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CO-CULTURING OF *Chlorella vulgaris* AND BACTERIA AT LABORATORIAL SCALE

by

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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ABSTRACT

The industrial production of axenic *Chlorella vulgaris* is very challenging as bacterial contaminations are common both in open and closed cultivation systems. Some microalgae and bacteria can establish symbiotic relationships in which both microorganisms benefit from one another, that can result in improved growth and culture robustness. This strategy would be advantageous to co-cultivate *C. vulgaris* with a well-known starter culture of bacteria that is able to promote the microalga's growth. The benefits would be: the establishment of a symbiotic relationship that could improve the growth rates of *C. vulgaris* and the presence of a beneficial bacteria consortium that would prevent the occupation of those niches by unknown and detrimental bacterial contaminants.

The aim of the present work was to find bacterial species that could establish a symbiotic relationship with *Chlorella vulgaris* strain*,* in co-culture, to improve the latter's growth and culture robustness. Thereby, bacterial strains that had previously been isolated from pilot scale cultures of *C. vulgaris* in mixotrophic conditions were co-cultivated with this microalga. Also, a commercially available culture of Lactic Acid Bacteria was tested in co-culture with *C. vulgaris.* The growth of these microorganisms and the constitution of the ecosystems was evaluated throughout time.

Results from this study suggested that two bacteria (LL_Bc07 and LL_Bc11) may have a positive impact on *C. vulgaris* in batch mode while one bacterial isolate (LL_Bc02) may have a negative impact, under the same conditions. On the contrary, in semi-continuous regime, bacterial consortia did not affect the growth of the microalga. It was clear that LAB did not grow well in the mixotrophic medium used, as they are fastidious bacteria. Therefore, the microalga's growth was not affected by the presence of LAB.

Regarding community stability, co-cultures containing two of the bacterial isolates LL_Bc02, LL_Bc07 and LL_Bc11, kept their composition stable from the beginning to the end of the test. On the contrary the monoalgal control and the mixed culture containing *C. vulgaris* and LAB suffered contaminations from one or more of the bacterial isolates.

Key words: Chlorella vulgaris, co-cultivation, bacterial consortium, DGGE, Lactic Acid Bacteria, mixotrophy.

RESUMO

A produção industrial de *Chlorella vulgaris* axénica é muito desafiante, visto que as contaminações bacterianas são comuns, tanto em sistemas de cultivo abertos como fechados. Algumas microalgas e bactérias podem estabelecer relações simbióticas, nas quais ambos os microrganismos beneficiam um do outro, podendo resultar na melhoria do crescimento e robustez da cultura. Esta estratégia poderia ser vantajosa para o co-cultivo de *C. vulgaris* com uma cultura bacteriana de arranque de composição bem conhecida e capaz de promover o crescimento da microalga. Os benefícios seriam: o estabelecimento de uma relação simbiótica que pudesse melhorar as taxas de crescimento da *C. vulgaris* e a presença dum consorcio de bactérias que poderiam prevenir a ocupação desses nichos por outros contaminantes desconhecidos.

O objectivo do presente trabalho foi encontrar espécies bacterianas que pudessem estabelecer uma relação com a estipe de *C. vulgaris*, em co-cultura, para melhorar o crescimento e robustez da última. Assim, estirpes bacterianas previamente isoladas duma cultura de *C. vulgaris*, em condições mixotróficas, à escala piloto, foram co-cultivadas com esta microalga. Para além destas, uma cultura comercial de Bactérias do Ácido Lático (BAL) foi testada em co-cultivo com *C. vulgaris.* O crescimento destes microrganismos e constituição dos ecossistemas foram avaliados ao longo do tempo.

Os resultados deste estudo sugerem que duas bactérias (LL_Bc07 e LL_Bc11) poderão ter um impacto positivo na *C. vulgaris*, em regime *batch*, enquanto um outro isolado bacteriano (LL_Bc02) poderá ter um impacto negativo, nas mesmas condições. Contrariamente, em regime semi-contínuo, os consórcios bacterianos não afectaram o crescimento da microalga. Foi claro que as BAL não cresceram bem no meio mixotrófico usado, visto que são bactérias fastidiosas. Deste modo, o crescimento da microalga não foi afectado pela presença das BAL.

Relativamente à estabilidade da comunidade, as co-culturas que continham dois dos isolados bacterianos – LL_Bc02, LL_Bc07 ou LL_Bc11 – mantiveram a sua composição estável do inicio ao fim do teste. Pelo contrário, o controlo unialgal e a co-cultura de *C.vulgaris* com BAL sofreram contaminações de um ou mais dos isolados bacterianos.

Palavras-chave: Chlorella vulgaris, co-cultivo, consórcio bacteriano, DGGE, Bactérias do Ácido Láctico, mixotrofia.

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LIST OF ABBREVIATIONS

ARA - Arachidonic Acid

BAL – Bactérias do Ácido Láctico

DGGE – Denaturating Gradient Gel Electrophoresis

DHA - Docosahexaenoic Acid

CI – Confidence Interval

EPA - Eicosapentaenoic Acid

FAO - Food and Agricultural Organization

GRAS – Generally Regarded as Safe

LAB – Lactic Acid Bacteria

NA – Nutrient Agar

PBR - Photobioreactor

PCR – Polymerase Chain Reaction

TAP – Tris Acetate Phosphate

PGPB – Plant Growth-Promoting Bacteria

PUFA - Polyunsaturated Fatty Acids

TVC – Total Viable Counts

TY – Triptone Yeast

TY A – Triptone yeast Agar

WHO - World Health Organization

1. INTRODUCTION

1.1. Microalgae: Definition and general characteristics and applications

Algae are a diverse group of photosynthetic eukaryotic organisms that colonise aquatic ecosystems and are accountable for up to 50% of the planet's atmospheric carbon fixation (Field *et al.*, 1998). All algae contain chlorophyll (Lin, 2005) and these organisms can be unicellular (microalgae) or multicellular (macroalgae) (Round, 1973). Algae physiology and metabolism is extremely varied (Ender *et al.*, 2002 and Croft *et al.,* 2006) and microalgae are one of the most primitive forms of plants (Vassilev and Vassileva, 2016).

Algae are categorized mostly into broad groups based on their photosynthetic pigmentation variations: green, blue-green, red, brown and golden algae. The most relevant categories and classes of microalgae in terms of their abundance are: diatoms (Bacillariophyceae), green (Chlorophyceae), blue and blue-green cyanobacteria (Cyanophyceae), golden (Chrysophyceae) and red (Rhodophyceae) algae (Vassilev and Vassileva, 2016). It is important to note that phycologists refer to cyanobacteria as blue-green microalgae, even though they combine characteristics of bacteria and algae (Spolaore *et al.*, 2006, Vassilev and Vassileva, 2016).

Microalgae present high production rates, are able to adapt to diverse environmental conditions and can colonise any aquatic environment providied there is a light and carbon source, nutrients and adequate temperatures (Shelef and Soeder, 1980) and they can also colonise soils (Lin, 2005). Microalgae colonising the planktonic region of the aquatic habitats are together with cyanobacteria, denominated phytoplankton (Field *et al.*, 1998 and Buchan *et al.*, 2014). Two examples of microalgae that colonise very different habitats are *Chlorella vulgaris*, which is a freshwater microalga and *Dunaliella* spp., which colonise hypersaline habitats and are considered halophiles (Ventosa and Nieto (1995).

As photoautotrophic organisms, microalgae use solar energy to synthetize organic matter, from inorganic subtracts such as carbon dioxide, water and nutrients and can perform photosynthesis 2 to 5 times more efficiently than higher plants (Thomas *et al.*, 1984). Nevertheless, these microorganisms can also grow under heterotrophic and mixotrophic conditions (Pérez-García *et al.* 2010).

Microalgae are one of the richest sources of proteins (Raposo *et al.*, 2013) and as their cells can synthesise all amino acids, they can provide all the essential ones to humans and animals. For example, *C. vulgaris* presents a total protein content up to 58% of dry weight and due to its high protein content and quality in the amino acid profile, has been proposed for human nutrition, by the World Health Organization (WHO) and the Food and Agricultural Organization (FAO) (Safi *et al.,* 2014). Also, the digestibility of dried whole microalgae is high (Spolaore *et al.* 2006). Furthermore, some microalgae and cyanobacteria species produce high-value compounds such as polyunsaturated fatty acids (PUFAs), carotenoids, phycobiliproteins, polysaccharides, vitamins, or sterols. Amongst PUFAs produced by microalgae, it is possible to find eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA). The latter is a ω-3 fatty acid found in tissues in the human body (Spolaore *et al.*, 2006). These PUFAs with more than 18 carbons are high value molecules that cannot be synthetized by higher plants or animals, hence, have to be obtained through the consumption of fish or fish oil. PUFAs are present in these products as fish feed on microalgae. These facts make microalgae a particularly interesting source of PUFAs (Spolaore *et al.*, 2006). DHA and EPA are two important PUFAs and are associated with the reduction of cardiovascular problems, the development and functioning of the nervous systems and are important as anti-inflammatory agents (Raposo *et al.*, 2013).

Due to their varied characteristics, microalgae have very diverse industrial applications. They can be used as nutraceutical foods and feeds as whole cells or certain compounds produced by microalgae can be incorporated in functional foods. For instance, some of the PUFAs produced are essential ω-3 and ω-6 that can be included in functional foods or feeds, such as feed for chickens, therefore supplying eggs enriched with those fatty acids (Raposo *et al.*, 2013). Microalgae and/ or their compounds can also be used in the cosmetic industry. In this case microalgae extracts can be incorporated in skin-care products with different functions, such as anti-aging and skin-whitening, among others (Wang *et. al.*, 2005; Spolaore *et al.*, 2006). Microalgae are also very important in aquaculture as they are required in larval nutrition for small live prey fed to fish and shrimp and for direct consumption of mollusks. Finally, some microalgae can be used in the production of biofuels, as an alternate source of energy, in wastewater treatment and as biofertilizers (Raposo *et al.*, 2013).

Besides all the characteristics that make microalgae interesting products, one major advantage of cultivating microalgae is not requiring agriculturally productive land and requiring much less land for their cultivation than terrestrial crops (Vassilev and Vassileva, 2016).

1.2. *Chlorella* spp.

1.2.1. Characteristics and metabolism

Microalgae belonging to the genus *Chlorella*, are unicellular eukaryotic microorganisms and green algae (Lin, 2005). These can inhabit both aquatic and terrestrial habitats (Liu et al., 2014).

Cells from most *Chlorella* species are spherical; however, rare cases present an ellipsoidal shape (Lin, 2005). The cell structure of *Chlorella* spp. is composed by a defined small nucleus, a large portion of cup-shaped chloroplasts, which contain chlorophyll, a cell wall composed by cellulose, a visible Golgi complex, mitochondria and can have accumulated substances, such as lipid granules or starch. Additionally, some species may contain a pyrenoid. (Lin, 2005). Figure 1.1 shows a detailed representation of the structures within a *C. vulgaris* cell.

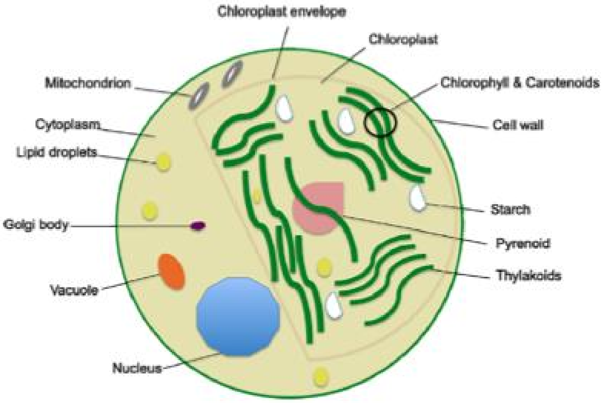


Figure 1.1 - Schematic structure of *Chlorella vulgaris*, representing diverse organelles (in: Yamamoto *et a*l., 2004).

Chlorella vulgaris is a freshwater microalga, grows rapidly and reproduces asexually through autosporulation. During this process, four daughters cells are formed inside their mother cell and after maturation the cell wall of the mother cell ruptures, releasing the newly formed cells (Yamamoto *et al.*, 2004). Figure 1.2 represents this process with more detail.

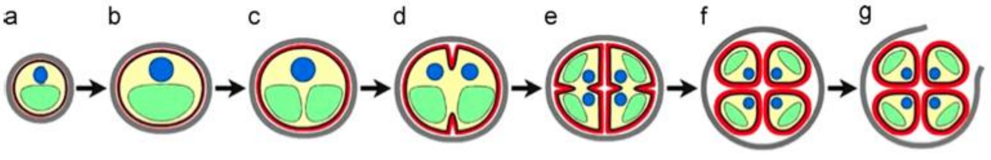


Figure 1.2 - Schematic representation of different phases of daughter cell wall formation in *Chlorella vulgaris*: (a) early cell-growth phase; (b) late cell-growth phase; (c) chloroplast dividing phase; (d) early protoplast dividing phase; (e) late protoplast dividing phase; (f) daughter cells maturation phase and (g) hatching phase (In: Yamamoto *et al*., 2005).

Chlorella vulgaris has a rapid growth rate and can grow in different modes: autotrophy, heterotrophy and mixotrophy. In autotrophic growth, cells perform photosynthesis, which requires light. On the contrary, heterotrophic growth does not require light but rather an organic carbon source. The carbon sources used to cultivate *C. vulgaris* are acetate, glycerol, glucose and glutamate. Among these, glucose allows obtaining the highest maximum specific growth rate. The main disadvantage of this growth mode is the price and availability of sugars, to add to the culture (Safi *et al.*, 2014). Finally, it is also possible to grow *C. vulgaris* under mixotrophic conditions, combining autotrophic and heterotrophic growth, by performing photosynthesis, since there is light while using an organic carbon source (Lee, 2001 and Liang *et al.*, 2009). In this way, cells are not completely dependent on light or on the organic carbon source, limiting the impact of biomass loss during dark respiration. Also, comparing to heterotrophic growth, mixotrophy reduces the quantity of organic substrates needed (Safi *et al.*, 2014).

Regarding *Chlorella* spp., the industry is focused primarily on human nutrition and animal feed (Liu et al., 2014) and *C. vulgaris, C. pyrenoidosa* and *C. luteoviridis* are authorized by the Novel Food Regulamentation (European Commission) as a food or food ingredient (www.ec.europa.eu). Nonetheless, some *Chlorella* spp. can also be used for biofuel production. For instance, the fatty acid profile of *C. vulgaris* and its high starch content, makes this microalga a good source for biofuel and bioethanol production, respectively (Safi *et al.*, 2014).

When selecting a cultivation system and growth mode to grow *Chlorella* spp., the intended profile of the final product must be considered.

Regarding cultivation conditions, microalgae have considerable plasticity and can be cultivated using different operating techniques for substract addition: batch, fed-batch and continuous (Wang *et al.*, 2015). As for microalgal culture systems, there are some options available: open ponds (Belay, 1997), tubular photobioreactors (Carlozzi, 2000; Hai *et al*., 2000; Molina *et al*., 2001), flat plate photobioreactors (Green Wall^{TM}) panels, column photobioreactors (Zittelli *et al*., 2006; Hulatt and Thomas, 2010), and stirred tank reactors (Li *et al*., 2003; Eriksen *et al*., 2007). Circular and raceway ponds are frequently used devices for cultivation of *Chlorella* spp. (Lin, 2005; Liu *et al.*, 2014). Even though open systems imply less building costs, they present disadvantages, namely difficulties managing temperature, rapid evaporation and by becoming more prone to microbial contamination, in comparison to more sophisticated PBRs (Liu *et al.*, 2014).

Regarding metabolism, studies have shown that the carbon source and growth modes can have an impact on the cellular composition (Ogbona *et al.*, 1997; Liang *et al.*, 2009). Furthermore, it is important to consider the fact that different growth modes and subtracts may impact the maximum specific growth rate of an algae. *Chlorella vulgaris* has been reported an exception as it was demonstrated its maximum growth rates were comparable in heterotrophic and autotrophic modes (Lee, 2001).

1.3. Relationships established between microalgae and other microorganisms

Algae and bacteria are numerically dominant organisms in the plankton of aquatic environments, such as oceans and lakes (Cole, 1982) and have coexisted since the early stages of evolution (Ramanan, *et al.*, 2016). Together, these microorganisms influence a variety of ecosystems from deep seas to lichens, exemplifying all types of interactions between microorganisms, from mutualism to parasitism (Ramanan, *et al.*, 2016).

In natural environments, microalgal growth is always related to the growth of other microorganisms, especially bacteria (Reynolds, 2016). In the 1970's, Bell & Micthel (1972) coined the term 'phycosphere' as a concept resembling the interactions between different microorganisms in the rizosphere. According to this concept, the phycosphere consists on the existence of a zone surrounding an algal cell or colony within which other microorganisms are stimulated by extracellular products from the alga. (Bell and Mitchel,1972).

More recently, numerous studies demonstrated heterotrophic bacteria are ubiquitously involved in the growth and survival of algae (Gonzalez and Bashan, 2000; Seyedsayamdost *et al.*, 2011; Kim *et al.*, 2014 and Amin *et al.*, 2015). Ramanan (2015) showed in a phylogenetic study that a predominant group of bacteria, including Proteobacteria and Bacteroidetes are more likely to be associated with green algae than other phylotypes. Moreover, this study also showed that these bacteria are functionally equipped to be associated with green algae. Bashan and Holguin (1998) had previously introduced the concept of Plant Growth Promoting Bacteria (PGPB), which consists in a group of bacteria able to enhance the growth of plants. This term was introduced to be used regardless of the bacterial genus or species and to be broad enough to include any kind of plant (Bashan and Holguin, 1998), whether terrestrial or aquatic.

Interactions between microalgae and bacteria include every type of symbiotic relationship possible. Therefore, as these interactions are ubiquitous in most ecosystems, it is often difficult to isolate, maintain and study algae in axenic cultures, due to their different metabolism and physiology when compared to a culture with their ubiquitous bacteria (Cho *et al.*, 2013; Amin *et al.*, 2015 and Ramanan, 2016). Moreover, interspecies interactions are often hard to observe and distinguish in nature, as ecosystems tend to be in equilibrium conditions and therefore, interactions are easier to be identified after long periods of observation or disturbance in the system (Amin, 2016).

1.3.1. Mutualism

Several cases of mutualistic interactions involving microalgae have been reported in the literature. These symbiotic relationships can be varied, ranging from obligate mutualism, in which symbionts cannot survive without each other, to facultative symbionts in which the environment can reduce the symbiotic effect (Medina and Sachs, 2010).

For instance, many algae are rich in cobalamin (vitamin B12) and require it for their growth, regardless the inability of many species to synthetize it. This fact implies many microalgae species are not truly autotrophic organisms and are indeed auxotrophs for vitamin B12 (Croft, 2005; Kazamia *et al.*, 2012). Furthermore, a survey revealed this requirement appears unrelated to established algal lineages, as every phyla contain species that need an exogenous source of this vitamin and others that do not (Croft, 2005). Therefore, in nature, vitamin-B12 dependent algae need to obtain this micronutrient from an external source of vitamin B12, through an interaction with bacteria. It has been proposed this interaction is based on the benefit of both microrganisms, in which the microalgae use the vitamin B12 produced by the bacteria and in return, supply fixed carbon to bacteria (Croft, 2005).

Kazamia *et al*. (2012) found a contaminating bacterium, *Mesorhizobium loti,* in a culture of the B12-dependent green microalga *Chlamydomonas nivalis* and could cultivate it with another green microalga – *Lobomonas rostrata.* These two strains (*M. loti* and *L. rostrata*) were then co-cultivated in a medium without a carbon source and could sustain each other's growth, by establishing an algal-bacterium symbiosis. This symbiosis consists in a facultative mutualism in which, in the absence of carbon and vitamin B12, the bacterium provides *L. rostrata* with vitamin B12 and the latter supplies fixed carbon to *M. loti*.

Other studies, including Gonzalez and Bashan (2000) and Watanabe *et al.* (2005) showed mutualistic relationships between *Chorella* spp. and bacterial symbionts, namely *Azospirillum* spp and a bacteria belonging to high G+C Gram-positive bacteria group (*Microbacterium* sp.), respectively. In the first study, *C. vulgaris* was coimmobilized with the PGPB *Azospirilum brasilense* in small alginate beads. Results showed the coimmobilization of the two microorganisms in the same bead, significantly increased the growth of the microalga. Besides presenting a significant increase in microalgal growth, there was also higher production of pigment in the microalgal cells. The authors speculated the effect of the bacterium in microalgal growth promotion could be due to the production of pytohormones by *A. brasilense* capable of stimulating the growth of *C. vulgaris* (Gonzalez and Bashan, 2000). Regarding Watanabe *et al.* (2005), a symbiotic association between *Chlorella sorokiniana* and five non-algal constituents of a natural microbial consortium maintained in a slant culture was studied. In this case, four individual bacterial strains and a fungal strain were individually co-cultivated with *C. sorokiniana* and results showed that, under photoautotrophic conditions, one of the bacterium, *Microbacterium* sp., and the fungal strain were able to significantly increase the growth rate of the microalga, hence promoting its growth (Watanabe *et al.*, 2005).

1.3.2. Commensalism

Commensalism, unlike mutualism, consists in a relationship in which only one partner benefits from the other. Nonetheless, there is a fine line between most cases of commensalism, mutualism and even parasitism, which depends on environmental factors. Therefore, it is understood that these interactions are a continual interface (Ewald, 1987; Johnson et al., 1997; van Ommeren and Whitham, 2002; Neuhauser and Fargione, 2004; Karst et al., 2008 and Valiente-Banuet and Verdú, 2008 Hu et al., 2010;). Thus, from this point of view, most of algal-bacterial associations studied are either mutualistic or parasitic, with fewer references to commensalism in the literature (Ramanan, 2016).

One example of commensalism in algal-bacterial interactions is the study of several mixed cultures with different bacterial symbionts, in which different types of algal-bacterial interactions were established. Amongst these, the growth of *C. sorokiniana* was not significantly affected by a strain of *Ralstonia* sp. and one of *Sphingomonas* sp., under photoautotrophic condition. Yet, these bacteria received nutrients from the microalga, hence demonstrating commensalism (Watanabe *et al.*, 2005).

1.3.3. Competition

Bacteria can induce negative effects on algal growth in various ways. Some bacteria are capable of infecting microalgae or causing lysis of algal cells (Cole, 1982).

Regarding competition, bacteria may compete with the microalgae for available nutrients, namely nitrogen and phosphorus (Hyenstrand *et al*. 2000; Joint *et al*., 2002). Since bacteria have faster growth rates, their proliferation can consume more nutrients, inhibiting microalgal growth (Lakaniemi *et al.*, 2012). Also, bacteria may produce metabolites that are inhibitory to microalgal growth (Cole, 1982).

A case of competition between microalgae and other microorganisms was demonstrated by Watanabe *et al.* (2005). In such study, *Chlorella sorokiniana* and a fungal strain were co-cultivated with 1% of glucose and the growth of the fungal strain was promoted, whereas the growth of *C. sorokiniana* was significantly inhibited. However, under photoautotrophic conditions, the same mixed culture demonstrated a mutualistic interaction since the fungal strain received nutrients from *C. sorokiniana*, while promoting its growth.

1.4. Improvement of *Chlorella* spp. Productivity in Mixed Cultures of Symbiotic Bacteria

It is most common to use pure cultures in industrial microbiology. However, symbiotic associations have been used in fermented food industries, wastewater treatment and activated sludge (Watanabe *et al.*, 2005).

In microalgal cultures, heterotrophic organisms are commonly considered contaminants and harmful for algal growth, in biomass production systems (Belay, 1997; Huntley and Redalje, 2007). Despite these views, some reports suggest that certain bacteria and fungi can have positive effects on algal growth (Watanabe *et al*., 2005, Park *et al*., 2008). Rivas *et al.* (2010) suggested the use of a beneficial bacterium found, associated with *Botryococcus braunii*, as inoculant for larger-scale production of microalgae. Hence, as in that study *Rhizobium* sp. promoted microalgal growth, the author proposed this bacterium could have positive effects on *B. braunii* in mass cultures, where the maintenance of axenic conditions is impossible (Rivas *et al.*, 2010).

It is common to have bacterial contaminations during large scale cultivation of *C. vulgaris*. However, the growth-promoting effects of bacteria on microalgal cultures have been described as a strategy to increase the production of microalgae as they increase biomass production by enhancing growth. (Rivas et al. 2010), This growth improvement can be originated through different mechanisms. For instance, by the interference of bacteria in the algal hormonal mechanism (Gonzalez and Bashan, 2000) or the bacterial production of substances, such as vitamin B12 that can stimulate microalgal growth (Cole, 1982; Croft *et al.*, 2005).

Several studies have demonstrated growth promoting effect of certain bacteria on the growth of *Chlorella* spp.. In the case of *C. vulgaris,* different plant growth promoting bacteria (PGPB) have been studied and their growth promoting effect has been verified when co-immobilized in alginate beads (Gonzalez and Bashan, 2000; Hernandez *et al*., 2009). Also, when in mixed culture, a *Pseudomonas* sp. had a growth-promoting effect on *C. vulgaris*, when comparing to a single algal culture. The effect of *Pseudomonas* sp. in co-culture resulted in about 1.4 times higher final cell concentration than in monoculture, after 8 days of cultivation (Guo and Tong, 2013).

Regarding other strains of *Chlorella* spp., Park *et al.* (2008), isolated eight bacterial strains from a long-term culture of *C. ellipsoidea* and co-cultivated each one of them with this microalga, finding out that all of them promoted the growth of *C. ellipsoidea.* The co-cultivation of each bacterial strain resulted in a 0.5 - 3 times higher growth of *C. ellipsoidea* in mixed culture than in single algal cultures. The most effective bacterium was *Brevundimonas* sp. and its growth was also positively affected by *C. ellipsoidea*, demonstrating a symbiosis between them (Park *et al.* 2008). Additionally, under photoautotrophic conditions, one bacterial isolate, identified as *Microbacterium* sp. and one fungal isolate were found to promote the growth of *C. sorokiniana* in mixed cultures. These microorganisms were also isolated from a non-axenic culture of *C. sorokiniana,* along with two other bacterial strains that did not seem to affect the growth behavior of the microalga, demonstrating commensalism (Watanabe *et al*., 2005).

Lastly, a better understanding of the bacterial role in mixed cultures may be invaluable to promote the growth of *Chlorella* sp., particularly for commercial cultivation (Tate *et al*., 2012).

1.5. Motivation/ Hypothesis

The industrial production of axenic *Chlorella vulgaris* is very challenging as bacterial contaminations are common both in open and closed cultivation systems (Guo and Tong, 2013). Nonetheless, some studies have shown that culturing microalgae in co-cultures with specific bacterial strains can be beneficial, improving the former's growth (Watanabe *et al.,* 2005; Liang *et al.*, 2014; Guo and Tong, 2013). Bacterial genera such as *Pseudomonas* sp., *Micrococcus* spp. and *Azospirilum* spp. were all identified as promoters of microalgae growth (Guo and Tong, 2013; Watanabe *et al.*, 2005; Gonzalez and Bashan, 2000).

Considering previous studies, it was hypothesize that it would be industrially advantageous to co-cultivate *C. vulgaris* with a well-known starter culture of bacteria that is able to promote microalgal growth. The benefits of such strategy would be the establishment of a symbiotic relationship that could improve growth rates and the presence of beneficial bacteria at the time of inoculation that would prevent the occupation of those niches by unknown and detrimental bacterial contaminants.

In order to study this hypothesis, in this thesis, results from co-cultivation studies carried out at laboratorial scale are presented. These co-cultures were set-up between a *C. vulgaris* strain and a set of five different bacterial strains previously isolated from an industrial mixotrophic *C. vulgaris* culture. In addition, a commercially available mix of Lactic Acid Bacteria was also tested in co-culture with *C. vulgaris*.

1.6. Objectives

The main objectives of the present work are:

1. To find a bacterial consortium able to establish a symbiotic relationship with *C. vulgaris*, in co-culture, improving the latter's growth and culture robustness.

To accomplish this objective several sub objectives were taken in consideration namely:

  a) To test the maximum number of bacterial consortia with two bacterial isolates, in co-culture with *C. vulgaris*;
  b) To test different microalgal - bacterial ratios and chose one for the following experiments;
  c) To co-cultivate *C. vulgaris* with bacterial consortia in batch and semi-continuous, in mixotrophic conditions;
  d) To test the co-cultivation of *C. vulgaris* with a commercially available consortium of Lactic Acid Bacteria.

2. To study the stability of the ecosystem throughout time.

To accomplish this objective several several sub objectives were taken in consideration namely:

  a) To find out if the bacterial consortia were maintained throughout time;
  b) To analyse possible contaminations;
  c) To use Denaturating Gradient Gel Electrophoresis (DGGE) for the analysis.

2. MATERIALS AND METHODS

This chapter will include the materials and methods used to carry out the assays comprised in this study.

2.1. Microorganisms

2.1.1. Microalga

Chlorella vulgaris used in this study was obtained from A4F's algoteca and was chosen since it had been the strain grown in A4F's photobioreactors where the bacterial isolates were isolated from. The cultivations in those photobioreactors had been in mixotrophic conditions with acetate as carbon source, hence this study kept such conditions.

2.1.2. Bacteria

Five bacterial strains that had previously been isolated by A4F, from a culture of *C. vulgaris* grown in a pilot scale photobioreactor, were used in this study. These isolates had been previously sequenced by A4F to determine their genera. Such results are presented in table 2.1.

Furthermore, a commercially available Lactic Acid Bacteria starter culture was used in this study. This mixed starter culture is BLC35 from CHR Hansen (Hoersholm, Denmark) and includes strains of *Lactobacillus curv*atus, *Staphylococcus xylosus* and *Pediococcus acidilactici*. In this thesis, BCL35 is hereafter identified as LAB.

Table 2.1 - Sequencing results of the bacterial strains isolated from a *Chlorella vulgaris* culture a pilote-scale.

A4F identification	Top Blast hit versus NR database
LL_Bc02	*Acidovorax* sp. (100% similarity)
LL_Bc07	*Sphingomonas* or *Sphingopyxis* sp. (99% similarity)
LL_Bc08	Uncultured bacterium (100% similarity)
LL_Bc10	Uncultured bacterium (100% similarity)
LL_Bc11	*Hydrogenophaga pseudoflava* strain PBR-28 (100% similatiry)

2.2. Maintenance of Cultures and Inoculum Preparation

2.2.1. Microalga and culturing conditions

To viably store the strain, *C. vulgaris* was kept in Tris Acetate Phosphate (TAP) agar slants at 4ºC. Also, colonies were grown on plates and picked onto fresh plates every two weeks, which were kept at 25 ºC.

Microalgal pre-inoculum was always prepared by picking an isolated *C. vulgaris* colony onto 5 mL of TAP medium (composition described in tables 7.1, 7.2, 7.3 and 7.4, in the appendix) and incubated in an orbital shaker at 25º C and 120 rpm, for 7 days. After this period, the pre-inoculum was used to inoculate other cultures intended for tests.

In every assay performed, *C. vulgaris* inoculum was prepared in the same way and before setting up the cultures for test, *C. vulgaris* cells from the pre-inoculum were quantified cusing Muse cell analyser (Millipore Corporation, Hayward, USA).

2.2.2. Bacteria

Bacterial isolates were maintained on Triptone Yeast agar (TYA), (containing 5 g/L triptone (Liofilchem, Italy), 3 g/L Yeast Extract (Oxoid, UK), 5,95 mM $CaCl_2.2H_2O$ (Panreac Applichem, Spain) and 15 g/L agar powder (Himedia, India)) and were picked onto fresh plates once or twice a week, depending on each isolate's growth. LAB were kept in Nutrient Agar (Himedia Laboratories, India) and picked to new plates twice a week.

Additionally, slants with all the bacteria in their respective media were kept at 4ºC, in triplicate.

To prepare the bacterial pre-inocula, an isolated colony from each bacterial strain was picked and put into a respective 15mL tube containing 5 mL of Triptone Yeast (containing 5 g/L triptone (Liofilchem, Italy), 3 g/L Yeast Extract (Oxoid, UK), 5,95 mM $CaCl_2.2H_2O$ (Panreac Applichem, Spain). These were then incubated for two days, at 25ºC and used to inoculate other pre-inocula, at 1%. Fresh pre-inoculum was incubated at 25ºC, for 24 hours and used as inoculum for the mono and co-cultures tested in the assays. For all the tests the, pre-inoculum was prepared beforehand as described here. Bacterial pre-inocula were also prepared in TAP medium, however, some isolates did not grow in such medium, reason why TY was used.

2.3. *Chlorella vulgaris* and bacteria Preliminary Ratio Assay

In this preliminary assay, three different bacterial strains were independently co-cultivated with *C. vulgaris* to determine the best ratio of *C. vulgaris* and bacterial inoculum. In order to do so, five proportions of inoculum were tested: 1 *Chlorella* cell: 10 bacterial cells; 1 *Chlorella* cell: 1 bacterial cell; 10 *Chlorella* cells: 1 bacterial cell and 100 *Chlorella* cells: 1 bacterial cell. Microorganisms used in this test were the following: *C. vulgaris*, LL_Bc02, LL_Bc08 and LL_Bc11.

Prior inoculation, 1 mL of each bacterial pre-inoculum was centrifuged, washed and resuspended in TAP medium. Furthermore, *C. vulgaris* cell counts were obtained using Muse cell analyzer (Millipore Corporation, Hayward, USA). Then, different co-cultures containing *C. vulgaris* and one bacterial strain were set up in a 24-well microplate in a final volume of 3 mL TAP medium. *C. vulgaris* was inoculated with a final concentration of $1{,}0 \times 10^6$ cells/mL and bacterial concentrations varied according to the tested ratios. This assay was performed in batch mode and lasted five days.

Daily sampling of the co-cultures was performed for OD_{600} measurements (SPECRTOstar Nano, BGM Labtech, Germany) and *C. vulgaris* cell counts, which were obtained using Muse cell analyser (Millipore Corporation, Hayward, USA). Furthermore, bacterial pre-inoculum was plated onto Nutrient Agar and TAP agar, in order to verify the number of bacteria inoculated.

Maximum growth rates of *C. vulgaris* were then estimated based on the growth curves obtained, as described in section 3.1.

Cultures were cultivated in mixotrophy, as TAP provides acetate as an organic carbon source, while in the presence of light. Therefore, both autotrophy and heterotrophy operate simultaneously in nutritional uptake (Lin, 2015). Furthermore, the organic source of nutrients provided in TAP can also be used to feed the bacteria in mixed culture.

2.4. Co-cultures in Batch Regime

To test the maximum number of bacterial consortia containing two bacterial strains, the maximum amount of combinations was tested. With five bacterial isolates, it was possible to test 10 consortia of two bacteria, in combination with *C. vulgaris.* Additionally, a co-culture of *C. vulgaris* was also tested.

List of combinations of *C. vulgaris* and bacteria tested:

- *C. vulgaris* + LL_Bc02 + LL_Bc07;
- *C. vulgaris* + LL_Bc02 + LL_Bc08;
- *C. vulgaris* + LL_Bc02 + LL_Bc10;

- *C. vulgaris* + LL_Bc02 + LL_Bc11;
- *C. vulgaris* + LL_Bc07 + LL_Bc08;
- *C. vulgaris* + LL_Bc07 + LL_Bc10;
- *C. vulgaris* + LL_Bc07 + LL_Bc11;
- *C. vulgaris* + LL_Bc08 + LL_Bc10;
- *C. vulgaris* + LL_Bc08 + LL_Bc11
- *C. vulgaris* + LL_Bc10 + LL_Bc11
- *C. vulgaris* + LAB.

The co-cultures were set up in mixotrophic medium TAP, in 50 mL T flasks (30 mL volume and 20 mL headspace). This test was performed in a batch regime for 7 days and cultures were incubated at 25ºC in an orbital shaker, at 120 rpm.

Furthermore, individual controls of each microorganisms (bacteria and *C. vulgaris*) were inoculated and analysed in the same way as co-cultures.

2.4.1. Preparation of Pre-inoculum and test conditions

Based on the ratio assay, the inoculation proportion used to inoculate co-cultures in this test and further ones, was 10 *C. vulgaris* cells to 1 bacterial cell. The reasons for such choice are discussed in chapter 3.1. In this case, as the bacterial portion of the co-culture was composed by two bacteria, the final concentration of bacteria intended for inoculation was divided by two and that was the amount of each bacterium inoculated.

Prior inoculation, 1 mL of each bacterial pre-inoculum was centrifuged at 3500 rpm for 10 min, washed and resuspended in TAP. *C. vulgaris* pre-inoculum cell counts were obtained using Muse cell analyser (Millipore Corporation, Hayward, USA) and pre-inoculum was observed under the microscope to look for any bacterial contaminations.

The initial concentration of *C. vulgaris* was set to approximately 2,25x10^5 cells/ mL and it was chosen based on previous studies and procedures from A4F. Based on this value, bacterial concentrations were then adjusted to work with the ratio of 10 *C. vulgaris* cells to 1 bacterial cell.

2.4.2. Monitoring of the cultures

During this experiment, several parameters of the controls and co-cultures were monitored: growth evolution of the community, microalgal growth, bacterial growth and microscopic aspect of the culture. In order to monitor these parameters, several methods and techniques were used.

Motorization of the community growth and microalgal growth were performed every day. Regarding the first, the growth of the whole community was quantified through OD_{600} measurements (SPECRTOstar Nano, BGM Labtech, Germany). *C. vulgaris* was quantified through cell counts, using Muse cell analyzer (Millipore Corporation, Hayward, USA). Furthermore, bacterial growth was monitored every other day, through total viable counts (TVC) in NA and TAP agar, using Miles and Misra method with serial dilutions prepared in Ringer solution. Plates were incubated at 25 ºC for 2 -3 days. The evolution of the cultures was also followed every other day through microscopic observation using BX53 microscope (Olympus, Japan).

Finally, at the end of this assay, the amount of acetate was quantified using an aquarium test kit from Hagen Nutrafin® (Canada), to assess if cultures had exhausted the organic carbon source.

The analysis methods, aims and frequency of analysis are described in table 2.6.

Table 2.2 - Methods used to monitor the development of mono and co-cultures and frequency of monitorisation throughout the assay in batch regime.

Methods and Equipment used	Aim	Frequency
Quantification through cell counts using Muse cell analyzer (Millipore Corporation, Hayward, USA)	Monitor the growth of *C. vulgaris*	Daily
Quantification of algal and bacterial growth through OD_{600} measurements (SPECRTOstar Nano, BGM Labtech, Germany)	Monitor the growth of the whole community (*C. vulgaris* and bacteria)	Daily
TVC in NA and TAP agar, through Miles and Misra method, using serial dilutions. Plates were incubated at 25 ºC for 2 -3 days. Serial dilutions were prepared in Ringer solution.	Monitor bacterial growth	Every other day
Microscopic observations using BX53 microscope, Olympus, Japan	Monitor the evolution of the co-cultures, qualitatively.	Every other day
Acetate quantification using an aquarium test kit from Hagen Nutrafin® (Canada)	Assess if cultures had consumed all of the acetate from the culture medium	Last day of test

2.5. Co-cultures in semi-continuous regime

2.5.1. Co-cultures inoculation and monitoring

Based on the previous assay, four co-cultures were chosen for further studies. These four co-cultures and monoalgal and bacterial controls were cultivated in this assay in a semi-continuous regime, with two weekly renewals, after reaching the exponential phase and in the same mixotrophic conditions as in previous assays. The co-cultures studied here were the following: *C. vulgaris* + LL_Bc02 + LL_Bc07, *C. vulgaris* + LL_Bc02 + LL_Bc011, *C. vulgaris* + LL_Bc07 + LL_Bc11 and *C. vulgaris* + LAB. Cultures were inoculated in T-flasks in a final volume of 100 mL and with 100 mL of headspace and were incubated for 17 days at 25º C, in an orbital shaker at 120 rpm.

As in the previous assays, the microalga: bacteria proportion used to inoculate co-cultures in this test and further ones, was 10 *C. vulgaris* cells to 1 bacterial cell. Moreover, pre-inocula preparation and analysis were performed as described in section 2.4.1.

The initial concentration of *C. vulgaris* was set to about $1{,}0\times10^{6}$ cells/ mL and it was chosen based on previous studies and procedures from A4F. Based on this value, bacterial concentrations were adjusted to work with the ratio of 10 *C. vulgaris* cells to 1 bacterial cell.

The evolution of the co-cultures was monitored as described in section 2.4.2, with the exception of the acetate quantification, which was not performed here, as the culture medium was renewed several times. In addition to the methods used in the previous assay, a DGGE analysis was performed at the end of this assay, with the aim of examining the composition of whole the community throughout time. To do this, samples were collected at the beginning and end of the assay and in every renewal point and at the end, three time points were chosen to be analysed.

The analysis methods, aims and frequency of analysis are described in table 2.7.

Table 2.3 - Methods used to monitor the development of mono and co-cultures and frequency of monitorisation throughout the assay in semi-continuous regime.

Methods and Equipment used	Aim	Frequency
Quantification through cell counts using Muse cell analyzer (Millipore Corporation, Hayward, USA)	Monitor the growth of *C. vulgaris*	Daily
Quantification of algal and bacterial growth through OD_{600} measurements (SPECRTOstar Nano, BGM Labtech, Germany)	Monitor the growth of the whole community (*C. vulgaris* and bacteria)	Daily
Total Viable Counts (TVC) in NA and TAP agar, through Miles and Misra method. Plates were incubated at 25 ºC for 2 -3 days. Serial dilutions were prepared in Ringer solution.	Monitor bacterial growth	Every other day
Microscopic observations using BX53 microscope, Olympus, Japan	Monitor the development of the co-cultures, qualitatively.	Every other day
DGGE analysis	Analyse the composition of whole the community throughout time	Samples collected at the beginning, end of assay and in every renewal point.

2.5.2. Molecular Biology Procedures

With the aim of performing a DGGE analysis of the samples from the co-cultures, the following steps of molecular techniques were necessary (described in chapters 2.5.2.1, 2.5.2.2, 2.5.2.3 and 2.5.2.4).

Although samples were collected at the beginning and end of the assay and also in every renewal, three-time points were chosen for co-cultures and *C. vulgaris* control: t0, t19 and t27. These were chosen to compare the content of the co-cultures at the beginning and end of the assay and also around the middle of the assay. Besides, the monoalgal control had suffered a bacterial contamination visible on agar plates, prior t19, reason why this time point was chosen. One additional time point was included from the co-culture containing *C.*

vulgaris and LAB as this had suffered a contamination from another bacterium that appeared on plates after t14. Therefore, t14 was also chosen for this analysis.

2.5.2.1. DNA Extraction

Genomic DNA was extracted from co-cultures samples, using FastDNA® SPIN Kit for Soil (MP Biomedics, Solon, Ohio), according to the manufactory's recommendations. DNA samples were kept at - 20°C until being used for PCR.

For bacterial isolates, a colony was picked and added directly to the PCR reaction (described below). In these cases, an additional 10 min cycle at 94ºC was added at the beginning of the PCR procedure.

2.5.2.2. PCR Amplification

PCR was performed using primers GC - 338F (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG) and 1800F (ACCTGCGGAAGGATCATTG) to amplify the highly variable V3 region of bacterial 16 rRNA gene fragments, according to Muyzer, 1993. These primers also amplified DNA from the microalga's chloroplasts due to the presence of 16S rRNA gene in chloroplasts (Andreote, *et al.* 2009).

PCR amplification was performed in 50 µL reaction mixtures containing 22 µL of autoclaved deionized water, 1 µL of each primer, 25 µL of Supreme NZTaq Polymerase (Nzytech, Portugal) and 1 µL of DNA or one colony.

The PCR temperature procedure consisted of a 5 min initial denaturation cycle at 94ºC, 30 cycles of denaturation at 92ºC, for 30 s, an annealing cycle of 55ºC for 30 s, an extension at 72ºC for 30 s and a final extension step at 72ºC for 30 min (Amorim *et al*, 2014). The reactions were performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA).

Amplification was confirmed with an electrophoresis gel of 1,5% agarose (Nzytech) using a 100bp ladder (Grisp – Production and Development of Laboratory Solutions, Porto, Portugal).

2.5.2.3. Denaturating Gradient Gel Electrophoresis (DGGE) Preparation

PCR products were separated by DGGE using DCode Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

Prior DGGE analysis, the amount of DNA in PCR-amplified fragments was quantified using Qubit Fluorometer, Thermo Fisher Scientific, EUA, according to Qubit Assay Kits and then *c.a.* 300 ng were loaded onto the gel.

PCR-amplified fragments containing about 300 ng of DNA were loaded onto the polyacrylamide gels with a denaturating gradient ranging from 35% to 70% for 16S rRNA gene. The composition of the gel prepared for 16S rRNA at such gradient was: 37.5:1, acrylamide:bisacrylamide in 0.5 TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA). These reagent amounts are based on the fact that a 100% denaturant contains 7 M urea and 40% formamide. Electrophoresis was carried out at 60ºC in a 1x TAE buffer, starting at 20 V for 15 min and then at 75 V for 960 min. The gels were stained in a 10 x GelGreen Nucleic Acid Stain solution (Biotium Inc., USA) in 0.1 M NaCl. The DGGE images were acquired using a Safe Imager" Blue- Light Transilluminator (Invitrogen", USA) and a microDOC gel documentation system (Cleaver Scientific Ltd, UK). Two gels were run, as there were not enough wells in only one to analyse all of the samples.

All of the procedures described for DGGE analysis, from gel preparation to analysis of the results were performed according to Amorim *et al.* (2014).

2.5.2.4. Analysis of DGGE Profiles

DGGE profiles obtained were analysed using Bionumerics software (Applied Maths, St-Martens-Laten, Belgium). Bandmatching and dendrograms were generated by cluster analysis (Jaccard coefficient). However, due to some problems discussed in chapter 3.3.2.2., dendrograms will not be presented and discussed.

2.6. Calculation Procedures and Statistical Analysis

2.6.1. *Chlorella vulgaris*: bacteria Ratio Assay

Maximum growth rate for each co-culture was estimated according to the following equation: $X = X0.e^{\mu max.t}$, in which, X is the cellular concentration, X0 the initial cellular concentration, t the time between X0 and X and μmax is the maximum growth rate of the microorganism.

Then, the μmax for each proportion was calculated with a confidence interval of 95% ($CI_{95\%}$) to the mean, using the data analysis toolpak from Microsoft Excel. Means were calculated considering that each combination of *C. vulgaris* and bacteria inoculated with the same ratio could be considered replicates. This was used since the goal of this assay was to have an idea of the best proportion of *C. vulgaris* and bacterial inoculum in general, rather than having a proportion for each bacterial strain.

2.6.2. Co-cultures in batch Regime

Maximum growth rate was calculated by applying a trendline to the exponential phase, and obtaining a linear regression equation. Results were processed using Microsoft Excel.

The equation obtained is $y = mx + b$, in which, m is the value of the maximum growth rate (μmax) obtained. Results from all of the μmax from were organised in a bar chart.

2.6.3. Co-cultures in a Semi-Continuous Regime

To calculate μmax and be able to statistically analyse the data obtained, a regression with an $CI_{95\%}$ was obtained using the data analysis toolpak from Microsoft Excel.

This allowed obtaining the values of μmax according to the equation mentioned in section 2.6.2 while obtaining a confidence interval to compare results.

These results were displayed in a bar chart and the error bar calculated was the $CI_{95\%}$ divided by two (one half for higher possible values and half for negative ones). Therefore, with the data displayed in this way it is possible to conclude if the values are statistically significantly different: if error bars do not intersect one another, results are statistically significantly different.

3. RESULTS AND DISCUSSION

In this chapter, results will be presented and discussed according to the goals set for this study and previously presented.

3.1. *Chlorella vulgaris*: bacteria Preliminary Ratio Assay

A preliminary ratio optimization experiment was performed with the aim of having an idea of the number of bacteria and microalga that should be inoculated in the co-cultures. To do so, co-cultures of bacteria and *C. vulgaris* were set up in five different proportions of *C. vulgaris*: bacteria and their growth was monitored. In this experiment, three bacterial isolates were used, namely LL_Bc02, LL_Bc07 and LL_Bc11. However, results were treated regardless of the bacterial species tested, since data was treated to allow comparison between different proportions, rather than different culture composition. The reason to disregard the nature of the bacteria tested was the fact that the objective of this assay was to have a general idea of the quantity of bacteria that should be inoculated in further experiments, regardless the strain.

In this experiment, the growth of *C. vulgaris* was monitored and maximum growth rates of the microalga were obtained for each ratio. Results presented in figure 3.1 show that ratios 1 *C.vulgaris* cell to 100 bacterial cells (1:100) and 1 *C.vulgaris* cell to 10 bacterial cells (1:10) are statistically significantly different from the remaining. However, these do not present significant differences between each other. Ratios 1:1, 10:1 and 100:1 are not statistically significantly different from one another or the control. This ratio optimization experiment showed that the inoculation ratios with highest *µmax* were 100 *Chlorella* cells to one bacterial cell (100:1) and 10 *Chlorella* cells to one bacterial cell (10:1) (figure 3.1). According to these results ratio 10 *C. vulgaris* cells to one bacterial cell (10:1) was chosen for further experiments.

Kazamia *et al., (*2012) studied stability of co-cultures between green alga *Lobomonas rostrata* and the soil bacterium *Mesorhizobium loti.* This association is based on the microalga dependency for vitamin B_{12} (cobalamin) and the capacity on *L. rostrata* to synthetize it, in return for the fixation of carbon by the microalga. Furthermore, in this study, ratios of algal and bacterial cells were tested in co-cultures, from 1: 10^6 to 10^5:1, and in all cases they equilibrated between 1:10 and 1:30 within 5 to 7 days, regardless of initial ratios. This happened both in semi-continuous and in batch.

Contrary to this study, in the present work, a ratio was chosen in order to standardize further assays, by maintaining the proportion of microalga and bacteria inoculated similar between tests.

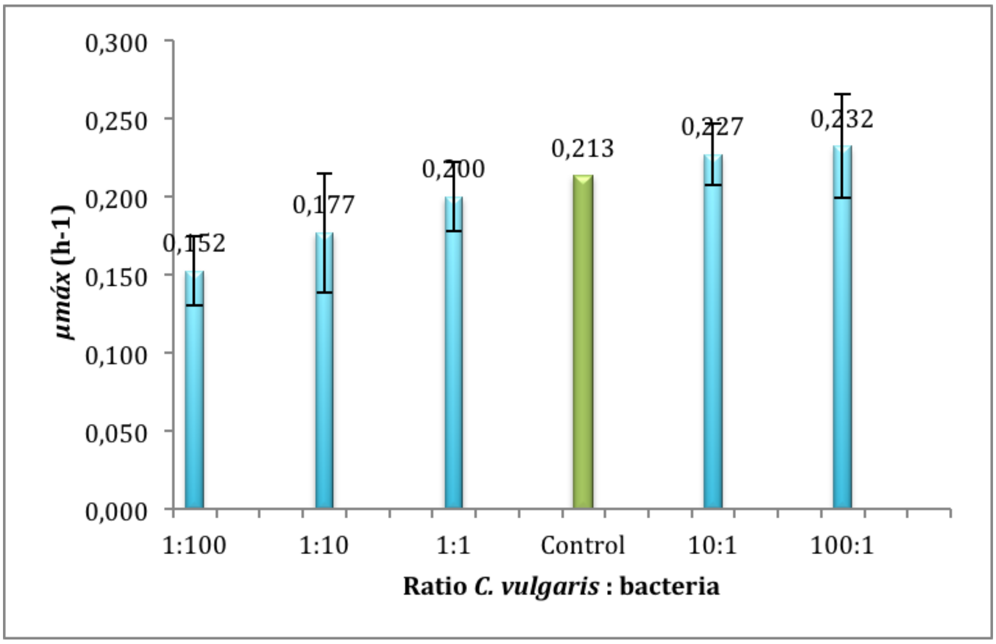


Figure 3.1 - Maximum growth rate of *Chlorella vulgaris*, inoculated in different microalga: bacteria ratios. Error bars were calculated based on 95% CI to the mean.

3.2. Co-cultures in Batch Regime

The aim of this experiment was to evaluate the effect of several bacterial consortia on the growth of *C. vulgaris.*

Since there were five bacterial isolates to be tested, multiple combinations of two strains were established in order to search for bacterial consortia capable of improving the microalgae's growth. This experiment was carried out for a week and ended when *C. vulgaris* cell counts started decreasing. During this time algal and bacterial growth was monitored as described in the methods (section2.4.2). Figure 3.2 shows the macroscopic appearance of the co-cultured immediately after inoculation and at the end of the assay.

Figure 3.2 displays growth curves of *C. vulgaris* obtained from cell counts of this microalga. It may be observed that two groups of growth curves stand out: one attaining higher cell concentrations of *C. vulgaris* (7,5 log cells/mL or higher), in which the monoalgal control is included and one attaining lower concentrations of *C. vulgaris* cells (6,13 log cells/mL or lower). The group of co-cultures reaching higher concentrations of *C. vulgaris* grew the most up to 40 hours of cultivation and after that its growth stabilised, until the end of the test. Results show that co-cultures that reached lower concentrations of algal cells have one bacterium in common - LL_Bc02. The remaining co-cultures, without LL_Bc02, display growth curves similar to the control, which may indicate the respective bacterial consortia did not have a negative impact on *C. vulgaris*.

From growth curves obtained and presented in figure 3.3, *μmax* of *C. vulgaris* were obtained for each culture. These results show that in this assay, co-cultures containing LL_Bc02 reached lower *μmax* values (from 0,041 to 0,138 h^{-1}) than the monoalgal control (0,156 h^{-1}) whereas co-cultures containing LL_Bc07 and LL_Bc11 attained higher *μmax* (from 0,178 to 0,200 h^{-1}).

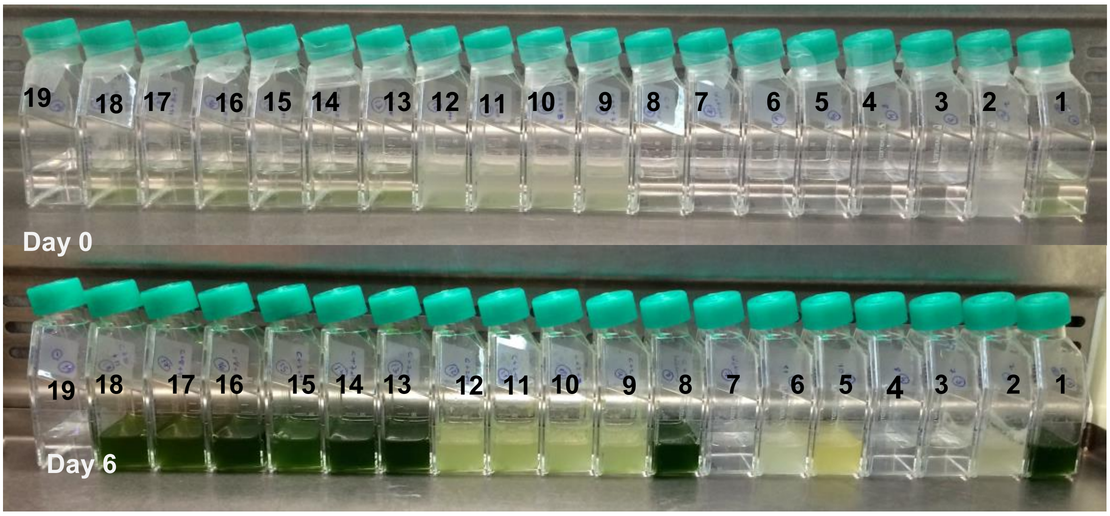


Figure 3.2 - Photos of T-flasks immendiatly after inoculation (day 0) with *Chlorella vulgaris* and bacteria and after six days of cultivation (day 6). Identification of T-Flasks: 1: *C.vulgaris;* 2: LL_BC02; 3: LL_Bc07; 4: LL_Bc08; 5: LL_Bc10; 6: LL_Bc10; 7: LAB; 8: *C. vulgaris* + LAB; 9: *C. vulgaris* + LL_Bc02 + LL_Bc07; 10: C. vulgaris + LL_Bc02 + LL_Bc08; 11: *C. vulgaris* + LL_Bc02 + LL_Bc10; 12: *C. vulgaris* + LL_Bc02 + LL_Bc11; 13: *C. vulgaris* + LL_Bc07 + LL_Bc08; 14: *C. vulgaris* + LL_Bc07 + LL_Bc10; 15: : *C. vulgaris* + LL_Bc07 + LL_Bc11; 16: *C. vulgaris* + LL_Bc08 + LL_Bc10; 17: *C. vulgaris* + LL_Bc08 + LL_Bc11; 18: *C. vulgaris* + LL_Bc10 + LL_Bc11; 19: negative control (TAP medium).

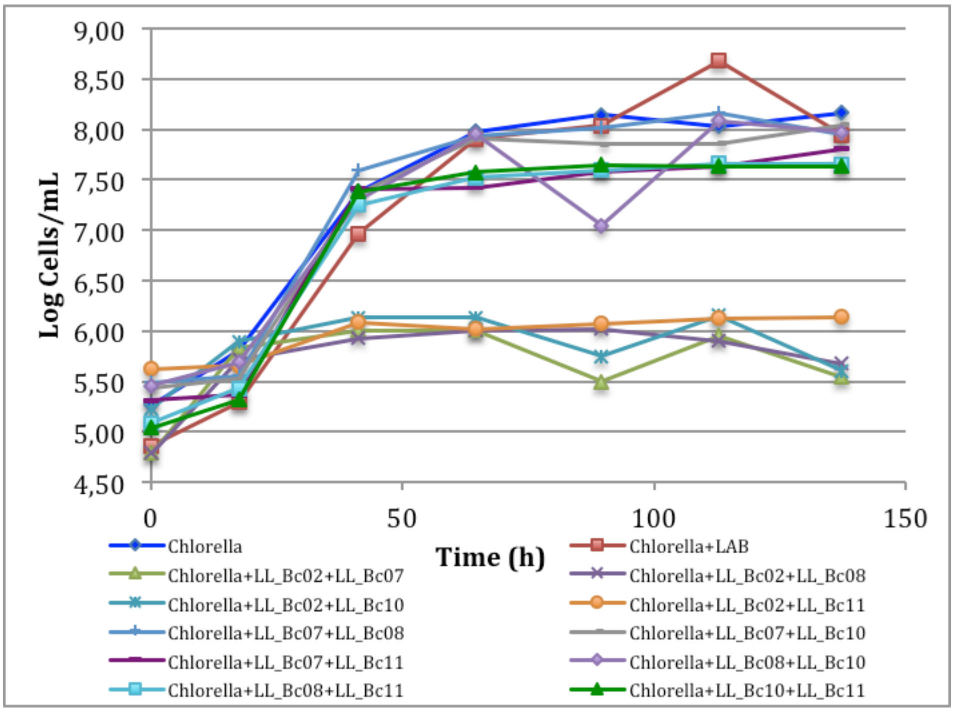


Figure 3.3 - *Chlorella vulgaris* growth curves in mono and mixed cultures, obtained through microalgal counts.

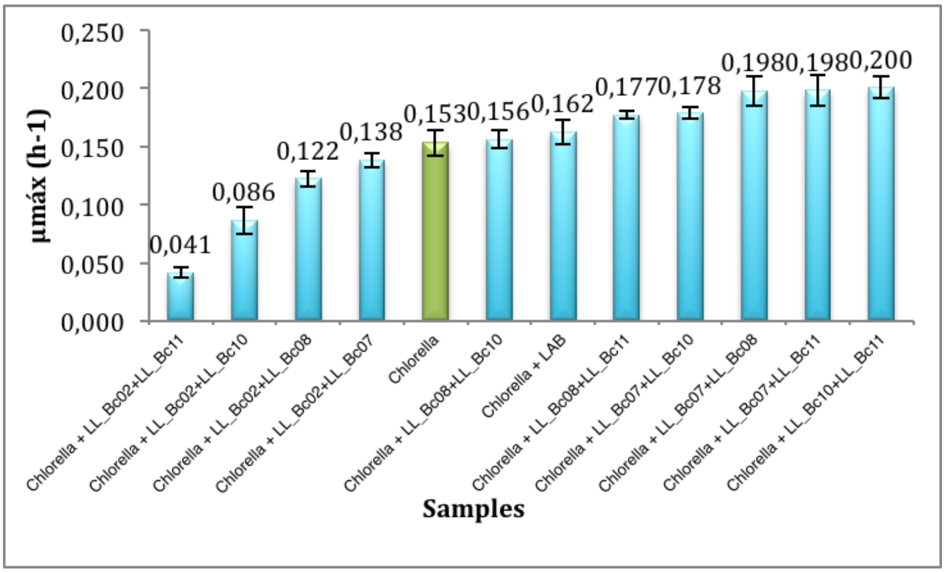
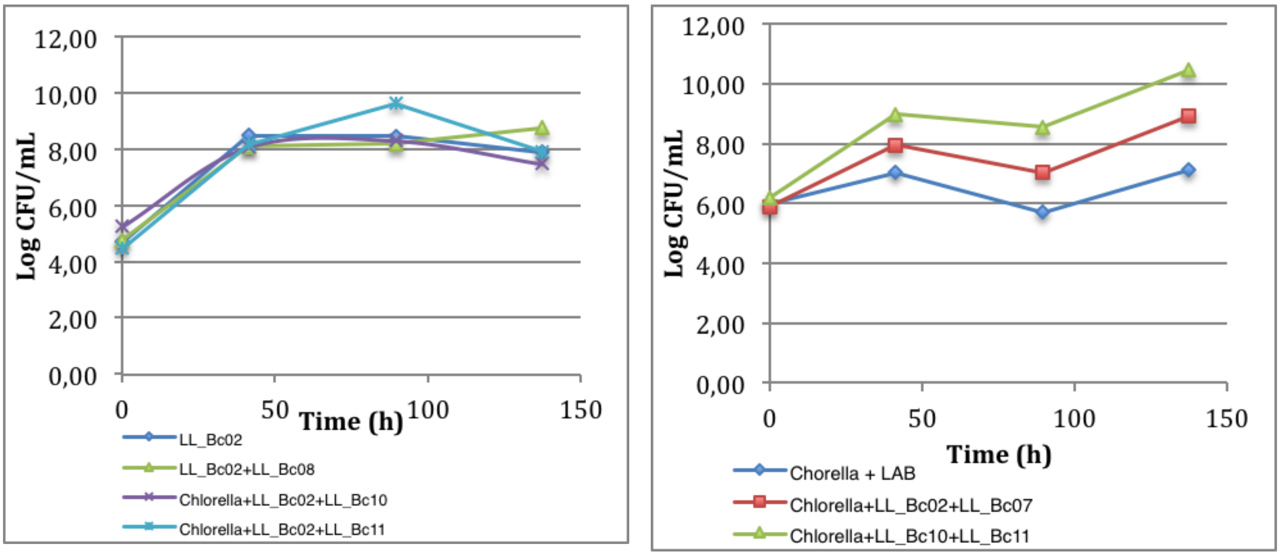


Figure 3.4 - Maximum growth rates of *Chlorella vulgaris* attained in each co-culture as well as in mono culture (control). Black vertical bars represent technical error.

Furthermore, as described in the methods (2.4.2), bacterial CFU/mL were obtained through plating and results are displayed in figure 3.5. Although samples from co-cultures were plated both on NA and TAP, not all bacteria grew well in TAP agar, reason why only results from NA are presented here (figure 3.5). An example is LAB that did not grow as a monoculture in TAP medium or TAP agar.

Figure 3.5 presents three charts grouped by similarity of bacterial behaviours. In chart a) bacteria grew until around 40 hours (41,25 h) of cultivation and then their concentration started decreasing. Bacteria from co-culture *Chlorella* + LL_Bc02 + LL_Bc11 grew until 89,5 h and only after this point did their concentration start declining. In chart b), bacteria from three co-cultures are presented and have the same initial concentration and same growth pattern, yet concentrations vary throughout time. Finally, chart c), displays six growth curves of bacteria in which, TVC increase throughout the assay. This is not the typical bacterial growth curve with a lag, log and stationary phase, which might indicate, these bacterial consortia were still growing by the time the experiment ended. This could suggest that these bacteria were feeding on organic matter from *C. vulgaris*. According to the literature, heterotrophic bacteria can uptake organic matter excreted by the microalgae, such as carbohydrates, lipids, vitamins and nitrogen amongst others (Haines, *et al.*, 1974; Chrost *et al.*, 1980). On the other hand, the microalga was in stationary phase due to nutrient depletion. This was assessed with an acetate test from a commercial kit. Results from this test showed that, with the exception of three bacterial controls (LL_Bc07, LL_Bc08 and LAB), others cultures had nearly or completely depleted the quantity of the carbon source (results in table 7.4 in Appendix). Considering this, results from co-cultures in graph c) suggest that even when *C.* vulgaris was in stationary phase, bacteria were growing and therefore could be thriving on nutrients from the microalga rather than from the medium. Regarding LAB, in graphic c), these bacteria decline from the first time point to the second but then increase its TVC again. However it was not possible to get the last time point. By then, LAB colonies did not show up on plates. For these reasons, the information obtained from this culture is inconclusive.


a) b)

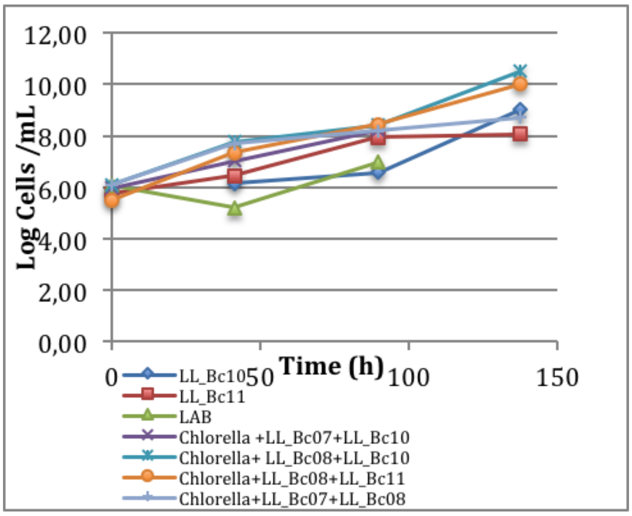

c)

Figure 3.5 - Growth curves obtained from TVC (in NA) of bacteria present in co-cultures and controls. Graphs a), b) and c) show bacteria with similar behaviours.

To summarise results and as previously mentioned, LL_Bc02 had a negative impact on the growth of *C. vulgaris* whereas LL_Bc07 and LL_Bc11 appeared to have a positive one. Co-cultures with the remaining bacteria presented similar *μmax* to the monoalgal control (figures 3.3 and 3.4). In figure 3.3 it is possible to observe that all co-cultures containing this bacterium reached lower concentrations of *C. vulgaris* than the monoalgal control, and in figure 3.4 that all the *μmax* lower than the control are from co-cultures containing this bacterium. LL_Bc02 is an *Acidovorax* sp., genus belonging to a diverse group of *β* – Proteobacteria, which contains both environmental species as well as phytopathogens responsible for some plant diseases (Willems *et al.*, 1990 and Bahar *et al.*, 2009). Thus there is a possibility that this bacterium is able to interfere with the microalga's behaviour.

Additionally, Chakraborty, *et al*. (2011) examined the growth of *Acidovorax* sp. and revealed that these bacteria are capable of utilising acetate as a carbon source. Since the carbon source included in TAP medium is acetate, this might explain why LL_Bc02 was able to negatively impact the growth of *C. vulgaris*, perhaps by competition with the microalga for the same carbon source.

Contrary to co-cultures containing LL_Bc02, the ones containing either LL_Bc07 and/ or LL_Bc11, presented favourable results, since the microalga's *µmax* was higher than the control's (figure 3.4). However, co-cultures containing either LL_Bc07 or LL_Bc11 in combination with LL_Bc02, reached lower cell concentrations and *µmax* of *C. vulgaris*, explained by the negative impact of LL_Bc02 on the microalga's growth. Any positive impact induced by LL_Bc07 or LL_Bc11 seemed to be overpowered by LL_Bc02.

LL_Bc07 was identified as *Sphingomonas* or *Sphingopyxis* sp. and LL_Bc11 as *Hydrogenophaga pseudoflava*. *Sphingomonas* sp. is a genus commonly present in natural occurring algae-bacterial consortia (Tate *et al.*, 2012) and Guo and Tong (2013) showed *Pseudomonas* sp. had a growth promoting effect on *C. vulgaris* when co-cultured with this bacterium, in comparison to a *C. vulgaris* monoculture. However, such study was performed under photoautotrophic conditions, thus it is not possible to directly compare the latter to the present study, in mixotrophic conditions. Regarding *Hydrogenophaga pseudoflava*, it has been reported that some strains from this species are capable of fixating nitrogen (Brenner *et al.*, 2005). This could be advantageous for the microalga's growth, similarly to the growth promoting effect of *Azospirillum brasilense* when co-cultured with *C. vulgaris*, as described in Gonzalez and Bashan (2000). *A. brasilense* is a nitrogen fixating bacterium, capable of promoting plant growth in symbiotic associations and has demonstrated a significantly increased growth of *C. vulgaris*, when coimmobilized in alginate beads (Gonzalez and Bashan, 2000). Regardless, this is just a hypothesis as there is no certainty about the capability of the strain used in the present study to fixate nitrogen.

Moreover, co-cultures containing LL_Bc08 or/ and LL_Bc10 presented a similar *µmax* to the monoalgal control. Also, when these bacteria were combined with LL_Bc07 or LL_Bc11, the microalga presented a higher *µmax* than the control. In contrast, when co-cultured with LL_Bc02 the algal *µmax* decreased. These observations reinforce the negative impact of LL_Bc02 and the positive impact of LL_Bc07 and LL_Bc11.

Finally, regarding LAB it was not possible to conclude anything about the impact of these bacteria on the microalga's growth. This is due to the facts that LAB did not always grow in TAP medium and that both growth curves (from the co-culture and the microalgae) are similar, as well as the microalgal *µmax*. Poor growth shown by LAB is not surprising, since these bacteria are fastidious organisms, requiring complex media (Vesanto *et al.*, 1996).

Given the results obtained in this experiment, four bacteria were selected for further experiments: LL_Bc02, LL_Bc07, LL_Bc11 and LAB. LL_Bc07 and LL_Bc11 were selected, as they appeared to have a positive effect on the growth of *C. vulgaris*. On the other hand, LL_Bc02 was chosen since it appeared to negatively impact *C. vulgaris* growth and it was decided to carry on studying co-cultures with this bacterium, to understand if the results were similar in a semi-continuous regime. Lastly, although LAB did not show any impact on the microalga's growth, it was selected for the experiment that followed due to its particular interest of being a commercially available consortium of bacteria and Generally Regarded as Safe (GRAS). This fact could add value to the final product and would not require additional virulence studies, to ensure food safety certification.

3.3. Co-cultures in Semi-continuous Regime

3.3.1. Growth behaviour of co-cultures

The purpose of this assay was to evaluate the effect of the four previously selected bacterial strains on the growth of *C. vulgaris.* In this experiment, the following combinations of *C.* vulgaris and bacteria were co-cultivated: *C. vulgaris* + LL_BC02 + LL_BC07; *C. vulgaris* + LL_Bc02 + LL_Bc11, *C. vulgaris* + LL_Bc07 + LL_Bc11 and *C. vulgaris* + LAB. These mixed cultures were set up in duplicate and were grown in a semi-continuous regime with two weekly renewals after reaching the exponential phase. The growth behaviour of the cultures was monitored for three weeks. T-flasks with the co-cultures and controls from the inoculation day (day 0) and the last day (day 27) are shown in figure 3.6. The photo from day 0 has 20 T-flasks, whereas the one from day 27 only has 16. This is explained by the fact that bacterial controls of LL_Bc07 (flasks 5 and 6) and LAB (flasks 9 and 10) were eliminated along the course of the experiment due to contamination by *C. vulgaris.*

In figure 3.7, results from *C. vulgaris* cell counts are presented. This graph does not show any data regarding bacterial controls, since bacteria were not counted using the same method as *C. vulgaris.* Results obtained from the growth curves and displayed in figure 3.7 show that *C. vulgaris* from all co-cultures have similar behaviours to the monoalgal control. Amongst these, the co-culture composed by *C. vulgaris* and LAB is the closest to the control. Furthermore, algal concentrations in the co-culture *C. vulgaris* + LL_Bc02 + LL_Bc07 decreased after reaching its highest value, going from the exponential phase to the lag one. However, after renewals, *C. vulgaris* kept on growing, as expected, always stabilising around the same values. Moreover, *C. vulgaris* + LL_Bc02 + LL_Bc11 was the first one reaching the lowest cell counts before renewals. Nevertheless, after the first one its behaviour became very similar to the monoalgal control.

Regarding the microalga's behaviour both in the control and in co-culture, even though the co-cultures were renewed twice a week, they did not grow a lot after reaching the exponential phase. The reason might be the fact they were renewed when the cell concentration was reaching the stationary phase. Therefore, it is possible that the microalga went into a lag phase again, before reaching another exponential phase. Thus, it is possible that with two weekly renewals, the microalga did not have enough time to reach a new exponential phase.

Based on the results from experiment in batch mode, it was expected that co-cultures containing LL_Bc02 would show a negative impact on *C. vulgaris*. However, in a semi-continuous regime, the microalga in mixed cultures with LL_Bc02 did not present differences to other cultures and *C. vulgaris* was able to grow, without statistically significant differences from the control.

Figure 3.6 - Macroscopic appearence of cultures after inoculation (day 0) and at the end of the assay (day 27). Identification of the T-flasks: 1 and 2: *Chlorella vulgaris*; 3 and 4: LL_Bc02; 5 and 6: LL_Bc07; 7 and 8: LL_Bc11; 9 and 10: LAB; 11 and 12: *C. vulgaris* + LAB; 13 and 14: *C. vulgaris* + LL_Bc02 + LL_Bc07; 15 and 16: *C. vulgaris* + LL_Bc02 + LL_Bc11; 17 and 18: *C. vulgaris* + LL_Bc07 + LL_Bc11; 19 and 20: negative control (TAP medium).

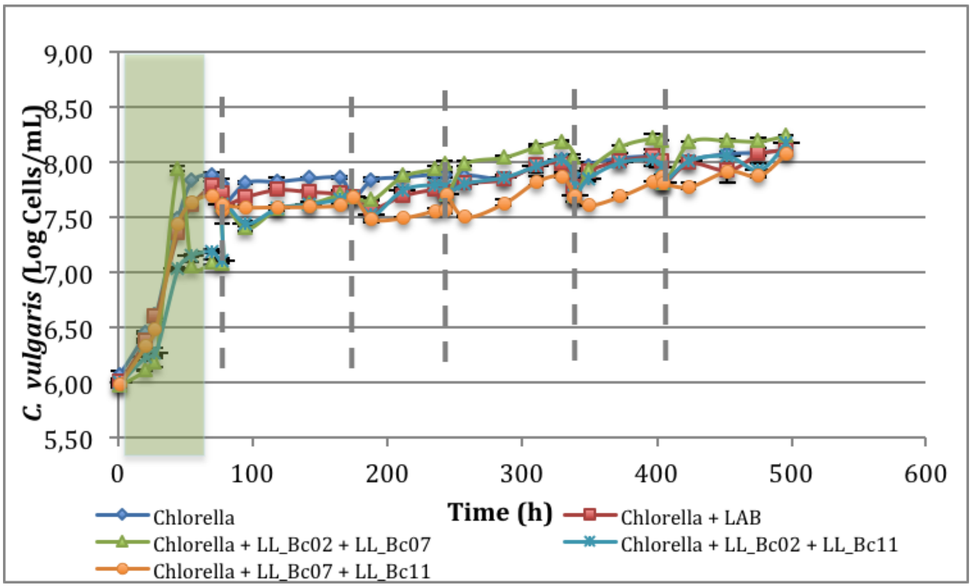


Figure 3.7 - Growth curves of *C. vulgaris* obtained through microalgal cell counts. Error bars represent the standard deviation between biological replicates. Grey dashed bars represent 30% (v/v) renewals. The Green rectangle represents the growth phase used to calculate maximum growth rate.

From the data presented in figure 3.7, maximum growth rate (μmax) of *C. vulgaris* was calculated and results are presented in figure 3.8.

Results show there are no statiscally significant differences between *μmax* of the co-cultures and the control, since the vertical bars (95% CI) always intersect one another.

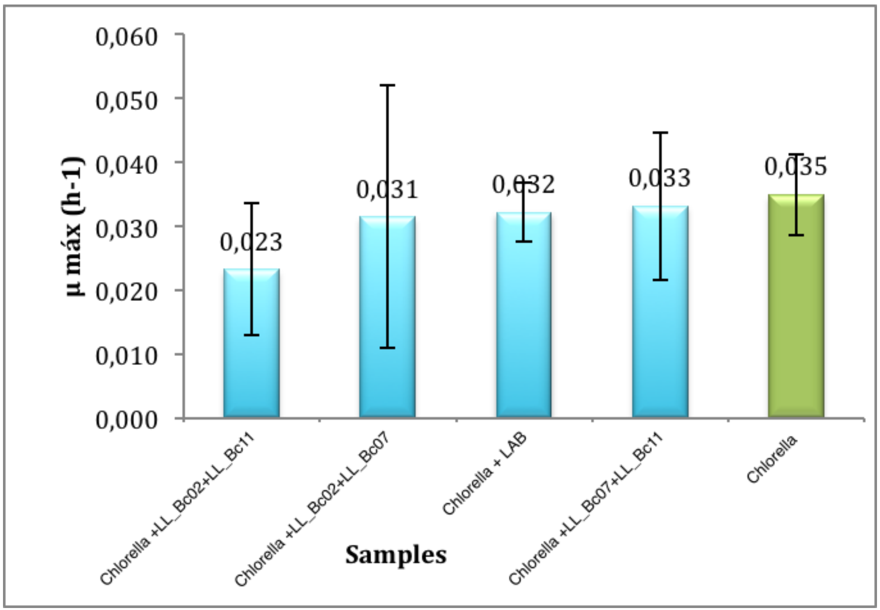


Figure 3.8 - Maximum growth rate of *Chlorella vulgaris* prior renewals, obtained from cell counts. Black vertical bars represent 95% CI based on regression. *μmax* attained by *C. vulgaris* are presented in blue and green bars, which represent samples from co-cultures and from the monoalgal control, respectively.

As mentioned in chapter 2.5.1, co-cultures were also monitored by OD_{600} measurements and such results are presented in figure 3.9. Contrary to figure 3.7, the graph in figure 3. Also presents data from bacterial controls, since these can also be measured at this wavelength. Hence, these results represent the evolution and behaviour of the mixed cultures as a community.

These results exhibit highly similar growth curves of all the co-cultures. Bacterial controls have similar behaviours among each other, however, after the first renewal LL_Bc02 grew more than LL_Bc11. It is important to note that LAB was not included in this graph since it did not grow in TAP medium. Such results were observed in the low OD_{600} measurements, all close to zero and in the lack of growth in the plates. Additionally, LL_Bc07 control was not included since it only started growing after 150 hours of cultivation but was then contaminated by *C. vulgaris,* spoiling the goal of a bacterial control in this assay.

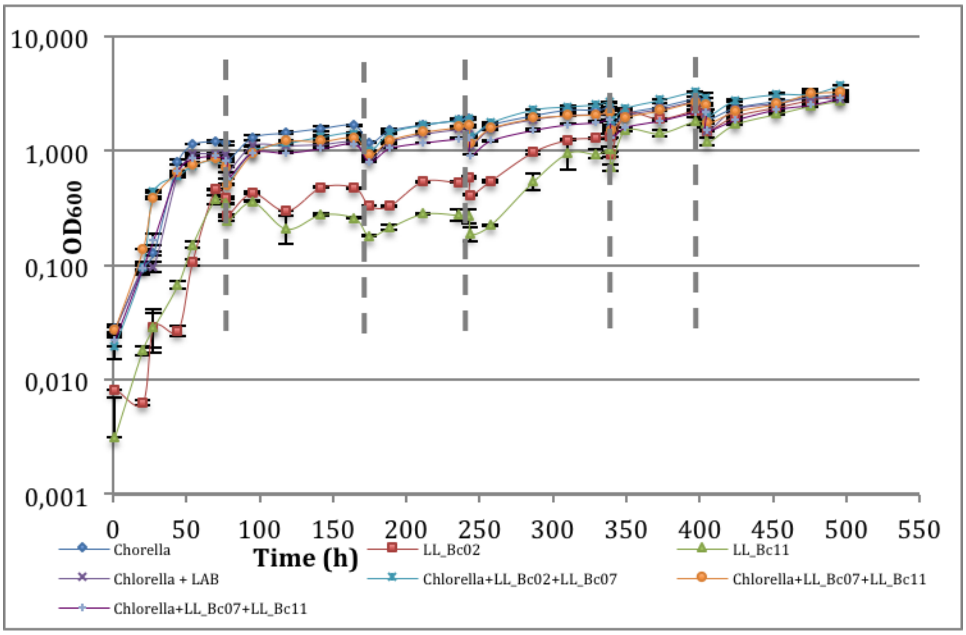


Figure 3.9 - Growth curves of mixed cultures, the monoalgal control of *Chlorella vulgaris* and two individual bacterial cultures, used as controls. Data obtained through OD_{600} measurements. Vertical black bars represent the standard deviation between biological replicates, while dashed bars represent 30% (v/v) medium renewals.

In the present assay, bacterial behaviour was monitored by TVC in NA and these results are presented (figure 3.10).

The graph presented in figure 3.10 exhibits the evolution of the bacterial fraction of the community, as well as two bacterial controls. It is important to note that when in co-culture, TVC do not differentiate bacterial species but rather account for the total amount of bacteria present in co-culture.

LL_Bc02 grew until 1,25 x 10^9 cells/ mL but after 211,25 h this number decreased, reaching 2,44 x 10^6 cells /mL by the end of the test. Bacteria from the co-culture *Chlorella* + LL_Bc07 + LL_Bc11 started at the highest initial concentration of all the co-cultures and maintained stable throughout time. Regarding *Chlorella* + LL_Bc02 + LL_Bc07, bacterial concentration decreased at the beginning of the cultivation but grew back to its initial concentration remaining stable, with very slight variations, until the end of the assay. This behaviour was similar to the one from *Chlorella* + LL_Bc07 + LL_Bc11, however in lower concentrations of bacteria. Furthermore, bacterial counts from *C. vulgaris* + LL_Bc02 + LL_Bc11 suffered some variations throughout time, increasing and decreasing several times.

Regarding the co-culture containing *C.* vulgaris and LAB, data presented in figure 3.10 only contains four time points since this culture suffered a contamination from another bacterium, which will be further discussed in chapter 3.3.2.1, regarding DGGE results.

Finally, LAB control did not grow in TAP medium and was contaminated with *C. vulgaris* along the cultivation, reason why such data is not presented in figure 3.10. The controls of LL_Bc07 were also eliminated as they were also contaminated, thus TVC results are not presented here.

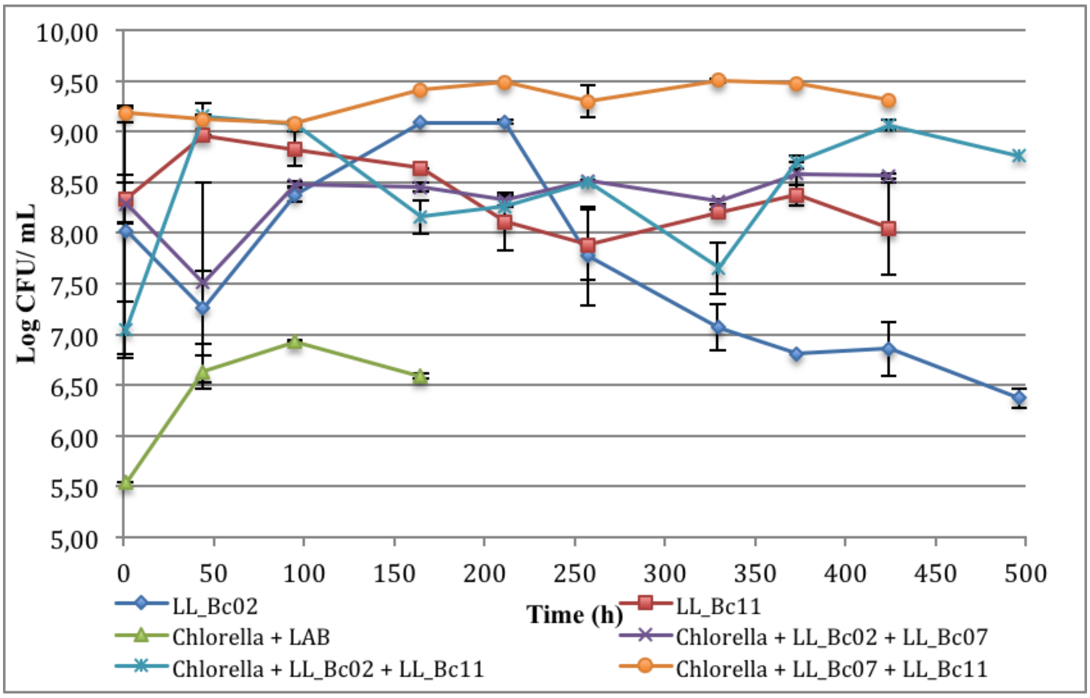


Figure 3.10 - Growth curves of bacteria present in the mixed cultures, as well as some bacterial controls. Error bars represent standard deviation between replicates. Samples were plated on NA.

3.3.2. Study of the communities of *C. vulgaris* and bacteria

In order to understand how the co-cultures in semi-continuous regime evolved as a community, a Denaturing Gradient Gel electrophoresis (DGGE) was performed to analyse the variation of the consortia along the course of cultivation. Samples for this purpose were collected at the beginning of the test, in every renewal and at the end of the test.

3.3.2.1. DNA amplification

In order to proceed with DGGE analysis, a DNA amplification step was required. In this step, not all samples were tested at the same time, to ensure the PCR program and primers were working, hence avoiding reagent losses. Also, in one case, (lane 6, figure 3.12) one of the samples did not show up on the gel, therefore had to be amplified again. For these reasons, samples were amplified in several groups and various agarose gels were run and are presented in figures 3.11 3.12 and 3.13. These gels show one DNA band at 200bp in each well, indicating successful amplification of the targeted sequence, which comprises the amplified V3 variable region, is 180 bp, and the GC-clamp.

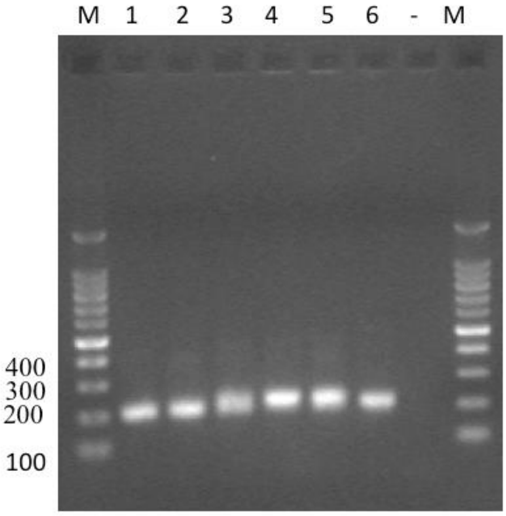


Figure 3.11 - Electrophoresis of DNA fragments; 1,5% agarose gel; Lanes M: DNA marker; lane 1: *C. vulgaris* + LL_Bc02 + LL_Bc07 at t19; lane 2: *C. vulgaris* + LL_Bc02 + LL_Bc11 at t19; lane 3: *C. vulgaris* + LL_Bc07 + LL_Bc11 at t19; lane 4: *C. vulgaris* + LAB at t14; lane 5: *C. vulgaris* + LAB at t19.

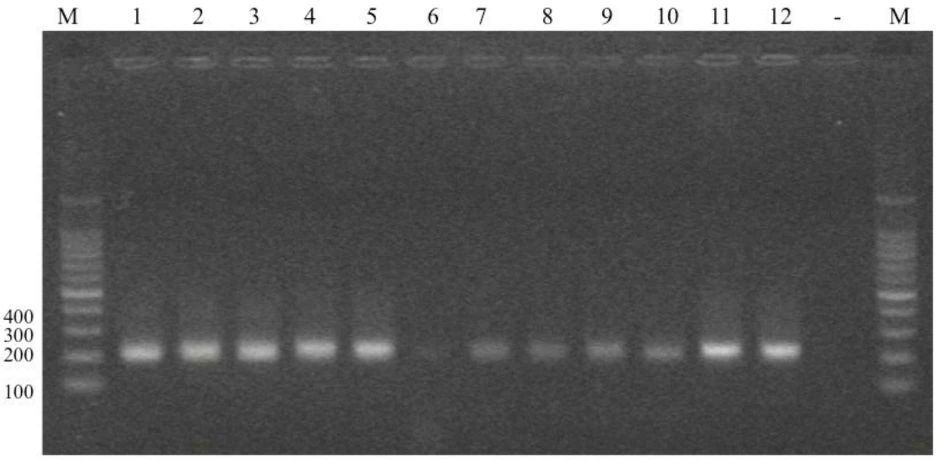


Figure 3.12 - Electrophoresis of DNA fragments; 1,5% agarose gel. Lanes M: DNA marker; lane 1: *C. vulgaris* at t27; lane 2:*C. vulgaris* + LAB at t27; lane 3: *C. vulgaris* + LL_Bc02 + LL_Bc11 at t27; lane 5: *C. vulgaris* +LL_Bc07 + LL_Bc11 ate t27; lane 6: *C. vulgaris* at t0; lane 7: *C. vulgaris* + LAB at t0; lane 8: *C. vulgaris* +LL_Bc02 + LL_Bc07 at t0; lane 9: *C. vulgaris* + LL_Bc02 + LL_Bc11 at t0; lane 10: *C. vulgaris* + LL_Bc07 + LL_Bc11 at t0; lane 11: LAB; lane 12: LL_Bc11; lane -: negative control.

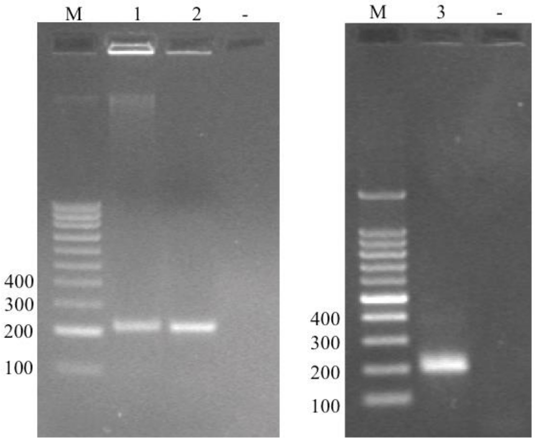


Figure 3.13 - Gel electrophoresis of DNA fragments; 1,5% agarose gels. Lanes M: DNA marker; lane 1: LL_Bc02; lane 2: LL_Bc07; lane 3: *C. vulgaris* at t0; lanes -: negative control.

3.3.2.2. DGGE Community Fingerprinting

DGGE is a technique that allows studying microbial diversity, offering an instant display of the elements from a population in two ways: qualitative and semi-quantitative. Moreover, DGGE analysis is a highly sensitive technique that allows the detection of sequence differences. (Muyzer *et al.*, 1993).

This molecular technique was used based on the amplification of the bacterial V3 region of 16S RNA, as described in Amorim *et al.* (2014). PCR products containing about 300 ng of DNA were loaded onto the polyacrylamide gels with a denaturating gradient from 35% to 70% for 16S rRNA gene, according to Amorim *et al.* (2014).

Regarding the samples included in this analysis, three time points of each co-culture and the algal control were chosen: t0 (day 0 - inoculation), t19 (day 19) and t27 (day 27, last day of cultivation). For the particular case of *C. vulgaris* + LAB, an extra sample was analysed, from day 14 (t14), since this co-culture suffered a contamination during the experiment, that was visible on plates. Furthermore, pure samples of the bacterial isolates were also included, in order to allow localisation and comparison of bacteria in the co-cultures. Finally, three time points of the *C. vulgaris* control were included instead of just one as a bacterial contamination showed up on plates, during the experiment. For this reason it was interesting to find out if the contamination was from any bacteria used in this study or an external contamination.

When proceeding with the technique, two gels were prepared, as there were not enough wells in one gel to analyse all of the samples. Untreated pictures (prior editing) of the gels can be observed in figure 3.14.

In a preliminary analysis of the gels obtained (figure 3.14) it is possible to observe one band for each bacterial colony tested and one in the *C. vulgaris* control from day 0 (t0). The remaining samples of this control, present more bands, likely due to bacterial contamination that occurred during the cultivation and that was visible on NA plates. Also, all co-cultures present more than one band. Moreover, the control of *C. vulgaris* on day 0 (inoculation day) shows one band, which is most likely from the chloroplast DNA of *C. vulgaris*. In fact, all of the co-cultures present a similar band. This occurs due to the presence of 16S rRNA gene in chloroplasts as these have a prokaryotic origin (Andreote, *et al.* 2009).

Gels from figure 3.14 were then aligned using Bionumerics software. This tool allows the alignment of gels, based on the position of bands from the DNA marker. This then allows a bandmathing analysis and the construction of dendrograms, based on the comparison of the relative position of bands, therefore allowing the comparison between the communities of microorganisms from each co-culture.

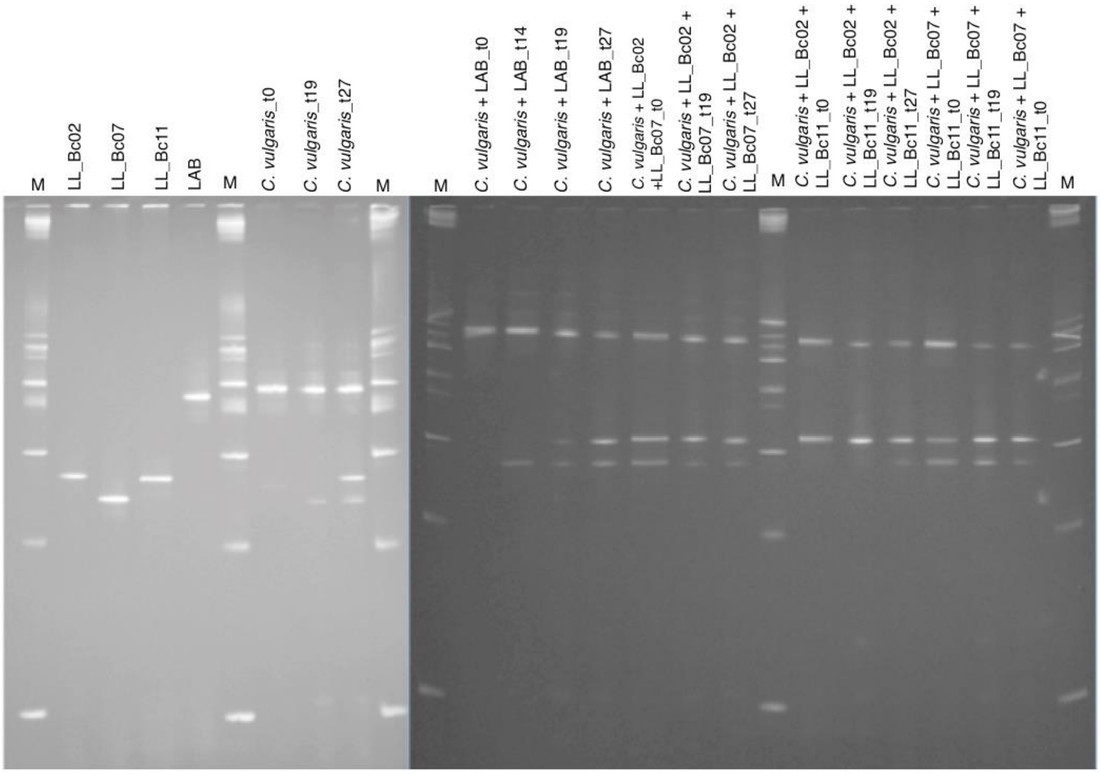


Figure 3.14 - DGGE community fingerprint of co-cultures composed by *C. vulgaris* and bacteria, the microalgal control and pure samples of the bacterial strains tested. The image presents two unedited pictures of the distinct gels, not yet aligned by Bionumerics. The name of the sample and time of collection is indicated above each lane. Lane M: DNA marker; t0: day 0; t14: day 14; t19: day 19 and t24: day 24.

Figure 3.15 shows the bandmatching pattern obtained during the Bionumerics analysis of the gels and it is possible to observe the bacterial controls and the microalgal control as well as the mixed cultures.

Bands in a green rectangle are likely from the chloroplast of the microalga, since they appear in all co-cultures or in all the microalgal control samples, including on day 0, when the culture was axenic. However, as it may be observed in figure 3.15, bands from the controls and the co-cultures are in distinct positions. Even though controls of *C. vulgaris* and individual bacteria were added to the analysis to allow comparison the between co-cultures' components, it is suspected that there has been a misalignment in the analysis of the gels. This suspicion is based on the fact that the controls from the microalga and bacteria do not match any of the bands from the co-cultures. For this reason, dendrograms generated through the same analysis, are not presented in the results as the misalignment originated dendrograms that do not correctly represent the evolution of the community, throughout time.

Regardless this misalignment, it can be observed that the band from LAB falls into a very similar position to the bands from *C. vulgaris*. LL_Bc07 and LL_Bc11 have very similar positions as well.

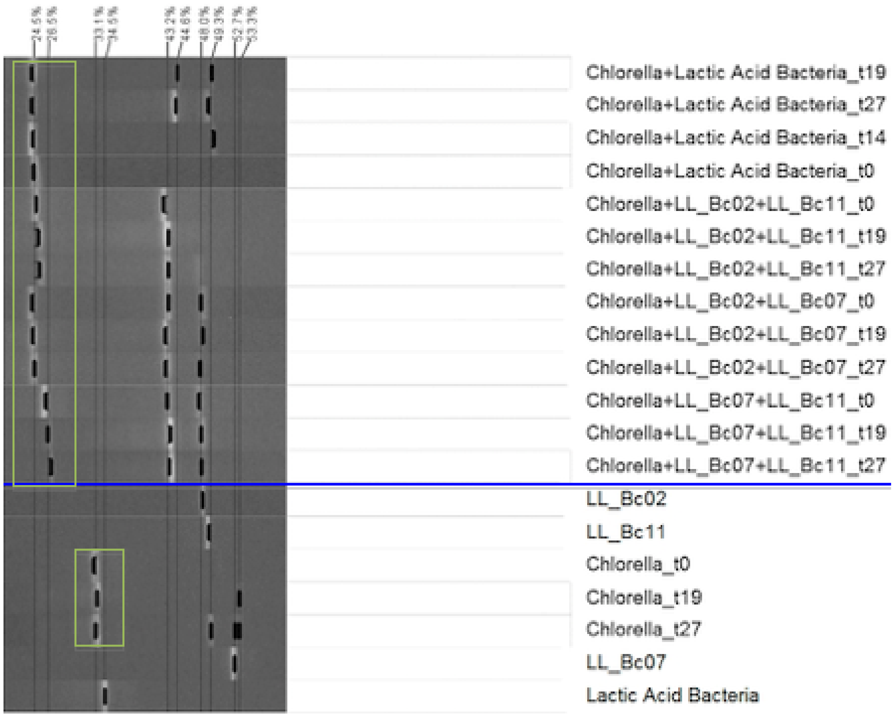


Figure 3.15 - Bandmatching analysis of bacterial communities in the co-cultures of *C. vulgaris* and bacteria, based on the DGGE profiles obtained. Relative positions of the bands are identified on top of the gel and sample names on the right side. The blue line separates the gel containing the controls from the gel containing samples from co-cultures.

Due to the misalignment, the analysis of the communities in co-cultures was done assuming the controls from *C. vulgaris* and bacteria appear in relative positions close to the positions from the strains in the gel with co-cultures. To perform the analysis, a simulated alignment of the gels was performed manually to mimic what would likely be the correct alignment. This constructed alignment can be observed in figure 3.16 and results will be analysed based on this figure.

As it can be observed in figure 3.16, the control of *C. vulgaris* on day 19 (t19) presents two bands – one from the chloroplast and another likely from a bacterial contamination. Moreover, the same control after 27 days (t27) presents four bands, one from the chloroplast and the others probably from bacterial contaminations. These results support the findings of bacterial contamination on plates, during cultivation. Furthermore, it is probable that contaminants in this culture were mainly from bacteria used in this study, as at least two of the bands correspond to relative positions of LL_Bc02 and/ or LL_Bc11 and LL_Bc07.

Regarding the bacterial controls, as previously mentioned, LAB is in a similar position to the band from *C. vulgaris* and LL_Bc02 and LL_Bc07 also have very similar relative positions. Regarding the mixed culture of *Chlorella* and LAB, a band for these bacteria does not show up in the DGGE. On the other hand, it is possible to observe is that after 14 days, a band from another bacteria shows up and after 19 and 27 days two bands may be observed, instead of one. This supports the observation made at the time of colony counting, of other bacterial colonies on agar plates rather than the characteristic colonies from LAB.

However, since both bands from *C. vulgaris* and LAB are in such similar positions, they may be overlapping and therefore be two instead of one. It is also possible that the DNA amount of LAB was much lower than the amount from *C. vulgaris* and consequently, only the latter be amplified and be visible on DGGE. To avoid this, a touchdown PCR program could have been used, as cycles in these types of programs allow equal amplification of DNA fragments, regardless the amount of DNA present in the sample. Even though touchdown PCR thermocycling parameters from Piterina and Pembroke (2010) were tested, these failed to amplify DNA from samples from this study. For this reason, PCR conditions used by Amorim *et al.* (2014) were tested and since they worked, DNA amplification was carried out using that protocol.

Additionally, the sample from this mixed culture at t14, presents one band that is not from LAB as it is at very different position (around 40% as opposed to near 20%) and samples from t19 and t27 present two additional bands, instead of one. These likely appeared due to a cross-contamination from two of the PBR isolates used in other mixed cultures, since samples were all processed at the same time and as their relative positions are equivalent to LL_Bc07 and/ or LL_Bc02 and LL_Bc11. Although there were evidences that LAB grew in co-culture, as its colonies were very characteristic and showed up on plates for some time, it is possible that the initial amount of DNA was not enough to be amplified during the PCR, therefore not appearing on the sample from the inoculation day. Furthermore, during the assay, based on plating for TVC, it was clear there was a contamination of this mixed culture by other bacteria, since LAB colonies ceased growing and others started appearing on NA plates. Hence, DGGE results support these observations.

Finally, regarding the remaining co-cultures (*C. vulgaris* + LL_Bc02 + LL_Bc07 and *C. vulgaris* + LL_Bc07+LL_Bc11), their communities were maintained throughout time, since all the bands remained in the same relative position from the beginning (t0) to the end of the test (t27). The mixed culture containing *C. vulgaris*, LL_Bc02 and LL_Bc11 only presents two bands, however, since LL_Bc02 and LL_Bc11 have identical relative positions in the gel the band that appears may, in fact, represent both bacterial isolates, due to their proximity. According to Muyzer, *et al.* (1993), bands at identical positions are not necessarily originated from the same species and are a problem that can be solved by optimization of DGGE gradients in order to improve resolution of the profile.

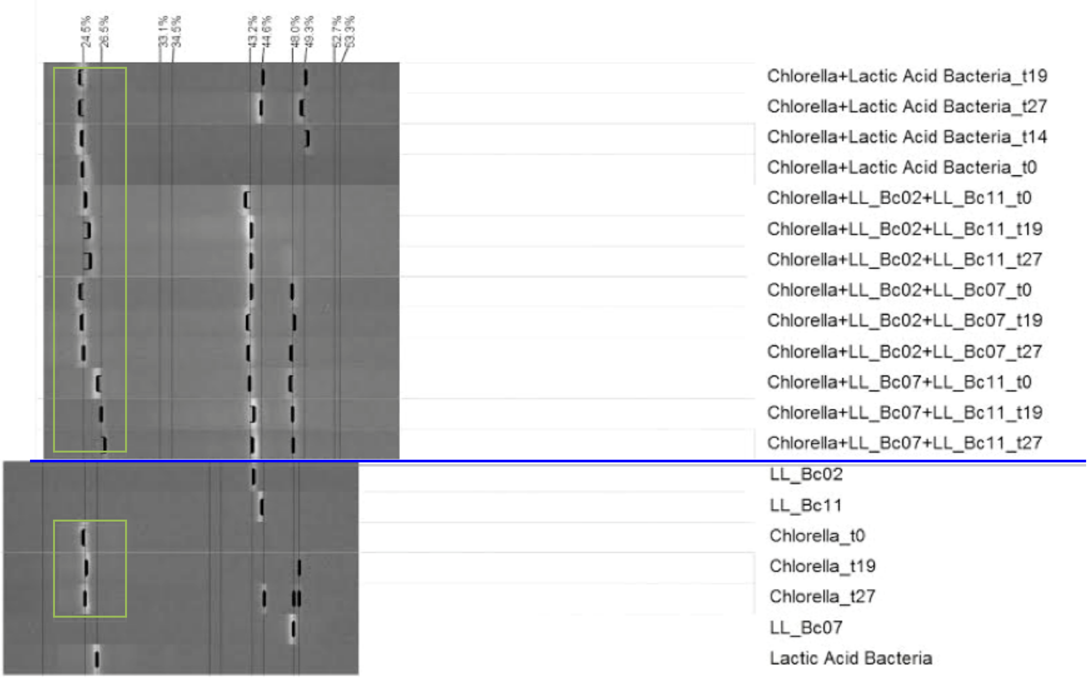


Figure 3.16 - Manual alignment of the gels with bands from the control of *C. vulgaris* matching the bands from this microalga in the mixed cultures, mimicking the expected alignment. The blue line separates the gel containing the controls from the gel containing samples from co-cultures.

Even though a cluster analysis would be interesting for this study, the analysis of communities through a dendrogram was not possible due to the misalignment.

Although DGGE is a powerful tool for monitoring microbial communities and their behaviour (Muyzer and Smalla, 1998), it is important to limit the number o samples so they can be analysed in one single gel or few (Valásková and Baldrin, 2009). Although the number of samples was reduced to limit the analysis to two gels, and DNA markers were used to reduce minor gel defects and allow between-gel comparison, as recommended by Valásková and Baldrin (2009), a processing error still occurred leading to misalignment of the gels.

In this study, the main goals of using DGGE analysis were to evaluate if bacteria and *C. vulgaris* were able to coexist in the same mixed culture and if the community established persevered throughout time or if one bacterium was able to overgrow the other.

Regarding these goals, co-cultures composed by *C. vulgaris* and combinations of two bacteria proved to be stable and co-exist throughout the cultivation time, whereas *C. vulgaris* + LAB did not prove favourable to LAB and these bacteria were quickly overpowered by other bacteria.

DGGE is useful in the study of community changes, since many samples taken at different times during a study, can be simultaneously analysed (Muyzer and Smalla, 1998). However, this is a challenging technique that requires practice and may require optimisation of parameters. For these reasons, in this work, it was not possible to further optimise the parameters that could have improved the analysis, due to the available time to conduct the experiments.

3.4. Co-cultures in Batch vs Co-culture in Semi-continuous

Results obtained with the co-cultures in batch showed there was one bacterial isolate (LL_Bc02) that had a negative impact on *C. vulgaris* and two bacteria (LL_Bc07 and LL_Bc11) that appeared to have a positive effect. Furthermore, co-cultures with the remaining bacteria presented maximum growth rates similar to the monoalgal control. These three isolates and LAB were chosen for the co-cultures in semi-continuous regime to verify if their behaviours were maintained when renewing the culture medium, hence replenishing the cultures with fresh medium and nutrients.

However, contrary to the co-cultures established in batch, co-cultures in semi-continuous exhibited a similar behaviour to the monoalgal control. In a previous study, Watanabe *et al.* (2005) found several types of relationships established between *Chlorella sorokiniana* and three different bacterial isolates. This investigation found two bacterial strains that showed commensalism with *C. sorokiniana*, by receiving nutrients from the microalga but not affecting its growth. From these, one belongs to the genus *Ralstonia* sp. and the other to *Sphingomonas* spp. In the co-cultures in semi-continuous from the present study, a similar relationship may have been established between bacterial consortia tested and *C. vulgaris*, except for LAB, since there was no decrease of microalgal growth but the bacteria remained in the mixed culture. Thus, this may indicate the bacteria inoculated were receiving nutrients from *C. vulgaris.*

Additionally, one of the isolates tested was from the genus *Sphingomonas* spp., a genus that has been isolated several times from non-axenic *Chlorella* spp. cultures, namely C. *saccharophila* (Ueda *et al*., 2009) and C. *sorokiniana* (Watanabe *et al*., 2005).

Results from Watanabe *et al*., 2005 revealed a mutualistic relationship between the microalga and an isolate of *Microbacterium* spp.. In this case, the microalga's growth was promoted by the bacterium while the latter received nutrients from *C. sorokiniana*. This was not verified in any of our mixed cultures in semi-continuous. However, in batch two bacteria – LL_Bc07 and LL_Bc11 - appeared to promote the growth of *C. vulgaris* since μmax of the microalga was higher, in the presence of these bacteria, than in the monoalgal control. Nevertheless, further studies would have to be pursued in order to verify the type of microbial interaction between these microrganisms.

The main goal of this study was to find out a possible bacterial consortium to use as inoculant for large-scale microalgal cultures. This idea resulted from the fact that unicellular microalgae usually grow in the presence of bacteria, especially in large-scale cultivations (Rivas *et al.*, 2010). Therefore, having a well-characterised bacterial community as inoculant could prevent undesired contaminants, by competition with the latter. In order for this to be viable, the inoculant consortium could not interfere negatively with the microalgae's growth, by competition, for instance. Ideally, this would even promote the microalgae's growth, acting as a probiotic. Similarly, Rivas *et al.* (2010) found that *Rhizobium* sp. acted as a probiotic bacterium by promoting the growth of *Botryococcus braunii* and considered using this bacterium as a large-scale inoculant for microalgal cultures.

In order to pursue this goal, growth rates of *C. vulgaris* in mixed cultures with bacterial consortia were compared to the monoalgal culture of this microalga and the population composition was studied throughout time, using DGGE as a tool to study communities.

From the results obtained, it is possible to conclude that the populations containing the bacterial isolates from the PBR were maintained throughout time, answering the question of population stability.

To summarise, this analysis suggests that consortia with the isolated bacteria are quite stable along the cultures growth. In contrast, LAB were not able to grow on the culture media used and thus had no effect on *C. vulgaris* growth rates.

4. GENERAL CONCLUSIONS

This study allowed to find two bacterial isolates (LL_Bc07 + LL_Bc11) that promoted the growth of *C. vulgaris* in batch regime and one bacterial isolate (LL_Bc02) that had a negative impact on microalgal growth, also in batch, regardless of other bacterial isolate in the consortium. As these results were not corroborated in semi-continuous regime, it is possible that the type of interactions between these bacteria and *C. vulgaris* are dependent on environmental conditions, which are affected by the growth regime. It was not possible to find a bacterial consortium that could be used as an inoculant alongside *C. vulgaris* in a large-scale production, where renewals are common. However, preliminary results in batch regime indicated LL_Bc07 and LL_Bc11 could have a positive effect on *C. vulgaris*, when in mixed culture.

Using the five bacterial isolates available and a commercially available mix of LAB, a total amount of eleven co-cultures were tested in batch and four of those were tested in semi-continuous regime. Regarding the use of LAB, it was conclusive that these bacteria do not grow well in the mixotrophic medium used and therefore did not make any impact on *C. vulgaris* in neither one of the growth modes tested.

Moreover, concerning the stability of communities throughout time, all of the co-cultures containing two bacterial isolates kept their initial composition until the end of the assay, without contaminations. On the contrary, the mixed culture containing *C. vulgaris* and LAB and the monoalgal control suffered contaminations by other bacteria, likely the ones present in the other consortia (LL_Bc02, LL_Bc07 and LL_Bc11). This analysis was possible due to the DGGE analysis performed. Nonetheless, it would have been important to optimize the gel gradient, had it been possible given the time constraints.

Finally, this work was important for the company as it allowed the development and establishment of methodologies to co-cultivate *C. vulgaris* and bacteria. This study can be used as a basis to test other bacteria in co-culture with *C. vulgaris* and even with other microalgae.

5. FUTURE WORK

This work is important as it can be a basis for future work at A4F, by adopting these methodologies to co-cultivate microalgae and bacteria. Nevertheless, these methodologies should be improved, especially regarding the inoculation ratio, as it was based on preliminary tests. Also, the quantification of the bacterial inoculum should be more specific to each strain used.

Considering the difference in the results obtained for different growth modes (batch and semi-continuous) it would be important to repeat the assays to confirm such results and depending on the intended large-scale cultivation plans. Given the results presented in this work, other bacteria could be studied, either isolates from non-axenic cultures available at the company, as well as others documented as PGPB.

It would be interesting to study the other commercially available bacteria for food purposes, as those are GRAS and could even add value to the final product.

Regarding DGGE it would be important to test other gel gradients as the one used did not allow the best resolution and separation of bands from different bacteria.

Furthermore, after finding a bacterium or bacterial consortia capable of promoting the growth of *C. vulgaris*, virulence tests should be performed, as the intended purpose of the production of this microalga is food. Even though part of the bacteria can be removed through centrifugation, the final product needs to meet the regulatory standards. Also, even if the amount of bacteria in the final product meets the standards, it is essential to assure there is not any release of toxins or toxic substances during the cultivation.

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7. APPENDIX

Preparation of Tris Acetate Phosphate (TAP) medium

Table 7.1 - Solutions and quantities required to prepare TAP medium.

Solutions used	Volume of each solution necessary to prepare TAP medium (Final volume = 1 L)
Tris	2,42 g
4xBeijerinck salts Solution	25 mL
Solution 1 M (K)PO_4 pH = 7	1 mL
Trace elements for TAP	1 mL
Deionized water	975 mL
Glacial Acetic Acid	Adjust to pH=7 (~1mL)

Table 7.2 - Reagents necessary to prepare 4x Beijerink salts solutions, required to prepare TAP medium.

4 x Beijerink salts	Molecular Weight	Concentration in solution	To prepare 500 mL of solution:
NH_4Cl	53,49	0,3 M	8 g
$CaCl_2 \cdot 2H_2O$	147,01	0,014 M	1 g
$MgSO_4 \cdot 7H_2O$	246,47	0,016 M	2 g

Table 7.3 - Reagents and amounts needed to prepare a 1M of (K)PO4, pH7, required to prepare TAP medium.

1 M (K)PO_4 pH 7	Molecular Weight	Preparing a 1M solution with a final volume of 500 mL	Volume needed in the final solution
K_2HPO_4 [1 M]	174,176	87,1 g	250 mL
KH_2PO_4 [1 M] (adicionar até ajustar o pH para 7)	136,09	68,0 g	~ 170 mL

Table 7.4 - Reagents and amounts required to prepare the trace elements solution used in TAP medium preparation.

Trace elements	Molecular Weight	Concentration in Solution	To prepare 1 L of solution
H_3BO_3	61,83	0,18 M	11,4 g
$ZnSO_4.7H_2O$	287,54	0,077 M	22 g
$MnCl_2.4H_2O$	197,91	0,026 M	5,06 g
$FeSO_4.7H_2O$	278,01	0,018 M	4,99 g
$CoCl_2.6H_2O$	237,93	0,0068 M	1,61 g
$CuSO_4.5H_2O$	249,68	0,0063 M	1,57 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	1235,86	0,0009 M	1,1 g

Co-cultures in Batch Regime

Table 7.5 - Results from the acetate quantification in the co-cultures in batch regime, at the end of the assay.

Sample	Acetic Acid concentrarion (mM)
Standard	2,000
Chlorella	0,000
LL_Bc02	0,093
LL_Bc07	5,523
LL_Bc08	5,377
LL_Bc10	0,286
LL_Bc11	0,000
LAB	4,858
Chlorella + LAB	0,453
Chlorella + LL_Bc02 + LL_BC07	0,330
Chlorella + LL_Bc02 + LL_BC08	0,305
Chlorella + LL_Bc02 + LL_BC10	0,377
Chlorella + LL_Bc02 + LL_BC11	0,000
Chlorella + LL_Bc07 + LL_BC08	0,300
Chlorella + LL_Bc07 + LL_BC10	0,367
Chlorella + LL_Bc07 + LL_BC11	0,698
Chlorella + LL_Bc08 + LL_BC10	0,402
Chlorella + LL_Bc08 + LL_BC11	0,353
Chlorella + LL_Bc10 + LL_BC11	0,533
Negative control (TAP medium)	6,005