beginning of the death phase. This kind of interaction is thought to occur within natural marine habits and similar results were also reported in a previous study where the microalgae *Chaetoceros calcitrans* and *Nannochloropsis oculata* were tested in co-culture with bacteria (*Pseudomonas* sp. and *Bacillus* sp.), which resulted in growth enhancement and delays in the death phase of the microalgae (Buchan et al., 2014; Sureshkumar et al., 2014).

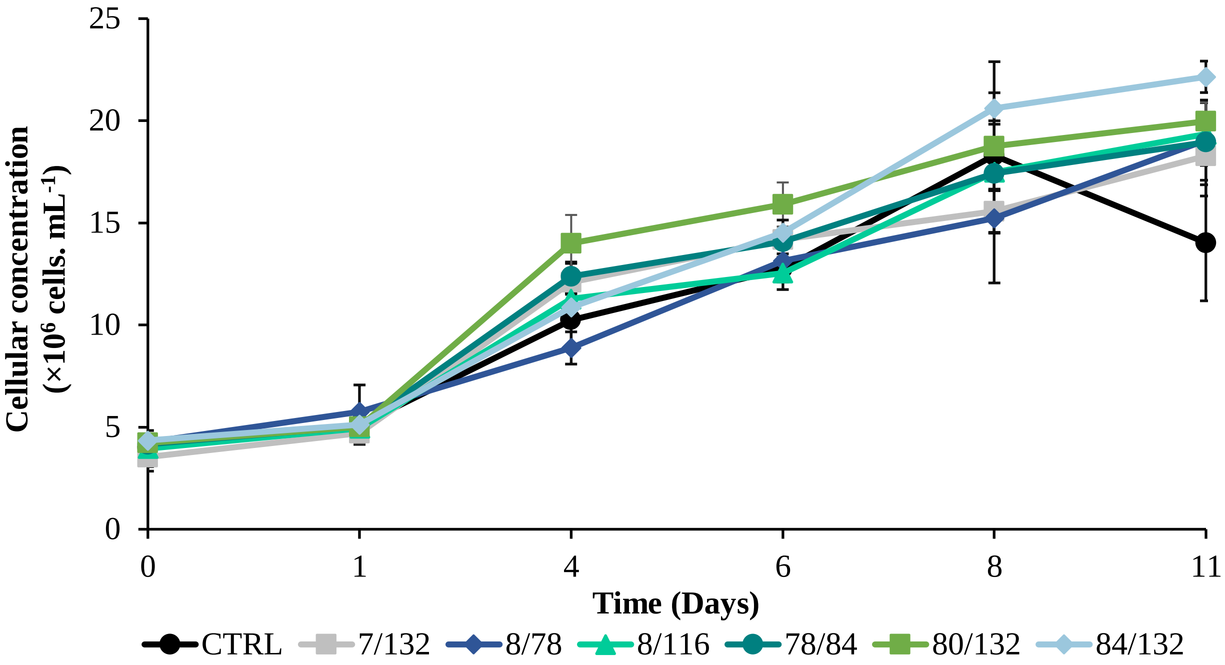

Figure 4.34-Cellular concentration of *T. lutea* (x10^6 cells. mL^{-1}), throughout the 11 days of the experiment, in the control cultures (CTRL), without the addition of bacteria, and in the cultures containing the bacterial mixes 7/132, 8/78, 8/116, 78/84, 80/132 and 84/132.

Considering these results, the nine best-performing bacterial mixes were selected for further biochemical analysis. The composition of these nine "tailor-made" bacterial blends is described in **Table 4.9**.

Table 4.9-Description of the best-performing bacterial combinations selected for biochemical analysis

Selected bacterial mixes	**ID of the bacterial isolates that make up the mix**
7/132	*Marinobacter nauticus*/*Pseudidiomarina maritima*
8/78	*Marinobacter* sp./*Microbacterium* sp.
8/116	*Marinobacter* sp./*Alcaligenes faecalis*
78/84	*Microbacterium* sp./*Haliea salexigens*
78/116	*Microbacterium* sp./*Alcaligenes faecalis*
80/84	*Roseovarius* sp./*Haliea salexigens*
80/132	*Roseovarius* sp./*Pseudidiomarina maritima*
84/116	*Haliea salexigens*/*Alcaligenes faecalis*
84/132	*Haliea salexigens*/*Pseudidiomarina maritima*

4.4.1. Biochemical analysis

4.4.1.1. Fatty acids profile

The effect of the bacterial pairs on the FAME profile of *T. lutea* was determined following 11 days of co-culture with the tailor-made mixes. The FAME profile of the CTRL cultures was also determined at T0 and T11 (**Table 4.10**).

Regarding the total FAME concentration, the replicates collected at T0 registered the lowest values overall (55.86±5.76 mg. g^{-1}). Unlike what occurred in the FAME concentration results obtained at the previous assay (**section 4.3.3.1**), the highest concentration in samples tested for this experiment corresponded to a treatment group that contained the tailor-made bacterial mix 78/116 (77.29±0.17 mg. g^{-1}), rather than the CTRL cultures (63.79±2.62 mg. g^{-1}) (**Table 4.10**). Inversely, the bacterial combination that reached the lowest concentration among the tested mixes (60.80±2.79) was with the pair 8/78, composed of the bacterial isolates identified as *Marinobacter* sp. and *Microbacterium* sp. (**Table 4.10**).

The determined FAME concentration of the CTRL cultures at stationary phase includes 13.99±0.74 mg. g^{-1} of SFA, 10.92±0.88 mg. g^{-1} of MUFA and 38.88±1.00 mg. g^{-1} of PUFA, corresponding to approximately 22%, 17% and 61% of total FAME content in SFA, MUFA and PUFA, respectively. At the same time, at T0, the analyzed samples contained 14.86±1.10 mg. g^{-1} of SFA (26.64±0.78%), and the lowest overall concentrations of MUFA (9.85±1.05 mg. g^{-1} or 17.63±0.06%) and PUFA (31.15±3.61 mg. g^{-1} or 55.73±0.72%) (**Table 4.10**).

Concerning the results obtained for the bacterial mixtures, the combination of bacteria 8/78 registered the lowest concentrations of SFA (13.50±0.81 mg. g^{-1}), MUFA (10.06±0.36 mg. g^{-1}) and PUFA (37.23±1.57mg. g^{-1}), equivalent to approximately 22, 16.5 and 61% of the total FAME contents, respectively. In contrast, the highest amount of SFA was found in the culture with bacterial mix 80/132 (15.97 mg. g^{-1} or 23.76%). In comparison, the highest concentration of MUFA was registered in combination that included the bacteria 80/84 (12.60±0.49 mg. g^{-1} or 19.02±0.86%). The pair designed with the bacteria 78/116 had the highest concentration of PUFA (37.23±1.57mg. g^{-1} or 64.30±0.49%) (**Table 4.10**).

Additionally, contrary to what occurred in the previous FAME analysis (**section 4.3.3.1**), none of the cultures containing the added bacteria correlated to a decrease in the total FAME concentration when compared to the values obtained at the beginning of the assay (T0) (**Table 4.10**).

The predominant SFA across all samples were the myristic (C14:0) and palmitic (C16:0) acids, while the principal MUFA are the palmitoleic (C16:1*n*-7) and oleic (C18:1*n*-9c) acids and the main PUFA were the SA (C18:4*n*-3) and DHA (C22:6*n*-3). These data are consistent with a prior report (Gnouma et al., 2017) and the results obtained in the previous analysis. Moreover, according to what is described in Table 4.10, the elaidic acid (C18:1*n*-9t) is also present in the samples in similar concentrations to that of palmitoleic acid (C16:1*n*-7).

Considering that aquaculture is the primary application of *T. lutea*, a nutritional profile enriched in essential FA for larval stage organisms (e.g., EPA and DHA) is of high interest for this industry nowadays (da Costa et al., 2017; García-García et al., 2024). According to **Table 4.10**, the concentration of EPA varied between 0.42±0.02 mg. g^{-1}, obtained in the cultures inoculated with the mix 8/78, and 0.53±0.01 mg. g^{-1}, registered in the replicates of the bacterial mixes 8/116 and 80/132 (**Table 4.10**), and regarding the concentration of DHA in the samples, ranged from 11.90±1.43 mg. g^{-1} at T0 to 21.99±0.16 mg. g^{-1}, for the bacterial combination 78/116 (**Table 4.10**). Despite no significant improvements were detected regarding these essential FA, compared to the CTRL, some of the tailor-made bacterial mixes showed the potential to increase the concentration of these compounds in the biomass of *T. lutea*.

However, adding some bacterial combinations to the cultures correlated with improvements in the content of other PUFA. For instance, the inoculation of the bacteria 78 and 116 with *T. lutea* matched up with significant increase in the concentration of hexadecadienoic acid (16:2*n*-6), *α*-ALA (18:3*n*-3α) and docosapentaenoic acid (DPA, 22:5*n*-3), while the addition of the bacterial mix 8/116 was associated with improvements only in 16:2*n*-6 and DPA. Other 2 bacterial combinations (8/78 and 78/84), when added to the cultures, enhanced the content of 16:2*n*-6 (**Table 4.10**).

Table 4.10-FAME profile of biomass collected at the beginning (T0) and end assay for the control cultures without the further addition of bacteria (CTRL) and each of the tested bacterial pairs (7/132 to 84/132). Values are expressed in FAME concentration (mg. g^{-1}) ± SD (n=2, except for the bacterial combination 80/132, where n=1). Different letters (a, b, c, d) represent significant differences among treatments (ANOVA One-way with Tukey post hoc test, $\rho<0.05$).

FAME (mg. g^{-1})	**T0**	**CTRL**	**7/132**	**8/78**	**8/116**	**78/84**	**78/116**	**80/84**	**80/132**	**84/116**	**84/132**
C12:0	0.05± 0.001^{b}	0.07± 0.01^{ab}	0.09± 0.003^{a}	0.07± 0.003^{ab}	0.06± 0.005^{ab}	0.06± 0.01^{b}	0.06± 0.002^{ab}	0.07± 0.01^{ab}	0.06	0.07± 0.003^{ab}	0.06± 0.001^{ab}
C14:0	7.01± 0.83^{a}	6.69± 0.24^{a}	7.15± 0.42^{a}	6.01± 0.30^{a}	6.51± 0.36^{a}	6.93± 0.46^{a}	7.47± 0.11^{a}	6.22± 0.06^{a}	6.97	6.34± 0.34^{a}	7.06± 0.25^{a}
C15:0	0.30± 0.03^{d}	0.37± 0.03^{abcd}	0.38± 0.02^{abcd}	0.36± 0.01^{bcd}	0.40± 0.03^{abc}	0.42± 0.01^{ab}	0.45± 0.01^{a}	0.38± 0.01^{abcd}	0.40	0.39± 0.02^{bc}	0.42± 0.02^{abc}
C16:0	6.13± 0.53^{a}	5.63± 0.29^{a}	5.59± 0.36^{a}	5.43± 0.22^{a}	6.09± 0.35^{a}	5.82± 0.24^{a}	6.31± 0.12^{a}	5.71± 0.11^{a}	6.64	5.43± 0.07^{a}	5.74± 0.25^{a}
C17:0	0.11± 0.01^{d}	0.13± 0.02^{cd}	0.15± 0.004^{abc}	0.16± 0.01^{abc}	0.17± 0.01^{a}	0.14± 0.001^{bcd}	0.15± 0.003^{abc}	0.17± 0.002^{ab}	0.17	0.14± 0.004^{abc}	0.14± 0.002^{cd}
C18:0	0.65± 0.28^{a}	0.57± 0.10^{a}	0.64± 0.04^{a}	0.84± 0.25^{a}	0.72± 0.03^{a}	0.78± 0.12^{a}	0.68± 0.08^{a}	0.95± 0.03^{a}	1.08	0.76± 0.04^{a}	0.71± 0.02^{a}
C20:0	0.19± 0.01^{a}	0.17± 0.01^{ab}	0.14± 0.0005^{b}	0.19± 0.01^{a}	0.17± 0.02^{ab}	0.17± 0.01^{ab}	0.18± 0.01^{ab}	0.18± 0.01^{ab}	0.19	0.16± 0.01^{ab}	0.15± 0.01^{ab}
C22:0	0.27± 0.004^{a}	0.24± 0.02^{a}	0.24± 0.02^{a}	0.28± 0.001^{a}	0.26± 0.04^{a}	0.24± 0.01^{a}	0.27± 0.03^{a}	0.29± 0.001^{a}	0.29	0.23± 0.02^{a}	0.25± 0.02^{a}
C23:0	0.14± 0.002^{ab}	0.14± 0.02^{ab}	0.12± 0.002^{b}	0.15± 0.01^{ab}	0.14± 0.01^{ab}	0.14± 0.002^{ab}	0.15± 0.002^{ab}	0.17± 0.01^{a}	0.16	0.15± 0.01^{ab}	0.14± 0.01^{ab}
ΣSFA	14.86± 1.10^{a}	13.99± 0.74^{a}	14.50± 0.86^{a}	13.50± 0.81^{a}	14.54± 0.84^{a}	14.71± 0.58^{a}	15.72± 0.29^{a}	14.16± 0.22^{a}	15.97	13.68± 0.33^{a}	14.67± 0.58^{a}
C14:1	0.09± 0.01^{ab}	0.09± 0.005^{ab}	0.09± 0.004^{ab}	0.09± 0.004^{ab}	0.08± 0.003^{ab}	0.08± 0.01^{ab}	0.10± 0.001^{a}	0.08± 0.001^{ab}	0.09	0.08± 0.002^{b}	0.08± 0.01^{ab}
C16:1 *n*-7	2.66± 0.25^{b}	2.78± 0.15^{ab}	2.83± 0.06^{ab}	2.71± 0.16^{ab}	3.06± 0.32^{ab}	3.02± 0.13^{ab}	3.38± 0.03^{a}	2.89± 0.07^{ab}	3.13	2.81± 0.11^{ab}	3.02± 0.20^{ab}
C17:1	0.19± 0.02^{a}	0.21± 0.0004^{a}	0.23± 0.02^{a}	0.23± 0.01^{a}	0.24± 0.01^{a}	0.23± 0.002^{a}	0.24± 0.004^{a}	0.22± 0.01^{a}	0.28	0.20± 0.003^{a}	0.22± 0.03^{a}
C18:1 *n*-9c	4.56± 0.81^{a}	4.71± 0.05^{a}	4.74± 0.77^{a}	4.04± 0.11^{a}	4.77± 0.42^{a}	4.53± 0.32^{a}	4.64± 0.16^{a}	4.51± 0.13^{a}	4.93	3.79± 0.22^{a}	4.42± 0.09^{a}

C18:1 *n*-9t	2.00±0.05^{c}	2.79±0.76^{bc}	2.92±0.11^{bc}	2.68±0.07^{bc}	3.27±0.54^{abc}	3.30±0.33^{abc}	3.18±0.04^{abc}	4.54±0.72^{a}	3.74	3.18±0.26^{abc}	3.75±0.30^{ab}
C20:1 *n*-9	0.11±0.003^{ab}	0.10±0.01^{ab}	0.09±0.003^{b}	0.11±0.001^{ab}	0.10±0.01^{ab}	0.10±0.003^{ab}	0.11±0.002^{ab}	0.12±0.001^{a}	0.12	0.10±0.01^{ab}	0.10±0.003^{ab}
C22:1 *n*-9	0.24±0.01^{a}	0.24±0.01^{a}	0.19±0.004^{b}	0.21±0.01^{ab}	0.22±0.01^{ab}	0.21±0.002^{ab}	0.22±0.001^{ab}	0.22±0.02^{ab}	0.25	0.19±0.02^{b}	0.20±0.01^{ab}
ΣMUFA	9.85±1.05^{a}	10.92±0.88^{a}	11.09±0.97^{a}	10.06±0.36^{a}	11.75±1.32^{a}	11.48±0.79^{a}	11.87±0.15^{a}	12.60±0.49^{a}	12.54	10.35±0.57^{a}	11.79±0.03^{a}
C16:2 *n*-6	0.15±0.01^{e}	0.23±0.02^{d}	0.22±0.01^{d}	0.42±0.002^{b}	0.29±0.03^{c}	0.46±0.01^{b}	0.55±0.01^{a}	0.23±0.004^{d}	0.24	0.27±0.01^{cd}	0.25±0.002^{cd}
C18:2 *n*-6	1.94±0.16^{a}	1.17±0.01^{b}	1.18±0.05^{b}	1.16±0.03^{b}	1.30±0.12^{b}	1.26±0.002^{b}	1.41±0.001^{b}	1.17±0.06^{b}	1.32	1.08±0.01^{b}	1.26±0.19^{b}
C18:3 *n*-3α	3.56±0.36^{b}	3.60±0.25^{b}	4.36±0.11^{ab}	4.05±0.04^{b}	4.47±0.21^{ab}	4.55±0.52^{ab}	5.38±0.04^{a}	3.93±0.33^{b}	3.45	4.14±0.28^{ab}	4.41±0.57^{ab}
C18:4 *n*-3	11.37±1.42^{c}	13.61±0.60^{abc}	14.24±0.53^{abc}	13.13±0.27^{bc}	15.43±2.38^{ab}	15.10±0.52^{abc}	17.18±0.003^{a}	13.32±0.45^{abc}	13.13	13.70±0.98^{abc}	14.54±0.68^{abc}
C20:3 *n*-6	0.16±0.01^{a}	0.15±0.01^{a}	0.13±0.004^{a}	0.16±0.00002^{a}	0.15±0.01^{a}	0.14±0.005^{a}	0.16±0.002^{a}	0.16±0.01^{a}	0.16	0.14±0.01^{a}	0.14±0.01^{a}
C20:4 *n*-6	0.20±0.01^{ab}	0.19±0.02^{ab}	0.17±0.01^{b}	0.20±0.004^{a}	0.21±0.01^{ab}	0.20±0.004^{ab}	0.22±0.02^{a}	0.20±0.01^{ab}	0.20	0.19±0.005^{ab}	0.18±0.002^{ab}
C20:5 *n*-3	0.45±0.05^{a}	0.48±0.01^{a}	0.47±0.00^{a}	0.42±0.02^{a}	0.53±0.01^{a}	0.45±0.01^{a}	0.52±0.05^{a}	0.48±0.01^{a}	0.53	0.43±0.02^{a}	0.48±0.02^{a}
C22:5 *n*-3	1.41±0.16^{c}	1.69±0.01^{bc}	1.77±0.13^{abc}	1.63±0.10^{bc}	2.06±0.34^{a}	1.98±0.05^{abc}	2.30±0.01^{a}	1.81±0.03^{abc}	1.83	1.84±0.17^{abc}	1.98±0.06^{ab}
C22:6 *n*-3	11.90±1.43^{b}	17.46±0.59^{ab}	17.64±1.38^{ab}	16.07±1.11^{ab}	20.32±3.77^{a}	19.04±0.51^{a}	21.99±0.16^{a}	18.19±0.44^{a}	17.85	18.45±1.51^{a}	18.97±0.11^{a}
ΣPUFA	31.15±3.61^{c}	38.88±1.00^{abc}	40.19±2.04^{abc}	37.23±1.57^{bc}	44.76±6.88^{ab}	43.17±0.50^{ab}	49.70±0.27^{a}	39.48±0.68^{abc}	38.71	40.24±2.97^{abc}	42.20±1.65^{abc}
ΣFAME	55.86±5.76^{b}	63.79±2.62^{ab}	65.78±3.87^{ab}	60.80±2.75^{b}	71.04±9.04^{ab}	69.35±1.87^{ab}	77.29±0.17^{a}	66.24±0.40^{ab}	67.22	64.26±3.87^{ab}	68.67±2.26^{ab}
Σ*n*-3	28.70±3.43^{c}	37.14±0.95^{abc}	38.48±1.98^{abc}	35.30±1.54^{bc}	42.81±6.71^{ab}	41.11±0.49^{ab}	47.37±0.26^{a}	37.73±0.60^{abc}	36.79	38.56±2.96^{abc}	40.38±1.44^{ab}
Σ*n*-6	2.46±0.19^{a}	1.74±0.04^{c}	1.70±0.06^{c}	1.93±0.03^{bc}	1.94±0.17^{bc}	2.06±0.01^{abc}	2.33±0.01^{ab}	1.75±0.07^{c}	1.92	1.68±0.01^{c}	1.82±0.20^{c}
Σ*n*-6/ Σ*n*-3	0.09	0.05	0.04	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.05

MUFA/ SFA	0.66	0.78	0.77	0.75	0.81	0.78	0.76	0.89	0.79	0.76	0.80
PUFA/ SFA	2.10	2.78	2.77	2.76	3.08	2.94	3.16	2.79	2.42	2.94	2.88

4.4.1.2. Protein

The protein content of *T. lutea* biomass was estimated in the samples of the 9 selected combinations, through an elemental analysis followed by an N-to-protein conversion, with the results presented as % DW (**Figure 4.35**).

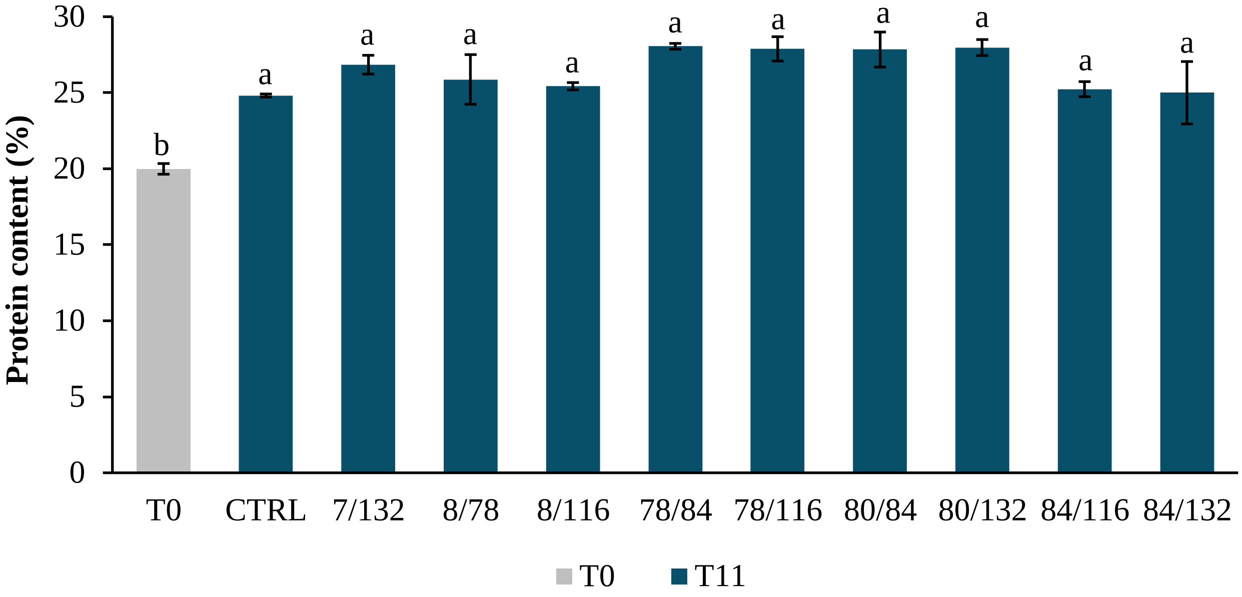

Figure 4.35-Protein content (%) in the biomass of *T. lutea*, harvested immediately after inoculation (T0) after 11 days of culture (T11) with the designed bacterial mixes or without the addition of bacteria (CTRL). Bars correspond to the mean values between biological replicates ± SD (*n*=2). Different letters (a, b) represent statistical differences between groups (ANOVA One-Way with Tukey post hoc test, ρ<0.05)

The protein content at the stationary phase in all the replicates ranged from 24.82±0.10% from the CTRL cultures to 28.05±0.19%, registered in the samples collected from the cultures containing the bacterial mix 78/84 (**Figure 4.35**). Like the previous protein content analysis (**section 4.3.3.2**) these results are marginally above those reported previously (Skrede et al., 2011; Gnouma et al., 2017).

While all samples had higher protein content at the stationary phase than at the beginning of this experiment (T0; 19.99%±0.35%), the addition of the bacterial combinations had no effects on the protein content compared with the CTRL cultures (**Figure 4.35**).

4.4.1.3. Pigment profile

The pigments profile was also assessed following the co-culture assays with the tailor-made mixes (**Table 4.11**).

The carotenoid profile of the microalga used in this assay at stationary phase contained fucoxanthin, lutein and *β*-carotene, the same as the pigment profile detected from Phase I samples (**Table 4.7** and **4.11**). Regarding *β*-carotene, the cultures with higher concentrations were also the CTRL (6.410±1.868 mg. g^{-1}). Still, the replicates containing the lowest content of this carotenoid were the ones to which the bacterial mix 84/116 was added (2.303±0.393 mg. g^{-1}). For both fucoxanthin and lutein, the lowest concentrations were registered in the co-cultures with the bacterial mix 78/116 (6.121±1.708 mg. g^{-1} and 0.437±0.170 mg. g^{-1}, respectively), while the highest was achieved in the CTRL cultures, which contained 9.199±2.456 mg. g^{-1} of fucoxanthin and 1.320±0.368 mg. g^{-1} (**Table 4.11**). From an industrial perspective, fucoxanthin is highly relevant for its bioactive properties and contributions to human and fish health, and following prior reports, *T. lutea* can accumulate approximately up to 18 mg. g^{-1} DW of this carotenoid pigment (Gao et al., 2020; Mohamadnia et al., 2021). However, these values are almost twice as much as the fucoxanthin content determined for the CTRL at T11. Thus, it is possible that the chosen culture conditions (e.g., culture media composition or irradiance) were inappropriate for the elevated production of this compound (H. Pereira et al., 2021; Mohamadnia et al., 2021).

After analyzing the results from the pigment analysis performed during Phase I, optimization was necessary since pigments usually found in *T. lutea* were not detected in the previously used conditions. For this reason, for the pigment analysis of Phase II, an alteration was made to the method (**section 3.7.3**): the dry extract was resuspended in 500 μL of HPLC-grade methanol, concentrating the extract 2x prior to injection in HPLC. This alteration allowed us to have a better overview of the pigment profile of the samples since we were able to quantify carotenoids and Chls, which were not detected previously but have been reported in literature to be present in this marine haptophyte (Beuzenberg et al., 2017; Gonçalves de Oliveira-Júnior et al., 2020).

Therefore, the carotenoid profile of this microalga also included other xanthophylls besides fucoxanthin, such as neoxanthin, violaxanthin, astaxanthin and zeaxanthin, although in lower concentrations. Simultaneously, the Chl *a* and *c* content varied from 0.404±0.028 mg. g^{-1} (bacterial mix 80/84) to 0.653±0.0002 mg. g^{-1} (CTRL) and from 0.047±0.002 mg. g^{-1} (bacterial combination 7/132) to 0.100±0.023 mg. g^{-1} (CTRL), respectively (**Table 4.11**).

Table 4.11-Pigment concentration (mg. g^{-1}) in *T. lutea* at the beginning (T0) and end of the assay without (CTRL) or in co-culture with the designed bacterial mixes (7/132 to 84/132). Values represent pigment concentration means ± SD (*n*=2). Different letters indicate statistical differences between treatments (ANOVA One-way with Tukey post hoc analysis, $\rho<0.05$)

Pigment (mg. g^{-1})	**T0**	**CTRL**	**7/132**	**8/78**	**8/116**	**78/84**	**78/ 116**	**80/84**	**80/132**	**84/116**	**84/132**
Fucoxanthin	7.260 ± 2.049^{a}	9.199 ± 2.456^{a}	7.127 ± 0.347^{a}	7.964 ± 0.035^{a}	6.989 ± 0.176^{a}	7.209 ± 0.608^{a}	6.121 ± 1.708^{a}	6.427 ± 0.043^{a}	7.602 ± 0.545^{a}	6.584 ± 0.159^{a}	7.007 ± 0.397^{a}
Neoxanthin	0.408 ± 0.196^{a}	0.409 ± 0.114^{a}	0.291 ± 0.006^{a}	0.335 ± 0.028^{a}	0.274 ± 0.021^{a}	0.298 ± 0.016^{a}	0.277 ± 0.106^{a}	0.277 ± 0.013^{a}	0.322 ± 0.007^{a}	0.290 ± 0.015^{a}	0.309 ± 0.037^{a}
Violaxanthin	1.418 ± 0.204^{a}	2.248 ± 0.612^{a}	1.478 ± 0.073^{a}	1.724 ± 0.030^{a}	1.546 ± 0.008^{a}	1.705 ± 0.010^{a}	1.373 ± 0.567^{a}	1.386 ± 0.008^{a}	1.664 ± 0.085^{a}	1.482 ± 0.006^{a}	1.486 ± 0.272^{a}
Astaxanthin	0.085 ± 0.030^{a}	0.063 ± 0.017^{a}	0.033 ± 0.0005^{a}	0.034 ± 0.0004 ^{a}	0.032± 0.0002^{a}	0.056 ± 0.033^{a}	0.095 ± 0.083^{a}	0.033 ± 0.003^{a}	0.037 ± 0.002^{a}	0.039 ± 0.006^{a}	0.039 ± 0.007^{a}
Lutein	0.416 ± 0.216^{b}	1.320 ± 0.368^{a}	0.641 ± 0.070^{b}	0.638 ± 0.021^{b}	0.640 ± 0.001^{b}	0.602 ± 0.045^{b}	0.437 ± 0.170^{b}	0.546 ± 0.057^{b}	0.689 ± 0.101^{b}	0.458 ± 0.009^{b}	0.626 ± 0.103^{b}
Zeaxanthin	0.012 ± 0.004^{b}	0.036 ± 0.011^{a}	0.021 ± 0.001^{ab}	0.024± 0.0004^{ab}	0.031 ± 0.005^{a}	0.033 ± 0.004^{a}	0.030	0.024 ± 0.002^{ab}	0.024 ± 0.002^{ab}	0.030 ± 0.003^{a}	0.022 ± 0.003 ^{ab}
***α*-Carotene**	0.020 ± 0.002^{ab}	0.038 ± 0.012^{a}	0.019 ± 0.002^{ab}	0.018 ± 0.002^{ab}	0.019 ± 0.005^{ab}	0.022 ± 0.002^{ab}	0.017 ± 0.011^{ab}	0.018 ± 0.001 ^{ab}	0.015 ± 0.003^{b}	0.015 ± 0.005^{b}	0.017 ± 0.003^{ab}
***β*-Carotene**	3.558 ± 0.491^{ab}	6.410 ± 1.868^{a}	3.567 ± 0.335^{ab}	3.379 ± 0.231^{ab}	3.396 ± 0.877^{ab}	3.752 ± 0.763^{ab}	2.982 ± 1.632^{ab}	3.060 ± 0.119^{ab}	2.676 ± 0.252^{b}	2.303 ± 0.393^{b}	2.598 ± 0.512^{b}
Chl *a*	0.316 ± 0.067^{c}	0.653 ± 0.0002^{a}	0.442 ± 0.022^{bc}	0.485 ± 0.064^{abc}	0.445 ± 0.038^{bc}	0.548 ± 0.111^{ab}	0.464± 0.011^{abc}	0.404 ± 0.028^{bc}	0.431 ± 0.019^{bc}	0.405 ± 0.015^{bc}	0.397 ± 0.032^{bc}
Chl *c*	0.047 ± 0.006^{b}	0.100 ± 0.023^{a}	0.047 ± 0.002^{b}	0.062 ±0.0004^{ab}	0.058 ± 0.008^{ab}	0.065 ± 0.003^{ab}	0.047 ± 0.035^{b}	0.049 ± 0.0003^{ab}	0.060 ± 0.002^{ab}	0.056 ± 0.003^{ab}	0.062 ± 0.008 ^{ab}

4.4.1.4. Vitamin B_{12}

Vitamin B_{12} concentration, in the form of MeCbl, was again evaluated in the biomass of the designed bacterial pair combinations (**Figure 4.36**).

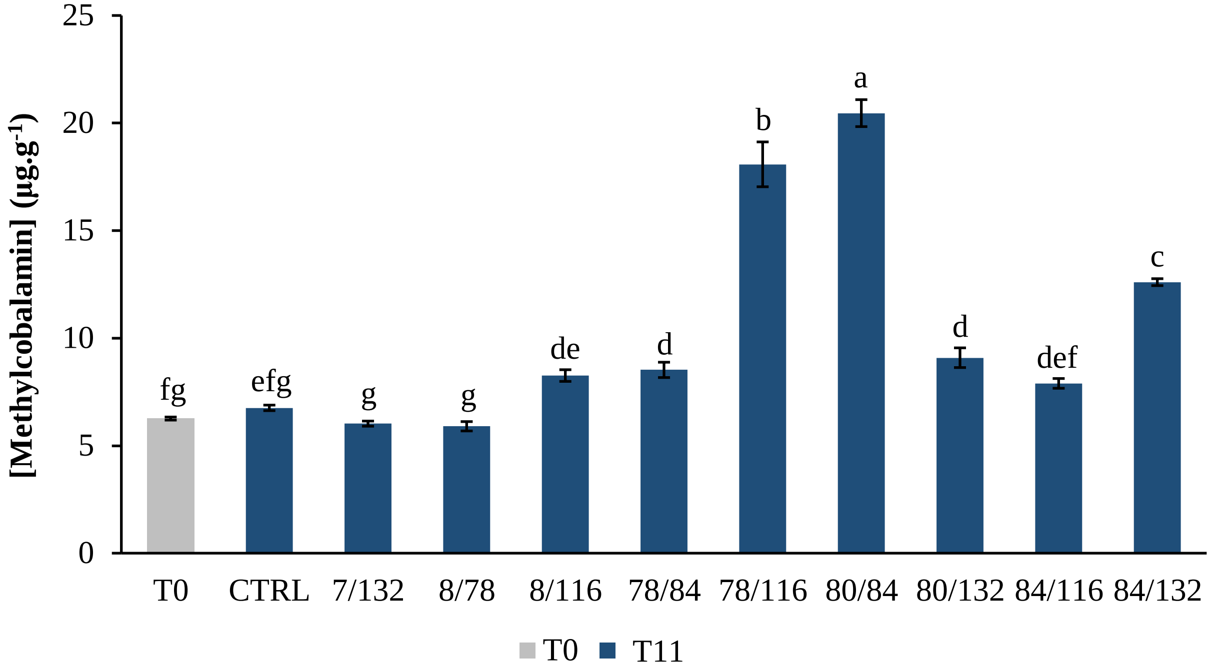

Figure 4.36 - Methylcobalamin concentration (µg. g^{-1}) in *T. lutea* at the beginning (T0) and end of the experiment without (CTRL) or in co-culture with the designed bacterial mixes (7/132 to 84/132). Values represent concentration means ± SD (n=2). Different letters represent differences between treatments (ANOVA One-way with Tukey post hoc test, ρ<0.05)

At the beginning of the experiment (T0), the concentration of MeCbl was 6.259±0.072 µg. g^{-1}, similar to the value measured in the Phase I analysis (6.154 µg. g^{-1}). On the other hand, the MeCbl content in the CTRL at stationary phase (6.757±0.129 µg. g^{-1}) was slightly below the measurements for the CTRL at Phase I (8.123 µg. g^{-1}). However, the second trial from Phase I only lasted 8 days, while this experiment continued until day 11, thus remaining 3 additional days in culture. Since B_{12} is an essential nutrient for the development and survival of this microalga, it is possible that during these 3 extra days, *T. lutea* consumed this active form of B_{12} in several biological processes, leading to a decline in the MeCbl concentration (Heal et al., 2014) .

Concerning the cultures containing the bacterial combinations, the lowest concentration of MeCbl was found on the replicates of the 8/78 group (5.901±0.217 µg. g^{-1}) and the highest on the cultures inoculated with the 80/84 mix, obtaining an average value of 20.460±0.627 µg. g^{-1} (**Figure 4.36**). Moreover, after 11 days of co-culture with *T. lutea*, 5 of the treatment groups with the designed bacterial pairs (78/84, 78/116, 80/84, 80/132 and 84/132) reached MeCbl concentrations significantly higher than of the CTRL (**Figure 4.36**). In 3 of these combinations (78/84, 80/132 and 84/132), the high concentration of this vitamin also was correlated to significant growth improvements, in comparison to the CTRL, maybe due to the better availability of this essential micronutrient (**Figures 4.33** and **4.36**).

On the other hand, the bacterial combinations 78/116 and 80/84 achieved the highest concentrations of MeCbl (18.080±1.043 µg. g^{-1} and 20.460±0.627 µg. g^{-1}, respectively). Still, after 11 days of co-culture with these mixes, *T. lutea* showed no improvement regarding growth performances (**Figures 4.33** and **4.36).** However, as previously mentioned, not all prokaryotes share vitamin B_{12} with microalgae, while some may only provide this nutrient under specific conditions, since the pathways of production of this compound are complex and extensive, having high metabolic and energetic costs for the producing bacteria (Nef et al., 2022; Sultana et al., 2023).

5.Conclusions and Future Perspectives

During this work, a wide variety of bacteria were identified, selected and co-cultivated with *T. lutea* to evaluate the potential of specific bacteria mixes to enhance the productivity and the production of high-value compounds in this microalga.

Initial co-cultures with individual bacterial isolates did not significantly enhance the nutritional profile of *T. lutea*. However, certain strains showed potential in specific areas. Notably, isolates 80 (*Roseovarius* sp.) and 84 (*Haliea salexigens*) were associated with significant improvements in biomass production.

In Phase II, the best-performing bacterial isolates were combined into 21 pairs for further testing. Six combinations (7/132, 8/78, 8/116, 78/84, 80/132 and 84/132) significantly increased *T. lutea* cellular concentration by approximately 30% to 58% in comparison to the control cultures of this microalga. Additionally, the combinations 78/84, 78/116, 80/84, 80/132 and 84/132 showed notable enhancements in vitamin B_{12} (methylcobalamin) concentrations. Furthermore, the pairs 8/78, 8/116, 78/84 and 78/116 showed increases in certain polyunsaturated fatty acids (PUFAs), such as hexadecadienoic acid (16:2*n*-6), α-linolenic acid (18:3*n*-3α) and docosapentaenoic acid (22:5*n*-3). Despite this, there were no significant improvements in protein content or pigment profile compared to those of the control.

Further studies employing metagenomics or whole-genome sequencing (WGS) are necessary to elucidate how the established microbial community of *T. lutea* is influenced by the introduction of specific bacterial mixes. These approaches can help identify shifts in microbial community composition and interactions that contribute to the observed biochemical changes in *T. lutea*. Expanding the biochemical analysis to include amino acids, additional vitamins, and different forms of vitamin B_{12} will provide a more comprehensive understanding of the nutritional profile changes and the interactions between this microalga and the co-cultivated bacteria.

While laboratory-scale results are promising, it is essential to test these co-cultures at an industrial scale to evaluate their practicality and reliability for large-scale production. This includes assessing the economic feasibility and scalability of the approach. Further genetic and biochemical analyses are required to understand the mechanisms behind the observed improvements and to optimize bacterial consortia for enhanced productivity and compound synthesis.

This research successfully demonstrated that the addition of “tailor-made” bacterial mixes to xenic *T. lutea* cultures can significantly enhance biomass production and the productivity of specific bioactive compounds. However, to fully understand the potential of this approach for industrial applications, further validation through large-scale testing and in-depth metagenomic, genetic and biochemical studies are essential. This represents a significant step towards improving the industrial production of *T. lutea*, with important implications for various biotechnological applications.

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Annex

Supplementary Data 1-Optical density measurements at 600 nm registered for the isolates with repeated identification that were selected for IT1

Isolated species	Nº of identified isolates	Highest achieved OD_{600nm}
Alcaligenes faecalis	11	5.864 (isolate 116)
Corynebacterium glyciniphilum	8	6.010 (isolate 17)
Cyclobacterium marinum	5	3.800 (isolate 103)
Flagellimonas marinaquae	2	3.950 (isolate 88)
Halomonas alkaliphila	3	3.040 (isolate 22)
Halomonas titanicae	2	2.664 (isolate 60)
Marinobacter adhaerens	5	5.280 (isolate 96)
Marinobacter nauticus	3	2.416 (isolate 7)
Tritonibacter mobilis	3	12.068 (isolate 123)